Exploration of the molecular mechanisms of cognitive dysfunction in schizophrenia using the sub-chronic PCP rodent model

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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Thesis Abstract

“Exploration of the molecular mechanisms of cognitive dysfunction in schizophrenia using the sub-chronic PCP rodent model” submitted for the degree of Doctor of Philosophy at The University of Manchester by James E Glasper (September 2014).

Cognitive dysfunction is a core symptom of schizophrenia, which is poorly treated by current antipsychotic medication. Deficits in the GABAergic system, as demonstrated by convergent genetic and $[^{25}]$I-iomazenil imaging evidence from patients, are thought to underlie these cognitive deficits. The sub-chronic PCP rodent model was used as it shows cognitive and behavioural parallels to schizophrenia and therefore provides a translational model for some aspects of the disease. However the neurobiological mechanisms responsible for the behavioural alterations in this model have not been fully elucidated.

The main aim of the studies presented in this thesis was to investigate the construct validity of the sub-chronic PCP model in relation to the GABAergic and sigma-1 (σ1) receptor systems. Transcriptional changes in gene markers were studied using qRT-PCR and proteomic alterations were investigated using radioligand binding, autoradiography and Western blotting. Finally, the cognitive enhancing potential of σ1 receptor modulators was tested using the novel object recognition (NOR) task.

Data presented in chapter 3 shows that sub-chronic PCP treatment in rats produces an increase in GABA$_A$ receptor α5-subunit mRNA and a decrease in α3 and δ subunit mRNA levels. No differences were observed in the mRNA levels of the other studied GABA$_A$ receptor subunits (α1, α2, α4 or γ2). No alterations in benzodiazepine site- or α5-subunit-containing GABA$_A$ receptors were seen following a 7-day washout period, although increased frontal cortical levels of α5-subunit protein were observed prior to the washout period. This suggests that sub-chronic PCP treatment affects extrasynaptic cortical GABA$_A$ receptor expression, as shown by the alterations in α5- and δ-subunits, which may contribute to the cognitive deficits observed in this model.

Studies in chapter 4 showed that sub-chronic PCP administration causes frontal cortical reductions in parvalbumin, GAD$_{67}$, GABA transporter-1 and calretinin mRNA levels. No alterations were observed for somatostatin, GAD$_{65}$, or GABA transporter-3 mRNA, although changes in the mRNA levels for the astrocytic marker glial fibrillary acidic protein were observed in the cerebellum, frontal cortex and hippocampus of sub-chronic PCP-treated animals. No differences in the frontal cortical protein levels of GAD$_{67}$, GAT-1 and calretinin were observed, suggesting that any proteomic differences in these markers which are present in the sub-chronic PCP model, they are limited in a layer- or cell-type-specific manner.

The NOR task is a translational cognitive test that measures recognition memory, which is known to be impaired in schizophrenia. Data in chapter 5 of this thesis showed that sub-chronic PCP-induced and delay-induced recognition memory deficits were ameliorated by acute administration of the σ1 receptor agonist (PRE-084) at 1 and 3mg/kg and by the σ1 receptor antagonist (NE-100) at 1mg/kg. NE-100 at 3mg/kg proved effective at ameliorating delay-, but not PCP-induced memory deficits. No procognitive effect was observed at lower concentrations of either compound or by co-administration of both compounds. These observations suggest that the improvement of recognition memory deficits is mediated, in part, by σ1 receptors in female rats.

The overall results of these studies suggest that sub-chronic PCP administration causes frontal cortical transcriptional alterations in GABAergic neuronal markers which correlate to clinical findings in schizophrenia patients, although these alterations were not observed at the proteomic level following the washout period. These findings also suggest that the σ1 receptor is a potential therapeutic target for recognition memory deficits in schizophrenia, as well as other disorders.
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# List of Abbreviations

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<th>Definition</th>
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<td>2-AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ABHD6</td>
<td>abhydrolase domain-containing protein 6</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>B.I.D</td>
<td>Bis in die (Twice daily)</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>Bmax</td>
<td>Binding site maximum</td>
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<tr>
<td>BOLD</td>
<td>Blood-Oxygen-Level-Dependent</td>
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<td>Cornu Ammonis area 1</td>
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<tr>
<td>CA3</td>
<td>Cornu Ammonis area 3</td>
</tr>
<tr>
<td>CALB2</td>
<td>Calretinin gene nomenclature</td>
</tr>
<tr>
<td>CaMK</td>
<td>Calcium/calmodulin-dependent kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaRE</td>
<td>Calcium Response Element</td>
</tr>
<tr>
<td>CATIE</td>
<td>Clinical Antipsychotic Trials of Intervention Effectiveness</td>
</tr>
<tr>
<td>CB</td>
<td>Calbindin</td>
</tr>
<tr>
<td>CBT</td>
<td>Cognitive behavioural therapy</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variant/Variation</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyl transferase</td>
</tr>
<tr>
<td>CR</td>
<td>Calretinin</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRT</td>
<td>Cognitive remediation therapy</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CUtLASS</td>
<td>Cost Utility of the Latest Antipsychotic drugs in Schizophrenia Study</td>
</tr>
<tr>
<td>DI</td>
<td>Discrimination Index</td>
</tr>
<tr>
<td>DISC1</td>
<td>Disrupted-in-schizophrenia 1</td>
</tr>
<tr>
<td>DLPFC</td>
<td>Dorsolateral prefrontal cortex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>DSM-V</td>
<td>Diagnostic and Statistical Manual of mental disorders v5</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>DUP</td>
<td>Duration of untreated psychosis</td>
</tr>
<tr>
<td>EDS</td>
<td>Extradimensional shift</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPS</td>
<td>Extrapyramidal symptoms</td>
</tr>
<tr>
<td>EuFEST</td>
<td>European First Episode Schizophrenia Trial</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>fMRI</td>
<td>functional magnetic resonance imaging</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanosine nucleotide-binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>GABRA1</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α1 subunit gene nomenclature</td>
</tr>
<tr>
<td>GABRA2</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α2 subunit gene nomenclature</td>
</tr>
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<td>GABRA3</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α3 subunit gene nomenclature</td>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor α4 subunit gene nomenclature</td>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor δ subunit gene nomenclature</td>
</tr>
<tr>
<td>GABRG2</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor γ2 subunit gene nomenclature</td>
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<tr>
<td>GAD1</td>
<td>Glutamate decarboxylase 1 gene nomenclature</td>
</tr>
<tr>
<td>GAD2</td>
<td>Glutamate decarboxylase 2 gene nomenclature</td>
</tr>
<tr>
<td>GAD&lt;sub&gt;65&lt;/sub&gt;</td>
<td>Glutamate decarboxylase protein (65KDa isoform)</td>
</tr>
<tr>
<td>GAD&lt;sub&gt;67&lt;/sub&gt;</td>
<td>Glutamate decarboxylase protein (67KDa isoform)</td>
</tr>
<tr>
<td>G&lt;sub&gt;ai&lt;/sub&gt;</td>
<td>G-protein inhibitory alpha subunit</td>
</tr>
<tr>
<td>G&lt;sub&gt;as&lt;/sub&gt;</td>
<td>G-protein stimulatory alpha subunit</td>
</tr>
<tr>
<td>GAT-1</td>
<td>GABA transporter type 1</td>
</tr>
<tr>
<td>GAT-3</td>
<td>GABA transporter type 3</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GluN</td>
<td>Glutamate [NMDA] receptor subunit</td>
</tr>
<tr>
<td>GRIN</td>
<td>Glutamate [NMDA] receptor subunit zeta</td>
</tr>
<tr>
<td>GRM</td>
<td>Metabotropic glutamate receptor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICD-10</td>
<td>International Classification of Diseases v10</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITI</td>
<td>Inter-trial interval</td>
</tr>
<tr>
<td>KA</td>
<td>Kynurenic Acid</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
</tbody>
</table>
LTD
Long term depression
LTP
Long term potentiation
MAM
Methoxymethanol
MAM
Mitochondrial-Associated Membrane
MATRICS
Measurement and Treatment Research to Improve Cognition in Schizophrenia
MCCB
MATRICS Consensus Cognitive Battery
mGlur
metabotropic glutamate receptor
MHC
Major Histocompatibility complex
MK-801
Dizocilpine
MLA
Methyllycaconitine
mPFC
Medial prefrontal cortex
MW
Molecular weight
NaCl
Sodium chloride
NE-100
4-methoxy-3-(2-phenylethoxy)-N,N-dipropylbenzeneethanamine
NICE
National Institute for Health and Care Excellence
NMKDA
N-methyl-D-aspartate
NOR
Novel object recognition
NOS
Nitric oxide synthase
Nox-2
Nicotinamide adenine dinucleotide phosphate oxidase type 2
NPY
Neuropeptide Y
NRG1
Neuregulin gene nomenclature
NVHL
Neonatal Ventral Hippocampal Lesion
OR
Odds Ratio
pa
Per annum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBST</td>
<td>Phosphate-Buffered Saline with TWEEN</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission topography</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polynosinic:polycytidilic acid</td>
</tr>
<tr>
<td>PPI</td>
<td>Pre-pulse inhibition</td>
</tr>
<tr>
<td>PRE-084</td>
<td>2-(4-morpholinethyl)-1-phenylcyclohexanecarboxylate</td>
</tr>
<tr>
<td>PV</td>
<td>Parvalbumin</td>
</tr>
<tr>
<td>PVALB</td>
<td>Parvalbumin gene nomenclature</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of Life</td>
</tr>
<tr>
<td>Ras</td>
<td>Abbreviation of rat sarcoma</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantification value</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SGA</td>
<td>Second generation antipsychotic</td>
</tr>
<tr>
<td>SIGMAR1</td>
<td>Sigma-1 receptor gene nomenclature</td>
</tr>
<tr>
<td>SLC6a1</td>
<td>GAT-1 gene nomenclature</td>
</tr>
<tr>
<td>SLC6a11</td>
<td>GAT-3 gene nomenclature</td>
</tr>
<tr>
<td>SNAP-23</td>
<td>Synaptosomal-associated protein 23</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOM</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-Photon Emission Computed Tomography</td>
</tr>
<tr>
<td>SST</td>
<td>Somatostatin gene nomenclature</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>Polymerase enzyme from the thermophilic bacteria <em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRIS-HCl</td>
<td>Tris(hydroxymethyl) aminomethane hydrochloride salt</td>
</tr>
<tr>
<td>TURNS</td>
<td>Treatment Units for Research in Neurocognition in Schizophrenia</td>
</tr>
<tr>
<td>TWEEN</td>
<td>Polyoxyethylene sorbitan monooleate</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
1.1 Schizophrenia

1.1.1 Aetiology

Schizophrenia is a complex, chronic, neuropsychiatric disease with multiple genetic, environmental and neurobiological factors. This disease affects up to 1% of the global population (Trivedi and Jarbe 2011), with an early age of onset (late teens to early twenties).

Men tend to have an earlier age-of-onset and higher prevalence of the disease compared to women (Castle et al. 1998, Angermeyer and Kuhn 1988). Recent studies have shown that males are 1.4 times more likely to develop schizophrenia than females (Aleman et al. 2003, McGrath et al. 2004), supporting Kraepelin’s observations that schizophrenia is a disorder that mostly affects young men (Kraepelin 1971). Studies have shown the age-of-onset of schizophrenia is gender-dependent, with the mean onset in males being in the early 20s compared to the late 20s in females (Angermeyer and Kuhn 1988, Salem and Kring 1998), although recent meta-analyses from first episode cohorts have shown modal age-of-onset values to be very similar between the genders (Abel et al. 2010). Males in the chronic stage of the illness also present with more negative symptoms (Zhang et al. 2012) and show reduced responses to typical (Canuso and Pandina 2007) and certain atypical antipsychotic medications (clozapine and olanzapine) (Usall et al. 2007). Decreased risk of developing schizophrenia in young females is thought to be associated with increased oestrogen levels. Epidemiological studies show that, in menopausal women, the risk of developing schizophrenia or relapse of existing symptoms increases compared to age-matched controls (Hafner 2003), and a small study showed an increase in psychotic symptoms in women during the “low-oestrogen” phase of the menstrual cycle (Riecher-Rossler et al. 1994). This hypothesis is supported by clinical studies showing reductions in psychotic and cognitive symptoms in male and female patients who take oestrogen (Kulkarni et al. 2002, Kulkarni 2009) or pregnenolone (Marx et al. 2011) as a supplement to their existing antipsychotic medication.

Schizophrenia has a severe economic and societal impact due to the inability of most sufferers to work consistently and the high rate of suicide (10x general populace rate and occurrence of 4-5% amongst patients) amongst sufferers compared to the
Schizophrenia and schizotypal disorders account for the longest admission duration of any psychiatric disorder in the UK for the year 2008-2009 (Pillay and Moncrieff 2011). Recent reports have shown that schizophrenia currently costs English society around £11.8bn/year in direct and indirect costs, translating into a cost of £60,000pa per person with the disorder (LSE report “Effective Interventions in schizophrenia” - Andrew et al. 2012, see Figure 1.1). Annual total treatment costs in the USA between 2005 and 2008 were $15.35bn, with $3.96bn being spent on direct treatment costs. This translated to an individual direct-treatment cost per patient per annum of $5,402 (Desai et al. 2013), although previous studies have placed the financial impact directly attributable to this disorder much higher ($19-22.7bn), showing that treatment costs are a small part of the financial impact of schizophrenia (Rice and Miller 1996, Wu et al. 2005).

*Figure 1.1:* Annual societal and public sector costs in pounds sterling (£) for treating schizophrenia in the UK for the year 2010/2011. Taken from the LSE economic report “Effective Interventions in Schizophrenia” (Andrew et al. 2012).

1.2 History and Diagnosis

Schizophrenia was first described as dementia praecox by Emil Kraepelin in 1887 (Kraepelin 1971), but was renamed as schizophrenia by Eugen Bleuler (Bleuler 1911). Schizophrenia comes from the Greek words skhizein (σχίζειν - “to split”) and phren (φρήν - “mind”), describing the detachment from reality caused by positive
symptoms rather than a ‘split mind’ which would be clinically characterised as multiple personality disorder. Currently two classification systems are used to characterise and diagnose psychiatric illnesses: the international classification of diseases in Europe (ICD-10) and the diagnostic and statistical manual of mental disorders (DSM-V®) criteria in the USA and UK.

Schizophrenia diagnostic symptoms are typically divided into two main categories: positive and negative symptoms. Cognitive deficits, whilst not part of the diagnostic criteria of schizophrenia, have been shown to be a key component in the disorder as described below.

1.2.1 Positive symptoms

Positive symptoms are thoughts and experiences that patients with schizophrenia suffer from that are not present in the general non-psychotic population. These include (ICD-10 guidelines, "ICD-10 classification" 2010):

**Delusions:** These take the form of strongly held beliefs of power, experience, influence or persecution (paranoia), regardless of contrary evidence. These are particular pronounced in the paranoid schizophrenia subtype.

**Hallucinations:** These mostly take the form of auditory, tactile or olfactory hallucinations as visual hallucinations are comparatively uncommon (ICD-10 guidelines, "ICD-10 classification" 2010).

**Disorganised speech:** Incoherence or difficulty ordering words/sentences, often resulting in the use of unnecessary or meaningless words; often called ‘word salad’.

1.2.2 Negative symptoms

Negative symptoms are feelings and behaviours that are absent or diminished in patients with schizophrenia, compared to the general populace. These are often difficult to diagnose and are not adequately treated by current medication i.e. typical and atypical antipsychotics (Keefe et al. 2007) with a minimum of 15% of patients (Buchanan 2007) showing persistent negative symptoms. Primary and secondary negative symptoms have been identified, with primary negative symptoms being a fundamental part of the disease and secondary negative symptoms arising from complications of other symptoms or medication. For example, avolition could be a
primary characteristic of the disease, a result of off-target sedative effects of antipsychotic medication or a delusional persecu
tional fear of leaving the house.

Negative symptoms include (ICD-10 guidelines, "ICD-10 classification" 2010):

**Alogia:** poverty of speech, manifesting as shortened non-fluent responses with a lack of the unprompted content usually seen in normal speech. Alogia is thought to be due to disrupted thought processes.

**Blunting of affect:** reduced or lack of emotional/facial responses, this can also manifest as catatonic symptoms.

**Avolition:** a lack of effort directed towards achieving reward-based personal goals/motivation, which is often confused with disinterest.

**Social withdrawal:** This could be due to reduced interest in personal hygiene or a dislike of crowds, resulting in more time spent alone.

Negative symptoms are thought to divide into two domains; alogic and affective blunting symptoms represent an ‘expression deficit’ domain, while avolition and social withdrawal represent an ‘avolition’ symptom domain (Liemburg et al. 2013). This reclassification of negative symptoms is reflected in the new DSM-V diagnostic criteria.

### 1.2.3 Cognitive dysfunction

Since dementia praecox was first described in 1887, cognitive dysfunction has been identified as a core schizophrenia symptom group, although they are not currently part of the diagnostic criteria for schizophrenia. The cognitive deficits in schizophrenia have been studied intensively over the last 2 decades as they have been shown to be present in the prodromal stage of the illness before the onset of psychotic symptoms (Niemi et al. 2003, Lencz et al. 2006, Whyte et al. 2006, Eastvold et al. 2007). Cognitive deficits also positively correlate with symptom severity and negatively with age-of-onset and response to pharmacological treatment (Green 2006). Cognitive deficits in schizophrenia include:

**Disorganised or slow thinking processes**
**Poor concentration and memory**

**Difficulty integrating thoughts, feelings and behaviours**

**Diminished theory of mind** (the ability to empathise with and attribute differing beliefs and desires to others)

These cognitive deficits manifest in a variety of domains including selective attention, working memory, executive control, episodic memory, language comprehension, social-emotional processing (Gonzalez-Burgos et al. 2011) and visual memory (Harvey 2006).

Working memory, in particular, is thought to be a core cognitive deficit in schizophrenia and working memory deficits are positively correlated with increased negative symptoms in patients (Brazo et al. 2002, Harvey et al. 2006). Imaging studies have shown reduced prefrontal cortical activation in mixed populations of patients during tasks that have a working memory component, which is hypothesised to explain part of these deficits (Ohi et al. 2011, Bleich-Cohen et al. 2013, Koike et al. 2013). Working memory is defined as the ability to hold and manipulate multiple items of information in order to successfully complete a goal-directed task in the presence of extraneous distractors or redundant information inputs. It is vital for a number of cognitive processes including problem solving and social interaction (Ericsson and Kintsch 1995, Forbes et al. 2009, Smith et al. 2013, Whitely and Colozzo 2013).

There is a great deal of evidence showing that cognitive deficits are present in unaffected first-degree relatives of schizophrenia patients, compared to second-degree relatives (Reviewed by Snitz et al. 2006, Bora and Pantelis 2013) suggesting a genetic basis to these deficits. These data are supported by functional magnetic imaging (fMRI) studies which show deficits in the activation of the dorsolateral prefrontal cortex (DLPFC) and cingulate and frontal gyri (Liao et al. 2012), as well as excessive dorsolateral and medial prefrontal cortical connectivity (Unschuld et al. 2013) in first-degree relatives of schizophrenia patients. This excessive connectivity, especially in the default-mode network, is thought to represent compensatory neuronal mechanisms in response to the disease and has been associated with impairment in working memory tasks (Meyer-Lindenberg et al. 2001). Excessive
cortical connectivity is also found in the first-degree relatives of schizophrenia patients (Whitfield-Gabrieli et al. 2009, Repovs and Barch 2012). Cognitive, but not emotional theory of mind deficits were also observed in first-degree relatives of schizophrenia patients (Montag et al. 2012, Bora and Pantelis 2013).

Schizophrenia symptom severity has been shown to correlate with decreased quality of life (QoL) as measured by the inability to form lasting relationships, function at work or in the community. These functional impairments are common in schizophrenia patients (Albert et al. 2011) and cause a huge burden on patients and their families (Bellack et al. 2007). Positive and negative symptoms, along with cognitive impairments are all correlated with reduced QoL (Savilla et al. 2008) and cognitive function was thought to be the best predictor of functional outcome (Green et al. 2000, Green et al. 2004). Recent work has shown that negative symptom severity is also a significant contributor to functional outcome, as improvement in negative symptom domains mediates neurocognitive improvement to give a significantly improved functional outcome (Schmidt et al. 2011). This highlights the importance of treating both cognitive impairment and negative symptoms in schizophrenia, as their relationship seems to be complementary.

Treating cognitive deficits was identified as a key challenge in the field of schizophrenia and schizophrenia-associated disorders, but problems such as a lack of consensus about how cognition should be measured in patients, and differing opinions as to the most promising pharmacological targets were seen as being likely to interfere with the development of novel pharmacological treatments. In order to address these issues the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative was established, with the aim of creating a consensus of how cognition in patients and the procognitive potential of new treatments should be measured. This, in turn, led to the development of the MATRICS Consensus Cognitive Battery (MCCB) which was designed to show the key cognitive domains affected in schizophrenia (Table 1.1) and aid the research and development of new pharmacological agents to treat these cognitive deficits (www.matrics.ucla.edu).
<table>
<thead>
<tr>
<th>Cognitive Domain</th>
<th>Test</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed of processing</td>
<td>Brief Assessment of Cognition in Schizophrenia (BACS): Symbol-Coding</td>
<td>Timed paper-and-pencil test in which respondent uses a key to write digits that correspond to nonsense symbols</td>
</tr>
<tr>
<td></td>
<td>Category Fluency: Animal Naming</td>
<td>Oral test in which respondent names as many animals as she/he can in 1 minute</td>
</tr>
<tr>
<td></td>
<td>Trail Making Test: Part A</td>
<td>Timed paper-and-pencil test in which respondent draws a line to connect consecutively numbered circles placed irregularly on a sheet of paper</td>
</tr>
<tr>
<td>Attention/Vigilance</td>
<td>Continuous Performance Test—Identical Pairs (CPT-IP)</td>
<td>Computer-administered measure of sustained attention in which respondent presses a response button to consecutive matching numbers</td>
</tr>
<tr>
<td>Working memory: (nonverbal)</td>
<td>Wechsler Memory Scale®—3rd Ed. (WMS®-III): Spatial Span</td>
<td>Using a board on which 10 cubes are irregularly spaced, respondent taps cubes in same (or reverse) sequence as test administrator</td>
</tr>
<tr>
<td></td>
<td>Letter-Number Span</td>
<td>Orally administered test in which respondent mentally reorders strings of number and letters and repeats them to administrator</td>
</tr>
<tr>
<td>Working memory: (verbal)</td>
<td>Hopkins Verbal Learning Test—Revised™ (HVLT-R™)</td>
<td>Orally administered test in which a list of 12 words from three taxonomic categories is presented and the respondent is asked to recall as many as possible after each of three learning trials</td>
</tr>
<tr>
<td>Verbal learning</td>
<td>Brief Visuospatial Memory Test—Revised (BVMT-R™)</td>
<td>A test that involves reproducing six geometric figures from memory</td>
</tr>
<tr>
<td>Visual learning</td>
<td>Neuropsychological Assessment Battery® (NABR): Mazes</td>
<td>Seven timed paper-and-pencil mazes of increasing difficulty that measure foresight and planning</td>
</tr>
<tr>
<td>Reasoning and problem solving</td>
<td>Mayer-Salovey-Caruso Emotional Intelligence Test (MSCEIT™): Managing Emotions</td>
<td>Paper-and-pencil multiple-choice test that assesses how people manage their emotions</td>
</tr>
</tbody>
</table>

**Table 1.1:** The key cognitive domains outlined by the MCCB and the tests used to measure cognitive performance in schizophrenia (Adapted from http://images.pearsonclinical.com/images/Products/MCCB/MCCB_Chart.pdf – accessed 27/11/14).
1.2.4 Prodrome of schizophrenia

It is established that there exists a prodromal stage of the disease, which features cognitive decline in processing speed, working memory, verbal episodic memory, executive functioning, general intelligence domains (Eastvold et al. 2007) and social function (Addington et al. 2008) which occur prior to the onset of psychotic symptoms. After the onset of psychosis, Kraepelin and others observed that the longer that psychotic symptoms went untreated, the greater the functional disabilities and symptom severity. This duration of untreated psychosis (DUP) is positively linked with symptom severity, cognitive dysfunction and poor response to antipsychotics (Marshall et al. 2005, Perkins et al. 2005, Boonstra et al. 2012).

1.3 Schizophrenia aetiology

1.3.1 Genetic studies

Twin studies have shown that schizophrenia has an 82-84% genetic component (Cardno and Gottesman 2000), and although genes such as neuregulin (NRG1) and catechol-O-methyl-transferase (COMT) have been implicated from multiple genome-wide association studies (Kleinman et al. 2011), none of them seem to show a large effect, suggesting that developing schizophrenia involves polygenic dysfunction across a number of cell signalling pathways. Schizophrenia shows a complex pattern of inheritance, although occasionally genes do link to families, such as the discovery of a translocation in the DISC1 gene in a Scottish family (Millar et al. 2000). This polygenicity, combined with a low relative risk for each pathogenic gene, makes the identification of “schizophrenia risk genes” very difficult indeed. Risk genes have been identified in a number of distinct domains, including catecholamine regulation, oxidative stress (Tosic et al. 2006), the MHC (major histo-compatibility complex) region of immune genes, GABAergic signalling (Volk and Lewis 2002), dysbindin, metabotropic glutamate receptor type 3 (GRM3) and DISC1 (Egan et al. 2004, Harrison and Weinberger 2005), although many of these genes have not shown reproducible association across multiple studies. The large number of genes identified and the lack of reproducible association suggests that schizophrenia is a complex genetic disorder which can result from a number of rare mutations in genes across a large number of potential signalling pathways. It has been estimated that >8000 SNPs independently contribute to developing schizophrenia, in combination with genetic and
environmental risk factors (Ripke et al. 2013), however sample size is thought to be one of the most important limiting factors when using GWAS technologies to study schizophrenia. Large scale meta-analyses (Schizophrenia Psychiatric Genome-Wide Association Study 2011, Rietschel et al. 2012, Ripke et al. 2013) were conducted in order to address the issue of small sample sizes, which subsequently yielded over 30 loci of interest which achieved genome-wide significance, including MIR137, TCF4, CACNA1C and SLC06A1. This approach culminated in the formation of the Schizophrenia Working Group of the Psychiatric Genomics Consortium (n=36989 cases and 113075 controls), who have combined published and unpublished results from a number of groups and revealed over 100 loci of interest, which achieve genome-wide significance (Schizophrenia Working Group of the Psychiatric Genomics 2014). These loci, while statistically significant, show very low odds ratio values associated with the risk of developing schizophrenia (OR=1.1-1.2) suggesting that each loci contributes a very small part of the genetic predisposition for developing schizophrenia. Inheriting multiple risk variants (Polygenic risk scores for schizophrenia) has been recently linked to reduced prefrontal cortical activation during working memory which is a well-documented observation in schizophrenia patients, compared to controls (Kauppi et al. 2014). The idea of a “schizophrenia risk gene/locus” is further complicated by a recent meta-analysis which demonstrates overlap of risk loci between psychiatric disorders, suggesting common pathways underlying disorders with similar aetiology (eg: COMT Val158Met in both schizophrenia and anxiety disorders) (Gatt et al. 2015). Table 1.2 shows a selection of the main susceptibility genes that have been identified in schizophrenia using large-scale GWAS/meta-analyses (Harrison 2014).
A number of gene loci which showed significant association with schizophrenia in previous studies have not achieved significance in more recent large-scale GWAS studies, including DISC1, ErBB4, DAO and NRG1. This is further complicated by the finding that “risk” loci such as NRG1 can also be associated with both increased and decreased risk of developing schizophrenia. Large (n>1000 patients) studies by Papiol and colleagues (Papiol et al. 2011) have suggested that certain NRG1 polymorphisms can be protective (SNP 8NRG243177, SNP 8NRG221533), despite also being identified as a risk gene in several association studies (Stefansson et al. 2003, Li et al. 2006). The SNP8NRG243177 polymorphism in particular was shown to correlate positively with age of onset and negatively with positive symptom severity, although not with cognitive or motor deficits; indicating a protective phenotype. NRG1 polymorphisms have, more recently, been linked with haemodynamic hippocampal activation during a working memory task (Kircher et al. 2009), as well as protecting against long-term

Table 1.2: Susceptibility genes (not CNVs) for schizophrenia that have shown significant association with schizophrenia in one or more GWAS studies. Adapted from Harrison 2014

<table>
<thead>
<tr>
<th>Locus</th>
<th>Implicated gene</th>
<th>Name of gene/product</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>12p13.33</td>
<td>CACNA1C</td>
<td>L-type calcium channel α subunit type 1c</td>
<td>Important in neuronal function</td>
<td>Bhat et al. 2012</td>
</tr>
<tr>
<td>11q23.3</td>
<td>DRD2</td>
<td>Dopamine D2 Receptor</td>
<td>Key target of antipsychotic drugs</td>
<td>Beaulieu and Gainetdinov 2011</td>
</tr>
<tr>
<td>5q33.2</td>
<td>GRIA1</td>
<td>AMPA receptor subunit 1</td>
<td>The subunit influences properties of the AMPA receptor, and synaptic plasticity and behaviour</td>
<td>Barkus et al. 2014</td>
</tr>
<tr>
<td>16p13.2</td>
<td>GRIN2A</td>
<td>NMDA receptor subunit 2A</td>
<td>The subunit influences properties of the NMDA receptor, including synaptic localisation and channel conductance</td>
<td>Paoletti et al. 2013</td>
</tr>
<tr>
<td>7q21.11-12</td>
<td>GRM3</td>
<td>Metabotropic Glutamate Receptor 3 (mGlu3)</td>
<td>Group II metabotropic glutamate receptor (along with mGlu2) acting primarily as inhibitory autoreceptors</td>
<td>Harrison et al. 2008</td>
</tr>
<tr>
<td>1p21.3</td>
<td>MIR137</td>
<td>MicroRNA 137</td>
<td>Non-protein-coding gene. A microRNA which regulates other genes by binding to the 3’ untranslated region of their transcripts</td>
<td>Pasquinelli 2012</td>
</tr>
<tr>
<td>17p13.3</td>
<td>SRR</td>
<td>Serine Racemase</td>
<td>Enzyme which synthesises D-serine from L-serine</td>
<td>Balu et al. 2013</td>
</tr>
<tr>
<td>18q21.2</td>
<td>TCF4</td>
<td>Transcription Factor 4</td>
<td>Basic helix-loop-helix transcription factor</td>
<td>Forrest et al. 2014</td>
</tr>
<tr>
<td>2q32.1</td>
<td>ZNF804A</td>
<td>Zinc Finger Protein 804A</td>
<td>Putative transcription factor</td>
<td>Hess and Glatt 2014</td>
</tr>
</tbody>
</table>
memory deficits (microsatellite 478B14-8480) and correlating with short-term memory deficits in schizophrenia (microsatellite 420M9-139500) (Alfimova et al. 2011).

1.3.1.2 Copy Number Variation
As well as SNPs, recent studies have shown that copy number variants (CNVs) are strongly associated with schizophrenia, as well as other neuropsychiatric disorders including autism and bipolar disorder (Kirov 2010, Van Winkel et al. 2010). Copy number variants are defined as micro-deletions or micro-duplications of segments of DNA ranging in size from hundreds to millions of base pairs (Grayton et al. 2012). A number of these CNVs have been linked to an increased risk of developing schizophrenia including micro-deletions at 1q21.1, 15q11.2, 22q11.2 and micro-duplications at 16q11.2 (Mowry and Gratten 2013). These CNVs are rarer than schizophrenia-associated SNPs but confer a much higher risk of developing schizophrenia (Kirov et al. 2014). A recent meta-analysis (Giaroli et al. 2014) showed that micro-duplications, but not micro-deletions, at 16q11.2 are associated with a very high risk of developing both psychosis (Odds ratio (OR)=14, 10 articles) and schizophrenia (OR=16, 5 articles), compared to SNPs which are associated with developing schizophrenia (OR=1.1-1.2).

Genetic studies have identified a number of loci (SNPs and CNVs) which show significant association with schizophrenia. These sites and the genes which they contain (or are linked with) are being studied in detail as they represent promising targets for pharmacological interventions for the treatment of schizophrenia, although whether the presence of these variants represents an increased risk of developing schizophrenia or certain clinical features of the disorder is not yet known. In addition, many of the genetic loci that have been associated with schizophrenia also show significant association with other mental disorders including autism and bipolar disorder (Cross-Disorder Group of the Psychiatric Genomics 2013, Cross-Disorder Group of the Psychiatric Genomics et al. 2013, Harrison 2014), which strongly suggests a degree of pleiotropy between different diseases. This pleiotropy, if it exists, would implicate key signalling pathways as being pathogenic for specific aspects of multiple disorders and therefore extend the therapeutic potential of any subsequent pathway-specific treatments.
1.3.2 Environmental risk factors

Despite having a high genetic component, strong environmental risk factors for developing schizophrenia have been identified including migrant status, urban living and adolescent drug use; however few explanations as to why these should be risk factors for developing the disorder have been proven. The evidence supporting the increased risk of developing schizophrenia following societal/environmental insults has led to the formation of the social defeat hypothesis (See this chapter, section 1.4.1).

1.3.2.1 Cannabis use

Adolescent cannabis use has been hypothesised to be linked with enhanced susceptibility to developing schizophrenia (van Os et al. 2002, Zammit et al. 2002), as well as being associated with grey and white matter loss (James et al. 2011) and deficits in attention and memory (Rabin et al. 2011) in patients. Cannabis use is also linked with an earlier age of onset of the disease in male patients (Large et al. 2011). Cannabis use and age of first-time use of cannabis have also been positively linked with the risk of psychosis in a number of studies (Henquet et al. 2005, Konings et al. 2008, Schubart et al. 2011), as well as higher relapse rates and reduced effectiveness of antipsychotic medication (Swartz et al. 2008, Rajji et al. 2009). Increased cannabis use amongst schizophrenia patients is thought to be a form of ‘self-medication’, with several studies showing cannabis use is correlated with diminished negative/depressive symptoms (Linszen et al. 1994) and increased cognitive performance (Rabin et al. 2011). Whether cannabis is a causal factor for developing schizophrenia or whether it demonstrates an underlying defect with the endocannabinoid signalling pathway has not been determined conclusively. Studies investigating this link have been inconclusive, with many studies showing that the neurocognitive deficits observed in patients are due to other factors such as premorbid functioning (Ringen et al. 2013) and differences in methodology (Segev and Lev-Ran 2012) rather than being due to cannabis use. Findings related to the effect of cannabis on cognition and schizophrenia risk have been compounded by a recent study showing illicit drug use is under-reported in schizophrenia and is also associated with greater cognitive deficits. This finding may be a confounding factor in studies where drug use frequency is measured using surveys (Bahorik et al. 2014), as well as reports
showing cognitive improvement in some groups of cannabis-using patients (Kumra et al. 2005, Coulston et al. 2007).

Genetic evidence for the “cannabis risk hypothesis” is limited; however Volk and colleagues (Volk et al. 2013) showed increased levels of α-β-hydrolase domain 6 (ABHD6) mRNA in the prefrontal cortex of schizophrenia patients. This enzyme is responsible for regulating endocannabinoid signalling and increased levels of ABHD6 would suggest that patients have increased production of the cortical endocannabinoid 2-arachidonoylglycerol (2-AG), increasing their susceptibility to the toxic effects of tetrahydrocannabinol (THC). The schizophrenia risk gene Neuregulin has also been shown to influence the effects of tetrahydrocannabinoids by hastening the onset of cannabis tolerance in rodent models (Boucher et al. 2007a, Boucher et al. 2007b), as well as in a small study of healthy humans (Han et al. 2012).

1.3.2.2 Socioeconomic/migrant status

Many studies have shown that being a first or second-generation migrant is a significant risk factor (Risk factor=4.5 for 2nd generation migrants vs 2.7 for 1st generation migrants) for developing schizophrenia (Cantor-Graae and Selten 2005, Veling et al. 2006). This risk is increased if the migrant is under 15 (Werbeloff et al. 2012) or the country of origin has a population which is mostly black, such as afro-caribbean countries (Cantor-Graae and Selten 2005, Fearon et al. 2006). The reasons for why this is a risk have not been established, but social stress (Selten and Cantor-Graae 2007), vitamin D levels (McGrath et al. 2010b) and poor living conditions caused by unemployment or substandard housing have all been implicated. People belonging to a low-ranking socioeconomic social group have been shown to be at an increased risk of developing schizophrenia (Muntaner et al. 2004). Urban living is also a known risk factor for schizophrenia (Marcelis et al. 1998, Pedersen and Mortensen 2001, Vassos et al. 2012) and by contrast, living in rural areas has been shown to be protective against schizophrenia in the UK and other European countries (March et al. 2008, Svensson et al. 2013).
1.4 Non-neurochemical hypotheses of Schizophrenia

1.4.1 Social defeat hypothesis

The social defeat hypothesis of schizophrenia is based upon findings in both rodents and humans indicating that social stress during adolescence increases the risk of developing schizophrenia (Reviewed in Selten and Cantor-Graae 2007). Studies in rodents have shown that rats that were defeated in a social defeat paradigm showed meso-limbic dopaminergic (but not nigrostriatal) hyperactivity and enhanced sensitisation to dopamine agonists such as amphetamine, as observed in patients with schizophrenia (Laruelle et al. 2003). This effect was exacerbated by isolation following the social defeat (Isovich et al. 2001). There are a small number of studies showing behavioural interactions between social defeat and known schizophrenia risk genes such as DISC1 (Haque et al. 2012) and neuregulin (Desbonnet et al. 2012) in mice, supporting a role for social defeat as a risk factor for schizophrenia, although these studies have yet to be carried out in clinical populations.

Human studies into social stress have cited low IQ, migrant status with a weak family support network and urbanicity as being socially disadvantageous and may contribute to the development of schizophrenia via unknown mechanisms (Selten and Cantor-Graae 2005, Selten and Cantor-Graae 2007).

1.4.2 Neurodevelopmental hypotheses

Schizophrenia was first described as a neurodevelopmental disorder by Kraepelin and Bleuler who both noted behavioural and neurological changes during childhood in patients who went on to develop schizophrenia. The neurodevelopmental hypothesis was extended by Weinberger (Weinberger 1987), who postulated that adolescent maturational changes would “unmask” a dysfunctional phenotype caused by genetic and environmental risk factors such as prenatal insults, giving rise to the onset of schizophrenia. A number of studies have implicated prenatal stress, reduced prenatal micronutrient levels, prenatal infection and season of birth as risk factors for developing schizophrenia, although these factors contribute to lower relative risk individually than genetic factors (Torrey et al. 2012).
1.4.2.1 Prenatal Stress

Khashan and colleagues (Khashan et al. 2008) showed that stressful events such as a first degree relative death or serious illness during the first trimester of pregnancy significantly increases the offspring’s risk of developing schizophrenia. Military invasion (van Os and Selten 1998) and natural disasters (Selten et al. 1999) also increased the likelihood of the child developing schizophrenia. Stress is known to affect glucocorticoid levels and neurodevelopment in rats (Mandyam et al. 2008), suggesting a mechanism by which these external factors influence brain development and the risk of schizophrenia. Drug use during pregnancy is another source of prenatal stress although studies investigating the risk of drug use during pregnancy have yielded mixed results. Prenatal exposure to tobacco has not been linked to increased incidence of schizophrenia (Baguelin-Pinaud et al. 2010), although exposure to analgesics during the second trimester is associated with a significant risk (odds ratio 4.75) of the offspring developing schizophrenia (Sorensen et al. 2004).

1.4.2.2 Vitamin D levels

Animal work has shown that restricting neonatal vitamin D in rats causes deficits in working memory, which are rescued by co-administration of a metabotropic glutamate receptor (mGluR5) agonist and the atypical antipsychotic risperidone, but not haloperidol (Becker and Grecksch 2006). McGrath and colleagues (McGrath et al. 2010b) showed an increased risk of schizophrenia in Danish patients who had low neonatal vitamin D levels. Vitamin D has been shown to increase differentiation of embryonic cell cultures and the vitamin D receptor is widespread throughout the human brain. Preclinical animal models, where female rats are vitamin D-depleted throughout pregnancy, show altered neurogenesis and enlarged ventricles in the subsequent neonates, which persist into adulthood. Vitamin D-deprived neonates also show altered response to pharmacological agents such as MK-801, amphetamine and haloperidol, as well as altered dopaminergic signalling and cognition (Reviewed in McGrath et al. 2010a). This suggests a potential mechanism of action of vitamin D deficiency in humans, where a reduction in vitamin D leads to reduced neuronal differentiation and increased proliferation. This would in turn lead to increased susceptibility to neuronal insult, as well as altered dopaminergic signalling which is known to be closely linked to schizophrenia (see this chapter, section 1.5.1).
1.4.2.3 Season of birth and infection

Numerous studies have shown increased risk of schizophrenia is associated with winter birth times (Torrey et al. 1997, Torrey et al. 2012). A number of explanations have been put forward for this including increased influenza exposure and decreased levels of vitamin D available to the foetus (McGrath et al. 2010a). Prenatal infection has been shown to increase the chance of developing schizophrenia for a number of common pathogens, including influenza, rubella virus (Brown et al. 2001) and the protozoan Toxoplasma gondii (Torrey and Yolken 2003, Torrey et al. 2012). A recent study by Landreau and colleagues (Landreau et al. 2012) has demonstrated that the influenza virus causes apoptosis preferentially in developing dopaminergic neurons, suggesting a link between prenatal infection and the dopaminergic dysfunction seen in schizophrenia. Various animal models have been used to model early-life insults in schizophrenia, including maternal exposure to the influenza virus or the dsRNA synthetic molecule poly I:C (see this chapter, section 1.6.1).

1.5 Neurochemical Hypotheses of Schizophrenia

1.5.1 Dopamine Hypothesis

The dopamine hypothesis of schizophrenia was first proposed in 1965 and remained the dominant theory until recently, although dopamine dysregulation still remains a key aspect of schizophrenia (Meltzer and Stahl 1976). It states that excess dopaminergic activity gives rise to schizophrenia symptomatology and is supported by a range of pharmacological and behavioural evidence.

There are four main dopaminergic pathways in the human brain: nigro-striatal, meso-limbic, meso-cortical and tuberoinfundibular pathways (Figure 1.2), each of which mediates different functions. A fifth dopaminergic pathway (retinal) transmits information from retinal amacrine cells to dopaminergic neurons in the optic nerve.
Figure 1.2: The major neuronal pathways implicated in the dopamine theory of schizophrenia. Taken from Scarr et al. 2013.

The nigro-striatal pathway projects from the substantia nigra to the striatum and is involved in motor control. Dysfunction of this pathway, either through pharmacological intervention (e.g., typical antipsychotic administration) or pathological loss of dopaminergic neurons (as observed in Parkinson’s disease), causes movement disorders (dyskinesias). The meso-limbic pathway projects from the ventral tegmentum in the mesencephalon to the limbic system, including the hippocampus and amygdala. Dysfunction in the meso-limbic dopaminergic pathway is thought to be involved in mediating the positive symptoms of schizophrenia. The meso-cortical pathway connects the mid-brain to the prefrontal and cerebral cortices and is involved in reward and motivation.

The positive and negative symptoms of schizophrenia are thought to be caused by dysfunction of the meso-cortical and meso-limbic pathways. Reduced dopaminergic activity in the prefrontal cortex gives rise to cognitive deficits, reduced motivation and other negative symptoms, whereas hyperactivity of the dopaminergic system in the meso-limbic circuitry mediates the positive symptoms (Laruelle et al. 2003).
The tuberoinfundibular pathway connects the medio-basal hypothalamus with the median eminence. Dopaminergic release at the median eminence regulates the secretion of prolactin; if dopaminergic activity is blocked (e.g., dopamine D<sub>2</sub> receptor blockade by antipsychotics), this leads to increased blood prolactin levels and subsequent reduced oestrogen levels, which can give rise to abnormal lactation and menstrual/sexual dysfunction (Meltzer and Stahl 1976). Further evidence for the role of dopamine in schizophrenia comes from clinical observations that cocaine and amphetamine give rise to psychotic-like symptoms which are very similar to the positive symptoms of schizophrenia. These psychoses are associated with increased dopamine levels in the brain. Classical antipsychotics (chlorpromazine and haloperidol) work extensively through blockade of D<sub>2</sub> receptors and reduce psychotic symptoms of schizophrenia adding weight to the involvement of dopaminergic dysfunction in schizophrenia symptomatology.

Genes downstream of D<sub>2</sub> receptors have also been linked to cognitive ability, including COMT and AKT phosphorylation signalling, indicating that dopamine dysfunction remains an integral part of schizophrenia symptomatology (Arguello and Gogos 2008, Yavich et al. 2007). Davis and colleagues (Davis et al. 1991) hypothesised that the negative symptoms of schizophrenia are caused by frontal cortical hypoactivity, whereas positive symptoms were more closely linked to striatal hyperdopaminergia. This “version II” hypothesis has been supported by PET and SPECT studies showing increased striatal dopaminergic transmission (Breier et al. 1997, Abi-Dargham et al. 1998, Laruelle et al. 1999) and elevated dopamine synthesis capacity (Howes et al. 2007) in patients. Reduced dopaminergic transmission in the prefrontal cortex (PFC), particularly via the D<sub>1</sub> receptor subtype has been linked with impairments in working memory, a core feature of schizophrenia, and has been shown in the brains of patients with schizophrenia (reviewed in Goldman-Rakic et al. 2004). COMT (catechol-O-methyltransferase) is responsible for methylating dopamine into 3-methoxytyramine in the prefrontal cortex, but has a much lower effect in the striatum, regulating dopamine availability in a region-specific manner. In the cortex COMT is highly expressed and it forms the main cortical pathway of dopamine elimination, by contrast, in the striatum COMT is expressed at a lower level. Therefore in the striatum the main pathway for removing dopamine from the synaptic cleft is achieved by pre-
synaptic dopamine re-uptake, rather than metabolism, explaining the region-specific importance of COMT in dopaminergic neurotransmission.

Recently the “final common pathway hypothesis” has emerged, expanding on the idea proposed by Davis and colleagues (Davis et al. 1991) that region-specific dopaminergic dysfunction gave rise to schizophrenia symptomatology. Howes and Kapur suggest that presynaptic striatal hyperdopaminergia is the point of convergence for multiple neurotransmitter dysfunctions including glutamate and GABA, as well as environmental and genetic risk factors, which in turn gives rise to psychosis (Howes and Kapur 2009). This “final common pathway” suggestion is supported by evidence from a variety of sources including molecular imaging studies and animal studies (Reviewed in Cannon et al. 2003, Howes et al. 2004, van Winkel et al. 2008).

Antipsychotic blockade of presynaptic D_2 autoreceptors may result in a compensatory increase in dopamine synthesis, worsening the underlying hyperdopaminergia, which would explain why patients show increased dopamine synthesis following chronic antipsychotic medication regimes (McGowan et al. 2004). This hypothesis also has the advantage over previous incarnations in that it accounts for how the numerous genetic and environmental risk factors that have been identified over the past 20 years could give rise to psychosis, which previous versions of the dopamine hypothesis do not adequately explain. The authors (Howes and Kapur 2009) make one novel distinction with this hypothesis, compared to previous iterations; that the dopaminergic abnormalities mentioned give rise to psychosis, not necessarily schizophrenia as a whole. The cognitive deficits and negative symptoms are suggested to be partially independent from psychosis and are likely to involve other neurotransmitter systems, a hypothesis strongly supported by clinical observations showing that dopaminergic drugs that show efficacy in treating psychosis, such as current anti-psychotics, show no significant effect in treating the cognitive and social deficits observed in schizophrenia (Naber and Lambert 2009).

Howes and Kapur (Howes and Kapur 2014) have recently suggested that there are two populations of patients with schizophrenia: ‘hyperdopaminergic’ patients who show elevated dopamine levels and who respond well to dopamine-depleting pharmacological interventions, and ‘normodopaminergic’ patients who show
unaltered dopamine levels and who do not respond to anti-psychotic medication, despite high levels of dopamine receptor blockade (Yoshimura et al. 2003, Reviewed in Demjaha et al. 2012). These observations suggest that dopamine dysregulation, while a commonly observed phenotype in schizophrenia, may not be the sole final common pathway for all patients in the pathogenesis of schizophrenia, and particularly psychosis.

1.5.1.1 Dopamine receptors

Dopamine receptors are metabotropic G-protein coupled receptors that fall into two categories: the D₁-like family and the D₂-like family. D₁-like (D₁ and D₅) receptors activate adenylyl cyclase as a second messenger through Gas activation, increasing the intracellular concentration of the signalling molecule cAMP (cyclic adenosine mono-phosphate). D₂-like receptors inhibit adenylyl cyclase as a second messenger through Gas activation, reducing the intracellular concentration of cAMP. cAMP has a wide range of effects in neurons, including neuronal regeneration during development (Cai et al. 2001) and wide ranging phosphorylation cascades (Girault and Greengard 2004). D₁ and D₄ receptors predominate in the cortical regions whereas D₂ receptors are found mainly in sub-cortical regions (Guillin et al. 2007). Increases in striatal D₂ receptor activity (Abi-Dargham et al. 2000) and cortical D₂ mRNA (Tallerico et al. 2001) levels have been reported in the brains of schizophrenia patients, supported by neuroimaging studies showing abnormal dopaminergic transmission in the striata of medicated and drug-naïve patients (Reviewed in Brunelin et al. 2013).

1.5.2 NMDA hypofunction hypothesis

Although traditional methods of treating schizophrenia (typical and atypical antipsychotics) have focused on the dopaminergic system, recent research has concentrated on the ‘glutamate hypothesis’, as an explanation for the cause of negative and cognitive symptoms, as these are poorly treated by dopaminergic antipsychotics. This hypothesis states that many of the symptoms in schizophrenia are caused by hypofunction of glutamate signalling at NMDA receptors (Stahl 2007b, Stahl 2007a). This theory does not replace the dopamine hypothesis and the two theories may be linked through circuitry based models of the disorder (Seamans et al. 2001, Sokoloff et al. 2013), as well as through the adenosine hypothesis of schizophrenia (Boison et al. 2012). Adenosine, in particular, offers a way to modulate the release of
both dopamine and glutamate in a region-specific manner from both neurons and glial cells. This hypothesis is discussed in more detail in this chapter, section 1.5.5.

Glutamate is the most abundant neurotransmitter in the brain (Choi 1992) and its ability to depolarise neurons has been recognised for over 50 years (Curtis et al. 1959), however it was not until the 1980s that glutamate signalling was directly implicated in schizophrenia (Kim et al. 1980), supporting the clinical observations of Luby and colleagues (Luby et al. 1959). Aside from being an important neurotransmitter, glutamate also functions as a putative gliotransmitter and an important neuronal metabolite involved in protein synthesis and energy production, as well as its vital role in excitatory neurotransmission. This direct regulatory effect on metabolism distinguishes it from ‘classical’ neurotransmitters such as dopamine and serotonin. The basis of the glutamate hypothesis arose from the observations that schizophrenia patients showed reduced levels of CSF glutamate (Kim et al. 1980) and that administration of PCP (phencyclidine), an NMDA receptor antagonist, gave rise to schizophrenia-like symptoms in humans (Luby et al. 1959). Administration of ketamine, another NMDA receptor antagonist, at sub-anaesthetic doses has also been shown to cause schizophrenia-like symptoms (positive, negative and cognitive) in healthy volunteers (Malhotra et al. 1996). Administration of ketamine has also been shown to exacerbate positive symptoms, depressive symptoms and executive function deficits in schizophrenia patients in a dose-dependent manner (Lahti et al. 2001, D’Souza et al. 2012, Tang et al. 2013). Preclinical studies have shown that acute and chronic ketamine induce working memory (Nakako et al. 2013) and context-processing (Blackman et al. 2013) deficits, similar to those present in schizophrenia patients, in non-human primates. In preclinical studies involving rodents, sub-chronic or chronic ketamine administration results in schizophrenia-like pathological changes including a reduction in GAD_{67} levels and loss of hippocampal and cortical parvalbumin (PV)-containing interneurons (Behrens et al. 2007, Kittelberger et al. 2012). The loss of hippocampal PV-positive interneurons in regions CA1 and CA3 in particular, was associated with loss of hippocampal theta and gamma oscillatory power (Kittelberger et al. 2012). In clinical studies, acute ketamine administration resulted in increased activity in the cortex of healthy volunteers (as measured by reduced blood-oxygen-level-dependent (BOLD) contrast signal), which correlated with increased psychotic

1.5.2.1 Glutamatergic Receptors

There are two classes of glutamate receptor, the subtypes and firing patterns of which are summarised in figure 1.3:

**Ionotropic receptors** - AMPA, kainate and NMDA receptor subclasses mediate a rapid short-term alteration in intracellular Ca$^{2+}$ concentration when agonists bind to the receptor. AMPA, kainite and NMDA receptors are so named because of their respective high-affinity agonists, although all classes are stimulated by glutamate.

**Metabotropic glutamate receptors (mGluRs)** - These mediate a longer term signalling cascade by means of an ion channel, activated by G protein linked secondary messages. There are 8 subtypes of mGluR (1-8) that were identified using genetic studies (Schoepp 1994). These receptors can be sub-divided into three main classes, based on their pharmacological characteristics and second messenger downstream signalling systems (Summarised in figure 1.4).

![Glutamate receptor subtypes and their transduction patterns](image)
1.5.2.2 NMDA receptors

NMDA receptors are non-selective cation channels with high permeability for Ca\(^{2+}\), Na\(^{+}\) and K\(^{+}\) ions, but are blocked by low concentrations of Mg\(^{2+}\). This blockade is voltage-dependent, so that upon depolarisation of the cell the Mg\(^{2+}\) ion moves out of the channel allowing pore opening. They require glycine as a co-agonist, so that glutamate and glycine must be bound before the channel can open. NMDA receptors have binding sites for a number of other molecules, including D-serine and PCP as shown in figure 1.5. All regulatory molecules bind at their respective labelled binding site and D-serine binds at the glycine modulatory site.
Figure 1.5: Comparative structures of the NMDA receptor and mGlu receptor complexes showing agonist and modulatory binding sites. Taken from Wieronska et al. 2011.

NMDA receptors are heterotetrameric complexes consisting of two GluN1 (glycine-binding) and two GluN2 (glutamate-binding) subunits. There are 8 different GluN1 subunits, formed by alternative splicing of the GRIN1 gene, whereas there are four GluN2 isoforms in vertebrates (NR2A, NR2B, NR2C and NR2D). These isoforms are coded for by 4 separate genes (GRIN2A, GRIN2B, GRIN2C, GRIN2D), which are thought to be a result of gene duplication (Teng et al. 2010), and each isoform possesses a different intracellular C-terminal domain which controls the receptors interaction with cellular signalling molecules.

Clinical evidence for the involvement of NMDA receptor hypofunction in schizophrenia comes from a variety of sources; post-mortem studies have shown decreases in glutamatergic receptor expression (mRNA and protein) and glutamate receptor binding in the hippocampus and prefrontal cortex of patients with schizophrenia (Reviewed in Clinton and Meador-Woodruff 2004). Genetic studies have also identified a number of risk loci with genome-wide significance (16p13.2-GRIN2A, 5q33.2-GRIA1, 7q21.11-GRM3 and 17p13.3-serine racemase) that could directly or indirectly modulate the action of glutamate receptors, including AMPA and NMDA receptor subunits (Harrison 2014). This evidence has been supported by the observation that the NMDA receptor modulators glycine or d-serine administered in conjunction with an antipsychotic (atypical, classical or combination regimes), significantly improved positive, negative and cognitive symptoms in schizophrenia patients (Rosse et al. 1989, Tsai et al. 1998). Both typical and atypical antipsychotics
were given during these studies. Kynurenic acid (KA), a tryptophan metabolite, acts as a non-competitive antagonist at the glycine site of the NMDA receptor and has been shown to be present in elevated concentrations in the CSF of drug-naïve schizophrenia patients (Erhardt et al. 2007).

1.5.3 GABAergic hypothesis

The GABAergic hypothesis of schizophrenia was first suggested as an amended version of the glutamate hypothesis (described in this chapter, section 1.5.2). Olney and Farber (Olney and Farber 1995) posited that NMDA receptor hypofunction on GABAergic interneurons reduces the amount of inhibitory firing, resulting in impaired feedback in neural rhythmic circuitry as shown in figure 1.6. This is then thought to disrupt the inhibitory feedback needed to generate synchronous rhythms, particularly in the gamma and theta bands, resulting in schizophrenia symptomatology.

![Figure 1.6: Proposed mechanism by which GABAergic inhibition of glutamatergic pyramidal cells generates oscillatory activity in the cortex and hippocampus. Dysregulation of the GABAergic system results in disinhibition of this feedback loop and is thought to be responsible for cognitive deficits in schizophrenia. Taken from Lisman et al. 2008.](image)

GABA (γ-amino butyric acid) was first discovered in 1950 as a non-alpha amino acid (Roberts and Frankel 1950) and was later proposed to have an inhibitory effect on
neuronal signalling (Curtis and Watkins 1960). GABA is the major inhibitory neurotransmitter in the mammalian brain (Schousboe and Waagepetersen 2007) although it plays a major excitatory role in the developing human brain (Taketo and Yoshioka 2000), with the switch between excitatory and inhibitory actions thought to be mediated in part by the neurosteroid oxytocin (Tyzio et al. 2006). This switch is mediated by changes in the internal chloride level of the developing neuron. High levels of chloride ions in developing neuronal cells means that when GABA receptors open, upon GABA binding, Cl- ions efflux from the cell thereby acting as an excitatory agent (Wang and Kriegstein 2009).

Evidence for the involvement of GABAergic interneuron dysfunction in schizophrenia comes from a variety of clinical and preclinical observations. Reduced levels of the rate-limiting GABA catabolic enzyme GAD_{67} (mRNA and protein) has been observed in schizophrenia patient frontal cortices (reviewed in Gonzalez-Burgos et al. 2011). Other gene markers of interneuron populations including somatostatin (SST), parvalbumin (PVALB) and GABA transporter 1 (GAT-1) are also reduced in the cortex of patients (reviewed in Lewis and Moghaddam 2006), suggesting that multiple interneuronal populations are affected in schizophrenia. Chronic administration of NMDA receptor antagonists such as PCP (phencyclidine), which are known to cause schizophrenia-like symptoms, results in decreased expression of GAD_{67} and parvalbumin in cortical GABAergic cells (Abdul-Monim et al. 2007, McKibben et al. 2010). Brain imaging data shows that an acute challenge with NMDA receptor antagonists results in hyperactivity of pyramidal cells in the cortex in both rodents (Miyamoto et al. 2000) and human subjects (Lahti et al. 1995). This counter-intuitive observation is supported by evidence in rats that shows working memory deficits, as well as cortical hyperactivity in animals that were given acute administration of NMDA receptor antagonists (Jackson et al. 2004).

Glial cells, in particular astrocytes, also play a vital role in regulating neuronal activity by interfacing with neurons at the synapse, forming a tripartite synapse (Figure 1.7). Originally astrocytes were thought of as simple supporting cells, lending structure to the neurons; this view was supported by the fact that astrocytes are electrically inactive. However Banaclocha and colleagues observed that as organisms and their
brain activities became more complex, the ratio of astrocytes:neurons increased beyond the need for increased metabolic support for the neurons (Banaclocha 2007). The number of astrocytes, relative to the number of neurons, in the frontal cortex of humans is also significantly increased in comparison to non-human primate frontal cortex (Sherwood et al. 2006). Astrocyte size, functionality and connectivity varies between species, with human astrocytes being larger and connecting to more neurons than the astrocytes of lower order animals such as mice or rats (Oberheim et al. 2009). A recent study shows that human astrocyte progenitors, when implanted into neonatal mice, result in a human astrocytic network. These mice had more rapid and sustained LTP, significantly faster calcium wave transmission and out-performed control mice in a variety of cognitive paradigms (Han et al. 2013).

Within a tripartite synapse, an individual astrocyte can contact with hundreds of synapses involving many neurons, allowing astrocytes to mediate neuronal activity across a wide area (Allen and Barres 2009). Astrocytes possess many of the same neurotransmitter system as neurons, including expressing the GABAergic markers GAD$_{67}$, GABA transporter 1 and both subtypes of GABA receptor (GABA$_A$ and GABA$_B$) demonstrating their GABAergic and GABAceptive properties in culture (Lee et al. 2011). Hippocampal astrocytes also express vesicular glutamate transporters (vGLUT1 and vGLUT2) in vivo (Bezzi et al. 2004) suggesting that astrocytes mediate both glutamatergic and GABAergic signalling. As well as direct point-to-point feedback synaptic communication with neurons (Agulhon et al. 2008), astrocytes, upon stimulation by neurons, activate long range calcium signalling cascades (Tian et al. 2006) which result in inhibition or enhancement of neuronal signalling, depending on the type of molecule that is released by the astrocyte. This evidence shows how astrocytes can modulate both local and widespread neuronal activity in a non-electrical manner, demonstrating the vital role that astrocytes play in modulating network activity.
**Figure 1.7:** Tripartite synapse showing the neuronal and astrocytic inputs that contribute to synaptic signalling. Taken from Allen and Barres 2009.

1.5.3.1 GABA receptors

GABA receptors are divided into three classes on the basis of their mode of action: GABA$_A$ receptors, GABA$_B$ receptors and GABA$_C$ receptors. GABA$_A$ and GABA$_C$ receptors are fast-acting ionotropic membrane-bound ion channels and are differentiated by their sensitivity (GABA$_A$) or insensitivity (GABA$_C$) to bicuculline antagonism, whereas GABA$_B$ receptors mediate a slow metabotropic inhibition of neuronal activity through G-proteins coupled to the receptor. The structures of these different subtypes are shown in figure 1.8, with GABA$_C$ receptors showing an almost identical structure to GABA$_A$ receptors.
1.5.4 Sigma (σ) receptors

σ receptors are a relatively little-studied class of receptor, despite first being identified in 1976 by the Martin group (Martin et al. 1976) as a subclass of opioid receptors, but were later reclassified as non-opiate and non-phencyclidine-binding receptors, as they did not respond to the classical opiate receptor antagonists naltrexone (Vaupel 1983) and naloxone (Su 1982).

There are two subtypes of σ receptor, which were originally characterised according to their different pharmacological responses to (+)-benzomorphans (Quirion et al. 1992): σ2 receptors have been poorly characterised and have not yet been cloned, but σ1 receptors have been implicated in the aetiology of neuropsychiatric disorders, including mood disorders, schizophrenia, depression and Parkinson’s disease (Ishikawa and Hashimoto 2010).

σ1 receptors are by far the better characterised subtype of σ receptor and they show a more widespread distribution throughout the brain than σ2 receptors. σ1 receptors are particularly abundant in the deeper laminar layers (IV-VI) of the cortex, dentate gyrus of the hippocampus and amygdala (Kitaichi et al. 2000); all regions that have been strongly implicated in learning and memory in rats (Yoon et al. 2008) and humans (summarised in Poch and Campo 2012). The σ1 receptor was cloned in 1996 (Hanner et al. 1996) and the molecular structure determined (Figure 1.9). The σ1 receptor is a 223 amino acid protein containing two transmembrane domains, which
shows little homology to any other mammalian proteins, although it shows 30% homology to a sterol isomerase found in yeast, although the σ1 receptor has no intrinsic isomerase activity (Hanner et al. 1996).

Figure 1.9: Predicted molecular structure of the σ1 receptor. Taken from Ishikawa and Hashimoto 2010

Post-mortem studies have shown a selective loss of σ1 receptors in the cerebral cortex of schizophrenia patients (Weissman et al. 1991, Shibuya et al. 1992) and combined imaging and genetic studies have shown an association between the homozygous Pro genotype at the Gln2Pro polymorphism site in the SIGMAR (σ1 receptor) gene and an increased risk of schizophrenia as well as reduced prefrontal haemodynamic activation during a verbal fluency task (Takizawa et al. 2009), and reduced bilateral prefrontal cortical activation (Ohi et al. 2011).

σ1 receptors can modulate neurotransmission by direct interaction with potassium channels (Aydar et al. 2002) and dopamine receptors (Peeters et al. 2004) or via an indirect SK-channel mediated interaction to modulate NMDA receptor activity (Hayashi and Su 2004, Ishikawa and Hashimoto 2010). In particular, the NMDA and dopaminergic signalling systems have been shown to be intimately involved in the pathophysiology of schizophrenia, as discussed in this chapter (section 1.5.1 and 1.5.2).
1.5.5 Adenosine hypothesis

The adenosine hypothesis of schizophrenia states that both dopaminergic and glutamatergic dysfunction in schizophrenia pathophysiology could be ultimately caused by dysfunction in adenosinergic receptors upstream of these two receptor groups. Adenosine is able to modulate multiple receptor groups in a variety of ways, including direct receptor interaction, epigenetics and bioenergetics (Reviewed in Boison et al. 2012). Adenosine acts as a neuromodulator, rather than as a neurotransmitter like dopamine or glutamate, as it acts through a variety of mechanisms including pre-synaptically (by controlling the release of neurotransmitters), post-synaptically (by directly affecting polarisation states of neurons) and non-synaptically (by modulating glial cell activity and gliotransmitter release) (Boison et al. 2012).

Evidence for the role of adenosine in cognition comes from the localisation of adenosine receptors in the striatum, hippocampus and neocortex (Hettinger et al. 2001, Ribeiro et al. 2003), all of which are brain regions that are known to be important for correct cognitive function (reviewed in Poch and Campo 2012). Adenosine receptors interact directly with dopamine receptors on both GABAergic striatopallidal and striatonigral neurons, as well as, upon activation, exerting a tonic inhibitory effect on NMDA receptor function (Reviewed in Boison et al. 2012).

The evidence shown above suggests a promising role for adenosine as a pharmacological target for the treatment of schizophrenia, particularly for cognitive deficits, although modulatory compounds to this receptor class are still in development. Figure 1.10 shows the potential role upstream that adenosine and adenosine receptor dysfunction could play in linking the dopaminergic and glutamatergic dysfunctions observed in schizophrenia.
1.6 Animal models of schizophrenia

Animal models are a vital preclinical step for the development of new pharmacological treatments, providing mechanistic and toxicological information for new compounds, however a single model cannot replicate an entire disease symptom spectra (Trivedi and Jarbe 2011). This necessitates breaking disorders down into individual symptom domains, which is difficult in complex disorders such as schizophrenia. Historically, animal models were developed in order to determine the pharmacology of the first serendipitously-discovered antipsychotic drugs, but more recent animal models have utilised a combined strategy of a genetic, neurodevelopmental or pharmacological “risk factor” combined with ethologically relevant behavioural testing.

Current animal models have aimed to study individual aspects of schizophrenia, such as its neurodevelopmental origins or neurotransmitter hypotheses such as the ‘dopamine’ or ‘glutamatergic dysfunction’ hypotheses, and these models have been used effectively to study the molecular mechanisms involved in the development and progression of schizophrenia. Current animal models have their limitations (reviewed in Moore 2010) and it is vitally important to continue to develop more refined animal models to further understand the causes of, and potential treatments for, schizophrenia. This process has been hindered by the lack of investment from various funding sources (Hendrie 2010).
1.6.1 Neurodevelopmental

The neurodevelopmental hypothesis of schizophrenia has been supported by preclinical animal work, showing that prenatal restricting of maternal thiamine (de Freitas-Silva et al. 2010), vitamin D (Eyles et al. 2009) or post-natal isolation rearing (Bianchi et al. 2006, Bloomfield et al. 2008) can all cause abnormal brain development in pups. The insults listed above all lead to schizophrenia-like pathology and cognitive deficits including memory deficits and cytoskeletal alterations (Bianchi et al. 2006), suggesting that both pre- and post-natal periods are vital for normal brain development.

Methylazoxymethanol (MAM) is also used to model the developmental aspect of schizophrenia. Administration of MAM to rodents on gestational day 17 results in schizophrenia-like cognitive deficits, including PPI deficits, impaired EDS in the attentional set-shifting task and working memory deficits in the Morris water maze. Pathological deficits, with relevance to schizophrenia, are also present in this model including loss of parvalbumin-positive interneurons, decreased hippocampal and prefrontal cortical volume and enlarged ventricles (reviewed in Jones et al. 2011). Disruptions in cortico-limbic signalling are also seen in this model (Grace 2010, Esmaeili and Grace 2013). Antipsychotic rescue of MAM-induced deficits has been carried out in a small number of studies, and neither acute classical (haloperidol) nor acute atypical (clozapine) antipsychotic treatment were able to reverse MAM-induced deficits in BDNF (Fiore et al. 2008), despite both classes of antipsychotic antagonising VTA dopaminergic neurons (Valenti et al. 2011). Typical and atypical antipsychotics are both effective at reversing MK-801-induced hyperlocomotion in MAM rats (Le Pen et al. 2011). The atypical antipsychotic sulpiride was able to rescue mPFC-hippocampal interactions that are absent in MAM-rodents (Belujon et al. 2013).

Preclinical animal models modelling prenatal infection such as the polyriboinosinic-polyribocytidylic acid (poly I:C) model or maternal influenza exposure model have been used in order to understand the mechanisms of prenatal infection as a risk factor for schizophrenia. Injection of the viral double stranded RNA mimetic poly I:C causes similar behavioural and pathological changes in adulthood to the offspring of maternal influenza exposed rodents (Macedo et al. 2012). These include deficits in working memory, social interaction, PPI and object recognition memory. Pathological
changes include reduced numbers of prefrontal cortical dopamine (D_1 and D_2) receptors and parvalbumin-expressing cells, as well as dopaminergic hyperfunction, which are all observed in patients with schizophrenia (reviewed in Patterson 2009, Macedo et al. 2012).

Administration of psychoactive drugs (PCP and ketamine) early in development can also give rise to schizophrenia-like cognitive deficits including object recognition and attentional set-shifting deficits (Broberg et al. 2009, Tan et al. 2011). Work in our lab (Rajagopal 2011) has shown that administration of PCP at specific points in development (post-natal days (PND) 7, 9 and 11), corresponding to the perinatal period (the period directly before and after birth) of human pregnancy (Bockhorst et al. 2008), gives rise to long-lasting cognitive deficits, with relevance to schizophrenia, indicating that these stages of development are vital to proper brain function. These findings are supported by the work of Wang and colleagues using the same postnatal (day 7, 9 and 11) PCP administration regime showing similar deficits including loss of frontal cortical parvalbumin interneurons (Wang et al. 2008), reductions in BDNF expression (Liu et al. 2011), disruptions in sensorimotor gating (Chen et al. 2011) and behavioural impairments (Tseng et al. 2009) in adult animals.

Lu and colleagues (Lu et al. 2011) have shown that prenatal exposure to PCP (5mg/kg) on PNDs 6-18 caused a deficit in recognition memory, which was attenuated by clozapine treatment. This deficit was correlated with a decrease in extracellular glutamate and increase in glutamate transporter (GLAST) expression in the PFC of post-pubertal mice. Similar studies have shown that PCP, given at certain developmental stages, can cause widespread neuronal cell death in the frontal cortex (Liu et al. 2011) and prolonged ketamine administration (20mg/kg every 2 hours) on PND7 causes up-regulation of NMDA receptor subunits, triggering increased apoptosis in developing neurons (Shi et al. 2010). This evidence suggests that prenatal administration of NMDA receptor antagonists at certain vital developmental time points causes long-lasting cognitive and pathological changes, which may be relevant to the disease in humans.
1.6.2 Lesion studies

Work by O’Donnell and colleagues has shown that neonatal rats lesioned in the ventral hippocampus on post-natal day 7-8 (NVHL rats) do not show the correct maturation of dopaminergic control of prefrontal cortical interneurons, resulting in a disinhibited cortex and a variety of schizophrenia-like cognitive deficits and pathological alterations (reviewed in O’Donnell 2010, O’Donnell 2012). NVHL rats show deficits in spatial learning and memory as measured by the radial arm maze task (Chambers et al. 1996) and Morris water maze task (Le Pen et al. 2000), as well as in executive function (Lipska et al. 2002), all of which are known to be disrupted in patients with schizophrenia. Executive function was not affected in adults lesioned in this region, but executive function was disrupted in adults lesioned in the medial prefrontal cortex (mPFC). Another prevalent feature of schizophrenia is disruption in sensorimotor gating (Braff et al. 2001a); a feature which is also observed in NVHL rats (Chambers et al. 1996, Le Pen et al. 2006).

1.6.3 Phencyclidine

1.6.3.1 - Pharmacology

Phencyclidine (PCP) is an arylcyclohexamine psychotomimetic drug, originally used as a dissociative anaesthetic, whose use was discontinued after it was found to cause schizophrenia-like symptoms in healthy humans (Collins et al. 1960) as well as exacerbating pre-existing symptoms in patients (Luby et al. 1959). Although principally known as a non-competitive NMDA receptor antagonist, PCP has been shown to affect a wide variety of signalling pathways including GABAergic, acetylcholinergic and dopaminergic signalling (Jentsch and Roth 1999, Morris et al. 2005), as well as being a σ1 receptor agonist in the brain. PCP treatment also influences oxidative stress (Radonjic et al. 2010), hypofrontality (Weinberger and Berman 1996) and glucose utilisation (Cochran et al. 2003) in the areas of the brain associated with neural plasticity and cognition, such as the prefrontal cortex, hippocampus and cerebellum. PCP-induced cognitive deficits have been shown to be improved by administration of compounds such as donepezil and fluvoxamine, both of which have strong affinity at the σ1 receptor site (Hashimoto et al. 2007, Kunitachi et al. 2009), despite PCP sites and σ1 sites possessing differing pharmacological profiles.
1.6.3.2 – Sub-chronic administration of PCP

Acute, chronic and sub-chronic administration of PCP to rats gives rise to schizophrenia-like cognitive deficits in a variety of tests including attentional set-shifting, object recognition memory and effects of relevance to negative symptoms such as social withdrawal; however, sub-chronic administration of PCP is thought to provide a more valid model than acute administration for modelling negative symptoms and cognitive deficits in schizophrenia (Reviewed in Neill et al. 2010, Neill et al. 2013). These sub-chronic PCP-induced effects are reversed by a variety of pharmacological agents including atypical, but not typical antipsychotics (McLean et al. 2008, Idris et al. 2010), GABA\textsubscript{A} receptor positive modulators (Damgaard et al. 2011) and α\textsubscript{7} nicotinic receptor positive modulators (McLean et al. 2011). Other pharmacological agents such as antidepressants and anxiolytics are ineffective at treating sub-chronic PCP induced deficits.

1.6.3.3 – Sub-chronic PCP-induced behavioural changes in rodents

Behavioural changes in the sub-chronic PCP rodent model (2mg/kg i.p. b.i.d) have been well characterised and parallel with deficits shown in patients. Work in our laboratory has shown that sub-chronic PCP administration to female rats causes deficits in tests of many cognitive domains which are known to be deficient in patients with schizophrenia. These include the reversal learning task, object recognition memory paradigm, 5-choice continuous performance test and attentional set-shifting (reviewed in Neill et al. 2010). This has been supported by other laboratories working in this area (Horiguchi et al. 2012, Horiguchi et al. 2013, Horio et al. 2013). Studies in our laboratory and others have shown that sub-chronic PCP administration also causes deficits in sociability, a known negative symptom of schizophrenia (reviewed in Neill et al. 2010, Gururajan et al. 2010, Neill et al. 2013). These findings are summarised in table 1.3:
<table>
<thead>
<tr>
<th>Animal cognitive test</th>
<th>Animal, Sex and Strain</th>
<th>Effect of sub-chronic PCP administration</th>
<th>PCP regimen</th>
<th>PCP administration duration</th>
<th>Reference</th>
<th>Cognitive domain</th>
<th>Human cognitive test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Holeboard test</strong></td>
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<td>5mg/kg</td>
<td>5 days continuous</td>
<td>Schroeder et al. 2000</td>
<td>Working Memory</td>
<td>N-back continuous performance task</td>
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<tr>
<td></td>
<td>Female Lister Hooded rat</td>
<td>Impairment</td>
<td>2mg/kg b.i.d</td>
<td>7 days</td>
<td>Grayson 2010</td>
<td></td>
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<tr>
<td><strong>5-Choice Continuous performance Test</strong></td>
<td>Male Wistar rat</td>
<td>Impairment</td>
<td>2mg/kg daily</td>
<td>5 days</td>
<td>Amitai et al. 2007</td>
<td>Attention</td>
<td>Continuous performance task</td>
</tr>
<tr>
<td></td>
<td>Female Lister Hooded rat</td>
<td>Impairment</td>
<td>5mg/kg b.i.d</td>
<td>7 days</td>
<td>Barnes et al. 2012</td>
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<tr>
<td><strong>NOR</strong></td>
<td>Female Lister Hooded rat</td>
<td>Impairment</td>
<td>2mg/kg b.i.d</td>
<td>7 days</td>
<td>Grayson et al. 2007</td>
<td>Visual learning and memory</td>
<td>Neurological Assessment Battery – Shape learning</td>
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<td>10 days</td>
<td>Hashimoto et al. 2005a</td>
<td>Neurological Assessment Battery - mazes</td>
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<td>10 days</td>
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<tr>
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<td>3mg/kg daily</td>
<td>Intermittent 5 weeks</td>
<td>Fletcher et al. 2005</td>
<td>Problem Solving</td>
<td>Wisconsin Card Sorting Test</td>
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<td></td>
<td>Male Long Evans rat</td>
<td>Impairment</td>
<td>10mg/kg daily</td>
<td>5 weeks</td>
<td>Deschenes et al. 2006</td>
<td>Problem Solving</td>
<td>CANTAB ID/ED task</td>
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<td>2.6mg/kg daily</td>
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<td>Egerton et al. 2008</td>
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<td></td>
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<td>7 days</td>
<td>McLean et al. 2008</td>
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<td>Goethebeuer and Dias 2009</td>
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<td>Rodefer et al. 2005</td>
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<td><strong>Reversal Learning</strong></td>
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<td>2mg/kg b.i.d</td>
<td>7 days</td>
<td>Abdul-Monim et al. 2006</td>
<td>Problem Solving</td>
<td>Brief Assessment of Cognition in Schizophrenia – Tower of London</td>
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<td>Idris et al. 2010</td>
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<td>McLean et al. 2010b</td>
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<td>5 days</td>
<td>Laurent and Podhora 2004</td>
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<tr>
<td></td>
<td>Male C57BL/6J mice</td>
<td>No effect</td>
<td>5mg/kg b.i.d</td>
<td>7 days</td>
<td>Brigman et al. 2009</td>
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</table>
Table 1.3: Effect of sub-chronic PCP administration on tests measuring cognitive performance in domains identified as being impaired in schizophrenia by the TURNS initiative.

1.6.3.4 – Sub-chronic PCP-induced pathological changes in rodents

Sub-chronic PCP treatment (2mg/kg i.p. b.i.d) has been shown to consistently cause widespread molecular changes in rodent brain regions associated with cognition, such as the hippocampus and prefrontal cortex. Reductions in the cortical and hippocampal protein levels of calcium-binding proteins such as parvalbumin (Abdul-Monim et al. 2007, Jenkins et al. 2008, McKibben et al. 2010), BDNF mRNA levels (Snigdha et al. 2011b) and dendritic spine density (Hajszan et al. 2006) have all been observed following sub-chronic PCP administration. These pathological changes, like the behavioural changes summarised in chapter 1.6.3.3, are very similar to the changes seen in post-mortem studies of schizophrenia patients including reduced prefrontal cortical dendritic spine density (Glantz and Lewis 2000, Kolluri et al. 2005), decreased levels of prefrontal BDNF mRNA (Snigdha et al. 2011b), decreased levels of parvalbumin-positive interneurons (Beasley and Reynolds 1997, Lewis et al. 2001), decreased numbers of striatal dopaminergic D₁ receptors and an increase in cortical serotonergic 5HT₁A receptor number (Choi et al. 2009).

1.7 Therapies

Positive symptoms are treated reasonably well with current antipsychotic medication, however cognitive and negative symptoms remain poorly treated with current pharmacological interventions (Keefe et al. 2007), with current atypical antipsychotics being shown to not be significantly more efficacious at treating cognitive deficits than typical antipsychotics (Lieberman et al. 2005). Large scale open-label trials such as the CATIE (Clinical Antipsychotic Trial of Intervention Effectiveness)
and CULASS (Cost Utility of the Latest Antipsychotic drugs in Schizophrenia Study) trials compared typical antipsychotics (perphenazine and 1 of 11 different typical antipsychotics respectively) with 2nd generation antipsychotics (SGA) (olanzapine, risperidone, quetiapine and ziprasidone) and found no significant difference in any outcome measure, although the reduction in extrapyramidal symptoms and other unwanted side effects such as elevated prolactin means SGAs are the preferred pharmacological treatment option (Naber and Lambert 2009). A later study trial focussing on first episode patients in Europe (EUFEST – European First Episode Schizophrenia Trial) showed the second generation antipsychotics tested (olanzapine, amisulpride, quetiapine and ziprasidone) were significantly more likely to lead to patients symptom remission than the first generation antipsychotic haloperidol (Boter et al. 2009), although SGAs were no more effective than first generation antipsychotics at treating cognitive deficits (Davidson et al. 2009). There are currently two alternative therapeutic strategies for treating cognitive impairments:

1.7.1 Non-pharmacological interventions

Non-pharmacological treatments for schizophrenia were developed in response to the discovery that around 30% of schizophrenia patients respond minimally to standard antipsychotic medication (Kane 1996), as well as poor medication compliance amongst patients, which is another commonly observed problem with antipsychotic medication treatment regimens (Morken et al. 2008).

1.7.1.1 Cognitive Behavioural Therapy

Cognitive behavioural therapy (CBT) utilises a goal-orientated approach to address dysfunctional emotions and cognitive processes, which is adapted from the patients own “coping processes” to deal with symptoms such as hallucinations (Tarrier 2005). This is achieved by using computer programs or therapy to alter specific modes of thinking or cognitive processes by the use of goal-driven exercises tailored to the patient’s individual needs and psychiatric diagnosis. CBT has shown such promise that it has become a recommended treatment for schizophrenia and people at risk of developing schizophrenia in the National Institute for Health and Care Excellence (NICE) guidelines for clinical practice (National Institute for Clinical Excellence 2014). A number of studies and meta-analyses have shown that CBT can be effective in managing and treating certain domains of schizophrenia, particularly in those patients
who do not respond to conventional pharmacological therapies (Wykes et al. 2007, Rathod et al. 2010), as well as preliminary studies showing that CBT can prevent the onset of full psychosis when administered to prodromal patients (Morrison et al. 2004).

1.7.1.2 Cognitive Remediation Therapy

Cognitive remediation therapy (CRT) is defined as a behavioural training based intervention that aims to improve cognitive processes (attention, memory, executive function, social cognition or metacognition i.e., regulation of how and when to use certain cognitive abilities) with the goal of durability and generalization (Wykes and Spaulding 2011). CRT is used to improve “real-world” cognitive processes in order to improve social functioning. Recent meta-analyses have shown CRT to be effective across multiple groups of patients in improving cognitive deficits, regardless of methodology used, as well as improving functional outcomes (Reviewed in McGurk et al. 2007, Wykes et al. 2011).

Both of these methods (CBT/CRT) have shown promise in improving cognition in the short-term, although longer term studies are required to ascertain whether the improvements seen are temporary or more permanent. Negative symptoms are significantly improved by psychosocial intervention (CBT/CRT) (Reviewed in Klingberg et al. 2011), as well as positive symptoms (Reviewed in Kern et al. 2009). Quality of life has been shown to be significantly improved by CBT (Bechdolf et al. 2010). The effect between different psychosocial therapies on positive and negative symptoms or quality of life measures has not been shown to be significant (Reviewed in Jones et al. 2012). Sample sizes of non-pharmacological therapies for cognitive deficits, although initially small, are now similar to those used in drug trials (Thornley and Adams 1998) and psychosocial intervention is seen as complementary to pharmacological treatments (Alvarez-Jimenez et al. 2011). Age of participants and baseline symptom severity have both shown to negatively correlate with CRT/CBT improvement, although remediation success is independent of typical/atypical antipsychotic medication (Wykes et al. 2007, Greenwood et al. 2005).
1.7.2 Pharmacological interventions:

1.7.2.1 Typical antipsychotics

Typical antipsychotics include fluphenazine, haloperidol and chlorpromazine and have a high affinity for dopamine D$_2$ receptors which is their site of action. This is an effective method of treating the positive symptoms of schizophrenia, but this class of drug has been shown to cause extra-pyramidal symptoms (EPS) such as tardive dyskinesia, dystonia and parkinsonian tremors in patients. These side-effects are caused by blockade of the D$_2$ receptors in the nigrostriatal dopaminergic pathway (see figure 1.2) and are a major reason given by patients for noncompliance to typical antipsychotic therapy, along with impaired or abnormal sexual function. This abnormal sexual function is caused by non-specific blockade of dopaminergic pathways involved in reward and desire or hyperprolactinaemia which is a common dose-dependent side effect of typical antipsychotic treatments (Reviewed in Cookson et al. 2012). Hyperprolactinaemia also occurs more often following risperidone or amisulpride administration than with other atypical antipsychotics (Haddad and Wieck 2004).

1.7.2.2 Atypical antipsychotics

Atypical antipsychotics were developed as therapies that aimed to treat the positive symptoms of schizophrenia by selectively antagonising the dopaminergic D$_2$ receptors of the meso-cortical and meso-limbic pathways (see Figure 1.2) without affecting the D$_2$ receptors of the nigro-striatal pathway that are implicated in causing the majority of the EPS of classical antipsychotics. Many atypical antipsychotics also antagonise the 5-HT$_{2A}$ receptor subtype, which has been shown to be important in reducing the likelihood of the compound causing unwanted EPS by modulating nigrostriatal dopaminergic signalling (Reynolds 2004). Recently developed antipsychotics (eg: lurasidone) combine this 5-HT$_{2A}$-D$_2$ receptor antagonistic effect with reduced α1 adrenergic, muscarinic acetylcholine type M1 and histamine H1 receptor antagonism. Antagonism at these receptors is associated with sedation, cognitive impairment and weight gain respectively (Kroeze et al. 2003) which are all unwanted side-effects of antipsychotic medication and contribute to drug non-compliance. 5-HT$_{2C}$ receptors have also been implicated in the weight gain associated with second generation antipsychotics (Reynolds et al. 2006, Reynolds and Kirk 2010).
Atypical antipsychotics (such as olanzapine and risperidone) are a preferred pharmacological treatment option to typical antipsychotics. This is because atypical drugs have been shown to cause fewer EPS (Essali et al. 2009) while maintaining similar efficacy to typical antipsychotics in ameliorating the positive symptoms of schizophrenia. Cognitive and negative symptoms are still poorly treated by both typical and atypical antipsychotics. Newer atypical antipsychotics are seen as preferable treatment options due to their improved metabolic profile compared to olanzapine (Citrome 2009, Citrome 2011, Stahl et al. 2013). These newer antipsychotics (3rd generation antipsychotics, eg: aripiprazole) show partial agonist activity at dopamine receptors, rather than antagonism, which is thought to be more effective at treating cognition without EPS. Lurasidone has shown promise in treating cognition in small trials involving acute schizophrenia and longer-term trials are ongoing (Samalin et al. 2011). Aripiprazole has also shown signs of being more efficacious at treating cognition in schizophrenia compared to first and second generation antipsychotics (Bervoets et al. 2012).

1.8 General Aim

NMDA receptor antagonists such as PCP are used extensively in animals to attempt to replicate the cognitive impairments seen in schizophrenia patients. The overall aim of this work is to provide evidence that the sub-chronic PCP model of cognitive deficits has strong construct validity. Extensive behavioural work and some neurobiological studies have demonstrated that sub-chronic PCP administration results
in behavioural changes in rodents of relevance to schizophrenia (Reviewed in Neill et al. 2010 and Neill et al. 2013). The work presented in this thesis aims to study the pathological changes associated with this model using a range of *ex vivo* methodologies and behavioural testing, focussing on the GABAergic and σ₁ receptor systems, as these systems are known to be dysfunctional in the disease, as well as being vitally important in cognition.

**Objectives:**

- This thesis will firstly investigate the effect of sub-chronic PCP administration on GABA<sub>A</sub> receptor number and composition in the following brain regions known to be involved in cognition and disturbed in schizophrenia: frontal cortex, hippocampus, striatum and cerebellum.

- qRT-PCR will be utilised to study transcriptional changes in GABA<sub>A</sub> receptor subunits and changes in protein levels will be investigated using radioligand binding and autoradiography.

- Secondly this work will look into the effects of sub-chronic PCP administration on different populations of GABAergic interneurons in these four brain regions. qRT-PCR will be used to study transcriptional changes in a variety of GABAergic marker genes including GABA transporters (GABA transporter 1 (GAT-1), GABA transporter 3 (GAT-3)), biochemical markers of GABAergic cell types (parvalbumin (PVALB), somatostatin (SST), calretinin (CALB2), glial fibrillary acidic protein (GFAP), GAD<sub>65</sub> (GAD2) and GAD<sub>67</sub> (GAD1)). In order to verify that any transcriptional changes that are observed translate into functional differences, Western blotting will be used to study changes in the protein levels of the markers listed above.

- Recognition memory is known to be disrupted in schizophrenia patients and the novel object recognition test is known to be an ethologically relevant test of object recognition memory in rodents. Performance in this task is known to be disrupted following sub-chronic PCP administration. σ₁ receptors are a novel potential therapeutic mechanism by which to improve cognition in schizophrenia. Therefore the pro-cognitive effects of σ₁ receptor-specific compounds will be investigated using the 1min ITI NOR paradigm in sub-chronic
PCP-treated female rats and in an alternative ‘natural forgetting’ 6hr ITI NOR paradigm using drug-naïve female rats.
Chapter 2

General Methods
2.1 Experimental animals

The experiments in this thesis used a total of 240 female (180-270g) hooded-Lister rats (Charles River, UK), in six cohorts (Tables 2.1 and 2.2), n=8-25 per treatment group. Rats were housed 5 per cage in solid floored plastic cages (as shown in figure 2.1) containing sawdust, paper sizzle nest (Datesand Ltd, Manchester, England) and fun tunnels (plastic environment enrichment tunnels, Datesand Ltd, Manchester, England). Food (standard rat chow - Special Diet Services, Essex, England) and water were available ad-libitum in the home cage. Rats were maintained in single-sex colonies under a constant temperature (21 ± 2°C), humidity (40-50%) and 12hr light:dark cycle (lights on at 7am). All experiments were conducted during the light phase between 9am and 4pm. All studies were compliant with the Animal Scientific Procedures Act 1986 and approved by the University of Bradford or the University of Manchester Ethical Review process respectively.

Figure 2.1: Diagram showing the different social housing conditions used in Manchester (a) and Bradford (b). A – personal communication from B.Grayson, B- taken from Grayson 2012

A flow diagram showing the dosing and experimental timescales used in these studies is shown in figure 2.2.
**Figure 2.2:** Flow diagram showing the timetable for animal dosing and ex vivo experiments

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Cohort no.</th>
<th>Total Number of Rats used</th>
<th>Assay</th>
<th>Brain regions</th>
<th>Gene/Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4</td>
<td>n=10 (Vehicle=5, PCP=5)</td>
<td>qRT-PCR</td>
<td>Frontal Cortex, Hippocampus, Striatum, Cerebellum</td>
<td>GABA$_{A_1-5, 6, 7}$</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>n=10 (Vehicle=5, PCP=5)</td>
<td>qRT-PCR</td>
<td>Frontal Cortex, Hippocampus, Striatum, Cerebellum</td>
<td>Parvalbumin, Calretinin, Somatostatin</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>n=10 (Vehicle=5, PCP=5)</td>
<td>qRT-PCR</td>
<td>Frontal Cortex, Hippocampus, Striatum, Cerebellum</td>
<td>GFAP, GAT-1, GAT-3, GAD1, GAD2</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>n=10 (Vehicle=5, PCP=5)</td>
<td>Western Blotting</td>
<td>Frontal Cortex</td>
<td>Somatostatin, Calretinin, GAT-1, GAT-3, GAD$_{67}$</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>N=12 (Vehicle=3, Vehicle no washout = 3, PCP=3, PCP no washout=3)</td>
<td>Autoradiography</td>
<td>Frontal Cortex, Cerebral cortex, Hippocampus, Striatum, Cerebellum, Thalamus</td>
<td>GABA$<em>{A_5}$, GABA$</em>{A_1-3, 5}$</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>N=28 (Vehicle=14, PCP=14)</td>
<td>Radioligand Binding</td>
<td>Frontal cortex</td>
<td>GABA$<em>{A_5}$, GABA$</em>{A_1-3, 5}$</td>
</tr>
</tbody>
</table>

**Table 2.1:** Cohorts of rats used throughout the ex-vivo studies
<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Cohort no.</th>
<th>Total Number of Rats used</th>
<th>Weight range (g)</th>
<th>Compound Tested (mg/kg)</th>
<th>NOR test ITI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>n=75 (Vehicle=15, PCP=60)</td>
<td>180-260</td>
<td>PRE-084 (0.3-3), NE-100 (0.3-3) and PRE-084 (1) + NE-100 (1)</td>
<td>1 minute</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>n=52 (Vehicle)</td>
<td>180-270</td>
<td>PRE-084 (0.1-3)</td>
<td>6 hours</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>n=53 (Vehicle)</td>
<td>190-265</td>
<td>NE-100 (0.3-3) and PRE-084 (1) + NE-100 (1)</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

**Table 2.2:** Cohorts of rats used throughout the behavioural studies

### 2.2 Sub-chronic PCP dosing regimen

Rats used in these experiments were administered with either sub-chronic PCP (Sigma-Aldrich) (2mg/kg b.i.d. i.p.) or vehicle (0.9% w/v saline b.i.d. i.p.) for 7 days in a volume of 1ml/kg, before a 7-day washout period prior to drug administration and NOR testing or genetic/proteomic analysis. This washout period following PCP administration is necessary to prevent any behavioural changes caused by either drug withdrawal or direct effects caused by residual PCP being present in the rats brain (Jentsch et al. 1998).

The sub-chronic PCP regimen used in these studies is adapted from earlier studies by Jentsch and Roth (Jentsch and Roth 1999) and has been shown in our laboratory to consistently induce robust cognitive and social behaviour deficits in various tasks of relevance to schizophrenia in female rats (reviewed in Neill et al. 2010 and Neill et al. 2013). These deficits, and the associated pathological changes such as reductions in cortical parvalbumin-positive neurons (Abdul-Monim et al. 2007, McKibben et al. 2010), reductions in cortical BDNF mRNA levels (Snigdha et al. 2011b), alterations in dopamine and serotonin receptor levels (Choi et al. 2009) and frontal cortical GABAergic alterations (See Chapter 3 and 4), are strongly associated with schizophrenia.

### 2.3 Sacrifice

For qRT-PCR studies the rats were sacrificed by an overdose of CO₂ followed by cervical dislocation. For autoradiography and radioligand binding studies the rats were sacrificed by stunning followed by cervical dislocation. Immediately after cervical dislocation, rats were decapitated using sharp scissors and the brain removed carefully from the skull on ice in preparation for dissection or slicing.
2.4 Brain dissection

Dissection of the regions of interest for qRT-PCR, Western blotting and radioligand binding used the following method, with all manipulations being carried out on ice:

2.4.1 Frontal cortex

Using a razor blade, the olfactory blubs were removed and discarded and a cut was made using a rat brain cutting block 8mm from the front of the cerebral cortex to remove the frontal cortex (Figure 2.3a).

2.4.2 Cerebellum

Dissection of the cerebellum was carried out using a razor blade between the cerebellum and cortex (Figure 2.3b). The cerebellum was lifted away from the pons and medulla using a sharp scalpel.

2.4.3 Striatum

Following removal of the frontal cortex and cerebellum, the brain was inverted (Figure 2.3c) and two cuts made in the plane shown, exposing the striated tissue. The striatum and cortex were separated by cutting in a straight line as shown in figure 2.3c. The cerebral cortex surrounding the striatal tissue was peeled away using a scalpel and a spatula to ensure that neither structure was damaged.

2.4.4 Hippocampus

To remove the hippocampus, a lateral cut was made between the two hemispheres. The left hemisphere was rotated so that the rear of the brain was uppermost (Figure 2.3d). A spatula was used to gently ease the white mid-brain material away from the remainder of the hemisphere. The hippocampus was ‘folded’ against the exposed surface and gently eased out using a spatula before cutting away from the thalamus and mid-brain using a scalpel (Figure 2.3e). The process was repeated for the right hemisphere and the two hippocampi combined for analysis.

All brain regions were stored at -80°C, except the regions used for qRT-PCR studies which were stored at 4°C in RNAlater™ solution (Sigma-Aldrich). RNAlater solution is a nontoxic reagent used to stabilise RNA in order to prevent any loss or degradation of RNA prior to RNA extraction.
Figure 2.3: Dissection of rat brain for qRT-PCR, Western blotting and radioligand binding analysis into frontal cortex (2.3a), cerebellum (2.3b), striatum (2.3c) and hippocampus (2.3d).

2.5 qRT-PCR

Following brain dissections on ice into frontal cortex, hippocampus, striatum and cerebellum (Figure 2.3a-e), these regions were stored in Sigma-Aldrich RNAlater™ solution at 4°C for <7 days, in preparation for RNA extraction and analysis.

RNAlater solution was removed using an RNase-free 1ml pipette tip and homogenised in 1ml Trizol™ reagent, using ≤5 x 1-second pulses of an X-1020 homogeniser on ice to prevent heat damage to the nucleic acids. RNA was extracted from this homogenate using the following Invitrogen mini-column method:

2.5.1 RNA Extraction

Homogenised brain regions were incubated in Trizol™ reagent for 5 mins to allow dissociation of nucleoprotein complexes. 0.2ml chloroform per 1ml of Trizol reagent was added and shaken vigorously by hand before incubation of the samples at room temperature for 3 mins. The samples were then centrifuged at 4°C in a Hettich mikro 22r centrifuge at 12,000g for 15 mins to separate out the RNA colourless phase from the protein and DNA-containing red/pink organic phase. This colourless phase (600-700μl) was transferred to a fresh RNase free tube for RNA purification.

2.5.2 RNA purification

An equal volume (600-700μl) of 70% ethanol was added to the colourless RNA-containing phase and mixed well by vortexing. The tubes were inverted 3-5 times to disperse any precipitate. 700μl of this mixture was transferred to a spin cartridge with collection tube and centrifuged at 12,000g for 15s at room temperature, discarding the flow-through but retaining the same collection tube. The remainder of the sample was then added to the spin cartridge and centrifuged at 12,000g for 15s at room temperature and the flow-through was discarded. 700μl of Wash Buffer I was added to the centre of the spin cartridge membrane and centrifuged at 12,000g for 15s at room
temperature. The flow-through was discarded and the collection tube was replaced. 500µl of Wash buffer II with 60% ethanol was added to the centre of the spin cartridge and centrifuged at 12,000g for 15s at room temperature and the flow-through was discarded. This wash step (Wash buffer II) was repeated and the flow-through was discarded. The dry spin cartridge, with the same collection tube, was then centrifuged at 12,000g for 1min at room temperature to dry the membrane. The spin cartridge was placed into a collection tube. 10µl of RNase free water was added to the centre of the spin cartridge membrane and left to incubate for 2mins at room temperature before spinning at 12,000g for 30s. This step was repeated twice and the sequential elutions were collected into the same collection tube for each sample.

All RNA was stored at -80°C after spectrophotometric analysis of purity/integrity by 260nm analysis of RNA concentration and RNA Integrity Number analysis of RNA purity. RNA samples were not used for qRT-PCR analysis if they had an RIN of less than 7 as this suggests that the extracted RNA sample is too fragmented to give reliable results.

2.5.3 cDNA production

After conversion to cDNA using the following Qiagen™ reverse transcription kit method, which is summarised below:

gDNA wipeout buffer, extracted template RNA and RNase-free water was mixed in a 2:2:10 ratio and incubated for 2mins at 42°C. The mixture was then stored on ice. Quantiscript Reverse Transcriptase, Quantiscript Reverse Transcriptase buffer (5x) and Reverse transcription primer were mixed in a 1:4:1 ratio and stored on ice. 6µl of the reverse transcription mixture was added to each 14µl of template RNA mixture. This combined mixture was incubated at 42°C for 15mins to produce the cDNA product, before inactivating the reverse transcriptase enzyme by incubating at 95°C for 3mins.

The cDNA produced was stored at -20°C prior to analysis by qRT-PCR.

2.5.4 qRT-PCR

Before the commencement of comparative qRT-PCR studies, primer efficiency tests for each of the primer sets were conducted. Although these primer assays have
been bioinformatically validated by Qiagen, an additional standardisation assay was carried out to show that no loss of primer efficiency had occurred in transit.

A four-point cDNA dilution series (0.5-0.05μg cDNA) was assayed for each set of primer reactions and all primer assays demonstrated good primer efficiency (88-127%) across these concentrations. Efficiency values were calculated by plotting Ct values against log10(cDNA concentration) in triplicate and testing for a linear relationship. The slopes of these charts were used to calculate an efficiency value for each PCR reaction. \( r^2 \) values for the linear relationship between log(cDNA concentration) vs Ct were all above 0.93 showing that Ct value outputs were closely correlated with cDNA concentration. The dilution curve efficiency values are shown in appendix 1.

0.1μg of cDNA (in triplicate) was used to quantify gene expression with a SYBR Green qRT-PCR kit using the following program:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Cycle repeats</th>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (mins:secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>95</td>
<td>15:00</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>1</td>
<td>94</td>
<td>00:15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>55</td>
<td>00:30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>72</td>
<td>00:30</td>
</tr>
</tbody>
</table>

**Table 2.3: qRT-PCR experimental conditions**

The 15min “Hot-Start” cycle at 95°C was required to activate the Taq Polymerase enzyme. Each reaction was made up of 0.1μg cDNA, 5μl of Qiagen SYBR Green Master mix, 1μl of Qiagen Quantitect primers and RNase-free water to a total volume of 10μl per well.

15 genes in total were studied: GABRA1-5 (GABA\(_A\) receptor subunits α1-5), (GABRD) (GABA\(_A\) receptor δ subunit), GABRG2 (GABA\(_A\) receptor γ2 subunit), the calcium-binding protein genes PVALB (parvalbumin) and CALB2 (calretinin), the peptide hormone gene (somatostatin), both isoforms of the glutamate decarboxylase enzyme responsible for the production of GABA (GAD\(_{65}\) and GAD\(_{67}\), coded for by the genes GAD2 and GAD1 respectively), GAT-1 (a neuronal GABA transporter), GAT-3 (an astrocytic GABA transporter) and GFAP (glial fibrillary acidic protein – a marker of astrocytes).
2.5.5 Statistical analysis

All data was normalised to a GAPDH housekeeping gene to generate a ΔCt value for each replicate. Analysis was carried out using the 2-ΔΔCt method as described by Livak and Schmittgen (Livak and Schmittgen 2001) and data is displayed as relative quantification (RQ) values. The vehicle animal mean mRNA level was set to 1 and sub-chronic PCP data is shown as a percentage of the mean vehicle value. Comparison between vehicle and sub-chronic PCP groups was done using an unpaired t-test on the RQ values.

2.6 Radioligand binding

2.6.1 Radioligands

The GABA<sub>A</sub> receptor specific radioligands [³H]-flumazenil and [³H]-L-655,708 were selected based on their relative binding affinities for different populations of GABA<sub>A</sub> receptors. [³H]-flumazenil has a high affinity for GABA<sub>A</sub> receptors containing α1, 2, 3 and 5 subunits, with >200x selectivity for these receptors containing α4 or α6 subunits (Table 2.4). [³H]-L-655,708 has a high specific affinity for receptors containing α5 subunits, with a greater than 30x relative affinity for receptors containing α5 vs α1, 2 or 3 subunits (Table 2.4), which minimises the possibility of any non-specific binding at other GABA<sub>A</sub> receptor subtypes affecting the assay.

Neither of these ligands have appreciable affinity for receptors containing α4 or α6 subunits, so this study will be confined to post-synaptic GABA<sub>A</sub> receptors and extrasynaptic α5-subunit containing receptors. The extrasynaptic GABA<sub>A</sub> receptors containing α4 subunits are thought to mediate the effects of ethanol (Liang et al. 2008) and α6 subunits are found almost exclusively in the cerebellum so are not thought to be especially important in cognitive functions.
### Table 2.4: A summary of the binding affinities of the compounds used in these studies for GABA<sub>A</sub> receptor subunits in humans (flumazenil and L-655,708). Values for rat brain are shown in brackets.

The dose ranges for [³H]-flumazenil and [³H]-L-655,708 in the radioligand binding assays were calculated from tissue dilution studies carried out during this study (see chapter 3, figure 3.5 and figure 3.13).

#### 2.6.2 Membrane preparation

5 regions of interest (frontal cortex, cerebral cortex, hippocampus, striatum and cerebellum) were dissected out using the method described in section 2.4 of this chapter, although only frontal cortex was assayed due to time constraints. The tissue was weighed and frozen at -80°C for one week in labelled Eppendorf tubes. Brain tissue was homogenised in 1:20 w/v 50mM Tris-HCl buffer on ice using an Ultra-turrax homogeniser in a clean 15ml centrifuge tube. The tissue was homogenised thoroughly in 2 second bursts before returning to ice to minimise heating of the samples. The homogeniser blade was washed thoroughly in clean 50mM Tris-HCl between samples. The homogenates were then centrifuged at 16,000rpm for 10mins at 4°C and the supernatant was discarded. An equal volume of ice-cold 50mM Tris-HCl as used in the homogenisation (1:20 w/v) was then added to the pellet and re-suspended thoroughly using a Sorvall mixer on ice. The cell suspension was then centrifuged at 16,000rpm at 4°C for 10mins and the supernatant was discarded. An equal volume of ice-cold 50mM Tris-HCl as used in the homogenisation (1:20 w/v) was then added to the pellet and re-suspended thoroughly on ice using a Sorvall mixer. After 3 washes the membrane pellet was re-suspended in 1:10 w/v of 50mM Tris-HCl and aliquoted into 1ml aliquots before freezing. Pooled brain regions were frozen at -80°C until they were needed and individual brain regions were assayed on the same day.
2.6.3 Radioligand binding assay

Previously prepared membrane preparations from pooled rat brains were thawed for 60-90mins on ice before being assayed. Thawed membrane preparations were stored on ice and the samples protein content determined using the Bio-Rad method.

Pure membrane samples were diluted 1:100, 1:167, 1:200, 1:400, 1:200, 1:267, 1:400 and 1:800 to a volume of 200µl in 50mM Tris-HCl, then to a final volume of 800µl in Milli-Q distilled water. All samples had 200µl of Bio-Rad protein determination reagent added and vortexed thoroughly. Protein values for each sample were calculated spectrophotometrically at 595nm using a reference standard curve of BSA diluted in water to 0, 2.5, 5, 7.5, 10, 12.5 and 15µg/ml.

2.6.4 Binding assay

Frozen membrane preparations were defrosted on ice and fresh membrane preparations were kept on ice throughout the experiment. Membrane protein concentrations were determined using the Bio-Rad protein determination assay, based on the principles of a Bradford assay (Bradford 1976). Defrosted membranes were then diluted down to 25µg/ml for [³H]-flumazenil saturation assays or 75µg/ml for [³H]-L-655,708 saturation assays in 5ml reaction tubes. 400µl of membranes was added to 50µl of radioligand and either 50µl 10µM flunitrazepam (blank) or 10mM Tris-HCl + 1mM EDTA (pH 7.4)(total binding) and incubated for 1hr on ice. After incubation on ice the reaction mixtures had 5ml of 10mM Tris-HCl + 1mM EDTA (pH 7.4) added and the diluted reaction mixture was poured into a well of a 40-well manifold containing GF/B filters ([³H]-L-655,708) or GF/C filters ([³H]-flumazenil) which had been pre-soaked in 10mM Tris-HCl + 1mM EDTA (pH 7.4) for 55mins. GF/B and GF/C filter papers were obtained from Whatman®. 2x5ml aliquots of 10mM Tris-HCl + 1mM EDTA (pH 7.4) were then added to each well before removal of the filter into an individual labelled scintillation vial. 3mls of scintillation liquid was then added to each vial, ensuring the filter was completely covered by the liquid. Vials were placed into 96-well racks and placed on a shaker before shaking vigorously for 10mins and then left overnight at room temperature. Counting of the radioactivity present in the sample (expressed as disintegrations per minute (DPM)) occurred in a Perkin Elmer TriCarb 2810TR scintillation counter.
2.6.5 Data analysis

Scintillation counts for bound radioactivity were plotted against radioligand concentration values to give rise to total binding, specific binding and blank values. Specific binding values were used to calculate $K_D$ and $B_{\text{max}}$. $K_D$ and $B_{\text{max}}$ values were calculated from a nonlinear regression analysis of specific binding using Graphpad Prism v5 as described previously (Atack et al. 2005). $K_D$ and $B_{\text{max}}$ comparisons between vehicle- and sub-chronic PCP-treated groups were done using an unpaired t-test analysis on IBM SPSS v20.

2.7 Autoradiography

2.7.1 Radioligands

$[^3]H$-flumazenil and $[^3]H$-L-655,708 were used in the autoradiographical studies. Binding affinities for these radioligands are described in chapter 2.6.1 of this thesis. The concentrations of radioligand used in these autoradiography studies were based upon previous studies in rat and mouse brain using these two radioligands (Sur et al. 1999, Atack et al. 2005). The dilution series graphs used in these studies to calculate $K_D$ and $B_{\text{max}}$ are shown in appendix 2.

2.7.2 Brain sectioning

Whole female rats were sacrificed and decapitated 24hrs after the final PCP or saline injection (non-washout animals, n=3 per group, see table 2.1) or 24hrs after the 7 day washout period (washout animals, n=3 per group, see table 2.1). Brains were immediately removed and rapidly frozen in dry-ice cooled isopentane (-40°C). 20µm-thick sections were cut on a Leica CM 3050 cryostat-microtome at -15°C and thaw-mounted onto glass microscope slides (Superfrost Plus slides). Three sagittal sections were collected per glass slide before storing at -20°C prior to analysis.


After thawing, slides were left to dry under a steady cold air-stream. Prior to radioligand incubation endogenous ligands were washed off by pre-incubating the saturation slides in buffer (50mM Tris-HCl, containing 1mM EDTA at pH 7.4) at room temperature. Slides were dried under a cold air-stream to prevent dilution of the radioligands during incubation. Slides were incubated in 0.05, 0.1, 0.48, 0.96 or 9.6nM $[^3]H$-flumazenil or 0.05, 0.1, 0.52, 0.99 or 10.2nM $[^3]H$-L-655,708 for 60mins at room
temperature. Concentrations of both radioligands were determined using liquid scintillation counting and non-specific binding was determined on adjacent sections using 10μM flunitrazepam. After incubation, slides were rinsed in ice-cold buffer (2x5mins at 4°C), followed by a dip into ice-cold distilled water. After drying, slides were placed in light-tight cassettes with a radioactive polymer standard and exposed to Fujix-IP plates for 1 week ([³H]-flumazenil) or 2 weeks ([³H]-L-655,708).

2.7.4 Development

Fujix-IP plates were scanned using a FLA-7000 laser scanner and digital autoradiograms were taken. These autoradiograms were analysed using AIDA software, with translation of the autoradiographic intensity into nCi/mg tissue was performed using co-exposed polymer standards. Areas of interest were defined using the anatomical figures from the brain atlas of the rat (Paxinos and Watson 1986). An example autoradiograph showing the definition of the areas of interest is shown in figure 2.3.

2.7.5 Data analysis

Scintillation counts for bound radioactivity were plotted against radioligand concentration values to give rise to total binding, specific binding and blank values. Specific binding values were used to calculate K_D and Bmax. K_D and Bmax values were calculated from a nonlinear regression analysis of specific binding using Graphpad Prism v5 as described previously (Atack et al. 2005). K_D and Bmax comparisons between vehicle- and sub-chronic PCP-treated groups were done using a one-way ANOVA analysis, followed by post-hoc Students’ t-test on IBM SPSS v20. Example autoradiograms for both [³H]-flumazenil and [³H]-L-655,708 are shown in appendix 3.
Figure 2.4: Representative rat brain autoradiogram showing the 6 main regions of interest. Frontal cortex – red, Cerebral cortex – purple, Hippocampus – green, Cerebellum – blue, Striatum – Tan and Thalamus – black.

2.8 Cognitive testing (NOR)

2.8.1 Animals and Dosing regimen

This study used 3 cohorts (table 2.2) totalling 188 female (180-270g) hooded-Lister rats (Charles River UK), n=7-25 per treatment group. Rats were housed as previously described (this chapter, section 2.1). All experiments were conducted during the light phase and the study was compliant with the Animal Scientific Procedures Act 1986 and approved by the University of Bradford Ethical Review Process or the University of Manchester Ethical Review process where appropriate.

Rats used in the 6hr study (cohorts 2 & 3, table 2.2) were not treated with any pharmacological agent prior to experimentation, with the cognitive deficit being induced by the extended ITI in a ‘natural forgetting’ paradigm.

The exclusion criteria for both the 6hr ITI study and the sub-chronic PCP study were as follows: If an animal failed to explore one or more of the objects for more than 1 second in either the acquisition or retention trial, or if the animal knocked over an object in either trial then it was excluded from the final data analysis.
**Table 2.5:** The number of rats excluded from the 6hr ITI NOR study following acute administration of PRE-084 (0.1-3mg/kg i.p.), NE-100 (0.3-3mg/kg i.p.), co-administered PRE-084 (1mg/kg, i.p.) + NE-100 (1mg/kg, i.p.) or vehicle (0.9% w/v NaCl, i.p.) to drug-naive rats.

Rats used in the PCP study (cohort 1, Table 2.2) were administered with either sub-chronic PCP (Sigma-Aldrich) (2mg/kg bi-daily i.p.) or vehicle (0.9% w/v saline bi-daily i.p.) for 7 days as described previously (this chapter, section 2.2).

**Table 2.6:** The number of rats excluded from the NOR study following acute administration of PRE-084 (0.3-3mg/kg i.p.), NE-100 (1mg/kg i.p.), co-administered PRE-084 (1mg/kg, i.p.) + NE-100 (1mg/kg, i.p.) or vehicle (0.9% w/v NaCl, i.p.) to sub-chronically PCP-treated rats (2mg/kg, i.p., twice daily for 7 days).

### 2.8.2 Drug administration

The σ1 receptor agonist PRE-084 and antagonist NE-100 were selected based on their high selectivity for the σ1 receptor as shown in Table 2.6; PRE-084 has a high affinity for the σ1 receptor, with very low affinity for a range of other receptors including σ2 receptor sites, the PCP binding site of the NMDA receptor and dopaminergic, serotonergic and acetylcholinergic receptor classes (Table 2.7). NE-100 also has high σ1 receptor affinity with a moderate affinity for σ2 receptors (Table 2.7). NE-100 has a much lower affinity for PCP binding sites and dopaminergic, serotonergic...
and acetylcholinergic receptor classes (Table 2.7) than either of the sigma receptor subtypes.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>σ-1 (nM)</th>
<th>σ-2 (nM)</th>
<th>PCP site (nM)</th>
<th>D₁ (nM)</th>
<th>D₂ (nM)</th>
<th>5-HT₁A (nM)</th>
<th>5-HT₂A (nM)</th>
<th>mACh (nM)</th>
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<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>N/A</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>N/A</td>
</tr>
<tr>
<td>NE-100</td>
<td>1.5</td>
<td>84.6</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 2.7: Binding affinities (nM) of PRE-084 and NE-100 for various receptor subtypes. Data are shown as IC₅₀ values (nM) and were obtained from Su et al. 1991 and Okuyama and Nakazato 1996.

The high selectivity of the agonist and antagonist for the σ₁ receptor minimises the possibility of off-target effects being involved in the observed effects. PRE-084 and NE-100 were not administered to vehicle animals in the PCP study.

The dose range for PRE-084 was chosen based on previous work showing reversal of MK-801-induced cognitive deficits in a variety of cognitive tests (Maurice et al. 1994b) as well as in vitro binding studies in rats (Peeters et al. 2004). The doses of NE-100 were chosen based on antagonism of σ₁ receptor agonist ((+)-pentazocine and SA4503) induced reversal of a PCP-induced deficit in mice (Noda et al. 2001), as well as an NE-100 induced reversal of an acute PCP-induced cognitive deficit, in the Morris water-maze, in male Wistar rats (Okuyama et al. 1995).

2.8.3 NOR apparatus

The NOR apparatus consists of five open Plexiglas boxes (L 52cm, W 52cm, H 31cm), each positioned 27cm above the floor on a moveable trolley (Figure 2.6). A camera was attached to frames above the boxes to record the rats' behaviour in each trial. The objects used in both of these studies were unopened Coca-Cola® cans and brown glass bottles filled with saline (Figure 2.5) to prevent them being easily moved by the animal. The heights of the objects were approximately equal (10cm ± 2cm) and the rats had previously been shown to have no preference for these objects when used in this paradigm (Grayson 2012). Objects were placed 6cm from walls in opposite corners (Figures 2.7b and 2.7c). Object placement was selected randomly using a pseudorandom Gellerman schedule (Gellermann 1933). After each trial, both objects and the box were cleaned using a 10% v/v solution of ethanol in an attempt to disrupt
any olfactory trails and dried using paper towels so as to not influence the behaviour of the animals during the test and therefore bias subsequent trials. Background radio music was played quietly within the behavioural room to lessen the distracting effects of other sounds within the room such as other rats exploring.

**Figure 2.5:** The objects used during the NOR experiments

**Figure 2.6:** The moveable trolley used to record behaviour with a camera mounted above the arena.
Figure 2.7: The empty NOR arena during the habituation trial (a), exploring two identical objects during the acquisition trial (b) and exploring a novel and familiar object during the retention trial (c).

2.8.4 NOR test

2.8.4.1 Habituation

Groups of 5 rats (per cage) were handled and exposed to the empty NOR arena and testing room daily for 20mins per session for 3 consecutive days prior to NOR testing, with testing always occurring on the day after the final habituation day. Habituation reduces the level of novelty of the arena itself, therefore increasing focus on the objects during testing. Rats were returned to their home cage after habituation until the test day.

2.8.4.2 Acquisition trial

Two identical objects (F1 and F2, Figure 2.8a) were placed in the arena at the pseudorandomised positions indicated above (Gellermann 1933) and the rats were allowed free access to the arena for 3mins of exploration before removal and return of the rat to the home cage. Cleaning of the arena and objects with 10% (v/v) ethanol occurred between trials as described above. At the start of the acquisition and retention trials, the rat was consistently placed into the arena facing the same corner as shown in figure 2.8.

2.8.4.3 Inter-trial interval

Following the 3min acquisition trial rats were returned to their home cage for 1min (sub-chronic PCP deficit experiment) or 6hrs (6hr ITI experiment). The 1min ITI has been shown to be a sufficient inter-trial interval (ITI) for vehicle rats to recall the familiar object, but PCP-treated animals are not able to, based on previous work in our laboratory (Grayson et al. 2007, Grayson 2012 and reviewed in Neill et al. 2010).
6hr ITI chosen for the drug-naïve NOR study is based on previous studies in our laboratory (Sutcliffe et al. 2007, Sood et al. 2011) and other laboratories (Ennaceur and Delacour 1988, King et al. 2004) showing that an ITI of greater than 3hrs is sufficient so that drug-naïve rats cannot recall the familiar object. During this period, the arena was cleaned with 10% ethanol as above. A triplicate copy of the familiar object (F3) and a novel object (N1) were then placed in the same positions as used in the acquisition trial (Figure 2.8b). This model also has the advantage of using naïve animals, preventing any non-specific behavioural effects caused by the administration of a drug even after the drug itself is no longer onboard, such as in PCP or MK-801 animal models.

2.8.4.4 Retention trial

Following the ITI, rats were then allowed to explore the arena with the novel (N1) and familiar (F3) object (Figure 2.8b) for 3mins. The position of the novel object was pseudorandomly assigned using a Gellerman schedule (Gellermann 1933) to reduce the effect of place preference. After the 3 minute exploration period, rats were returned to their home cage.

![Diagram showing the relative positions of the objects](image1)

**Figure 2.8:** Diagram showing the relative positions of the identical objects during the acquisition (a) and the novel and familiar objects during the retention (b) trial of the NOR test.

2.8.5 Behavioural analysis

Exploration of the objects was defined by the following criteria (Ennaceur and Delacour 1988, Grayson et al. 2007, Grayson 2012), such that licking or sniffing of the object counted as exploratory behaviour, but turning around, sitting or standing on or leaning against the objects was not counted as object exploration. Sniffing or licking of
the object while touching it with the forepaws was also counted as object exploration. All exploration was recorded digitally to reduce the presence of experimenters during the study and scored at a later time using a stopwatch by an experimenter blind to the treatments.

The exclusion criterion for the NOR test was defined as described previously: If an animal failed to explore one or both of the objects (<1s of exploration per object) in the acquisition or retention trial, or if the animal knocked over any of the objects during either trial it was excluded from the final data analysis. The following factors were calculated from the exploration data:

\[ E_1 = \text{Total exploration time of both left (F1) and right (F2) identical objects in the acquisition phase of the NOR test (E_{1F1} + E_{1F2})} \]

\[ E_2 = \text{Total exploration time of both novel (N1) and familiar (F3) objects in the retention trial of the NOR test (E_{2F3} + E_{2N1})} \]

Exploration times for the novel (N1) and familiar (F1-3) objects were also shown separately to allow a direct comparison of within-trial object exploration (eg: novel vs familiar object exploration).

\[ \text{Discrimination Index} = \frac{(E_{2N1}-E_{2F3})}{E_{2N1}+E_{2F3}} \]

The DI shows the difference in time spent exploring the novel object and the familiar object, expressed as a proportion of the time spent exploring the two objects during the retention trial.

Line crossings were measured by counting the total number of sectors (lines) crossed by the rat during the acquisition or retention trials. One line crossing was recorded when the base of the rats tail crossed a line, either horizontally, vertically or diagonally. There are 9 square sub-sections in the NOR arena, measuring 17.3cm x 17.3cm each (Figure 2.8)

2.8.6 Statistical analysis

All data are expressed as the mean ± S.E.M. and were analysed using IBM SPSS v20. Exploration data were analysed by a repeated measures 2-way ANOVA. This detected the main effect of drug treatment, main effect of the test (exploration of both objects) and the interaction between drug treatment and the two trials.
(acquisition trial and retention trial). Further analysis by Students` t-test was used to compare the time spent exploring the novel and familiar objects. Analysis of DI, exploration times and total locomotor activity were carried out using a 1-way ANOVA, followed by a post-hoc LSD t-test.

2.9 Western blotting

Figure 2.9: Flow-chart showing the Western blotting process used in these studies

2.9.1 Protein lysate preparation

Brain regions (Frontal cortices and hippocampi) were dissected as previously described (this chapter, section 2.2) and weighed in pre-weighed and labelled 2ml microfuge tubes. RIPA buffer (Sigma-Aldrich) with protein inhibitor cocktail (1x final concentration, Sigma-Aldrich) was added at a ratio of 1ml buffer per 100-150mg tissue and lysed using 3x3s pulses of an X-1020 homogeniser. Protein homogenates were centrifuged at 12000rpm for 20min to remove cellular debris and the supernatant was removed into labelled Eppendorf tubes and stored at -20°C for Western blotting.

Protein supernatants were defrosted on ice and their total protein concentration determined by using a Bradford assay (Bradford 1976).
2.9.2 Bradford Assay

The Bradford assay was performed as described in chapter 2.6.3, with the following alterations: Bovine serum albumin (BSA) standards were used at the following concentrations (0, 0.1, 0.2, 0.5, 0.75, 1, 2.5mg/ml BSA in water and 10% v/v lysis buffer). 10μl of each concentration and 10μl 1:10 protein lysate were analysed with 200μl Bio-Rad protein determination reagent at 595nm on a Biotek Elx 800 spectrophotometric plate reader.

2.9.3 Antibody optimisation

To determine an optimal protein load for this assay, a dilution series was prepared for each antibody. 10, 20, 30 and 40μg of total frontal cortex protein was loaded onto a gel as described below and probed with the appropriate primary and secondary antibody concentrations as shown in table 2.8, using the method described in chapter 2.9.4, 2.9.5 and 2.9.6 in this thesis. These concentrations were taken from the literature provided with each antibody. As expected each antibody optical density increased in a highly linear fashion (r² values = 0.93-0.96) across the concentration range and particularly (r² values = 0.93-1) between 20 and 40μg therefore 30μg of frontal cortical protein was loaded into each well. Parvalbumin and GFAP antibodies could not be optimised to the standards above and so were not used in these studies.

2.9.4 Gel running and transfer

30μg of each protein lysate was aliquoted into an equal volume of 2x Laemelli sample buffer with 10% β-mercaptoethanol before boiling at 75°C for 5mins. Centrifugation of each sample at 2000rpm for 10 seconds ensured no loss of sample by evaporation before being loaded into each well of a 10-well 4-20% Bio-rad pre-cast acrylamide gradient gel. Each gel had 1μl of a Bio-Rad WesternC protein ladder aliquoted into one of the wells. Each gel was run for 40mins (30mins for low MW proteins such as somatostatin) at 200V, 0.4A until the blue ‘front’ had reached the bottom of the plate. The gel cassette was then cracked open and the gel peeled from the cassette before imaging on a Chemidoc imaging system (Stain free gel program) to ensure correct protein migration. After imaging, proteins were transferred to a nitrocellulose membrane using a Bio-Rad Trans-blot kit in a turbo-transfer blot machine (Mixed MW program – 2.5A, 25V, 7mins). Membranes were then imaged using a Chemidoc imaging system (Stain free blot program) to ensure complete protein
transference, as well as to calculate total protein for normalisation of the blot, before blocking.

### 2.9.5 Membrane probing

After blocking overnight (o/n) at 4°C in 3% milk solution membranes were incubated with a primary antibody according to the conditions in table 2.8.

<table>
<thead>
<tr>
<th>1° Antibody</th>
<th>Supplier</th>
<th>Host species</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calretinin</td>
<td>Abcam</td>
<td>Rabbit</td>
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<tr>
<td>GAD67</td>
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<td>Rabbit</td>
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<td>o/n</td>
<td>4°C</td>
</tr>
</tbody>
</table>

**Table 2.8:** Incubation conditions for Western blotting primary antibodies.

After incubation of the membrane with the primary antibody, the membrane was washed 3x10mins with PBST before incubating with a goat anti-rabbit HRP-conjugated secondary antibody (Abcam®) diluted 1:10000 in 3% milk (w/v) at room temperature for 1hr on a shaker. After incubation with the secondary antibody, membranes were washed in PBST 3x10mins before developing on a shaker for 5mins in Bio-Rad ECL clarity substrate.

### 2.9.6 Data analysis

Membranes were analysed using Chemidoc MP imaging station software, normalising to total lane protein as determined by TGX-stain free imaging. Normalised intensity values were calculated and data is shown as normalised intensity values ± S.E.M. Comparisons between vehicle and sub-chronic PCP groups were analysed using IBM SPSS v20 using an unpaired t-test.
Chapter 3

Investigation into GABA_A receptor alterations in the sub-chronic PCP model
3.1 Introduction

The GABAergic dysfunction hypothesis of schizophrenia arose from the NMDA hypofunction hypothesis and postulates that dysfunctions in GABAergic signalling gives rise to the pathophysiology of schizophrenia. Patients with schizophrenia show prefrontal cortical reductions in several GABA_A receptor subunits (Table 3.1), which are thought to contribute to the diseases symptomatology. The pathological and cognitive deficits in schizophrenia have been modelled preclinically by administering NMDA receptor antagonists such as phencyclidine or ketamine, which has been shown to cause a number of behavioural and pathological changes similar to those seen in schizophrenia.

3.1.1 GABA_A receptor subtypes

3.1.1.1 Benzodiazepine binding site-containing receptors

Around 75% of the GABA_A receptors that are found in the brain are classified as benzodiazepine-binding GABA_A receptors (McKernan and Whiting 1996). This is due to the presence of a benzodiazepine binding site, between the α and γ2 subunits of the receptor, where the subunit composition of the receptor is α1βγ2, α2βγ2, α3βγ2 or α5βγ2 (Sieghart and Sperk 2002). Due to a single amino acid substitution (His-Arg) in α4/α6 subunits, receptors with the composition α4βγ2 or α6βγ2 show no binding affinity for benzodiazepines (Wieland et al. 1992). If the GABRG2 gene, which encodes the GABA_A γ2 subunit, is knocked down using antisense nucleotide methods, the animals show significantly decreased numbers of αβγ GABA_A receptors and decreased GABAergic tone (Karle 2002). Post-transcriptional splicing of the GABRG2 gene product yields two splice variants, long (γ2L) and short (γ2S) γ2 subunits (Whiting et al. 1990). These splice variants show spatially distinct expression profiles, with γ2S subunits predominating in the cortex and hippocampus and γ2L subunits being found mostly in the cerebellum and medulla of the rat brain (Gutierrez et al. 1994). Both of these subunits can form functional receptors and compensate for down-regulation of the other variant, as demonstrated by γ2L KO mice having no changes in γ2 protein levels, due to a compensatory increase in γ2S subunit production (Homanics et al. 1999). γ1 and γ3 subunits are very restricted in their expression profiles (D'Hulst et al. 2009).
Aside from simply conferring affinity for benzodiazepines, the α1, 2, 3 and 5 subunits confer different pharmacological properties to the receptor. Using knockout models and pharmacological methods, the sedative effects of benzodiazepines are thought to be mediated by the α1 subunit, while the anxiolytic properties of these compounds are due to affinity at α2/3 subunits (D’Hulst et al. 2009). Evidence for this comes from knockout mouse models, where loss of the GABA<sub>A</sub> α1-containing receptors in mice resulted in increased sensitivity to diazepam-induced sedation and compensatory tonic inhibitory current (Ortinski et al. 2006), with no loss of PPI or change in conditioned place preference or modulation of other (NMDA/dopamine) receptors (Reynolds et al. 2003). Apart from the proposed anxiolytic role for GABA<sub>A</sub> receptors with α2 or α3 subunits, receptors containing α3 subunits have been implicated in sensorimotor gating. Mice where the GABRA3 gene is knocked out show decreased startle responses and PPI dysfunction, with PPI dysfunction being a consistently observed symptom of schizophrenia (Braff et al. 2001b). Absence of α3 subunit-containing receptors in mice induces a hyperdopaminergic phenotype that results in a serious sensorimotor gating deficit (Yee et al. 2005), suggesting that these receptors are found mainly on dopaminergic neurons. Pharmacological studies have also shown sedative, anxiolytic and cognitive effects are generally mediated by α1, α2/3 and α5 subunits respectively (Atack 2005, D’Hulst et al. 2009).

GABA<sub>A</sub> receptors with α5 subunits are of particular interest in the field of cognition and especially cognitive enhancement due to their localisation pattern within the brain and encouraging data from preclinical studies (described in chapter 3.1.2). Immunoreactivity for the α5 subunit is localised in the hippocampal CA1 and CA3 regions, with medium levels of expression in the deeper layers (V-VI) of the cortex. Even though α5 subunits are found in only 5% of GABA<sub>A</sub> receptors, they are present in 15-20% of receptors in the hippocampus (Sur et al. 1999, Ali and Thomson 2008). As these regions are intimately involved in cognition, this suggests a vital role for GABA<sub>A</sub> receptors containing α5 subunits in cognitive processes. Electrophysiology experiments have implicated the bitufted class of interneurons (including Martinotti cells) in mediating the neocortical effects of α5 subunit-containing receptors (Ali and Thomson 2008), suggesting a cell-specific mechanism for the effects of α5 subunit-
selective pharmaceuticals, although further experimentation is needed to confirm this hypothesis.

3.1.1.2 Neurosteroid binding site-containing receptors

Another major class of GABA<sub>A</sub> receptors are those that bind and potentiate the actions of neuroactive steroids. Replacement of the γ2 subunit by a δ subunit has been shown to increase the affinity of the resulting receptor for neurosteroids (Belelli and Lambert 2005) and δ subunit KO mice show reduced responses to the neurosteroids alphaxalone, pregnanolone and ganaxalone (Mihalek et al. 1999). GABA<sub>A</sub> receptor subunits α4 and α6 preferentially assemble with the δ subunit (Korpi et al. 2002) in an anatomically distinct manner, with α4δ receptor predominating in the forebrain and α6δ receptors forming a large proportion of receptors in the cerebellum. The delta subunit is expressed at high levels in the cerebellum, hippocampus and thalamic regions (Laurie et al. 1992a, Laurie et al. 1992b). As well as colocalising, these receptor subunits (α4, α6, δ) also influence the expression of the other subunits. Support for this comes from observations that knocking out the genes for the α4 or α6 subunit cause reductions in the levels of the δ subunit in the hippocampus (Sabaliauskas et al. 2012), and cerebellum (Peng et al. 2002) respectively. A similar result is seen when the δ subunit is knocked out, as this reduces the levels of the α4 subunit brain, without altering the levels of a non-extrasynaptic subunit (α1).

Neurosteroids are steroids that are synthesised from a cholesterol precursor within neurons and glial cells and which influence neurotransmission. Neurosteroids such as allopregnanolone and pregnenolone are negative allosteric modulators of the GABA<sub>A</sub> receptor and are strongly linked with cognition. Hippocampal neurosteroid binding site receptors (α<sub>4</sub>βδ) have been implicated in spatial learning and memory, as well as in LTP induction in preclinical rodent models (Shen et al. 2010). The expression of α<sub>4</sub>δ GABA<sub>A</sub> receptors increases during puberty in both rodents and humans, making these receptors a target for neurological insults during this critical period of development. In line with α<sub>4</sub>δ receptors being dysfunctional in schizophrenia, a small clinical study (n=58 patients) (Ritsner 2010) showed that administering pregnenolone to patients improves positive symptoms, attention and working memory.
### 3.1.2 GABA<sub>A</sub> receptor modulators and cognition

Post-mortem studies in patients with schizophrenia have shown reductions in cortical GABA<sub>A</sub> subunit expression compared to matched controls, as shown in table 3.1.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Brain Region</th>
<th>Observation</th>
<th>Antipsychotics</th>
<th>References</th>
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<td>11/14</td>
<td>Hashimoto et al. 2008£</td>
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<td>Huntsman et al. 1998*</td>
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<td></td>
<td></td>
<td>11/14</td>
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</tr>
<tr>
<td>δ</td>
<td>DLPFC</td>
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<td></td>
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<td>11/14</td>
<td>Hashimoto et al. 2008£</td>
</tr>
</tbody>
</table>

**Table 3.1:** Alterations in GABA<sub>A</sub> subunit transcription in patients with schizophrenia.

Notation: $ - determined by microarray, £ - determined by microarray and qRT-PCR, # - determined by immunoreactivity * - shortened γ2 transcript only, NSI – no specific antipsychotic information was given

The large amount of post-mortem evidence supporting the GABAergic hypothesis of schizophrenia (Tables 3.1 and 4.2), has led to the hypothesis that GABA<sub>A</sub> receptor-specific pharmacological compounds could be used as potential treatments for the disease. Historically, large doses of the benzodiazepine diazepam were used to treat schizophrenia (Carpenter et al. 1999), although recent research has focused on subunit-specific compounds as these show fewer side-effects, such as sedation, which is often caused by compounds having affinity at the GABA<sub>A</sub> α1 subunit (Atack 2005).

Currently no GABAergic compounds have been approved for the treatment of schizophrenia; BL-1020 showed promise in early stage clinical trials (Geffen et al. 2012), however this compound was discontinued following a phase II/III trial due to low efficacy.
Specific agonists for the α5 subunit have shown promise in increasing cognition in animal models, especially in the domain of working memory (Collinson et al. 2006, Dawson et al. 2006) and fear associative memory (Crestani et al. 2002). RO4882224 and RO4938581 have shown promise as α5 inverse agonists in treating spatial working memory deficits (Knust et al. 2009) in rodents. Other GABA_A receptor agonists have shown mixed results in reversing impairments in cognition in animal studies: work in our group has shown that a positive modulator of extrasynaptic GABA_A receptors can ameliorate sub-chronic PCP-induced recognition memory deficits in rats (Damgaard et al. 2011). Gabapentin (a GABA analogue) has shown promise in improving spatial and social learning in rats (Celikyurt et al. 2011) and CGP-36742 (GABA_B receptor antagonist) improves performance of both rodents and monkeys in learning and memory paradigms (Mondadori et al. 1993). BL-1020, a GABA_A receptor agonist with dopaminergic antagonist activity, improves recognition memory and reversal learning deficits in the sub-chronic PCP rodent model of cognitive deficits with relevance to schizophrenia (Neill et al. 2010). However clinical results for MK-077 (a GABA α2/α3 partial agonist) have been mixed, with one study showing no effect on visual memory and problem-solving task success (Buchanan et al. 2011) and a previous study of similar size (using similar drug dosages) finding pro-cognitive benefits of MK-077 administration on working memory in patients (Lewis et al. 2008). An inverse agonist at α5 receptors (α5IA) improves alcohol-induced recall memory deficits in healthy volunteers (Nutt et al. 2007), but it was prevented from entering clinical trials due to renal toxicity issues (Atack 2010).

### 3.2 Aim

As dysfunction in GABAergic transmission (Cho et al. 2006, Gonzalez-Burgos and Lewis 2008) and gene expression (Hashimoto et al. 2008) is thought to be central to schizophrenia pathophysiology, the aim of this study is to investigate the effect of sub-chronic PCP administration on GABA_A receptor subunit transcription and the number and composition of functional GABA_A receptors in regions of the brain known to be affected in schizophrenia. Transcription of the GABA_A receptor subunit genes GABRA1-5, GABRD and GABRG2 was measured using subunit-specific qRT-PCR primer assays. GABA_A receptor number was measured using the general BZ-site specific radioligand [3H]-flumazenil (specific for GABA_A receptors containing α1, 2, 3 or 5
subunits) and the α5 subunit-containing receptor specific radioligand \[^{3}H\]-L-655,708. GABA\(_{A}\) receptors containing α5 subunits were of particular interest because they have been strongly implicated in cognition, as described above, due to their strong localisation in areas important for cognition such as the hippocampus and cortex, and preclinical studies showing that α5-subunit inverse agonists have a cognitive enhancing effect, particularly in the domain of working memory (Collinson et al. 2006).

3.3 Methods

3.3.1 Animals and dosing regimen

Animals used in these studies were housed (chapter 2.1) and treated with sub-chronic PCP or vehicle (chapter 2.2) as previously described in chapter 2 of this thesis. Following drug administration (and washout period), rats were sacrificed by overdose of CO\(_{2}\) (chapter 2.3).

3.3.2 qRT-PCR

Brains from vehicle- (n=5) and sub-chronic PCP- (n=5) treated female rats were removed and dissected on ice into four regions for qRT-PCR studies using the methods described in detail in chapters 2.3 and 2.4. RNA was extracted and purified using the protocols in chapters 2.5.1 and 2.5.2 before being converted to cDNA as described in chapter 2.5.3. qRT-PCR analysis of GABA\(_{A}\) receptor subunit gene mRNA levels was carried out as described in chapter 2.5.4. Statistical analyses to compare sub-chronic PCP and vehicle groups were performed as described in chapter 2.5.5.

3.3.3 Radioligands

Binding affinities for \[^{3}H\]-flumazenil and \[^{3}H\]-L-655,708 for the different GABA\(_{A}\) α subunits is shown in table 2.4 (chapter 2). \[^{3}H\]-flumazenil was chosen because it is a highly specific radioligand with very low non-specific binding which is able to detect changes in benzodiazepine site numbers at similar sensitivity to \textit{in vivo} studies (Atack et al. 2006). \[^{3}H\]-L-655,708 was chosen as it has a high specificity for GABA\(_{A}\) α5 subunits, which have been closely linked to cognitive ability.
3.3.4 Radioligand binding

Brains from vehicle (n=14) and sub-chronic PCP (n=14) treated female rats were removed and dissected on ice for radioligand binding studies as described in chapters 2.3 and 2.4.

Membrane fractions were prepared from all regions using the method described in chapter 2.6.2. Radioligand binding studies in frontal cortex using \[^{3}\text{H}]\)-flumazenil and \[^{3}\text{H}]\)-L-655,708 were carried out as described in chapter 2.6.3 and 2.6.4.

Statistical analysis of the binding characteristics (K_D and Bmax) of the two radioligands was carried out using the method described in chapter 2.6.5.

3.3.5 Autoradiography

Brains from vehicle- (n=3 pre-washout and n=3 post-washout) and sub-chronic PCP- (n=3 pre-washout and n=3 post-washout) treated female rats were removed and frozen in iso-pentane at -30°C for autoradiographical analysis as described in chapters 2.3 and 2.7.2. Sagittal slices were cut to a thickness of 20μm and mounted onto glass microscope slides as previously described (Chapter 2.7.2). Autoradiographical analysis of \[^{3}\text{H}]\)-flumazenil and \[^{3}\text{H}]\)-L-655,708 binding was carried out using the method described in chapters 2.7.2 and 2.7.3 of this thesis.

3.3.6 Data analysis

Specific regions were delineated according to figure 2.3 and analysed using the method described in chapter 2.7.4 of this thesis. Statistical analyses comparing sub-chronic PCP and vehicle groups, as well as pre- and post-washout were carried out according to the details in chapter 2.7.5.

3.4 Results

3.4.1 qRT-PCR

Effect of sub-chronic PCP administration on GABA_A subunit gene (GABRA1-5, GABRG2 and GABRD) mRNA levels in the frontal cortex of female hooded-Lister rats

Unpaired t-test analysis revealed that sub-chronic PCP administration caused significant reductions in the mRNA levels of the GABRA3 (t7=6.49; p<0.01) and GABRD (t8=3.32; p<0.05) genes in the frontal cortex of female rats. A significant increase (t8=2.57; p<0.05) in the mRNA level of the GABRA5 gene was also observed in the
frontal cortex of sub-chronic PCP treated animals, when compared to vehicle animals. No significant differences were observed in the mRNA levels of the GABRA1, GABRA2, GABRA4 or GABRG2 genes between vehicle and sub-chronic PCP-treated groups (Figure 3.1a).

**Effect of sub-chronic PCP administration on GABA\(_A\) subunit gene (GABRA1-5, GABRG2 and GABRD) mRNA levels in the hippocampus, striatum and cerebellum of female hooded-Lister rats**

Unpaired t-test analysis of RQ values revealed that sub-chronic PCP administration did not affect the hippocampal, striatal or cerebellar transcription of any of the GABA\(_A\) receptor subunit genes measured in this study. No significant differences were observed in the mRNA levels of any of the genes assayed between sub-chronic PCP and vehicle-treated animals (Figure 3.1b-d).
a) GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRG2, GABRD

Relative Quantification

Frontal Cortex

Gene

Vehicle | PCP

b) GABRA1, GABRA2, GABRA3, GABRA4, GABRA5, GABRG2, GABRD

Relative Quantification

Hippocampus

Gene

Vehicle | PCP
Figure 3.1: The effect of sub-chronic PCP (2mg/kg i.p.) and vehicle (0.9% NaCl i.p.) on the transcription of a variety of GABA<sub>A</sub> receptor subunit genes in female hooded-Lister rat frontal cortex (a), hippocampus (b), striatum (c) and cerebellum (d). Data are shown as RQ (relative quantification) values ± S.E.M. (n=3-5 per treatment group). *p<0.05, **p<0.01; significant difference in mRNA levels compared to vehicle treated animals.
3.4.2 [³H]-flumazenil radioligand binding

Effect of membrane concentration (0-40µg/assay) on the binding of 1nM [³H]-flumazenil in vehicle-treated female hooded-Lister rat vehicle-treated and PCP-treated frontal cortex membrane preparations

Before saturation assays could be carried out it was necessary to determine the optimal protein concentration to use. Figure 3.2 shows a tissue dilution curve for vehicle membranes. These data show very low blank values which are typical of the [³H]-flumazenil assay. In this tissue dilution study, 10µg of protein per assay was the minimum amount sufficient to generate a high specific binding value, relative to the blank conditions (cold flunitrazepam at 10µM). Subsequently, this was the amount of protein that was used in the saturation assays.

![Graph showing the effect of membrane concentration on binding](image)

**Figure 3.2**: The effect of increasing vehicle-treated frontal cortical membrane preparation concentration (0-40µg/assay) on the binding of 1nM [³H]-flumazenil. Data are shown as mean ± S.D. 10µg of protein per assay was determined to be optimal for the saturation assays.

Effect of sub-chronic PCP administration on the binding of the tritiated benzodiazepine-site specific radioligand [³H]-flumazenil in female hooded-Lister rat frontal cortex membrane preparations

Unpaired t-test analysis revealed that sub-chronic PCP treatment produced no significant alterations in $K_D$ (NS, $t_6$= 1.36, Figure 3.3a) or $B_{max}$ (NS, $t_7$=0.74, Figure 3.3b) in female rat frontal cortex compared to vehicle animals. This indicates that there are no gross changes in the composition of, or number of benzodiazepine site-containing
(α1, 2, 3 or 5 subunit-containing) GABA<sub>A</sub> receptors in the sub-chronic PCP animals when compared to the vehicle group.

**Figure 3.3**: The effect of sub-chronic PCP and vehicle on [³H]-flumazenil binding in female rat frontal cortices. Data are shown as the mean ± S.E.M (n=4-5 per treatment group). No significant differences were observed between treatment groups in either K<sub>D</sub> (a) or B<sub>max</sub> (b) values (Figure 3.3a and 3.3b).
3.4.3 [³H]-flumazenil autoradiography

Effect of sub-chronic PCP administration, and presence or absence of a washout period, on the binding of the tritiated benzodiazepine-site specific radioligand [³H]-flumazenil in frontal cortical, cerebral cortical, hippocampal, striatal, thalamic and cerebellar regions of sagittal sections of female hooded-Lister rat brain

One-way ANOVA analysis of the $K_D$ values revealed no significant drug or washout effect on the total number of benzodiazepine-site containing GABA$_A$ receptors in the frontal cortex ($F_{3,12}= 0.79$; NS; Figure 3.4a), cerebral cortex ($F_{3,12}=0.83$; NS; Figure 3.5a), hippocampus ($F_{3,12}=0.3$; NS; Figure 3.6a), striatum ($F_{3,12}=0.31$; NS; Figure 3.7a), thalamus ($F_{3,12}=0.97$; NS; Figure 3.8a) or cerebellum ($F_{3,12}=1.07$; NS; Figure 3.9a). Planned post-hoc Students’ t-test analyses showed no significant difference between the $K_D$ values for the vehicle animals (pre-washout) and the $K_D$ values for the other treatment groups in any of the regions tested.

One-way ANOVA analysis of the $B_{max}$ values revealed no significant drug or washout effect on the total number of bz-site containing GABA$_A$ receptors in the frontal cortex ($F_{3,12}= 0.23$; NS; Figure 3.4b), cerebral cortex ($F_{3,12}=0.51$; NS; Figure 3.5b), hippocampus ($F_{3,12}=0.36$; NS; Figure 3.6b), striatum ($F_{3,12}=2.85$; NS; Figure 3.7b), thalamus ($F_{3,12}=1.02$; NS; Figure 3.8b) or cerebellum ($F_{3,12}=0.22$; NS; Figure 3.9b). Planned post-hoc Students’ t-test analyses showed no significant difference between the $B_{max}$ values for the vehicle animals (pre-washout) and the $B_{max}$ values for the other treatment groups in any of the regions tested.
Vehicle no washout  
PCP no wash-out  
PCP washout  
Vehicle washout

Frontal cortical $K_D$ values for PCP and vehicle-treated rats, pre- and post-washout

Treatment

Vehicle no washout  
PCP no wash-out  
PCP washout  
Vehicle washout

Frontal cortical $B_{max}$ values for PCP and vehicle-treated rats, pre- and post-washout

Treatment

Figure 3.4: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on frontal cortical $[^3H]$-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
**Figure 3.5:** The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on cerebral cortical $[^3]$H-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or Bmax (b) values.
a) Hippocampal $K_D$ values for PCP and vehicle-treated rats, pre- and post-washout

b) Hippocampal $B_{max}$ values for PCP and vehicle-treated rats, pre- and post-washout

**Figure 3.6:** The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on hippocampal $[^3H]$-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
Figure 3.7: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on striatal $[^3]H$-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
Figure 3.8: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on thalamic $[^3]$H-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
Cerebellar $K_D$ values for PCP and vehicle-treated rats, pre- and post-washout

![KD Values Graph](image)

Treatment

Cerebellar $B_{max}$ values for PCP and vehicle-treated rats, pre- and post-washout

![Bmax Values Graph](image)

Treatment

**Figure 3.9:** The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on cerebellar $[^3]$H-flumazenil binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
3.4.4 $[^3]H\text{-}L\text{-}655,708$ radioligand binding

**Effect of membrane concentration (0-100µg/assay) on the binding of 2nM $[^3]H\text{-}L\text{-}655,708$ in vehicle-treated female hooded-Lister rat vehicle-treated and PCP-treated frontal cortex membrane preparations**

Before saturation assays could be carried out it was necessary to determine the optimal protein concentration to use. Figure 3.10 shows a tissue dilution curve for vehicle membranes. These data show blank values typical of the $[^3]H\text{-}L\text{-}655,708$ radioligand binding assay. According to this data, 75µg of protein per assay was the minimum amount sufficient to generate a high specific binding value, relative to the blank conditions (cold flunitrazepam at 10µM), subsequently this was the amount of protein used in the saturation assays.

![Figure 3.10](image)

Figure 3.10: The effect of increasing vehicle-treated female hooded-Lister rat frontal cortical membrane preparation concentration (0-100µg/assay) on the binding of 2nM $[^3]H\text{-}L\text{-}655,708$. Data are shown as mean ± S.D. 75µg of protein per assay was determined to be optimal for the saturation assays.

**Effect of sub-chronic PCP administration (2mg/kg i.p.) on the binding of the tritiated GABA$_A\alpha5$-subunit-specific radioligand $[^3]H\text{-}L\text{-}655,708$ in female hooded-Lister rat frontal cortex membrane preparations**

T-test analysis revealed that sub-chronic PCP caused no significant changes in either $K_D$ (NS, $t_7=0.63$, Figure 3.11a) or $B_{max}$ (NS, $t_7=0.39$, Figure 3.11b) values, indicating that there was no significant change in the number or composition of GABA$_A\alpha5$ subunit-containing receptors in the female rat frontal cortex after sub-chronic PCP treatment.
Figure 3.11: The effect of sub-chronic PCP and vehicle on $[^3\text{H}]$-L-655,708 binding in individual female hooded-Lister rat frontal cortices. Data are shown as the mean ± S.E.M. (n=3-5 per treatment group). No significant differences were observed between treatment groups in either $K_D$ values (a) or $B_{\text{max}}$ values (b).

3.4.5 $[^3\text{H}]$-L-655,708 autoradiography

Autoradiography analysis of the striatum and thalamus with $[^3\text{H}]$-L-655,708 could not be carried out due to very low radioligand binding in these regions. This was anticipated due to the low number of $\alpha_5$-subunit containing receptors in these brain regions so these regions were not included in the analyses.
Effect of sub-chronic PCP and presence or absence of a washout period on the binding of the tritiated GABA α5-subunit-specific radioligand [³H]-L-655,708 in the frontal cortical region of sagittal sections of female hooded-Lister rat brain

One-way ANOVA analysis of the Bmax values revealed no significant drug or washout effect on the total number of α5-subunit containing GABA<sub>A</sub> receptors in the frontal cortex (F<sub>3,12</sub>= 0.72; NS; Figure 3.12a). Planned post-hoc Students` t-test analysis showed no significant difference between the Bmax values for the vehicle animals (pre-washout) and the Bmax values for the other treatment groups.

Analysis by one-way ANOVA of the K<sub>D</sub> data revealed that drug administration or the presence of a washout period had a significant effect on the affinity of the receptors in the frontal cortex for [³H]-L-655,708 (F<sub>3,12</sub>= 6.48; p<0.01; Figure 3.12b). Planned post-hoc analysis by Students` t-test showed a significant reduction in K<sub>D</sub> (p<0.01) in [³H]-L-655,708 binding in the frontal cortices of sub-chronic PCP-treated animals compared to vehicle-treated animals before the washout period. This difference did not reach significance in animals that had completed the washout period.

Effect of sub-chronic PCP and presence or absence of a washout period on the binding of the tritiated GABA α5-subunit-specific radioligand [³H]-L-655,708 in the cerebral cortical region of sagittal sections of female hooded-Lister rat brain

One-way ANOVA analysis of the Bmax values revealed no significant drug or washout effect on the total number of α5-subunit containing GABA<sub>A</sub> receptors in the cerebral cortex (F<sub>3,12</sub>= 1.66; NS; Figure 3.13a). Planned post-hoc Students` t-test analysis showed no significant difference between the Bmax values for the vehicle animals (pre-washout) and the Bmax values for the other treatment groups.

Analysis by one-way ANOVA of the K<sub>D</sub> data revealed a significant drug or washout effect on the affinity of the receptors in the cerebral cortex for [³H]-L-655,708 (F<sub>3,12</sub>= 5.29; p<0.05; Figure 3.13b). Planned post-hoc analysis by Students` t-test showed a significant reduction in K<sub>D</sub> (p<0.05) in the sub-chronic PCP animals before the washout, compared to the vehicle animals at the same time point. This difference did not reach significance in animals after the washout period has elapsed.
Effect of sub-chronic PCP and presence or absence of a washout period on the binding of the tritiated GABA α5-subunit-specific radioligand \[^3\text{H}\]-L-655,708 in the hippocampal region of sagittal sections of female hooded-Lister rat brain

One-way ANOVA analysis of the Bmax values revealed no significant drug or washout effect on the total number of hippocampal α5-subunit containing GABA\(_A\) receptors (\(F_{3,12} = 1.89\); NS; Figure 3.14a). Planned post-hoc Students’ \(t\)-test analysis showed no significant difference between the Bmax values for the vehicle animals (pre-washout) and the Bmax values for the other treatment groups.

Analysis by one-way ANOVA of the \(K_D\) data revealed no significant drug or washout effect on the affinity of the receptors in the hippocampus for \[^3\text{H}\]-L-655,708 (\(F_{3,12} = 1.2\); NS; Figure 3.14b). Planned post-hoc analysis by Students’ \(t\)-test showed no significant difference between the \(K_D\) values of the vehicle animals (pre-washout) and the \(K_D\) values of the other treatment groups.

Effect of sub-chronic PCP and presence or absence of a washout period on the binding of the tritiated GABA α5-subunit-specific radioligand \[^3\text{H}\]-L-655,708 in the cerebellar region of sagittal sections of female hooded-Lister rat brain

One-way ANOVA analysis of the Bmax values revealed no significant drug or washout effect on the total number of α5-subunit containing GABA\(_A\) receptors (\(F_{3,12} = 0.44\); NS; Figure 3.15a). Planned post-hoc Students’ \(t\)-test analysis showed no significant difference between the Bmax values for the vehicle animals (pre-washout) and the Bmax values for the other treatment groups.

Analysis by one-way ANOVA of the \(K_D\) data revealed no significant drug or washout effect on the affinity of the receptors in the cerebellum for \[^3\text{H}\]-L-655,708 (\(F_{3,12} = 1.57\); NS; Figure 3.15b). Planned post-hoc analysis by Students’ \(t\)-test showed no significant difference between the vehicle animals (pre-washout) and the other treatment groups.
**Figure 3.12**: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on frontal cortical $[^3]$H-L-655,708 binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). **p<0.01; significant reduction in the sub-chronic PCP $K_D$ value (a) compared to the vehicle group at the same time point, no significant difference between the groups was observed in Bmax (b) values.
Figure 3.13: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on cerebral cortical [3H]-L-655,708 binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). *p<0.05; significant reduction in the sub-chronic PCP $K_D$ value (a) compared to the vehicle group at the same time point; #p=0.06; reduction in the sub-chronic PCP Bmax value (b) compared to the vehicle group at the same time point which approached statistical significance.
Figure 3.14: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on hippocampal $[^3]$H-L-655,708 binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
Figure 3.15: The effect of sub-chronic PCP (2mg/kg b.i.d. i.p. for 7 days followed by a 7-day washout period) or vehicle (0.9% NaCl b.i.d. i.p. for 7 days followed by a 7-day washout period) on cerebellar $[^3]$H-L-655,708 binding on 20µm sagittal thaw-mounted female hooded-Lister rat brain sections. Data are shown as the mean ± S.E.M (n=3 per treatment group). No significant differences were observed between treatment groups at the same time points in $K_D$ (a) or $B_{max}$ (b) values.
3.5 Discussion

The data presented in this chapter shows that the α3 subunit is transcriptionally down-regulated specifically in the frontal cortex, although no changes in receptor binding were observed, as measured by [3H]-flumazenil binding. As α3 subunit-containing receptors only make up a small percentage of synaptic GABA<sub>A</sub> receptors in the cortex compared to receptors containing an α1 subunit (McKernan and Whiting 1996), the [3H]-flumazenil assay may lack the specificity to detect subtle alterations in α3-subunit levels. No changes in GABA<sub>A</sub> receptor subunit transcription or GABA<sub>A</sub> receptor composition were observed in the hippocampus, striatum, thalamus or cerebellum of sub-chronic PCP-treated animals, compared with vehicle-treated animals.

The data presented in this chapter demonstrates that the α5 and δ subunits are alternatively transcriptionally up- and down-regulated following sub-chronic PCP administration. Significant differences were observed in autoradiographical α5-subunit receptor binding, prior to the washout period, but no differences were observed after the washout period. These data suggests that the composition of α5-subunit containing extrasynaptic GABA<sub>A</sub> receptors changes during the washout period immediately following the cessation of sub-chronic PCP administration. No differences were observed between sub-chronic PCP and vehicle-treated animals in the transcription of the α5 or δ subunits in the hippocampus, striatum or cerebellum and no alterations in receptor binding were seen in these regions.

3.5.1 qRT-PCR

The qRT-PCR data presented in this chapter shows sub-chronic PCP administration causes reductions in the mRNA levels of the α3 and δ subunits and an increase in the level of α5 subunit mRNA in the frontal cortex of female rats. There were no significant changes in the mRNA levels of the α1, α2, α4 and γ2 subunits after sub-chronic PCP administration. No alterations in mRNA levels were observed in the cerebellum, striatum or hippocampus. Larger errors were observed in the hippocampus and cerebellum, which could contribute to the lack of effect seen in these regions. High errors that were observed in the cerebellum, especially in the expression of the α5 subunit, which may be due to the low expression of this subunit in cerebellar GABAergic receptors. In the cerebellum the expression of α6 subunit
containing receptors predominates over other subtypes. Subtle changes in mRNA levels have been recently shown to be difficult to demonstrate accurately with a single reference gene, especially using tissue samples as were used in this study (Bustin et al. 2013). Future qRT-PCR studies should utilise a second brain-specific reference gene such as CYC1 or Rpl13a to improve reproducibility and accuracy in assays where mRNA has been extracted from tissue.

Preclinical studies involving rodents have shown mixed results with regards to alterations in cortical GABA<sub>A</sub> receptor mRNA levels after prolonged NMDA receptor antagonism. Chronic PCP (7.5mg/kg i.p. for 14 days) administration resulted in decreases in the levels of the β2/3 subunit mRNAs in cortical regions in rats, with α subunit expression only being affected by an acute PCP regime (Abe et al. 2000). Tan and colleagues demonstrated that chronic NMDA antagonism (30mg/kg ketamine, 1 month) resulted in transcriptional up-regulation of the GABA<sub>A</sub> α5 subunit in the prefrontal cortex of mice (Tan et al. 2011), with no increase in α1, 2 or 4. These findings are very similar to the findings in this chapter and were confirmed by microarray, as well as by western blotting in the Tan study (Tan et al. 2011). The differences in the findings of the studies listed above could be attributed to dosing time (7 days vs >7 days), the nature of the NMDA receptor antagonist used (ketamine vs PCP) or species used (rats vs mice). There are further difficulties when comparing the findings in these studies, due to confounding factors such as behavioural testing and varying washout periods. In the Tan study mice were left for four days before they were sacrificed, during which they underwent behavioural analysis using the Morris water maze, which may have influenced gene expression. The animals in the Abe study were sacrificed 24hrs after the final administration of PCP, whereas the data in this chapter is from animals that had a 7-day drug-free washout period. The Abe study data may therefore be confounded by the acute effects of PCP being present. This highlights the need for consistency across studies, as well as confirmation of findings using a variety of techniques in order to better understand the mechanisms of schizophrenia.

3.5.2 Radioligands

[<sup>3</sup>H]-flumazenil has a nanomolar affinity for the benzodiazepine site of GABA<sub>A</sub> receptors with an α1, 2, 3 or 5 subunit and a low affinity for α4/α6 subunit containing receptors, indicating specific binding at these receptor subtypes. [<sup>3</sup>H]-L-655,708 has a
>30x higher affinity for GABA<sub>A</sub> receptor α5 subunits over α1, α2 or α3 subunits with a negligible affinity for α4 and α6 subunits of the GABA<sub>A</sub> receptor, indicating that both radioligands that were used in this study have very specific binding profiles, minimising the possibility of detecting alterations in receptors other than the target populations.

### 3.5.3 [<sup>3</sup>H]-flumazenil

The results presented here show that [<sup>3</sup>H]-flumazenil binding is not significantly affected by sub-chronic PCP treatment, demonstrated by the lack of significant differences between the K<sub>D</sub> and B<sub>max</sub> values in the vehicle and PCP groups, as measured by autoradiography and radioligand binding. This lack of significant change was observed before and after the washout period. These findings are in agreement with other studies showing that chronic administration of PCP (14 days, 7.5mg/kg once daily, 24hr washout) does not alter the number or composition of GABA<sub>A</sub> receptors containing a benzodiazepine site, measured using [<sup>3</sup>H]-flunitrazepam, in any of the regions studied in this chapter (Abe et al. 2005). Alterations in [<sup>125</sup>I]-iomazenil (a flumazenil analogue) binding were observed in specific cortical areas (parietal and piriform cortices) after chronic (14 days, once daily, 24hr washout) phencyclidine in rats (Kaneko et al. 1996), but no alterations were observed in frontal cortical regions. This suggests that localised cortical areas are being affected by PCP in the period immediately following the final injection, but no widespread alterations are occurring in the frontal cortex as a whole. The changes observed in these two studies were seen after prolonged PCP exposure (14 days vs 7 days), although the areas affected in the Kaneko study were not investigated in other studies, making comparisons between these studies difficult. These studies also only investigated the effects of sub-chronic PCP prior to the washout period, but both studies showed no alterations in benzodiazepine-site containing GABA<sub>A</sub> receptors, supporting the findings of this chapter. Administration of PCP for 7 days (4.5mg/kg b.i.d. i.p., followed by a 10-14 day washout) did increase the total number of GABA<sub>A</sub> receptors (Beninger et al. 2010), measured by [<sup>3</sup>H]-muscimol binding in male Sprague-Dawley rats. This PCP regime is almost identical to the one used here and suggests that GABA<sub>A</sub> receptors without a benzodiazepine site may be preferentially affected by sustained PCP administration.
3.5.4 [³H]-L-655,708

The data shown in this chapter shows that, immediately after sub-chronic PCP dosing was complete, there were increases in the affinity of [³H]-L-655,708 for frontal and cerebral cortical GABA_A receptors in the sub-chronic PCP-treated animals compared to the vehicle animals, indicating an increase in the proportion of α5 subunits per receptor. There was also a decrease in the number of α5-subunit-containing receptors in the cerebral cortex, although this did not achieve statistical significance (p=0.06). These alterations were not observed in animals following the washout period, as measured by either autoradiography or radioligand binding (Figures 3.8-3.12). This indicates there was no significant change in the composition or number of GABA_A α5-subunit containing GABA_A receptors in the frontal cortex of sub-chronic PCP treated animals after the washout period.

Preclinical studies involving alterations in α5 subunit expression following drug treatment in models of schizophrenia are not common, with the focus being directed towards the pro-cognitive effects of α5 subunit modulators. Chronic administration (30mg/kg for 1 month) of ketamine (an NMDA receptor antagonist) resulted in increased production of α5 subunits, as measured by Western blotting, in mice immediately after the final administration of ketamine, with no concurrent increase in α1 subunit production (Tan et al. 2011), supporting the findings in this chapter, as measured by quantitative autoradiography. The apparent decrease in the number of α5-subunits observed following the washout period in our sub-chronic PCP model was not investigated in the Tan study, making comparisons between the studies difficult.

3.5.5 Synaptic GABA_A receptors

The decrease in the transcription of the α3 subunit in the frontal cortex was observed in the sub-chronic PCP group, compared to the vehicle-treated animals. This reduction in mRNA levels was observed specifically in the GABRA3 gene. Knocking out the GABRA3 gene in mice leads to dopaminergic dysfunction and impairments in PPI (Yee et al. 2005), suggesting that a subset of GABA_A receptors containing an α3 subunit could play a role in the pathology of the sub-chronic PCP model, as well as in schizophrenia. No other significant differences were observed in other synaptic GABA_A receptor subunits (eg: α1 and α2) in the frontal cortex of the sub-chronic PCP-treated animals. This, combined with the [³H]-flumazenil binding data, indicates that sub-
chronic PCP treatment does not have a general effect on synaptic GABA<sub>A</sub> receptors. This observation is supported by clinical [\(^{125}\text{I}\)]-iomazenil (a flumazenil analogue) imaging studies which showed no significant differences in cortical BZ-binding GABA<sub>A</sub> receptor numbers between patients and controls (Abi-Dargham et al. 1999, Verhoeff et al. 1999, Ball et al. 1998). Binding studies in clinical populations have shown a consistent increase in total GABA<sub>A</sub> receptor number as measured by [\(^{3}\text{H}\)]-muscimol binding (Hanada et al. 1987, Dean et al. 1999, Verdurand et al. 2013), but when benzodiazepine-sites were specifically measured, findings have proved inconsistent with studies having shown increases (Verdurand et al. 2013) and decreases (McLeod et al. 2010) in benzodiazepine binding in the frontal cortex of schizophrenia patients. These differing clinical observations are not clarified when mRNA data is investigated. Studies have shown increases (Impagnatiello et al. 1998), decreases (Lewis et al. 2008) and no changes (Duncan et al. 2010) in synaptic receptor subunit mRNA expression.

The clinical data in this area has yielded mixed results thus far, so the role of the BZ-site containing class of synaptic GABAergic receptors in schizophrenia remains unclear. Data from preclinical studies, as described above, infers that extrasynaptic or a specific class of synaptic GABA<sub>A</sub> receptors (e.g., \(\alpha_3\beta x\) receptors), rather than benzodiazepine binding GABA<sub>A</sub> receptors in general, are more promising potential targets for symptom amelioration in schizophrenia.

### 3.5.6 Extrasynaptic GABA<sub>A</sub> receptors

The opposing transcriptional effects of sub-chronic PCP on markers of extrasynaptic GABA<sub>A</sub> receptors (\(\alpha_5, \delta\)) may be a compensatory mechanism. The autoradiography data shown in this chapter suggests an increase in the production of \(\alpha_5\) subunits in both cerebral and frontal cortical areas before the washout period, as shown by a significant increase in the affinity of GABA<sub>A</sub> receptors for the \(\alpha_5\) subunit-specific radioligand [\(^{3}\text{H}\)]-L-655,708. Data from a number of studies has shown that a decrease in \(\alpha_5\) subunit-mediated tonic GABAergic neurotransmission is associated with an increase in cognitive abilities (Atack et al. 2006, Collinson et al. 2006) and transcriptional down-regulation of the \(\delta\) subunit would be a way of reducing tonic GABAergic tone, helping to prevent a hyperGABAergic state in the cortex. The reverse scenario could also be possible, where increases in the number of \(\alpha_5\) subunits are a response to persistent transcriptional downregulation of the \(\delta\) subunit. This hypothesis
is supported by work from our laboratory showing that activation of δ-subunit-containing receptors improved sub-chronic PCP-induced recognition memory deficits in female rats (Damgaard et al. 2011). These preclinical studies suggest different modes or sites of action for the two extrasynaptic GABA$_A$ receptor modulators used in the studies highlighted above. GABA$_A$ receptors containing α5 subunits are found mostly in the hippocampus, highlighting this region as a likely site of action for α5-subunit receptor modulators, whereas the δ subunit is more ubiquitously expressed. Differential regulation of α5 and δ subunit expression is possible due to their differing chromosomal locales (GABRA5-Chr1q:22, GABRD-Chr5q:36). Further studies are needed to investigate if alterations in the binding characteristics of δ subunit-containing receptors are present before the washout period in this model, as well as studying any transcriptional differences in the α5 and δ subunits that are present before the washout period between sub-chronic PCP and vehicle animals. The lack of significant differences in the level of α4 subunit mRNA, post-washout, between PCP and vehicle-treated animals indicates that the expression of this subunit is not affected by sub-chronic PCP administration. Clinical studies of the pro-cognitive effects of inverse agonism at α5 subunit-containing receptors have shown positive findings in relation to alcohol-induced recall memory deficits (Nutt et al. 2007), although advanced clinical studies have been halted by toxicity issues with these compounds (Atack 2010). Further investigation is needed to determine the mechanisms behind extrasynaptic GABAergic neurotransmission and the therapeutic potential for modulators of extrasynaptic GABA$_A$ receptors in treating cognitive deficits in schizophrenia.

3.5.7 Conclusions

The data from this chapter show that sub-chronic PCP administration causes cortical transcriptional alterations in specific GABA$_A$ receptor subunits (α5, δ) related to extrasynaptic GABAergic neurotransmission. These transcriptional alterations are present after a 7 day washout period, although the cortical increase in α5 subunit binding observed before the washout is not present after the washout period, suggesting a post-transcriptional mechanism to reduce α5-receptor mediated neurotransmission in the cortex of female rats. A sub-chronic PCP-induced increase in α5 subunit-containing receptor number and consequent decrease in δ receptor levels,
as suggested by this data, may not have an effect on overall tonic GABAergic transmission levels, however δ-subunit-containing receptors are responsible for mediating the effects of many neuroactive steroids. A reduction in δ subunits would make cortical regions less susceptible to the effects of these steroids, which may help to explain the cognitive deficits observed in the sub-chronic PCP model. Previous behavioural work has shown that sub-chronic PCP-induced recognition memory deficits can be ameliorated by administration of a positive modulator of extrasynaptic neurotransmission (Damgaard et al. 2011), suggesting that dysfunctional tonic GABAergic neurotransmission plays a role in the cognitive deficits in this model. The data presented in this chapter suggest that this dysfunction is due to alterations in α5 or δ subunit-containing receptors, not receptors containing an α4 subunit. Further experimentation is required to ascertain if any pre-washout alterations in the composition of synaptic GABA_A receptors (α3 subunit-containing receptors) occurs in this model.

The phenotypes observed in the sub-chronic PCP model and in schizophrenia may be due to changes in localised concentrations of receptors or disruption of connectivity between certain populations of pyramidal cells and interneurons, rather than a widespread loss of GABA_A receptors. This disruption in GABAergic tone would lead to incorrect control of pyramidal cell outputs and subsequent disruptions in cortical gamma and theta rhythms, which are known to be dysfunctional in schizophrenia (Curley and Lewis 2012).
Chapter 4

Investigation into GABAergic marker alterations in the sub-chronic PCP model
4.1 Introduction

Working memory deficits are a key symptom domain of schizophrenia and have been linked to increased negative symptom severity in patients (Brazo et al. 2002, Harvey et al. 2006). Gamma (γ) and theta (θ) oscillatory activity is induced during working memory tasks in humans and the power of these two frequencies increases as working memory load increases (Howard et al. 2003). Schizophrenia patients show reduced levels of θ and γ oscillatory power during working memory tasks, compared to controls, and these reductions are correlated with poorer task performance (Cho et al. 2006, Minzenberg et al. 2010). θ and γ oscillations, which are neuronal network activities at 4-7Hz and 30-80Hz respectively (Fries 2009), are controlled by the synchronised activity of excitatory pyramidal cells and inhibitory GABAergic interneurons. The duration of the inhibition of the pyramidal cells is inversely related to the frequency of the oscillation (Gonzalez-Burgos and Lewis 2008).

4.1.1 GABAergic Interneurons

All GABAergic interneurons are defined by having the catabolic GABAergic enzyme glutamate decarboxylase (GAD_{65} or GAD_{67}), with GAD_{67} supplying most of the cellular non-synaptic GABA in the cortex (Battaglioli et al. 2003). GABAergic interneurons are located in all cortical layers, as well as throughout the hippocampus, and show a large degree of variability in morphology, firing patterns and expression of biochemical markers (summarised in table 4.1) (reviewed in Petilla Interneuron Nomenclature et al. 2008, Druga 2009, DeFelipe et al. 2013).
<table>
<thead>
<tr>
<th>Immunochemical marker</th>
<th>Morphological Type</th>
<th>Co-expressed molecules</th>
</tr>
</thead>
<tbody>
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<td>CB</td>
</tr>
<tr>
<td></td>
<td>Chandelier cells</td>
<td></td>
</tr>
<tr>
<td>CB</td>
<td>Double-bouquet cells</td>
<td>PV</td>
</tr>
<tr>
<td></td>
<td>Bipolar cells</td>
<td>SOM</td>
</tr>
<tr>
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<td>Bi-tufted cells</td>
<td>NPY</td>
</tr>
<tr>
<td>CR</td>
<td>Bipolar cells</td>
<td>VIP</td>
</tr>
<tr>
<td></td>
<td>Bi-tufted cells</td>
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</tr>
<tr>
<td></td>
<td>Double-bouquet cells</td>
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</tr>
<tr>
<td></td>
<td>Multipolar cells</td>
<td></td>
</tr>
<tr>
<td>SOM</td>
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<td>NPY</td>
</tr>
<tr>
<td></td>
<td>Fusiform cells</td>
<td>NOS</td>
</tr>
<tr>
<td></td>
<td>Martinotti cells</td>
<td>CR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CB</td>
</tr>
<tr>
<td>NOS</td>
<td>Bipolar cells</td>
<td>NPY</td>
</tr>
<tr>
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<td>Double-bouquet cells</td>
<td>CR</td>
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</tr>
<tr>
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<td>Martinotti cells</td>
<td>NOS</td>
</tr>
<tr>
<td></td>
<td>Small basket cells</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Morphology and expression of biochemical markers in different populations of cortical GABAergic interneurons. Adapted from Druga 2009. PV = parvalbumin, CB = calbindin, CR = calretinin, NPY = neuropeptide Y, SOM = somatostatin, NOS = nitric oxide synthase, CCK = cholecystokinin and VIP = vasoactive intestinal polypeptide.

4.1.2 Interneuron subtypes

Different populations of GABAergic interneurons, as defined by their calcium-binding protein expression profiles, innervate pyramidal cells at different regions (Figure 4.1) and are expressed at varying levels throughout the cortex (Rudy et al. 2011). In rat cortex, almost 100% of the interneurons express one of the following neurochemical markers: parvalbumin (PV), calretinin (CR) or somatostatin (SST), whereas in humans 85-100% of cortical interneurons express parvalbumin, calretinin or calbindin (CB). Parvalbumin- and calretinin-expressing interneurons form a major part of the inhibitory neuronal network in both rodents and humans.

Interneurons expressing parvalbumin are part of the perisomatic-innervating class of interneuron, which contains the basket and chandelier/axo-axonic classifications of cells. These parvalbumin-expressing interneurons innervate the axon
initial segment and cell body of pyramidal cells (see figure 4.1) (Melchitzky et al. 1999). A major group of somatostatin-containing interneurons consist of Martinotti cells that form connections with distal dendrites of pyramidal neurons (see figure 4.1) (Ma et al. 2006). Calretinin-expressing interneurons are thought to act as dis-inhibitory cells by forming connections to other interneuron types in rodent (Gulyas et al. 1996), non-human primate (Melchitzky et al. 2005) and human brains (Toth et al. 2010) (see figure 4.1).

Inhibition at the dendrites of pyramidal cells seems to regulate the plasticity of synaptic inputs, however inhibitory actions targeting the perisomatic region of the excitatory cell regulate control output, helping to synchronise the firing output of large numbers of cells. This pattern of regulation is observed in both the cortex (Somogyi et al. 1998) and the hippocampus (Freund and Buzsaki 1996). Preclinical studies have shown important roles for parvalbumin, somatostatin and calretinin-containing interneurons in oscillatory control and in cognition (Gulyas et al. 1996, Caputi et al. 2009, Lodge et al. 2009, Epelbaum et al. 2009, Lepousez et al. 2010). Decreases in the levels of parvalbumin- and somatostatin-containing interneurons, along with reductions in GAD$_{67}$, are also the most consistently observed deficits in the frontal cortex of patients with schizophrenia (see table 4.2).

Figure 4.1: Diagram showing how different populations of interneurons innervate pyramidal cells. Taken from Hashimoto et al. 2008. P – pyramidal cell, G – GABAergic interneuron, AIS – axon initial segment.
Populations of GABAergic interneurons change throughout the developmental life of humans, with somatostatin, calretinin and neuropeptide Y-containing interneurons decreasing post-natally, with a concomitant increase in parvalbumin and cholecystokinin-expressing interneurons. Calbindin and vasoactive intestinal peptide-containing interneuron numbers increase post-natally, reaching a peak at around 2 years of age and then declining (Figure 4.2 - Fung et al. 2010). Parvalbumin-positive cells proliferate rapidly around the time of puberty, compared to other types of interneurons, making them temporally vulnerable to insults occurring during adolescence, in accordance with the neurodevelopmental hypotheses of schizophrenia (see chapter 1.4.2).

Figure 4.2: Developmental transcriptional profiles of three populations of GABAergic interneuron markers in humans (Parvalbumin, Calretinin and Calbindin). Taken from Fung et al. 2010.

4.1.2 Astrocytes

Recent work has shown that astrocytes can release transmitter molecules (gliotransmitters) in a Ca$^{2+}$-dependent and exocytotic manner, as well as by reversing transporter direction (in the case of glutamate and GABA). Supporting evidence for vesicular gliotransmission comes from molecular genetic studies showing that astrocytes express vesicular transporters for neurotransmitters such as glutamate (Bezzi et al. 2004), exocytose D-serine (Mothet et al. 2005) and adenosine triphosphate (Zhang et al. 2007), as well as expressing SNARE (Soluble NSF (N-ethylmaleimide Sensitive Factor) Attachment Receptor) complex proteins such as synaptobrevin II, SNAP23 and complexin 2 (Montana et al. 2004, Zhang et al. 2004).
4.1.2.1 Astrocytes as GABAergic cells

Astrocytes, as well as releasing a number of compounds that act at glutamatergic receptors (D-serine, glutamate), have also been shown to release GABA which causes tonic inhibition of pyramidal cell activity (Lee et al. 2010). This release of astrocytic GABA is dose-dependently linked to the level of tonic inhibition in the hippocampus and cerebellum (Yoon et al. 2011). The release of GABA from astrocytes is thought to occur through reversal of the GAT-3 GABA transporter, which is present exclusively on astrocytes (Minelli et al. 1996), in contrast to GAT-1, which is preferentially located on neurons (Radian et al. 1990). This bi-directionality of the GAT-3 transporter allows astrocytes to terminate or enhance inhibitory neurotransmission by increasing or reducing the amount of GABA in the extrasynaptic space available to activate extrasynaptic GABA_A receptors or GABA_B receptors, which are both involved in tonic inhibitory neurotransmission.

Exogenous ATP depolarises both calretinin-containing interneurons and astrocytes, resulting in enhanced synaptic inhibition via P_X and P_Y receptors (Bowser and Khakh 2004). Astrocytic ATP also stimulates a wave of astrocytic excitation, resulting in long distance modulation of gliotransmission and subsequently neurotransmission over a greater distance than simple point-to-point synaptic transmission (Bowser and Khakh 2004). Through these two mechanisms astrocytes, like inhibitory interneurons, add an extra layer of complexity and control to the binary signalling of a single set of excitatory neurons.

4.1.3 Clinical findings

Various post-mortem studies conducted on tissue from schizophrenia patients have shown robust and consistent reductions in prefrontal cortical GABAergic markers (summarised in table 4.2). Reductions in GAD_67, parvalbumin and somatostatin mRNA are the most robust changes that are observed in the schizophrenia patient frontal cortex and reductions in GAT-1 mRNA are also consistently seen across multiple study groups showing that GABAergic dysfunction is a key component of schizophrenia pathophysiology.

Post-mortem studies from schizophrenia patients studying astrocytic markers, despite being rarer than those studies investigating interneuron markers, have shown
decreases in the expression of GFAP and GAT-3 in the cingulate and prefrontal cortices of schizophrenia patients (See table 4.2), as well as reduced astrocyte numbers in the PFC of schizophrenia patients (Stark et al. 2004).
<table>
<thead>
<tr>
<th>Marker</th>
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<th>Antipsychotics</th>
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<tr>
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</table>

**Table 4.2:** Cortical alterations in GABAergic markers and antipsychotic medication information of schizophrenia patients. All protein measurements were made using western blotting techniques except the following exceptions: <sup>a</sup> – autoradiography, <sup>b</sup> – immunoblotting/immunohistochemistry. (#) - includes risperidone, ($) – includes fluoxetine (---) - no details given.
4.2 Aim

Chapter 3 of this thesis shows the effect of sub-chronic PCP administration on the transcription of certain GABA<sub>A</sub> receptor subunits, as well as the composition and number of specific GABA<sub>A</sub> receptor populations. This chapter will expand on the GABA<sub>A</sub> receptor data in chapter 3 by studying the effects of sub-chronic PCP administration on markers of GABA<sub>A</sub>ergic interneurons in the rodent brain. The markers were chosen based on their clinical significance in schizophrenia, with the selected markers known to be altered in the frontal cortex of schizophrenia patients (see table 4.2). This dysfunction in inhibitory neurotransmission is hypothesised to contribute to the cognitive dysfunction seen in schizophrenia via impaired network synchronicity. The experiments presented in this chapter will investigate the effect of sub-chronic PCP administration on GABA<sub>A</sub>ergic gene transcription, in order to improve the construct validity of the sub-chronic PCP model, as well as studying any sub chronic PCP-induced protein level alterations in these markers. These studies will attempt to elucidate potential mechanisms for the cognitive dysfunction observed in this model.

4.3 Methods

4.3.1 Animals and PCP/vehicle administration

Animals used in these studies were housed (chapter 2.1) and treated with sub-chronic PCP or vehicle (chapter 2.2) as previously described in chapter 2 of this thesis. Following 7-day drug administration (and 7-day washout period), rats were sacrificed by overdose of CO<sub>2</sub> (chapter 2.3).

4.3.2 Brain dissection and cDNA production

Brains from vehicle (n=5) and sub-chronic PCP (n=5) treated female rats were removed and dissected on ice into four regions for qRT-PCR studies using the methods described in detail in chapters 2.3 and 2.4. RNA was extracted and purified using the protocols in chapter 2.5.1 and 2.5.2 and converted to cDNA as described in chapter 2.5.3.

4.3.3 qRT-PCR

qRT-PCR analysis of GABA<sub>A</sub>ergic and astrocytic gene (GFAP, GAD2, GAD1, GAT-1, GAT-3 PVALB, CALB2 and SST) mRNA levels was carried out as described in chapter 2.5.4.
4.3.4 Western blotting

Western blotting analysis of the GAD$_{67}$, calretinin, somatostatin, GAT-1 and GAT-3 protein levels was carried out as described in chapter 2.9. Analysis of GFAP and parvalbumin levels could not be analysed due to problems with the optimisation of the respective primary antibodies.

4.3.5 Data Analysis

All qRT-PCR data was normalised to a GAPDH housekeeping gene and analysed after conversion to RQ values using the 2-ΔΔCt method as described in chapter 2.5.5 of this thesis. RQ comparison between vehicle and sub-chronic PCP-treated groups was done using an unpaired t-test as described in chapter 2.5.5 of this thesis.

Western blotting data was normalised to total lane protein and analysed using Chemidoc MP imaging software as described in chapter 2.9.5.

4.4 Results

Effect of sub-chronic PCP administration on GABAergic and astrocytic marker gene (GFAP, GAD2, GAD1, GAT-1, GAT-3, PVALB, CALB2 and SST) mRNA levels in the frontal cortex of female hooded-Lister rats

Unpaired t-test analysis of RQ values revealed that sub-chronic PCP administration caused a significant reduction in the level of PVALB ($t_7=2.7$; $p<0.05$), CALB2 ($t_7=2.96$; $p<0.05$), GAD1 ($t_7=10.75$; $p<0.001$) and GAT-1 ($t_7=2.58$; $p<0.05$) mRNA in the frontal cortex, compared to vehicle levels. A significant increase ($t_6=5.86$; $p<0.01$) in GFAP mRNA was also observed in the frontal cortex of sub-chronic PCP-treated female rats, compared to vehicle-treated rats. No significant differences were observed in SST, GAT-3 and GAD2 mRNA levels between sub-chronic PCP- and vehicle-treated animals (Figure 4.1).

Effect of sub-chronic PCP administration on GABAergic and astrocytic marker gene (GFAP, GAD2, GAD1, GAT-1, GAT-3, PVALB, CALB2 and SST) mRNA levels in the hippocampus of female hooded-Lister rats

Unpaired t-test analysis of RQ values revealed that sub-chronic PCP administration caused a significant reduction in the level of GAD1 ($t_8=4.68$; $p<0.01$), GAD2 ($t_8=2.37$; $p<0.05$) and GFAP ($t_8=3.82$; $p<0.01$) mRNA in the hippocampus of sub-chronic PCP-
treated animals, compared to vehicle-treated animals. No significant differences were observed in mRNA levels of any of the other markers assayed between sub-chronic PCP- and vehicle-treated animals (Figure 4.2). There was an increase in CALB2 mRNA levels in sub-chronic PCP-treated animals that approached significance (tₙ=2.12; p=0.08).

**Effect of sub-chronic PCP administration on GABAergic and astrocytic marker gene (GFAP, GAD2, GAD1, GAT-1, GAT-3, PVALB, CALB2 and SST) mRNA levels in the striatum of female hooded-Lister rats**

Unpaired t-test analysis of RQ values revealed that sub-chronic PCP administration did not significantly affect the mRNA levels of any of the GABAergic genes in the striatum, as measured in this study. No significant differences were observed in mRNA levels of any of the markers assayed between sub-chronic PCP- and vehicle-treated animals (Figure 4.3). There was a reduction in GFAP mRNA levels in sub-chronic PCP-treated animals that closely approached significance (tₙ=2.37; p=0.06).

**Effect of sub-chronic PCP administration on GABAergic and astrocytic marker gene (GFAP, GAD2, GAD1, GAT-1, GAT-3, PVALB, CALB2 and SST) mRNA levels in the cerebellum of female hooded-Lister rats**

Unpaired t-test analysis of RQ values revealed that sub-chronic PCP administration caused a significant increase in the mRNA levels of the PVALB (tₙ=3.49; p<0.05) and GFAP (tₙ=2.56; p<0.01) genes in sub-chronic PCP-treated animals. No significant differences were observed in mRNA levels of any of the other markers (GAD1, GAD2, CALB2, SST, GAT-1 and GAT-3) between sub-chronic PCP- and vehicle-treated animals (Figure 4.4).
Figure 4.1: The effect of 7-day sub-chronic vehicle (0.9% NaCl b.i.d. i.p.) or PCP administration (2mg/kg b.i.d. i.p.) on the mRNA levels of various GABAergic cell marker genes in the frontal cortex of female hooded-Lister rats after a 7-day washout period. Data are expressed as RQ values ± S.E.M. (n=3-5 per group). *p<0.05, **p<0.01, ***p<0.001; significant alteration in mRNA levels compared to the vehicle group.

Figure 4.2: The effect of 7-day sub-chronic vehicle (0.9% NaCl b.i.d. i.p.) or PCP administration (2mg/kg b.i.d. i.p.) on the mRNA levels of various GABAergic cell marker genes in the hippocampus in female hooded-Lister rats after a 7-day washout period. Data are expressed as RQ values ± S.E.M. (n=4-5 per group). *p<0.05, **p<0.01; significant decrease in mRNA levels compared to the vehicle group, #p=0.08; non-significant increase in mRNA levels compared to the vehicle group.
Figure 4.3: The effect of 7-day sub-chronic vehicle (0.9% NaCl b.i.d. i.p.) or PCP administration (2mg/kg b.i.d. i.p.) on the mRNA levels of various GABAergic cell marker genes in the striatum of female hooded-Lister rats after a 7-day washout period. Data are expressed as RQ values ± S.E.M. (n=4-5 per group). #p=0.06; non-significant decrease in mRNA levels compared to the vehicle group.

Figure 4.4: The effect of 7-day sub-chronic vehicle (0.9% NaCl b.i.d. i.p.) or PCP administration (2mg/kg b.i.d. i.p.) on the mRNA levels of various GABAergic cell marker genes in the cerebellum of female hooded-Lister rats after a 7-day washout period. Data are expressed as RQ values ± S.E.M. (n=4-5 per group). *p<0.05, **p<0.01; significant increase in mRNA levels compared to the vehicle group.
Effect of sub-chronic PCP administration on GABAergic marker (GAD1, GAT-1, GAT-3 and SOM) protein levels in the frontal cortex of female hooded-Lister rats

Unpaired t-test analysis of densitometry values revealed that sub-chronic PCP administration did not affect the frontal cortical protein levels of GAD$_{67}$, GAT-1, GAT-3, somatostatin or calretinin, as measured in this study. No significant differences were observed in the protein levels of any of the markers studied between sub-chronic PCP- and vehicle-treated animals (Figure 4.5).

![Graph](image)

**Figure 4.5:** The effect of 7-day sub-chronic PCP (2mg/kg b.i.d. i.p.) or vehicle (0.9% NaCl b.i.d. i.p.) administration on calretinin, somatostatin, GAD$_{67}$, GAT-1 and GAT-3 protein levels in the frontal cortex of female hooded-Lister rats after a 7-day washout period. Data are expressed as relative densitometry levels after normalisation to total protein $\pm$ S.E.M. (n=4-5 per treatment group).

4.5 Discussion

The data presented in this chapter shows that there are significantly reduced levels of GAD1, PVALB and GAT-1 gene mRNA in the rat frontal cortex following sub-chronic PCP treatment, although no concomitant reduction in GAD$_{67}$ or GAT-1 protein levels were observed, as assayed by Western blotting. A significant reduction in GAD1 and GAD2 mRNA levels were also observed in the hippocampus of sub-chronic PCP-treated rats, as well as a non-significant increase in CALB2 mRNA levels.
Sub-chronic PCP treatment also significantly increases the transcription of the GFAP gene in the frontal cortex and cerebellum, with a significant down-regulation of the same gene observed in the hippocampus. A non-significant reduction in GFAP transcription was observed in the striatum of sub-chronic PCP-treated rats. Expression of the astrocytic transporter GAT-3 was unchanged at the transcriptional level in the hippocampus, frontal cortex, striatum and cerebellum and at the protein level in the frontal cortex.

4.5.1 qRT-PCR

4.5.1.1 Frontal cortex and striatum

The qRT-PCR data presented in this chapter shows that sub-chronic PCP administration causes a transcriptional down-regulation of the PVALB, CALB2, GAD1 and GAT-1 genes in the frontal cortex of female hooded-Lister rats. There were no significant differences between the levels of SST or GAD2 mRNA observed after sub-chronic PCP administration. This closely reflects the clinical mRNA findings in patients (table 4.2). The magnitude of the mRNA reductions observed in the frontal cortex of PCP-treated rats were generally larger than those observed in patients (eg: PVALB PCP – 34% reduction vs PVALB patients - 17-30% reduction), although this could be explained by the regional specificity of the post-mortem studies (DLPFC) compared to the work presented in this chapter, where the whole frontal cortex was studied. No significant changes in mRNA levels were observed for any of the GABAergic markers in the striatum.

While reductions in frontal cortical mRNA levels of GABAergic markers such as parvalbumin and GAD$_{67}$ are well-replicated findings in post-mortem studies of schizophrenia (table 4.2), preclinical studies looking at cortical mRNA levels of GABAergic markers in NMDA receptor antagonist rodent models have focussed on the parvalbumin subset of interneurons, with few studies having looked at transcriptional alterations in other gene markers such as GAD1 or GAT-1 in these models.

In agreement with the findings of this chapter, Cochran and colleagues showed that male hooded Long-Evans rats which were administered chronic intermittent PCP (2.58mg/kg/day i.p. for 14 days out of 26, with a 72hr washout) showed reductions in parvalbumin mRNA in the prelimbic cortex, with losses coming in all layers of the
prelimbic cortex, without a reduction in parvalbumin containing interneurons, suggesting that this reduction was caused by a reduced cellular production of parvalbumin mRNA rather than a loss of parvalbumin neurons (Cochran et al. 2003), an observation that was not seen in rats treated acutely with PCP (Cochran et al. 2002). This reduction in frontal cortical parvalbumin mRNA was replicated in male rats (5mg/kg/day for 5 days followed by a 3 day washout period) and male mice (10mg/kg/day for 10 days with a 3 day washout period, Thomsen et al. 2009) treated with sub-chronic PCP, as well as in cortical cell cultures (Behrens et al. 2007).

4.5.1.2 Hippocampus

The qRT-PCR data presented in this chapter shows that sub-chronic PCP administration causes a significant transcriptional down-regulation in the GAD1 and GAD2 genes in the hippocampus. There was also a non-significant (p=0.08) increase in calretinin gene transcription in the hippocampus of sub-chronic PCP treated animals. There were no observed differences in the levels of somatostatin, GAT-1 or parvalbumin transcription between vehicle and PCP-treated female rat hippocampus. Preclinical studies have focussed on transcriptional alterations in the expression of the parvalbumin gene and the few studies that have been conducted support the findings in this chapter. No alterations in hippocampal parvalbumin mRNA levels following sub-chronic NMDA receptor antagonism were observed in male hooded Long-Evans rats which were administered sub-chronic intermittent PCP (2.58mg/kg/day i.p. for 14 days out of 26, with a 72hr washout, (Cochran et al. 2003). This finding was also observed in male rats treated acutely with PCP (Cochran et al. 2002), but not in male Wistar rats acutely treated with MK-801 (1mg/kg i.p., Romon et al. 2011).

4.5.1.3 Cerebellum

Sub-chronic PCP administration also caused significant transcriptional up-regulation of the parvalbumin gene in the cerebellum. The mRNA levels of all of the other markers tested were not affected by sub-chronic PCP treatment. Few preclinical studies have been conducted into the effects of prolonged NMDA receptor antagonism on the cerebellum, with the majority of research being conducted in regions such as the hippocampal formation and cortical brain regions. However, a study by Bullock and colleagues (Bullock et al. 2009) showed that male Long-Evans rats treated with intermittent PCP (2.58mg/kg/day for 14 days out of 26, followed by a 72hr washout
period) showed reduced levels of GAD2, GAD1 and GAT-1 mRNA in cerebellar Golgi cells compared to vehicle-treated cells. This finding is in agreement with clinical post-mortem study results showing reduced cerebellar levels of GAD2, GAD1 and GAT-1 in schizophrenia patients when compared to controls (Bullock et al. 2008). This is in contrast to the findings in this chapter, which showed no transcriptional alterations in GAD1, GAD2 or GAT-1 gene expression, although this may be due to differences in gender as male rats require higher doses of PCP to cause behavioural alterations than females (Grayson 2004). These contrasting findings may also be explained by the longer duration of PCP administration (and shorter washout period) in the Bullock study, compared to this study, which is likely to have a more pronounced effect on gene transcription. No GABAergic gene expression time-course studies have been carried out in the sub-chronic PCP model so the shorter washout period used in the Bullock study (3 days instead of 7 days) may represent transcriptional alterations that do not persist following a longer washout period.

4.5.1.4 Astrocytes

Astrocytes are known to be both GABAergic and GABAceptive cells and a number of alterations in GFAP (an astrocytic marker belonging to the intermediate filament family) transcription were observed following sub-chronic PCP administration. Increased GFAP transcription was observed in the frontal cortex and cerebellum, while a significant decrease in GFAP mRNA was observed in the hippocampus. No significant changes in GAT-3 mRNA levels were seen following sub-chronic PCP administration compared to vehicle in any of the regions tested. No studies, to the authors knowledge, have been conducted that have investigated the effect of sub-chronic PCP on GFAP and GAT-3 gene transcription in rats, although work by Barzilay and colleagues showed that male mice treated with sub-chronic PCP (10mg/kg/day s.c. for 14 days) showed no hippocampal change in GFAP mRNA levels after an 8 day washout period (Barzilay et al. 2013). The difference between the findings in the Barzilay study and the study in this chapter may be due to differences in species and the gender of the animals used (female rats vs male mice) as female rodents are known to metabolise PCP differently to males (Shelnutt et al. 1999). Further confounds include the mice in the Barzilay study undergoing sham surgery, as well as behavioural testing
during the washout period; both of these may have influenced gene expression and may explain the differing conclusions of the two studies.

4.5.2 Western blotting

The results presented here show no differences in protein levels of any of the markers studied (GAT-1, GAT-3, GAD$_{67}$, calretinin and somatostatin) in the frontal cortex of female rats that were administered sub-chronic PCP, compared to vehicle rats, as measured by Western blotting.

Preclinical studies looking at the effects of repeated NMDA receptor antagonist administration on GABAergic interneuron populations have focussed mainly on the parvalbumin class of interneurons as these have consistently shown transcriptional reductions in schizophrenia patient populations. A number of studies have reported a reduction in parvalbumin-reactive interneurons in both the hippocampus and the frontal cortex of hooded-Lister rats which had been administered sub-chronically with PCP according to the same regimen used in this thesis (2mg/kg i.p. b.i.d for 7 days followed by a 7-day washout) (Abdul-Monim et al. 2007, Jenkins et al. 2008, Jenkins et al. 2010, McKibben et al. 2010). A further study by Amitai and colleagues (Amitai et al. 2012) showed male Wistar rats treated with an alternative sub-chronic PCP regimen (2mg/kg for 2 days, followed by a 10 day vehicle treatment, then 2mg/kg PCP for 5 days with a 1 day washout) also showed significant reductions in frontal cortical parvalbumin and GAD$_{67}$ reactivity, suggesting less parvalbumin and GAD$_{67}$ per cell (Amitai et al. 2012). However with this study, it is impossible to rule out the acute effects of PCP, despite the increased metabolic clearance of PCP in male rats (Shelnutt et al. 1999). This parvalbumin-specific loss of phenotype was observed in the CA3 region of the hippocampus of male Sprague Dawley rats, following sub-chronic PCP administration (3 days of 1.25mg/kg PCP followed by a 10 day washout period, Pollard et al. 2012). This selective loss of cortical parvalbumin-containing interneurons is also observed in rats treated with PCP postnatally (10mg/kg s.c. on PND 7), with this treatment having no effect on calretinin-containing interneurons in cortical or hippocampal regions (Wang et al. 2008). This observation is not limited to PCP models of schizophrenia pathology: postnatal treatment with another NMDA receptor antagonist, MK-801, on PND 7 (1mg/kg), combined with social isolation, also gives rise to cortical reductions in the number of parvalbumin-positive and GAD$_{67}$-positive
interneurons (Gilabert-Juan et al. 2013). Sub-chronic administration of ketamine (30mg/kg i.p. for 2 days followed by a 0, 3 or 10 day washout in male mice (Behrens et al. 2008), and MK-801 (0.02mg/kg for 21 days followed by a 1 day washout period in juvenile male Long-Evans rats (Braun et al. 2007), also cause reductions in parvalbumin-positive interneuron levels. The Behrens and Braun studies also support the findings in this chapter, in that they showed no alterations in calretinin-positive interneurons. Zhang and colleagues demonstrated that sub-chronic ketamine administration (30mg/kg for 2 days, followed by a 1 day washout in male Long-Evans rats) causes reductions in GAD$_{67}$ and PV in the PFC without a reduction in PV neurons suggesting a reduction in both GAD$_{67}$ and parvalbumin levels in the cell rather than a loss of parvalbumin interneurons. (Zhang et al. 2008). Parvalbumin protein levels were not studied in this body of work, making direct comparisons with these studies difficult, although future experiments should certainly include the study of parvalbumin protein levels in NMDA receptor antagonist animal models. The proteomic findings in the above studies generally agree with the work presented in this chapter, showing no alterations in calretinin-positive interneurons. Reductions in GAD$_{67}$ levels observed in previous studies have utilised densitometric techniques, combined with microscopy to ascertain the levels of GAD$_{67}$ per interneuron, whereas the work in this chapter utilised Western blotting to calculate the total amount of GAD$_{67}$ protein in the frontal cortex. The work presented in this chapter, supported by previous studies, suggests that the loss of GABAergic interneurons in the frontal cortex of rodents treated sub-chronically with NMDA receptor antagonists is limited to the parvalbumin class of interneurons, with no significant differences observed in other classes. All GABAergic interneurons express the GAD$_{67}$ protein, therefore if the loss of GAD$_{67}$ in the sub-chronic PCP model is confined to the parvalbumin class of interneuron, the global change in GAD$_{67}$ levels (as measured by Western blotting) may be too subtle to be detected by this technique. The lack of alteration in total GAD$_{67}$ protein level in the frontal cortex of sub-chronic PCP rodents supports the hypothesis that reductions in this enzyme are cell-type specific. Given that parvalbumin-expressing interneurons are expressed predominantly in deeper cortical layers (Rudy et al. 2011), any insult to this population of neurons would result in increased dysfunction in circuitry originating from deeper cortical layers.
The work shown in this chapter shows significant alterations in GFAP expression at the transcriptional level, however protein levels were not investigated in the current study. The astrocyte-specific transporter GAT-3 was investigated and showed no alterations in either mRNA or protein levels in the regions that were studied. Very few studies have looked at the impact of sub-chronic NMDA antagonism on astrocyte number or GFAP expression in rodents. Female Sprague-Dawley rats that were administered 20mg/kg i.p. PCP for 5 days, followed by a 72hr washout period and a final challenge of 3.2mg/kg PCP showed an increase in GFAP-positive cells in layer 2 of the piriform cortex (Johnson et al. 1998), but this study did not look at the impact of sub-chronic PCP administration without the final acute PCP challenge, making it difficult to draw accurate conclusions about the impact of sub-chronic PCP treatment alone. Other models, including prenatally administered PCP have shown no alterations in frontal cortical GFAP protein levels at PND9, 16 or 22 following sub-chronic PCP exposure (gestational day 9-20 in Sprague Dawley rats), although a significant increase in GFAP protein levels was observed in the frontal cortex at PND2 (Lindahl et al. 2008). A study by Vandame and colleagues demonstrated that acute MK801 (1 or 5mg/kg i.p.) administration in adult female Sprague Dawley rats increases the number of GFAP-positive cells in the cingulate cortex. However the level of the increase observed in the cortex was dependent on the NMDA receptor antagonist used, with GK-11 (a PCP derivative, 1-5mg/kg i.p.) yielding much smaller increases than memantine (20-50mg/kg i.p.) or MK-801 (Vandame et al. 2013). This finding may go some way to explaining why preclinical studies into the effects of PCP on astrocyte number have not produced conclusive findings. Clinically, alterations in astrocyte number or alterations in GFAP expression have also not been reproducibly demonstrated in the cortex of schizophrenia patients at the transcriptional or proteomic levels (table 4.2).

**4.5.3 Conclusions**

The data in this chapter show that sub-chronic PCP administration causes transcriptional alterations in specific populations of cortical and hippocampal GABAergic interneurons (parvalbumin and calretinin). Interneurons are particularly vulnerable to NMDA antagonism compared to glutamatergic neurons (Coyle 2004). The presence of these reductions in mRNA levels 7 days following the last PCP injection is indicative of the sub-chronic PCP model giving rise to long-lasting alterations in the
brains of rats. Administration of an NMDA receptor antagonist has been shown to reduce the expression of GABAergic genes 30 minutes after a single dose in female Wistar rats (Jacobs et al. 1994), demonstrating that NMDA receptor antagonist-mediated gene suppression is not simply a delayed or long-term consequence of the treatment, but is persistently suppressed by the 7-day treatment regimen. Many of the cortical qRT-PCR findings in this chapter are in agreement with those observed in patients with schizophrenia with reductions in GAT-1, GAD1 and parvalbumin mRNA being consistently reported in a number of patient cohorts (see table 4.2). This adds to the construct validity of the sub-chronic PCP model of cognitive deficits and further implicates the GABAergic system in being in part responsible for cognitive deficits in both the sub-chronic PCP model and schizophrenia.

Reductions in parvalbumin-positive interneuron numbers and fluorescence per cell have also been consistently been reported in the hippocampus and frontal cortices of a number of NMDA receptor antagonist rodent models. The work presented in this chapter supports the hypothesis that parvalbumin-containing interneurons are preferentially affected by sub-chronic PCP administration, as other markers of interneuron populations (calretinin and somatostatin) are not affected at the protein level in the frontal cortex or at the transcriptional level in the hippocampus, supporting the work of other groups. This reduction in parvalbumin levels is thought to be mediated by nox2 and IL-6, although the exact mechanism remains to be determined (Behrens et al. 2008). Prolonged NMDA receptor antagonist administration seems to cause a loss of phenotype in parvalbumin interneurons, rather than cell death, as suggested by Zhang and colleagues (Zhang et al. 2008). This lack of phenotype results from severe reductions in the level of GAD67 and parvalbumin in the cell, impairing the cells GABAergic neurotransmission and intracellular calcium buffering capabilities. Loss of fast spiking parvalbumin interneurons in the areas of the brain that are essential for cognition, such as the cortical and hippocampal regions, would result in a loss of synchrony, particularly in the gamma frequency. This, combined with theta frequency oscillations, has long been recognised as being fundamentally important to a variety of cognitive processes, thus dysfunction in gamma oscillatory activity would lead to cognitive impairment, which is present in both the sub-chronic PCP model (Chapter 5
of this thesis and reviewed in Neill et al. 2010) and schizophrenia (Minzenberg et al. 2010).

Previous data (chapter 3 of this thesis) has suggested that the protein levels of GABA$_A$ receptor subunits are reduced during the washout period, but are not significantly different following the washout period, despite sustained reductions in mRNA levels, suggesting a compensatory increase in protein synthesis, trafficking or reduced catabolism of these subunits. As these transcriptional and protein studies were conducted after the washout period, this makes it possible that other subtypes of GABAergic interneuron do contribute to the cognitive and social deficits observed in the sub-chronic PCP models during the washout period, possibly through a lack of connectivity, rather than a reduction in cell number. Support for the role of GABAergic interneuron dysfunctional connectivity in the sub-chronic PCP model comes from a study carried out by Hajszan and colleagues, which showed that male rats treated with sub-chronic PCP (5mg/kg i.p. b.i.d. for 7 days followed by a 7-day washout period) period showed reduced prefrontal cortical spine synapses, accompanied by a comparable increase in the number of astroglial processes (Hajszan et al. 2006). Further study by Morrow and colleagues also showed a loss of axo-axonic connectivity in parvalbumin interneurons, following sub-chronic PCP treatment (0.3mg/kg b.i.d for 14 days, followed by an 8 day washout) in male non-human primates (Morrow et al. 2007). Further experimentation using a combined PCR-immunohistochemistry protocol across a number of time points during the washout is essential to investigate this hypothesis further and answer many of the questions that were raised in this chapter.
Chapter 5

Effect of \( \sigma_1 \) receptor ligands in two novel object recognition paradigms
5.1 Introduction

The predominant hypotheses of schizophrenia are the dopaminergic and glutamatergic dysfunction models (Jentsch and Roth 1999, Laruelle et al. 2003), with many current medications for schizophrenia being based on targets relating to these pathways, eg: antagonism at D₂ receptors. Recent work has revealed pathways upstream of neurotransmission dysfunction such as the adenosine hypothesis (Reviewed in Boison et al. 2012) or circuitry models (Lisman et al. 2008) that have been implicated in glutamatergic and dopaminergic dysfunction, as summarised in chapter 1 of this thesis. Investigation into these targets, which modulate cellular processes prior to neurotransmission, may uncover novel efficacious treatment options for cognitive deficits in schizophrenia which are poorly treated by current antipsychotics (Keefe et al. 2007).

Previous chapters in this thesis have investigated the effect of sub-chronic PCP administration on the GABAAergic system, in an attempt to test the glutamate:GABA hypothesis and add to the construct validity of the sub-chronic PCP rodent model. The work presented in this chapter will investigate the effect of modulating σ₁ receptors on short term memory using the ethologically relevant novel object recognition (NOR) cognitive task. These receptors have been shown to modulate NMDA, GABA_A and D₁ receptor mediated neurotransmission (Kourrich et al. 2012), as well as cellular calcium levels (Hayashi and Su 2007), acetylcholine (ACh) levels in rat hippocampus (Horan et al. 2002) and cortex (Kobayashi et al. 1996) and striatal dopamine levels (Patrick et al. 1993), making them an attractive potential pharmacological target in the treatment of cognitive deficits in schizophrenia.

5.1.1 σ₁ receptor modulators in clinical trials

With a growing body of evidence supporting the role of σ₁ receptors in neuropsychiatric disease, a number of drug trials have been initiated using drugs with high σ₁ receptor affinity in Alzheimer’s disease and schizophrenia patient groups (Volz and Stoll 2004, Di Santo et al. 2013). Two popular pharmacological interventions for the treatment of Alzheimer’s disease: donepezil and memantine show high σ₁ receptor affinity (Cobos et al. 2008) as well as clinical efficacy in treating cognitive deficits in patients (Roman et al. 2010, Di Santo et al. 2013). Single treatment trials using drugs with high σ₁ receptor affinity are less common in schizophrenia patient
groups with the results being much more varied. Clinical trials involving panamasine, eliprodil and fluvoxamine have shown great promise, but despite this panamasine was not progressed to more advanced stages of clinical testing (Volz and Stoll 2004, Hayashi and Su 2004), due to mild dyskinesic symptoms in some patients. Small subject numbers has limited the impact of eliprodil and fluvoxamine, but signs are encouraging, particularly regarding negative symptoms. Despite this variability in clinical efficacy, adjunctive treatment with drugs with high \( \sigma_1 \) receptor affinity, such as the selective serotonin-reuptake inhibitor (SSRI) antidepressant fluvoxamine (Ki at the \( \sigma_1 \) receptor = 36nM, Narita et al. 1996), along with atypical antipsychotics have been shown to significantly improve cognitive deficits and negative symptoms in small groups of patients with schizophrenia (Silver et al. 2000, Silver 2001, Iyo et al. 2008).

5.1.2 Sub-chronic PCP and NOR task

The NOR task was developed based on the natural propensity of rodents to explore novel objects (Ennaceur and Delacour 1988). This task is ethologically relevant and is known to correlate well with visual learning and recognition memory tests in humans such as the Hopkins verbal learning test and neurological assessment battery – shape learning task (Hagan and Jones 2005). The NOR task is relatively simple and requires no prior training or food reward so is routinely used as a relatively quick method of testing the efficacy of potential novel pharmacological treatments for the cognitive deficits found in schizophrenia. Administration of sub-chronic PCP has been shown to cause deficits in recognition memory in female rats (reviewed in Neill et al. 2010) with oestrus cycle stage having little impact on the performance of female rats in this task (Sutcliffe et al. 2007). Furthermore the novel antipsychotics asenapine (Snigdha et al. 2011a), sertindole (Idris et al. 2010), clozapine and risperidone (Grayson et al. 2007), as well as the GABA \( \alpha_5 \) inverse agonists AA29504 and gadoxadol (Damgaard et al. 2011), the dopamine \( D_4 \) receptor agonist PD168077 (Sood et al. 2011) and the dopamine \( D_1 \)-like receptor agonist SKF-38393 (McLean et al. 2009) have all been shown to ameliorate the sub-chronic PCP-induced deficit in this task.

5.1.3 Neuroanatomy of the NOR task

Preclinical studies involving both rodents and primates have revealed the importance of cortical and limbic structures in visual/object recognition memory. Cortical areas, especially the perirhinal cortex, play an important role in object
recognition tasks with a short ITI such as the standard NOR task (reviewed in Antunes and Biala 2012). Lesioning of the perirhinal (Aggleton et al. 1997, Malkova et al. 2001) or prefrontal cortical (Browning et al. 2013) regions impairs recognition memory in non-human primates and rodents. The hippocampus is functionally connected to both the perirhinal and prefrontal cortices and the correct function of this axis is considered vitally important in recognition memory. Lesioning of <75% of the dorsal hippocampus seems to have no effect on short-term recognition, but excitotoxic lesioning of >90% of the hippocampus impairs both short term (<15min) and long term (>3hr ITI) memory (Dere et al. 2007). This, and other studies, suggests that correct cortical-hippocampal function is vital for correct recognition memory, but that in the case of partial hippocampal dysfunction or loss, intact parahippocampal structures such as the perirhinal cortex are able to mediate short-term, but not long term, recognition memory.

In order to use the NOR task to assess different forms of memory, it is possible to vary the inter-trial-interval (ITI) of the NOR task. Extending the ITI of the NOR task alters the task from studying short-term to longer-term memory processes, with hippocampal involvement becoming more pronounced at longer ITIs, particularly hippocampal glutamate release following exposure to novelty (Stanley et al. 2012). Hippocampally-lesioned rats show impairments in recognition in tasks with longer ITIs (>15mins), but not shorter ITIs (<15mins) (Clark et al. 2000). The importance of the hippocampus in the NOR task is supported by studies showing that excitotoxic lesioning of the whole hippocampus disrupts recognition memory in both rodents and primates (Zola et al. 2000, Clark et al. 2001).

5.2 Aim

The ability of σ1 receptor compounds to modulate dopaminergic and NMDA receptors makes them a promising target for the treatment of schizophrenia as these receptors are known to be dysfunctional in the disease, in line with the dopamine/glutamate hypotheses. As drugs with high σ1 receptor affinity have also shown efficacy in small trials of patients with cognitive dysfunction, the effect of highly selective σ1 receptor ligands on cognitive dysfunction in this well-established rodent model will be studied. The principal aim of the work presented in this chapter is to investigate the effect of the highly selective σ1 receptor agonist PRE-084 and selective
σ1 receptor antagonist NE-100 in two models of cognitive dysfunction, using the novel object recognition (NOR) test. Receptor affinities of these compounds are described in chapter 2.8.2 of this thesis. These experiments will investigate the dose response effects of these two compounds, PRE-084 and NE-100, in reversing the object recognition memory deficit seen in sub-chronic PCP treated female rats using a 1min ITI NOR test to study short term memory and also in improving memory in drug-naïve female rats using a “natural forgetting” 6hr ITI NOR paradigm to study long term memory storage and consolidation.

5.3 Methods

5.3.1 Animals and drug administration
All animals were housed and experiments carried out according to the conditions and under the appropriate legislation as detailed in chapter 2 of this thesis. Sub-chronic treatment with either vehicle (0.9% NaCl) or PCP (2mg/kg b.i.d.) was carried out as previously described (Chapter 2.2). Sigma-1 receptor-specific drugs were administered at the previously determined doses via the intraperitoneal route (Chapters 2.2 and 2.8.1).

5.3.2 NOR test
Both the 1min ITI and 6hr ITI versions of the NOR task were performed as previously described in chapter 2.8. NOR equipment set up and experimental procedure is detailed in chapter 2 of this thesis (Chapters 2.8.3 and 2.8.4).

5.3.3 Behavioural analysis
Analysis of object discrimination and locomotor activity in the acquisition and retention trials of the novel object recognition task was carried out as described in chapter 2.8.5 of this thesis.

5.3.4 Statistical analysis
Statistical analyses were carried out as described in chapter 2.8.6. The 6hr ITI experiment was carried out using two groups of rats who were tested at separate times, generating two sets of data, including two groups of vehicle animals (N=25). These two data sets were then combined into a total data set and analysed as previously described (Chapter 2.8.6).
5.4 Results

5.4.1 6hr ITI NOR task

Effect of acute administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on object exploration following a 6hr ITI in the home cage in the acquisition trial in the NOR test.

An overall two-way ANOVA revealed that administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) or PRE-084 (1mg/kg) + NE-100 (1mg/kg) had no significant effect on object exploration in the acquisition trial of the NOR test (F_{8,92}=0.46, NS; Figure 5.1). Rats from all of the treatment groups spent similar times exploring both of the objects (Figure 5.1).

![Graph showing the effect of PRE-084, NE-100, and their co-administration on object exploration time]

**Figure 5.1**: The effect of PRE-084 (0.1-3mg/kg i.p), NE-100 (0.3-3mg/kg i.p.) and co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) on exploration times (s) of two identical objects in the 3min acquisition phase of the 6hr ITI NOR test. Data are expressed as the mean ± S.E.M. (n=9-25 per group).

A one-way ANOVA on the total object exploration times in the acquisition trial revealed a significant drug treatment effect (F_{8,92}=2.93, p<0.01; Table 5.1). Further analysis by post-hoc Students’ t-test showed a significant (p<0.05) decrease in
exploration times following treatment with NE-100 (0.3mg/kg) and PRE-084 (1mg/kg) + NE-100 (1mg/kg), compared with the vehicle group, as shown in table 5.1.

**Effect of acute administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on object exploration following a 6hr ITI in the home cage in the retention trial in the NOR test.**

An overall two-way ANOVA revealed that administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) or PRE-084 (1mg/kg) + NE-100 (1mg/kg) produced a significant effect on object exploration in the retention trial of the NOR test ($F_{8,92}=3.97$, $p<0.001$; Figure 5.2). Further post-hoc analysis by Students’ t-test showed that vehicle rats spent comparable amounts of time exploring novel and familiar objects after a 6hr ITI. Rats that were pre-treated with PRE-084 (1 and 3mg/kg) spent significantly ($p<0.05$ and $p<0.01$ respectively) more time exploring the novel object compared with the familiar object during the retention trial. However this effect was not observed at doses of 0.1mg/kg and 0.3mg/kg of PRE-084 (Figure 5.2). Rats that were treated with NE-100 (1 and 3mg/kg) spent significantly ($p<0.05$) more time exploring the novel object compared to the familiar object during the retention trial, whereas this effect was not observed at the lowest dose of NE-100 (0.3mg/kg) or when NE-100 (1mg/kg) and PRE-084 (1mg/kg) were co-administered (Figure 5.2).
**Figure 5.2:** The effect of PRE-084 (0.1-3mg/kg i.p.), NE-100 (0.3-3mg/kg i.p.) and co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) on exploration times (s) of a novel object and familiar object in the 3min retention phase of the 6hr ITI NOR test. Data are expressed as the mean ± S.E.M. (n=9-25 per group).

* *p<0.05, **p<0.01; significant increase in time spent exploring the novel compared with the familiar object.

A one-way ANOVA analysis of the total exploration times in the retention trial of the NOR test revealed no significant effect of drug treatment. (F<sub>8,92</sub>=1.80, NS; Table 5.1). However planned post-hoc Students` t-test analysis revealed a significant (p<0.05) decrease in exploration times in the acquisition trial following treatment with the NE-100 (0.3mg/kg) and the NE-100 (1mg/kg) groups, compared to vehicle controls.

A one-way ANOVA analysis of total exploration times over the two trials revealed a significant drug effect (F<sub>8,92</sub>=2.02, p<0.05; Table 5.1). Planned post-hoc analysis by Students` t-test revealed that exploration times were significantly reduced (p<0.05), in the NE-100 (0.3mg/kg) group compared to the vehicle group.
Effect of acute administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on the DI following a 6hr ITI in the home cage in the retention trial of the NOR test

A one-way ANOVA on the DI data showed a significant effect of drug treatment on the rats’ ability to discriminate between the novel and familiar objects ($F_{8,92}=3.77; p<0.001$; Figure 5.3). Planned post-hoc analysis showed a significant ($p<0.05$) increase in the DI in the PRE-084 higher dose groups (1 and 3mg/kg), but not at lower doses of PRE-084 (0.1 and 0.3mg/kg) when compared to vehicle control. A significant ($p<0.05$) increase in the DI of the higher dose NE-100 groups (1 and 3mg/kg) compared to vehicle control was also observed, which was not observed in the lower dose group (0.3mg/kg). This significant increase in DI was also not observed in the group treated with PRE-084 (1mg/kg) + NE-100 (1mg/kg) (Figure 5.3).

**Figure 5.3:** The effect of PRE-084 (0.1-3mg/kg i.p), NE-100 (0.3-3mg/kg i.p.) and co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) on the DI in the 3 min retention phase of the 6hr ITI NOR test. Data are expressed as the mean ± S.E.M. (n=9-25 per group). *$p<0.05$; significant increase in DI when compared to the vehicle group.
Effect of acute administration of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on the total number of line crossings in the acquisition and retention trials of the 6hr ITI NOR test.

Figure 5.4 shows the effect of drug treatment on the total number of line crossings during both the acquisition and retention trials of the NOR test. A one-way ANOVA analysis showed a significant effect of drug treatment on the number of line crossings ($F_{8,92}=2.51$, $p<0.05$; Figure 5.4). Planned post-hoc analysis revealed a significant ($p<0.05$) decrease in the number of line crossings in the PRE-084 (1mg/kg) group compared to the vehicle group.

**Figure 5.4:** The effect of PRE-084 (0.1-3mg/kg i.p), NE-100 (0.3-3mg/kg i.p.) and co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) on the total number of line crossings in the acquisition and retention phases of the NOR test. Data are expressed as the mean ± S.E.M. (n=9-25 per group). * $p<0.05$; significant decrease in number of line crossings compared to the vehicle group.
<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Total Exploration time (s)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acquisition Trial</td>
<td>Retention Trial</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>18.43 ± 1.95</td>
<td>21.48 ± 2.11</td>
<td>39.18 ± 4.28</td>
<td></td>
</tr>
<tr>
<td>PRE-084 0.1</td>
<td>17.3 ± 1.92</td>
<td>14.4 ± 2.54</td>
<td>31.7 ± 4</td>
<td></td>
</tr>
<tr>
<td>PRE-084 0.3</td>
<td>21.9 ± 3.34</td>
<td>17.1 ± 2.25</td>
<td>39.1 ± 5.05</td>
<td></td>
</tr>
<tr>
<td>PRE-084 1</td>
<td>15.78 ± 2.81</td>
<td>20.67 ± 3.07</td>
<td>38.63 ± 6.25</td>
<td></td>
</tr>
<tr>
<td>PRE-084 3</td>
<td>20.8 ± 2.5</td>
<td>20.5 ± 2.6</td>
<td>41.3 ± 3.37</td>
<td></td>
</tr>
<tr>
<td>NE-100 0.3</td>
<td>10.3 ± 1.33 *</td>
<td>12.6 ± 1.09 *</td>
<td>22.9 ± 2.09 *</td>
<td></td>
</tr>
<tr>
<td>NE-100 1</td>
<td>15.89 ± 1.54</td>
<td>14.11 ±1.57 *</td>
<td>30 ± 2.35</td>
<td></td>
</tr>
<tr>
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<td>15.7 ± 0.88</td>
<td>18.9 ± 2.18</td>
<td>34.6 ± 2.71</td>
<td></td>
</tr>
<tr>
<td>PRE-084 1 ± NE-100 1</td>
<td>9.9 ± 1.13 *</td>
<td>19.2 ± 1.9</td>
<td>29.1 ± 2.6</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.1:** The effect of PRE-084 (0.1-3mg/kg), NE-100 (0.3-3mg/kg) and co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on total object exploration time in the acquisition and retention trials of the NOR test. Data are expressed as the mean ± S.E.M. (n=9-25 per group). *p < 0.05; significant decrease in exploration times compared to the vehicle group.

5.4.2 Sub-chronic PCP 1min ITI NOR task

**Effect of acute administration of PRE-084 (0.3-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) in sub-chronic PCP treated rats on object exploration in the acquisition trial of the NOR test**

An overall two-way ANOVA revealed that sub-chronic PCP, PRE-084 (0.3-3mg/kg), NE-100 (0.3-3mg/kg) or PRE-084 (1mg/kg) + NE-100 (1mg/kg) had no significant effect on object exploration in the acquisition trial of the NOR test (F$_{8,71}$=1.26, NS; Figure 5.5). Rats from all of the treatment groups spent similar times exploring both of the objects (Figure 5.5).
A one-way ANOVA on the total exploration times in the acquisition trial revealed a significant effect of drug treatment on exploration times. \( (F_{8,71}=3.90, p<0.01; \text{Table 5.2}) \). Planned post-hoc analysis by Students’ t-test revealed a significant increase \( (p<0.01) \) in exploration time in the acquisition trial of the NOR task in the NE-100 (0.3mg/kg) group, compared to the vehicle group.

**Effect of acute administration of PRE-084 (0.3-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) in sub-chronic PCP treated rats on object exploration time in the retention trial of the NOR test.**

A two-way ANOVA revealed that administration of PRE-084 (0.3-3mg/kg) or NE-100 (0.3-3mg/kg) to rats treated with sub-chronic PCP produced no significant effect on object exploration times in the retention trial of the NOR test \( (F_{8,71}=1.22, \text{NS}; \text{Figure 5.6}) \). Planned post-hoc Students’ t-test showed that vehicle treated rats spent
significantly (p<0.05) more time exploring the novel object compared to the familiar object during the retention phase of the NOR test. This ability to discriminate novel from familiar objects was not observed in sub-chronic PCP-treated rats.

Rats that were pre-treated acutely with PRE-084 at 1mg/kg and 3mg/kg significantly attenuated the sub-chronic PCP induced recognition memory deficit, resulting in rats spending significantly (p<0.01 and p<0.05 respectively) more time exploring the novel object than the familiar object during the retention trial. This attenuation of the sub-chronic PCP deficit was not observed in the group treated acutely with PRE-084 at 0.3mg/kg. Acute treatment with NE-100 (1mg/kg) significantly attenuated the sub-chronic PCP-induced impairment such that a significant (p<0.001) increase in time spent exploring the novel object compared to the familiar object was again observed. However, acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) resulted in the loss of the attenuation of the sub-chronic PCP recognition memory deficit. Administration of NE-100 (3mg/kg) resulted in an effect that was very close to significance (p=0.053).
Figure 5.6: The effect of acute administration of PRE-084 (0.3-3mg/kg i.p), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) in rats treated sub-chronically with PCP (2mg/kg i.p b.i.d for 7 days, followed by a 7-day washout period) on exploration times (s) of a novel object and familiar object in the 3min retention phase of the 1min ITI NOR test. Data are expressed as the mean ± S.E.M. (n=7-10 per group). *p < 0.05, **p<0.01, *** p<0.001; significant increase in time spent exploring the novel object compared with the familiar object.

One-way ANOVA on total exploration times in the retention trial revealed a significant effect of drug treatment ($F_{8, 71}=6.111$, p<0.001; Table 5.2). Planned post-hoc analysis by Students’ t-test showed a significant increase in exploration times in the retention trial of the NOR task in the NE-100 (0.3mg/kg) group, compared to the vehicle control group.

Effect of acute administration of PRE-084 (0.3-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) in sub-chronic PCP treated rats on the DI in the retention trial of the NOR test.

A one-way ANOVA of the DI data showed a significant effect of drug treatment on the ability of the rats to discriminate between the novel and familiar objects ($F_{8,72}=2.61$, p<0.001; Table 5.2). Planned post-hoc analysis by Students’ t-test showed a significant increase in DI in the NE-100 (0.3mg/kg) group, compared to the vehicle control group.
Planned post-hoc comparisons showed a significant (p<0.05) decrease in the DI in sub-chronic PCP treated animals compared to vehicle control. Administration of PRE-084 (0.3mg/kg) was unable to attenuate the reduction in DI following sub-chronic PCP treatment, however, following treatment with the higher doses of PRE-084 (1 and 3mg/kg), a significant increase in DI was observed when compared to sub-chronic PCP treated animals (p<0.05 and p<0.01 respectively). Also following treatment with NE-100 (1mg/kg) a significant (p<0.05) increase in the DI was also observed compared to sub-chronic PCP treated animals. Acute dosing of NE-100 at 3mg/kg was also very close to being significantly different to sub-chronic PCP DI values (p=0.052). However, administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg), produced no significant increase in DI when compared to sub-chronic PCP treated animals (Figure 5.7). Post-hoc data from the NE-100 (0.3mg/kg) group was not significantly different from either vehicle or sub-chronic PCP groups.
Figure 5.7: The effect of acute administration of PRE-084 (0.3-3mg/kg i.p), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) in rats treated sub-chronically with PCP (2mg/kg i.p. b.i.d for 7 days, followed by a 7-day washout period) on the DI in the 3min retention phase of the 1min ITI NOR test. Data are expressed as the mean ± S.E.M. (n=7-10 per group).* p<0.05 significant decrease in DI compared to vehicle controls, # p<0.05, ##p<0.01; significant increase in DI compared to sub-chronic PCP-treated rats.

Effect of acute administration of PRE-084 (0.3-3mg/kg), NE-100 (0.3-3mg/kg) and acute co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) in sub-chronic PCP treated rats on the number of line crossings in the acquisition and retention trials of the NOR test.

Figure 5.8 shows the effect of drug treatment on the total number of line crossings during both the acquisition and retention trials of the NOR test. A one-way ANOVA showed a significant effect of drug treatment on the number of line crossings (\(F_{8,72}=4.12, p<0.01\); Figure 5.8). Planned post-hoc Students’ t-test analysis revealed a significant increase in line crossings in the 0.3mg/kg NE-100 group (p<0.01), compared to vehicle animals. No significant differences were observed in the number of line crossings between any other individual treatment groups when compared to vehicle controls.
**Figure 5.8:** The effect of acute administration of PRE-084 (0.3-3mg/kg i.p), NE-100 (0.3-3mg/kg i.p) and acute co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) in rats treated sub-chronically with PCP (2mg/kg i.p. b.i.d for 7 days, followed by a 7-day washout period) on the total number of line crossings in the acquisition plus retention phase of the NOR test. Data are expressed as the mean ± S.E.M. (n=7-10 per group). **p<0.01; significant increase in locomotor activity compared to the vehicle group.
Table 5.2: The effect of acute administration of PRE-084 (0.3-3mg/kg i.p.), NE-100 (0.3-3mg/kg i.p.) and acute co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) in rats treated sub-chronically with PCP (2mg/kg i.p. b.i.d for 7 days, followed by a 7-day washout period) on the total exploration time in the acquisition and retention trial of the NOR test. Data are expressed as the mean ± S.E.M. (n=7-10 per group). **p<0.01, ***p<0.001; significant increase compared to the vehicle group.

The findings from these studies are summarised below in table 5.3 (6hr ITI) and table 5.4 (1min ITI):

Table 5.3: Summary table showing the effect of acute administration of PRE-084 (0.1-3mg/kg i.p.), NE-100 (0.1-3mg/kg i.p.) and acute co-administration of PRE-084 (1mg/kg i.p.) + NE-100 (1mg/kg i.p.) on the performance of female rats in the 6hr-ITI NOR test. *p<0.05, **p<0.01: significant change compared to the vehicle group.
<table>
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<th>Treatment</th>
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<th>Deficit in DI?</th>
<th>Effect on locomotor activity?</th>
<th>Effect on object exploration time</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PCP + PRE-084</td>
<td>2</td>
<td>Yes</td>
<td>Yes (*)</td>
<td>No</td>
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</tr>
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<td>Yes (*)</td>
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<td>No</td>
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<tr>
<td>PCP + PRE-084</td>
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<td>No (#)</td>
<td>No</td>
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</tr>
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<td>No</td>
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<td>No</td>
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<tr>
<td>PCP + NE-100</td>
<td>2 + 0.3</td>
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<td>No</td>
<td>Yes ↑ (***)</td>
<td>Yes ↑ (***)</td>
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<td>2 + 1 + 1</td>
<td>Yes</td>
<td>Yes (*)</td>
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</tr>
</tbody>
</table>

Table 5.4: Summary table showing the effect of sub-chronic treatment with PCP (2mg/kg i.p. b.i.d. for 7 days, followed by a 7-day washout period) and acute administration of PRE-084 (0.3-3mg/kg i.p.), NE-100 (1mg/kg) and co-administration of PRE-084 (1mg/kg) + NE-100 (1mg/kg) on the performance of female rats in the 1min-ITI NOR test. *p<0.05, **p<0.01, p<0.001; significant change compared to the vehicle group, #p<0.05, ##p<0.01, ###p<0.001; significant change compared to the sub-chronic PCP group.

5.5 Discussion

5.5.1 Pharmacology

PRE-084 has a high specific affinity for the σ1 receptor, with very low affinity at other receptor groups, including the NMDA receptor PCP-binding site and dopaminergic and serotonergic receptor classes (Su et al. 1991), indicating that its primary mode of action is through the σ1 receptor. This hypothesis is supported by the observation that the pro-cognitive effects of PRE-084 in both versions of the NOR task are antagonised by the specific σ1 receptor antagonist NE-100. However, the pro-cognitive effects of NE-100 at this dose (1mg/kg), as shown in both the 6hr and 1min ITI versions of this task, make making conclusive statements about this antagonistic activity difficult, without other studies looking at the effects of using a sub-therapeutic dose of NE-100 (<1mg/kg) to block the effects of σ1 receptor agonism.

The σ1 receptor antagonist NE-100 has nanomolar affinity for both σ receptor subtypes, with 55x selectivity for the σ1 receptor over the σ2 receptor (see table 2.7 in chapter 2 of this thesis). The mode of action for NE-100 is therefore thought to be via the σ1 receptor, although the role of σ2 receptors cannot be ruled out (see section 5.5.5).
5.5.2 Delay-induced deficit (6hr ITI)

The results presented here show that an ITI of 6 hours is sufficient to give rise to a delay-induced cognitive deficit in female rats, as these animals were unable to differentiate between the novel and familiar objects in the NOR task. This observation is supported by previous work in our laboratory (Sutcliffe et al. 2007) and other groups' findings with male rats (King et al. 2004). These findings add to the evidence for the use of the 6hr ITI NOR task as an ethologically valid task for the study of novel treatments for cognitive dysfunction caused by a time-delay.

5.5.2.1 PRE-084

The data here show that acute administration of the σ1 receptor agonist PRE-084 at 1 and 3mg/kg to the drug-naive vehicle rats was effective at significantly ameliorating the delay-induced object recognition deficit in the 6hr ITI NOR task. Studies in other laboratories have found similar effects of σ1 receptor agonists; work by Giorgetti and colleagues (Giorgetti et al. 2010) showing that intraperitoneal administration of the σ1 receptor agonist (donepezil) ameliorates 24hr ITI-induced deficits in object recognition memory in male Sprague-Dawley rats, a finding which is supported by results from our laboratory using female rats (McLean 2011). The σ1 receptor agonist PRE-084 also ameliorates spatial memory deficits in aged Wistar rats using the water-maze paradigm (Maurice 2001). Further in vivo studies have demonstrated that σ1 receptor agonists (donepezil and PRE-084) can be neuroprotective against Aβ25-35-mediated toxicity and the resulting spatial working memory deficits in a Y-maze paradigm in mice. This amelioration was antagonised by the administration of the σ1 receptor antagonist BD-1047 (Meunier et al. 2006b). This pro-cognitive effect of σ1 receptor agonists is further supported by studies in mice showing amelioration of long term memory dysfunction, using a passive avoidance test, caused by Aβ25-35 administration by the specific σ1 receptor agonists SA4503, PRE-084 and (+)-pentazocine (Maurice et al. 1998). Sub-chronic (7-8 day) administration of the endogenous neurosteroid and σ1 receptor agonist DHEAS was able to ameliorate olfactory bulbectomy (OBX)-induced object recognition deficits in the 1hr ITI NOR test in mice (Moriguchi et al. 2011). This effect was antagonised by acute pre-treatment with 1mg/kg of the σ1 receptor antagonist NE-100. In all the above cases the amelioration in cognitive performance was antagonised by administration of a σ1
receptor antagonist; an effect which is also seen in the present study; despite the need for further studies into the effects of sub-therapeutic doses of NE-100.

Locomotor activity analysis showed a significant decrease in locomotor activity in the 1mg/kg treatment group, although there was no effect on object exploration in the acquisition trial for any of the doses tested, indicating specificity of PRE-084 for the retention trial. This is contrary to other studies which have demonstrated no reduction in locomotor activity of σ1 receptor agonists in rats (Skuza et al 2002, Skuza and Rogoz 2006) or mice (Maurice et al 2003, Hashimoto et al 2006).

5.5.2.2 NE-100

There are a limited number of studies into the efficacy of specific σ1 receptor antagonists in time-induced cognitive deficit/long term cognitive paradigms. Studies by Frye and colleagues (Frye et al. 2009) and Harburger and colleagues (Harburger et al. 2008) have shown the endogenous neurosteroid and σ1 receptor antagonist progesterone to ameliorate 4hr-ITI induced object recognition memory deficits in female rats and 24hr-ITI induced object recognition memory deficits in female mice. More specific ligands such as NE-100 were also able to attenuate the acute PCP and time-induced combined deficit in working memory in rats as measured by the Morris water maze (Okuyama et al. 1995), supporting the pro-cognitive role of NE-100 shown in the present study; with NE-100 at 1 and 3mg/kg causing significant increases in DI and novel object exploration compared to vehicle animals in this paradigm. The lowest dose of NE-100 used in this study also caused a significant reduction in object exploration with no concurrent effect on locomotor activity. This suggests that the administration of NE-100 (0.3mg/kg) causes an increased tendency for the animal to be distracted from the objects or reduces attentiveness for the task, without any sedation, as there is no alteration in locomotor activity. It is very difficult to ascertain exactly what effect NE-100 is having on the animal at 0.3mg/kg without the use of a specialised test of attention like the 5 choice-serial reaction time task (5C-SRTT) or 5-choice-continuous performance task (5C-CPT).

5.5.3 Sub-chronic PCP-induced deficit

Administration of sub-chronic PCP resulted in a robust deficit in cognitive performance in the NOR task, as animals were unable to distinguish between novel and familiar objects after only a 1min ITI. The sub-chronic PCP-induced recognition
memory deficit in this task is well documented by our group and in other laboratories (reviewed in Neill et al. 2010, Horiguchi et al. 2012, Horiguchi et al. 2013, Horio et al. 2013). These findings add to the evidence supporting the use of the PCP deficit in the 1min ITI NOR task as a robust method for the development of novel treatments for cognitive dysfunction in schizophrenia.

5.5.3.1 PRE-084

Results presented in this chapter show that acute administration of the σ1 receptor agonist PRE-084 at 1 and 3mg/kg to the sub-chronic PCP treated rats ameliorated the sub-chronic PCP-induced object recognition memory deficit. There was no effect on exploration in the acquisition trial or on the number of line crossings suggesting specificity of PRE-084 for the retention trial without any reduction in locomotor activity.

These findings are supported by work undertaken in other laboratories where sub-chronic (10 days) treatment with the σ1 receptor agonist (SA4503) or an selective serotonin reuptake inhibitor (SSRI) with σ1 receptor agonist properties (fluvoxamine) was able to reverse the sub-chronic PCP-induced impairments in mice (Hashimoto et al. 2007), although acute administration of these agonists was not sufficient to reverse the sub-chronic PCP-induced deficits in this study. This could be attributed to the use of higher-dose PCP administration (10mg/kg) over a longer period of time (10 days) in this study than was used in the study presented in this chapter (2mg/kg b.i.d, 7 days), therefore requiring increased dosing regimens of the σ1 receptor agonist in order to reverse the object recognition memory deficit in the NOR test. The short washout period (2 days) may also play a role in these differences as the effects of having PCP present in the brain at the time of testing cannot be discounted. σ1 receptor agonist administration has also been shown to ameliorate ethanol-induced cognitive deficits in mice (Meunier et al. 2006a) These data presented in this chapter are the first, to the author’s knowledge, to show σ1 receptor-mediated amelioration of sub-chronic PCP-induced object recognition deficits in rats.

σ1 receptor agonists have been shown to ameliorate recognition memory deficits caused by administration of MK-801 which, like PCP, is an NMDA receptor antagonists, in working and recognition memory tasks such as the Y-maze and NOR
paradigms. A study by Maurice and colleagues (Maurice 2001) showed administration of the α1 receptor agonists PRE-084 and DHEAS ameliorated the memory deficits in the Y-maze induced by acute MK-801 treatment in mice. Further studies have shown that the α1 receptor agonists PRE-084 (Maurice et al. 1994a), (+)SKF-10,047 and SA4503 (Zou et al. 1998) are effective at ameliorating recognition memory deficits caused by NMDA antagonism at similar dose ranges to those used in this chapter (0.1-5mg/kg agonist).

5.5.3.2 NE-100

Acute administration of the α1 receptor antagonist NE-100 at 1mg/kg to the sub-chronic PCP treated rats ameliorated the PCP-induced object recognition memory deficit. Administration of NE-100 at 3mg/kg was very close to producing a significant cognitive improvement (p=0.053). There was no reduction in exploration in the acquisition trial or in the number of line crossings suggesting specificity of NE-100 for the retention trial without any other behavioural effects. NE-100 administered at 0.3mg/kg resulted in a significant increase in LMA and exploration times in both acquisition and retention trials of the NOR suggesting that low dose administration of NE-100 induces hyperactivity in female rats, although this increase in exploration and locomotion did not result in any discrimination between novel and familiar objects suggesting no pro-cognitive effect of a low acute dose of a α1 receptor antagonist. This was particularly interesting given that in the previous studies in drug-naïve animals an identical dose of NE-100 had significantly reduced exploration times after 6 hours, without having a significant effect on locomotor activity. This would suggest that the effects of NE-100 at 0.3mg/kg on exploration are time- or drug-dependent, although further experimentation is required to provide an explanation for this hypothesis. Previous studies into hyperactivity in mice and rats have shown that the α1 receptor antagonist BD-1047 (0.5-10mg/kg) has no effect on acute amphetamine, MK-801, phencyclidine (Skuza and Rogoz 2006) or methamphetamine-induced hyperactivity (Kitanaka et al. 2009), whereas the α1 agonist SA4503 (2.7-27μmol/kg) was able to reverse cocaine-induced hyperactivity (Rodvelt et al. 2011). In these studies the effect of the α1 compounds was not investigated by themselves which would have revealed any hyperactivity induced by the acute administration of the α1 receptor antagonist, as
studies from the 6hr ITI variation of the NOR task are unlikely to show acute effects of NE-100 due to its high clearance rate (Yamamoto et al. 2005).

No studies have been carried out to the authors knowledge that investigate the ability of σ1 receptor antagonists to attenuate sub-chronic PCP-induced cognitive deficits, although an acute PCP (15mg/kg) study showed efficacy of NE-100 in ameliorating the PCP-induced cognitive deficit in the Morris water maze in rats (Okuyama et al. 1995). A recent study by Seminerio et al (Seminero et al. 2013) showed neuroprotective effects and cognitive enhancement after administration of the σ1 receptor antagonist (AZ66). AZ66 administration was able to ameliorate object recognition memory deficits in mice. Work by Szuka and Rogoz (Skuza and Rogoz 2006) and Takahashi (Takahashi et al. 1999) showed that the σ1 receptor antagonist BD-1047 and MS-377 ameliorates PCP-induced head twitching at similar doses to those used in this study and high doses (10mg/kg) significantly reduced apomorphine-induced climbing behaviour in mice, through a proposed blockade of hippocampal/cortical dopaminergic (non-D2) activity. The mechanism of action by which NE-100 acts is currently unknown; NE-100 has affinity to sigma-2 receptors and these may play a role in the pro-cognitive effect of NE-100 but further study is required to confirm this hypothesis.

5.5.4 Co-admin of PRE-084 and NE-100

Acute co-administration of PRE-084 (1mg/kg) and NE-100 (1mg/kg) prevented any cognitive improvement in both the 6hr ITI and sub-chronic PCP-induced deficits in recognition memory in the NOR test in this chapter. This suggests that both PRE-084 and NE-100 are acting through a σ1 receptor mediated mechanism, as both are highly specific for the σ1 receptor and both antagonise the positive cognitive effect of the other. This is understandable given the criteria used to define ‘agonist’ and ‘antagonist’ activity is, respectively, the ability to dissociate and maintain the association between the σ1 receptor and binding immunoglobulin protein (BiP). This would prevent any molecule from producing an enhanced cognitive effect from both agonism and antagonism of the σ1 receptor. This pro-cognitive effect of both agonists and antagonists of the same receptor has also been demonstrated for 5HT6 receptors in scopolamine and MK-801-induced memory deficits in the conditioned emotional response (CER) test in rats (Woods et al. 2012), with SB-271046 (5HT6 receptor
antagonist), E-6801 and EMD 386088 (5HT6 receptor agonists) all showing pro-cognitive properties in the CER test. This effect is also observed with α7 nicotinic receptor (α7 nAChR) compounds, with EVP-6124 (α7 nAChR agonist) and methyllycaconitine (MLA – α7 nAChR antagonist) both ameliorating a delay-induced object recognition memory deficit using the NOR test in rats (van Goethem 2013). Therefore these ‘pro-cognitive’ effects of σ1 receptor antagonist and agonists are not limited to this receptor class, although the mechanism responsible remains to be elucidated.

5.5.5 Pharmacology
Although modulation of σ1 receptor-mediated signalling has been shown to be beneficial in both clinical and preclinical studies of cognition, the precise mechanisms by which this is achieved have not yet been fully elucidated. This is in part due to σ1 receptors influencing a wide variety of intracellular (Ca2+) and neurotransmitter (dopaminergic, acetylcholinergic, glutamatergic) systems, making individual pathway contributions difficult to pinpoint.

5.5.5.1 σ1 receptor agonism
σ1 receptor agonists have been shown to increase acetylcholinergic neurotransmission in the hippocampus without affecting acetylcholine levels in the striatum in rat brain using in vivo microdialysis (Horan et al. 2002). This is most likely due to the higher numbers of σ1 receptors in the hippocampus and cortex compared to the striatum. Activation of systemic, but not cortical acetylcholinergic receptors by the α7 nicotinic acetylcholine receptor agonist PNU-120596 causes an increase in dopamine release in the mPFC (Livingstone et al. 2009), suggesting involvement of acetylcholinergic signalling in a region outside the frontal cortex. σ1 receptor binding sites also interact directly with NMDA receptors causing an increase in glutamate in the hippocampus (Chaki et al. 1998), which is known to be important in mediating the cognitive processes associated with novelty (Stanley et al. 2012). Impaired cortical dopaminergic transmission is known to be a key feature of object recognition memory deficits in the sub-chronic PCP model of cognitive deficits (Harte 2011) and increased acetylcholinergic tone in the hippocampus, leading to increased cortical dopamine neurotransmission could explain the pro-cognitive effects of PRE-084. σ1 receptor agonism in the hippocampus could be a plausible site of action for PRE-084 to act in
the 6hr ITI version of the NOR task, as the involvement of the hippocampus in the NOR
test is well established in rodents (Broadbent et al. 2004, Jessberger et al. 2009,
Broadbent et al. 2010) as well as in human recognition memory tasks (Burgess et al.
2002). Increased activity in cortical D₁ receptors could be a potential mechanism for
the pro-cognitive effects of σ₁ receptor agonism in the 1min ITI NOR task. D₁ receptor
activation in the cortex is known to be strongly associated with the ability for rats to
successfully complete this task (Snigdha et al. 2011a) and σ₁ receptors have been
shown to modulate D₁ receptors directly, although without further testing, it is
impossible to draw definite conclusions about these potential mechanisms.

5.5.5.2 σ₁ receptor antagonism

The mechanism by which σ₁ receptor antagonists function in cognition is not
well understood, however some studies have shed light on some of the potential
pathways involved. σ₁ receptor antagonism is known to prevent hyperactivity (and
subsequent cytotoxicity) at NMDA receptors in the rat hippocampus (Smith et al.
2010). Glutamatergic/GABAergic signalling is known to be disrupted, both in
schizophrenia and the sub-chronic PCP model, and σ₁ antagonism may help to restore
balance to this system. The role of the hippocampus, and especially hippocampal
glutamate, in the recognition of novelty in this task was shown elegantly by in vivo
microdialysis (Stanley et al. 2012). As previously described, cortical dopamine levels
are known to be a key determinant in successful completion of the NOR task and σ₁
receptor antagonism has also been shown to restore dopaminergic tone during object
recognition tasks after administration of methamphetamine (Seminero et al. 2013).

Although the exact mechanism by which σ₁ receptor agonists and antagonists
exert their pro-cognitive effect is unclear, the role of σ₂ receptors in the pro-cognitive
effects of NE-100 in the sub-chronic PCP and 6hr ITI NOR tests cannot be ruled out as
NE-100 has significant affinity for the σ₂ receptor, unlike PRE-084 which has a much
lower affinity for the σ₂ receptor (Table 5.4). The data in this chapter support the role
of σ₁ receptors in cognitive enhancement; building on the existing clinical data
showing σ₁ receptor-mediated improvements in attention and cognition, as measured
by the letter fluency test, category fluency test, trail-making test and Stroop test in
small schizophrenia patient groups (Iyo et al. 2008, Niitsu et al. 2010).
5.5.6 Comparison between the 6hr ITI and 1min ITI NOR tasks

As previously discussed, by varying the parameters of the NOR task, especially the ITI, it is possible to test both long-term and short-term recognition memory, processes that utilise different aspects of the cortico-limbic network. Long-term processes are more dependent on hippocampal integrity, while short-term processes are governed largely by the perirhinal and frontal cortices, although both memory forms require hippocampal inputs to function correctly. Possible reasons why systemic co-administration of equal amounts of σ1 receptor agonists and antagonists reversed any pro-cognitive effects have already been discussed (section 5.5.4), however the mechanisms behind both σ1 receptor agonists and antagonists being pro-cognitive in two different paradigms at the same doses remain to be elucidated.

σ1 receptor agonism has been shown to affect a number of neurotransmitter systems that are intimately involved in cognition, including acetylcholine, glutamate and dopamine. Modulation of these systems would produce a pro-cognitive effect, as detailed in section 5.5.1. The pro-cognitive mechanisms of σ1 receptor antagonists are more uncertain, as maintaining the σ1 receptor-BiP complex effectively prevents translocation of the σ1 receptor. The antagonist that was used in these studies (NE-100), as well as being a very potent σ1 receptor antagonist, also has appreciable affinity at the σ2 receptor site (~55x selectivity for σ1 receptors), so modulation of this pathway is a potential mechanism for the actions of NE-100. Further studies involving a selective σ2 receptor antagonist would help to ascertain the role of the σ2 receptors in recognition memory.

Studies from our own laboratory have shown that sub-chronic PCP administration reduces the transcription of the SIGMAR1 gene in the hippocampus, without affecting cortical transcription (unpublished data). A reduction in hippocampal σ1 receptors would result in central administration of σ1 receptor ligands having a comparatively stronger impact in cortical regions in PCP-treated animals, due to loss of hippocampal σ1 receptors, whereas administering the same doses of σ1 receptor modulators in drug-naïve animals would preferentially affect hippocampal neurotransmission, as there is a high concentration of σ1 receptors in this region. PCP-induced changes in receptor numbers may suggest why the same dose of a compound
could show region-specific amelioration of cognitive deficits in two different cognitive paradigms.

5.5.7 Conclusions

The experimental data in this chapter shows that selective agonism and antagonism at σ1 receptors is able to reverse the sub-chronic PCP-induced deficit and 6hr ITI delay-induced deficit in recognition memory. Clinically some drugs with σ1 receptor activity have shown promise in improving cognition in small schizophrenia patient groups and more selective compounds are thought to act, according to the literature, through modulation of dopaminergic/glutamatergic pathways, which are both known to be important in recognition memory. Larger clinical trials and further preclinical experimental work is needed to ascertain the exact role σ1 receptors play in cognitive processes in both animal models and clinical populations.
Chapter 6

General Conclusions
6.1 General Discussion

The work presented in this thesis aims to contribute to the large body of work produced by neurobiological researchers with the goal of developing an animal model that shows considerable construct and face validity to aspects of schizophrenia. Development of a translational animal model for cognitive deficits in schizophrenia is of paramount importance as these deficits are poorly treated by current antipsychotics. The need for well-validated animal models for cognitive deficits and negative symptoms in schizophrenia was highlighted by the MATRICS committee as an essential step to treating patients’ symptoms more effectively. Second generation antipsychotics have been shown to be no more effective at treating cognitive deficits than first generation medications by large-scale clinical trials including CaTIE and CUTLASS (Keefe et al. 2007, Naber and Lambert 2009), a finding that highlights the urgent need for animal models with good construct and face validity. The lack of a ‘gold standard’ antipsychotic medication to act as a positive control is a further obstacle to the development of animal models with good predictive validity for schizophrenia. An important caveat to consider when comparing preclinical and clinical data is that all animal models (with the exception of transgenic models) will involve the use of genetically similar animals kept under optimal housing conditions (lighting, heat and food levels), whereas schizophrenia patients are genetically diverse, frequently have drug abuse co-morbidity and are often taking some form of antipsychotic medication. These confounding factors are present in many patient populations and need to be considered when translating findings from preclinical models into the clinic.

One of the animal models commonly used is the sub-chronic PCP model in rats which produces robust behavioural deficits of relevance to schizophrenia. Previous studies using this model have demonstrated that sub-chronic PCP treatment causes deficits in social interaction, working memory, recognition memory and attention (reviewed in Mouri et al. 2007, Neill et al. 2010, Meltzer et al. 2011, Neill et al. 2013). The behavioural alterations observed in the sub-chronic PCP model are accompanied by pathological changes in the GABAergic system of these animals. Loss of cortical parvalbumin-positive interneurons has been consistently shown in a number of studies (Abdul-Monim et al. 2007, McKibben et al. 2010), as well as a selective cortical reduction in parvalbumin mRNA levels (Cochran et al. 2003). The data presented in this
thesis attempts to expand on this knowledge by studying levels of other GABAergic interneuron population markers as well as GABA_A receptors and investigating their potential role in restoring the cognitive deficits observed in this model. GABAergic markers such as somatostatin and GAT-1 were investigated as they, like parvalbumin and GAD67, have been shown to be consistently reduced in the DLPFC of patients with schizophrenia (Table 4.2). Post-mortem prefrontal cortical studies have not shown a consistent alteration in the number of calretinin-containing interneurons in patient frontal cortices. This class of interneurons was studied in order to add construct validity to the sub-chronic PCP model for a variety of GABAergic interneuron populations, rather than simply focussing on parvalbumin. Clinical studies have also shown altered levels of GABA_A receptor subunits in patients (summarised in table 3.1) and GABA_A receptor subunits, particularly the GABA_A receptor α5 subunit, have been implicated in cognition. GABA_A receptor α5-subunit specific inverse agonists have yielded positive results in enhancing working memory (Collinson et al. 2006, Dawson et al. 2006), spatial memory (Knust et al. 2009) and recognition memory (Damgaard et al. 2011) in preclinical animal models, as well as ameliorating alcohol-induced recall memory deficits in healthy humans (Nutt et al. 2007).

The data presented here demonstrate that sub-chronic PCP causes transcriptional alterations in a number of GABAergic genes relevant to schizophrenia. The GABAergic system has been strongly implicated in causing cognitive dysfunction in accordance with the NMDA receptor hypofunction hypothesis of schizophrenia and this system formed the focus of this thesis. NMDA receptor antagonism at GABAergic interneurons blocks the NMDA receptor-dependent influx of calcium into the cell (Jiang et al. 2010). Calcium-binding proteins such as parvalbumin buffer neuronal calcium levels to prevent Ca^{2+}-mediated cell damage (Pauls et al. 1996) or dysfunctional neuronal plasticity (Lisman 2001). Parvalbumin has been shown to be deficient in schizophrenia patient frontal cortex (Reynolds and Beasley 2001, Hashimoto et al. 2008, Volk et al. 2012) as well as in the frontal cortex of sub-chronic PCP-treated rodents at the protein level (McKibben et al. 2010), suggesting that these cells have impaired calcium buffering ability. We hypothesised that modulators of intracellular calcium stores, in particular sigma-1 receptors (via IP_3 receptors), could be a novel mechanism for ameliorating sub-chronic PCP-induced cognitive deficits. This
hypothesis is supported by clinical findings showing cognitive improvement in small cohorts of patients with schizophrenia who were given compounds with significant \( \sigma_1 \) receptor activity (Silver 2001, Iyo et al. 2008), as well as by post-mortem studies that have shown reductions in the cortical levels of the sigma-1 receptor in schizophrenia patients (Weissman et al. 1991, Shibuya et al. 1992). Sigma-1 receptor function has also been linked to prefrontal cortical activation and NMDA receptor function (Ohi et al. 2011), which are both known to be dysfunctional in schizophrenia. These findings strongly implicate a role for intact sigma-1 receptor function in both schizophrenia, in line with the NMDA hypofunction hypothesis, and cognition. Therefore to help clarify the role that sigma-1 receptors play in impaired cognition in this model, behavioural studies were also carried out to investigate the potential of selective \( \sigma_1 \) receptor modulation as a novel mechanism for the treatment of cognitive dysfunction in this model and therefore in schizophrenia.

Investigation of the impact of sub-chronic PCP administration on the GABAergic system in female rats forms the majority of the work contained in this thesis, with the findings summarised in table 6.1. As the aim of these studies is to try and find a molecular basis for the cognitive deficits observed in this model, female rats were used as they show robust sub-chronic PCP-induced cognitive deficits in the NOR task compared to male rats (Grayson 2012), as well as showing consistent sub-chronic PCP-induced deficits in working memory, attentional set-shifting and social interaction (reviewed in Neill et al. 2010) In order to maintain consistency across these studies, female rats were used for both the ex vivo and in vivo experiments contained in this thesis. The stage of the oestrus cycle has no effect on performance in the NOR task (Sutcliffe et al. 2007, Grayson 2012). The main finding of this study was that sub-chronic PCP induces significant transcriptional alterations in a number of GABAergic genes in the frontal cortex, hippocampus and cerebellum of female rats. No significant PCP-induced transcriptional alterations were observed in the striatum. However autoradiographical and radioligand binding studies demonstrated no significant sub-chronic PCP-induced alterations in the number and composition of either benzodiazepine site- or \( \alpha_5 \) subunit- containing GABA\(_A\) receptors in any of the regions studied, after the washout period, in this model. Further autoradiographical investigation revealed an increase in the affinity for the \( \alpha_5 \)-specific radioligand \([^3H]-L-\)
655,708 in frontal and cerebral cortical regions without an increase in the number of α5-subunit-containing receptors in these areas prior to the washout period. These data suggests that there are an increased proportion of α5-subunits in the GABA\textsubscript{A} receptors in these regions immediately following the cessation of PCP treatment, an effect that was not seen following a 7-day washout period. This effect was not observed in hippocampal, cerebellar or striatal regions.

The effect of σ1 receptor ligands was also investigated using the NOR paradigm, with the principal findings summarised in table 6.2. In summary the σ1 receptor agonist PRE-084 was effective at ameliorating both sub-chronic PCP- and delay-induced recognition memory deficits at 1mg/kg and 3mg/kg, but not at lower concentrations (0.1-0.3mg/kg), as measured by the 1min ITI NOR task for sub-chronic PCP studies and the 6hr ITI NOR task for drug-naïve animals respectively. The sigma-1 antagonist NE-100 also showed pro-cognitive effects in the 1min ITI NOR test at 1mg/kg but not at 0.3mg/kg or 3mg/kg and in the 6hr ITI natural forgetting NOR paradigm at 1 and 3mg/kg but not at 0.3mg/kg. Enhanced cognition observed in both variants of the NOR task with PRE-084-treated animals (1mg/kg) were reversed by co-administration of the specific σ1 receptor antagonist NE-100 at 1mg/kg. These data support the hypothesis that the cognitive improvement observed in these experiments is mediated by σ1 receptors, although as both PRE-084 and NE-100 show pro-cognitive effects, the exact role of σ1 receptors in cognitive enhancement remains undetermined.
<table>
<thead>
<tr>
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<th>Measurement</th>
<th>Brain region</th>
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<tr>
<td>qRT-PCR</td>
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<td>mRNA levels</td>
<td>Frontal cortex</td>
<td>Reduction in GABA(_A) receptor subunit (\alpha 3) and (\delta) mRNA post-washout</td>
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<td>Increase in GABA(_A) receptor subunit (\alpha 5) mRNA post-washout</td>
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<td></td>
<td>Reduction in parvalbumin, calretinin, GAD1, and GAT-1 mRNA post-washout</td>
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<td></td>
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<td></td>
<td>Increase in GFAP mRNA post-washout</td>
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<tr>
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<td>Hippocampus</td>
<td>Reduction in GAD2, GAD1 and GFAP mRNA post-washout</td>
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<td>Frontal cortex</td>
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<td>Frontal cortex</td>
<td>Reduction in (K_o) pre-washout</td>
</tr>
</tbody>
</table>

**Table 6.1**: Summary table showing the effects of sub-chronic PCP administration on the GABAergic system in female rats.

Abbreviations: \(K_o\) - dissociation constant, \(B_{max}\) - maximal level of a ligand which can bind specifically to the receptors in a preparation, mRNA - messenger ribonucleic acid, GFAP - glial fibrillary acidic protein, GAD1 - gene expressing the 67KDa isoform of glutamate decarboxylase, GAD2 - gene expressing the 65KDa isoform of glutamate decarboxylase.
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<th>Antagonist effect (NE-100)</th>
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<td>No effect</td>
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<td>Increased locomotor activity at 0.3mg/kg</td>
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<td>Increased object exploration at 0.3mg/kg</td>
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<tr>
<td>6hr ITI NOR</td>
<td>6hr ITI</td>
<td>Amelioration of deficit at 1 and 3mg/kg</td>
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<td>Decreased acquisition phase exploration</td>
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<td>Reduced locomotor activity at 1mg/kg</td>
<td>Reduced object exploration at 0.3mg/kg</td>
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</tbody>
</table>

**Table 6.2:** Summary table showing the effects of the σ1 receptor agonist (PRE-084) or antagonist (NE-100) in the 1min ITI NOR task used in the sub-chronic PCP studies and the 6hr ITI NOR task used in the drug-naive ‘natural forgetting’ studies.

The main findings from the studies listed above are as follows:

Sub-chronic PCP administration causes a transcriptional reduction in the α3 and δ subunit genes in the frontal cortex, as shown by the reduced mRNA levels in the sub-chronic PCP-treated group compared to the vehicle-treated group. The opposite effect was observed for the α5 subunit gene, which showed increased levels of mRNA in the sub-chronic PCP animal frontal cortices, compared to vehicle animals. It was also found that sub-chronic PCP administration caused a reduction in the dissociation constant ($K_D$) of [3H]-L-655,708 in the frontal and cerebral cortices prior to the washout period, suggesting that there is an increase in the proportion of α5-subunits in the GABA$_\alpha$ receptors in the frontal and cerebral cortical regions. No concomitant increase in α5-subunit-containing receptor number was observed, as shown by the lack of change in Bmax values in all of the regions tested in these studies. This reduction in the $K_D$ value, compared to vehicle animals, was not observed after the 7-day washout period suggesting that post-transcriptional compensatory mechanisms such as reduced trafficking or increased degradation are reducing the expression of the cortical GABA$_\alpha$ receptor α5 subunit during the PCP washout. An increased level of α5 subunits before the washout period in the sub-chronic PCP animals may be indicative of compensatory measures because of a reduction in the levels of another α subunit such as α3. The lack
of a significant difference in GABA$_A$ $\alpha$5 subunit protein levels between sub-chronic PCP- and vehicle-treated animals does not rule out a difference in synaptic location (synaptic or extrasynaptic) due to the influence of ancillary subunits ($\gamma$2 or $\delta$) which could contribute to the cognitive deficits observed in this model.

Sub-chronic PCP administration causes transcriptional reductions in the parvalbumin, calretinin, glutamate decarboxylase isoform 1 and GABA transporter-1 genes in the rodent frontal cortex, as shown by the reduced mRNA levels in sub-chronic PCP-treated animals, compared to the vehicle-treated animals. Increases in GFAP mRNA were observed in the frontal cortex and cerebellum, with a significant reduction being observed in the hippocampus. None of these changes were observed in the protein levels of somatostatin, calretinin, GAD$_{67}$ and GAT-1 suggesting that compensatory mechanisms are maintaining protein levels immediately following the washout period, although parvalbumin and GFAP were not investigated in this study due to problems with the optimisation of the respective antibodies.

The results from chapter 5 show that both sub-chronic PCP administration and a 6hr ITI both cause robust deficits in recognition memory in female rats, as demonstrated by the lack of significant differences between the exploration values for both novel and familiar objects in the respective NOR paradigm. Administration of a selective $\sigma$1 receptor agonist (PRE-084) or a selective $\sigma$1 receptor antagonist (NE-100) attenuated a time-induced deficit in recognition memory at 1mg/kg and 3mg/kg. Administration of PRE-084 at 1mg/kg and 3mg/kg or NE-100 at 1mg/kg also attenuated the sub-chronic PCP-induced recognition memory deficit. The effects of NE-100 at 3mg/kg to attenuate the deficit in this paradigm approached, but did not achieve, statistical significance. The PRE-084-mediated amelioration of recognition memory observed in both NOR paradigms was not observed when NE-100 was administered concomitantly, strongly suggesting a $\sigma$1 receptor-mediated mechanism for this improvement. The doses of both the agonist and antagonist were selected based on the criteria outlined in chapter 2, section 2.8.2 of this thesis. Lower concentrations of these compounds proved ineffective at improving the recognition memory deficits described in this chapter. The mechanism by which PRE-084 and NE-100 exert their effects in the NOR task is not fully understood, although it is thought that the
dopaminergic and glutamatergic signalling pathways are intimately involved. Sigma-1 receptor agonists directly increase frontal cortical dopamine D1 receptor mediated signalling, which has been shown to be involved in recognition memory (Snigdha et al. 2011a). Sigma-1 receptor antagonism has also been shown to increase striatal dopamine levels, which in turn increases hippocampal glutamatergic signalling, which is essential for recognising novelty in the NOR task (Stanley et al. 2012). Further experimentation is required to determine the precise roles of prefrontal cortical dopaminergic and hippocampal glutamatergic signalling, as well as calcium signalling in the mechanism by which sigma-1 receptor modulation produces procognitive effects in the NOR paradigm.

6.2 Construct validity of the sub-chronic PCP model

Animal models have been used to model certain aspects of schizophrenia, although it is unlikely that a single animal model would be able to model such a complex disease, as these often involve dysfunction in complex cognitive processes such as verbal memory and hallucinatory activity which has not yet been successfully modelled in lower organisms. Attempts have been made to study hallucinations in rodents using behavioural effects such as the head-twitch response (HTR) in response to hallucinogen administration (Halberstadt and Geyer 2013) and “impaired-reality” behavioural tasks (McDannald et al. 2011), however animal models are mostly used to model specific symptom clusters in schizophrenia, such as social dysfunction and deficits in certain cognitive domains. The sub-chronic PCP model has been shown, in a number of studies, to induce behavioural alterations of relevance to schizophrenia, including deficits in working memory, social interaction and object recognition memory. The work in this thesis investigates the pathological alterations caused by this regime; table 6.3 compares the findings in this thesis with clinical post-mortem studies.
Table 6.3: Comparison of pathological transcriptional alterations observed in the DLPFC of schizophrenia patients, with the pathological transcriptional changes observed in the frontal cortex of the sub-chronic PCP model of cognitive deficits.

According to the data shown in table 6.3, the interneuron population marker deficits which are present in the sub-chronic PCP model most closely mirror those seen in patients. This adds to the weight of evidence showing that the sub-chronic PCP model has good face validity for some of the pathological deficits observed in schizophrenia. An important point to consider is that throughout these studies the rat ‘frontal cortical’ region has been defined as the area rostral from Bregma +3.2mm, excluding the olfactory bulb. This area includes the prelimbic and infralimbic cortices as well as parts of the anterior cingulate. The DLPFC in humans has no direct anatomical equivalent in rodents; however some of the functionality of the human DLPFC is present in the rat frontal cortex such as modulating working memory processes. This would suggest that modelling GABAergic deficits in the rat frontal cortex would have a comparable detrimental effect on memory processes as the GABAergic deficit observed in the DLPFC of schizophrenia patients.

6.3 Limitations of the current studies

The current studies have shown that the sub-chronic PCP rodent model accurately models aspects of schizophrenia, as shown by the data contained within this thesis. However conclusions drawn from the data are subject to the following limitations:
• Low n numbers for qRT-PCR
• Genetic and proteomic techniques describe whole regions
• NE-100 has significant sigma-2 receptor affinity

The qRT-PCR studies described in chapter 3 and 4 of this thesis demonstrate that the sub-chronic PCP model shows reductions in genes relevant to schizophrenia such as parvalbumin and GAD_{67}, however due to the low numbers of samples in some assays (n=3) some tests showed large errors, making it difficult to detect subtle or small-scale changes. Increasing the sample size to 6 for each group would increase the power of the qRT-PCR assays described in this thesis.

The qRT-PCR, radioligand binding, autoradiography and western blotting described in chapters 3 and 4 of this thesis shows changes in whole brain regions (eg: striatum and hippocampus), not subfields (eg: CA3 vs CA1) of these regions. A lack of a significant difference in a region does not necessarily mean that PCP has no effect there, as PCP-induced increases in a particular marker in certain subregions may be masked by concomitant reductions in others or alterations may be too subtle or localised to be detected using the techniques listed above.

It is impossible to discount the possibility of NE-100 acting through the sigma-2 receptors to give rise to the behavioural effects that are described in chapter 5. The specificity of NE-100 suggests that it would act through the sigma-1 receptor, however without the co-administration of a specific sigma-2 receptor antagonist this cannot be determined for certain in these studies.

Possible solutions to these limitations and potential future studies are addressed later in chapter 6 of this thesis (chapter 6.5).

6.4 Possible mechanism of sub-chronic PCP on gene transcription with relevance to cognition

The data presented in this thesis shows that sub-chronic PCP administration reduces the transcription of a number of GABAergic genes in the frontal cortex. Many of these genes, especially parvalbumin, somatostatin and GAD1, have also been shown to be down-regulated in the cortex of schizophrenia patients. These post-mortem studies were carried out on post-mortem tissue obtained from patients who were on a
variety of typical and atypical antipsychotic medications at the time of death, as shown in table 4.2, which is replicated below.
<table>
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<tr>
<th>Marker</th>
<th>Brain Region</th>
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<td>Reduction</td>
<td></td>
<td>---</td>
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<td>Reduction</td>
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<td>Reduction</td>
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</tr>
<tr>
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<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>PFC</td>
<td>Protein</td>
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<tr>
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<td>Protein + mRNA</td>
<td>Unchanged</td>
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Table 6.4: Replication of table 4.2 showing cortical alterations in GABAergic markers and antipsychotic medication information of schizophrenia patients. All protein measurements were made using western blotting techniques except the following exceptions: ^a – autoradiography, ^b – immunoblotting/immunohistochemistry. (#) - includes risperidone, ($) - includes fluoxetine (---) - no details given
As shown in table 6.4, antipsychotic medication regimens are often not reported in detail in studies looking at GABAergic alterations in schizophrenia patients, with only 6 of the 16 studies providing drug names. Work by Curley et al. (Curley et al. 2011) suggested no significant effect of antipsychotic medication on GAD67 mRNA levels (36 medicated patients and 6 drug-naive patients), although further comparative studies with larger drug-naive patient populations would lend greater support to this conclusion. However a study by Cochran and colleagues (Cochran et al. 2003) showed that chronic administration of the atypical antipsychotic clozapine but not the typical antipsychotic haloperidol was able to reverse PCP-induced reductions in parvalbumin mRNA in rats. Further studies using animal models are required to determine whether antipsychotic medications play a role in the gene transcription alterations that are observed in schizophrenia patients.

The mechanism by which sub-chronic PCP-mediated NMDA antagonism alters GABAergic gene transcription is still unclear, although a potential mechanism for this reduction in gene transcription is explored below. This mechanism is supported by findings in the current literature, but further experimentation is required to confirm whether it is causative in producing the changes observed in the sub-chronic PCP model.

PCP is a NMDA receptor antagonist with an affinity of 2μM (Kapur and Seeman 2002) which blocks NMDA receptors following ligand- and voltage-dependent activation by binding to the PCP site within the membrane channel (See figure 1.5 in chapter 1 of this thesis). The most common groups of NMDA receptors consist of two NR1 subunits (subtypes NR1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B) combined with a further two NR2 subunits (subtypes NR2A-D). The NR2 subunit controls receptor localisation and therefore receptor mechanics, with the NR2A subunit being localised at synaptic sites, while NMDA receptors containing an NR2B subunit are predominantly extrasynaptic (Vanhoutte and Bading 2003). NR2A-subunit-containing receptors show an increased opening probability in the presence of NMDA or glutamate, compared to NR2B-subunit-containing receptors (Erreger et al. 2005). As PCP requires an open NMDA pore in order to exert its antagonistic action, this would mean that PCP preferentially blocks NR2A-subunit-containing receptors. A post-natal phencyclidine administration regime (10mg/kg on PND 7, 9, 11) has been shown to preferentially act through NR2A-subunit-
containing NMDA receptors in rats (Anastasio et al. 2009), lending evidence to the NR$_2$ subunit-selective action of PCP. GABAergic interneurons express higher levels of NR$_{2A}$-subunit-containing NMDA receptors, compared with pyramidal cells, making interneurons a preferential target for PCP (Kinney et al. 2006, Xi et al 2009). Reductions in GAD$_{67}$ would selectively affect chandelier cells as they preferentially express GAD$_{67}$ over GAD$_{65}$ in their axons (Fish et al. 2011). This is supported by findings in chapter 4 of this thesis which show that PCP-induced alterations occur in multiple populations of interneurons, including parvalbumin- and somatostatin-containing cells.

Blockade of NR$_{2A}$ subunit-containing NMDA receptors on GABAergic interneurons by PCP would lead to reduced calcium influx into the cell. This activity-dependent influx of calcium through NMDA receptors results in the activation of calmodulin (West et al 2001). Activated calmodulin activates a wide range of calcium-mediated signalling pathways including the PKC, Ras/ERK, CaMK and cAMP/PKA pathways, which phosphorylate and activate the transcription factor CREB (cAMP response element binding protein). Activated CREB translocates to the nucleus and helps to initiate the transcription of genes containing a cAMP-response element (CRE) sequence in their promoter (Sanchez-Munoz et al. 2010). Therefore suppression of NMDA signalling in interneurons (by sub-chronic PCP administration) is likely to reduce calmodulin activation and therefore CREB activation. This would reduce the activity-dependent transcription of CRE-responsive genes such as PVALB, SST and GAD1, which is observed in the frontal cortex of sub-chronic PCP-treated rats in chapter 4 of this thesis. However this direct reduction in the transcription of CRE-responsive genes would likely only apply while PCP was in the system. The data in this thesis shows that alterations in GABAergic gene mRNA levels in the sub-chronic PCP model are present 7 days after the final PCP injection, suggesting that there are other signalling pathways behind the transcriptional alterations observed in chapters 3 and 4 of this thesis.

PCP, apart from its primary action at the NMDA receptor, also acts to increase the binding affinity of calcium channel antagonists (Bolger et al. 1985) and competitively inhibits the Ca$^{2+}$ATPase enzyme (Pande et al. 1999), as well as reducing the expression of CaMKII subunits and calcineurinA $\alpha$ and $\gamma$ subunits (Wesseling et al. 2013). This demonstrates that PCP disrupts intracellular calcium signalling, beyond its
blockade of the NMDA receptor. Evidence for the role of disrupted calcium signalling affecting GABAergic gene transcription is supported by findings from Kinney and colleagues who demonstrated that antagonism at NR2A-subunit-containing NMDA receptors resulted in significant decreases in the expression of GAD67 and parvalbumin (Kinney et al. 2006) which could be rescued by the application of a calcium channel opener.

This hypothesis of a calcium-mediated mechanism of gene transcriptional downregulation in the sub-chronic PCP model is also supported by observations in the BDNF gene. BDNF is a neuronal transcription factor which is highly expressed in the frontal cortex and hippocampus that has also been implicated in the formation of short- and long-term memory (Alonso et al. 2002), making it a plausible target for both the cognitive and transcriptional effects of PCP. Acute PCP administration causes reductions in BDNF levels in as little as 30mins (Katanuma et al. 2014) and a sub-chronic PCP regime (2mg/kg i.p. b.i.d) has been shown to cause a reduction in frontal cortical BDNF mRNA levels in rats (Snigdha et al. 2011b). BDNF binds to the TrkB receptor, which auto-phosphorylates and induces the transcription of genes containing a CREB promoter site such as PVALB, GAD1 and BDNF, but the activity-dependent transcription of BDNF is regulated by calcium response elements (including calcium response element 1 (CaRE1)), further linking intra-cellular calcium signalling and the transcription of GABAergic genes.

PCP-mediated reductions in BDNF levels in parvalbumin-positive interneurons would therefore further reduce the transcription of genes with a cAMP response element (CRE) at or near its promoter sequence, such as the PVALB, SST and GAD1 genes, as well as reducing auto-activation of BDNF gene transcription. Reduced TrkB signalling due to a reduction in BDNF levels would further impair NMDA receptor mediated signalling, as TrkB receptors have been shown to induce the phosphorylation of the NR2A and NR2B subunits of the NMDA receptor. This would exacerbate the antagonistic effects of PCP after the removal of PCP from the system. Unpublished data from our group has revealed that the reduction in hippocampal parvalbumin immunoreactivity observed after a 1 week washout period following PCP administration recovers after 3 weeks, which supports the hypothesis that sub-chronic
PCP administration causes a reversible region-specific loss of phenotype in parvalbumin-positive interneurons.

This proposed calcium- and BDNF-mediated transcriptional impairment (Figure 6.1) would also be a likely candidate for the pro-cognitive effects of σ1 receptor stimulation seen in chapter 5 of this thesis, as σ1 receptor agonism has been shown to stimulate the release of BDNF (Fujimoto et al. 2012), as well as increasing calcium flow through IP₃ receptors (Hayashi and Su 2007). This suggested mechanism is shown in figure 6.1 although further ex vivo experimentation is required to confirm whether this hypothesis is accurate in the rodent and human brain.

**Figure 6.1**: A simplified diagram showing the proposed mechanism of action for sub-chronic PCP-mediated gene repression and a potential site of action for the pro-cognitive effects of σ1 receptor agonism. aCaM – activated calmodulin, CaM – calmodulin, CaMKII – calmodulin-dependent protein kinase II, cAMP/PKA – cAMP-dependent protein kinase signalling pathway, CRE – cAMP response element, CREB – cAMP response element binding protein, CREB-P – activated CREB, ER – Endoplasmic reticulum, PKC – protein kinase C signalling pathway, PMCA – plasma membrane calcium ATPase, SERCA – sarco/endooplasmic reticulum calcium ATPase.
6.5 Future Studies

Many of the post-mortem studies that have been conducted into alterations in GABAergic markers in schizophrenia have been carried out using PCR technologies, which have a number of advantages including high specificity and accuracy. Many of the changes that were observed in the sub-chronic PCP model have also been observed at the transcriptional/gene level. However, as the findings in chapters 3 and 4 suggest, alterations in mRNA levels do not automatically result in equivalent alterations in protein levels and therefore functionality. This highlights the need for multi-disciplinary studies involving genetic, proteomic and imaging technologies in order to distinguish between causative, noncausative and confounding factors/results, and therefore to elucidate the dysfunctional pathways involved in cognitive impairment.

In order to gain a more complete understanding of the pathological changes caused by sub-chronic PCP administration, and therefore the molecular basis of cognitive deficits in schizophrenia, the answers to four main questions are required:

1) What is the relationship between the transcriptional and functional deficits observed in the sub-chronic PCP model and how can this be applied to the transcriptional deficits observed in patients?

2) Is the reduction in the apparent number of certain populations of GABAergic interneurons caused by a loss of GABAergic phenotype?

3) By what mechanism does sub-chronic PCP cause transcriptional alterations?

4) Does chronic antipsychotic medication play a role in the GABAergic deficits observed in patients and/or the sub-chronic PCP rodent model?

1) What is the relationship between the transcriptional and functional deficits observed in the sub-chronic PCP model and how can this be applied to the transcriptional deficits observed in patients?

As shown in this thesis, sub-chronic PCP administration causes a number of transcriptional alterations in GABA$_A$ receptor subunits (Chapter 3) and GABAergic cell markers (Chapter 4) that are present 7 days after the cessation of PCP administration, however it remains to be proven that these alterations are responsible for the cognitive dysfunction observed in this model. These frontal cortical transcriptional
alterations in GABAergic markers (Chapter 4) show close similarity to those found in the frontal cortical regions of patients with schizophrenia, however the lack of difference at the protein level suggests that the GABAergic changes reported in this chapter are too subtle to be detected by whole-region western blotting. This highlights the differences in sensitivity between the strongly quantitative qRT-PCR method and the semi-quantitative western blotting techniques. Causative links between differences in protein and mRNA levels in patients is very difficult to achieve due to numerous confounding factors in clinical populations including differing antipsychotic medication regimens, genetic variability, drug use, lifestyle as well as duration of the illness. Given these difficulties, it is essential that well-established animal models, such as the sub-chronic PCP model, are used to study the link between genetic and proteomic alterations.

Data from chapter 3 of this thesis shows that the composition of extrasynaptic GABA<sub>A</sub> receptors in the frontal cortex alters during the washout period; increased α5 subunit levels were observed in sub-chronic PCP-treated animals compared to vehicle animals immediately following the final injection of PCP, an effect which was not seen following the washout period, despite the presence of a transcriptional increase in this subunit in PCP-treated animals compared to vehicle animals post washout. Autoradiographical or immunogold electron microscopy analysis of α5- and δ-subunit-containing receptors using the specific radioligands [³H]-L-655,708 (α5-subunit specific) and [³H]-Ro154513 (δ-subunit specific) or antibodies specific to these subunits, combined with mRNA analysis of these subunit genes would reveal how extrasynaptic GABA<sub>A</sub> receptor composition and subunit transcription is altered within the frontal and cerebral cortices of sub-chronic PCP-treated animals before and after the washout period. This would help to elucidate the role of extrasynaptic GABA<sub>A</sub> receptors in the cognitive and pathological deficits observed in the sub-chronic PCP model, although the pre-washout comparisons would be complicated by the presence of PCP.

Data from chapter 4 of this thesis shows that sub-chronic PCP administration causes frontal cortical alterations in a number of GABAergic marker genes including parvalbumin, somatostatin, GAD<sub>67</sub> and GAT-1. In order to determine whether these transcriptional changes have a functional impact on the frontal cortical GABAergic
interneuron network of rats who have been administered PCP sub-chronically, concurrent *ex vivo* analysis of mRNA levels (using qRT-PCR) and densitometric immunohistochemical staining would be carried out in the prelimbic, infralimbic and anterior cingulate regions of the frontal cortex for the markers described above. This data would suggest whether the transcriptional alterations correlate with a functional loss of specific GABAergic interneurons in the rodent frontal cortex. Identifying region(s) of the frontal cortex that are particularly vulnerable to sub-chronic PCP administration will also be useful as it will help to guide future studies including the placement of microdialysis probes or intracerebroventricular injection of pharmacological interventions prior to cognitive testing. The experiments required to determine whether any apparent loss of the markers described above is due to a loss of the relevant cells or a loss of phenotype are detailed below in answer 2.

Cognitive testing using the NOR paradigm, as described above, in conjunction with frontal cortical *in vivo* electrophysiology could then be conducted on a separate cohort of animals when the pathological deficit(s) of interest manifests. Dysfunctional oscillatory activity in the gamma frequency band, caused by GABAergic interneuron dysfunction is thought to be responsible for cognitive impairment in both schizophrenia patients and sub-chronic PCP-treated rats (Kissler et al. 2000, Minzenberg et al. 2010, McLean et al, *in preparation*). These studies described above have the ability to causatively link pathological changes in GABAergic interneurons, with impaired oscillatory activity and cognitive performance, if such a link is present in this model. The identification of a transcriptional and functional deficit in this model and subsequent correlation with cognitive impairment would help to explain the molecular basis of the cognitive deficits observed in this model, as well as suggesting potential pharmacological intervention targets. The use of a second cohort of animals to perform the NOR/electrophysiological experiments is necessary to prevent the act of performing the NOR task from affecting the pathological findings, especially the qRT-PCR results as gene transcription could potentially alter within the time frame of the task or as a result of performing the task.
2) Is the reduction in the apparent number of certain populations of GABAergic interneurons caused by a loss of GABAergic phenotype?

Question 2 could be answered by immunohistochemical staining analysis for parvalbumin, GAD$_{67}$ and a general GABAergic interneuron marker such as ErbB4 which is known to be co-expressed in parvalbumin-containing interneurons in rodent cortex (Neddens et al. 2011) and hippocampus (Vullhorst et al. 2009). Microscopy techniques such as confocal or fluorescence microscopy would allow co-staining of these three markers and would also allow quantitative analysis of the number of interneurons and the percentage of these that contained parvalbumin in rat frontal cortex. These studies would reveal if the loss of parvalbumin reactivity, which is commonly observed following NMDA antagonist treatment, is due to a reduction in the number of parvalbumin-containing cells (through apoptosis) or due to a specific loss or reduction of parvalbumin in these cells. The use of slice immunohistochemistry also permits the cortical layer-specific analysis of these markers to study alterations in interneurons in individual cortical layers. Further studies could be conducted in somatostatin- or calretinin-containing interneuron populations in the same manner to complement and expand on the existing knowledge around individual GABAergic interneuron populations.

Due to the increases in GFAP that were observed in the studies presented here, analysis of the numbers and morphology of GFAP-positive cells would also be important. An increase in GFAP levels could be indicative of astrogliosis in response to the neurotoxic insult of repeated PCP administration or of astrocytic dysfunction/proliferation. Astrocytes play a significant role in glutamatergic signalling by removing glutamate from the synaptic cleft, therefore preventing neurotoxicity and excessive neuronal excitation, as well as providing glutamine and GABA to neurons as part of the “glutamate-glutamine-GABA metabolic cycle”. Clinical studies have shown alterations in astrocyte-specific proteins in the DLPFC and ACC of schizophrenia patients compared to matched controls: these include an increase in GLT-1 mRNA and protein in patient prefrontal cortex (Matute et al. 2005) and decreases in patient ACC and superior temporal gyrus glutamine synthetase protein levels (Steffek et al. 2008). These enzymes are all intimately involved in glutamatergic neurotransmission, which is
known to be dysfunctional in schizophrenia. Increases in the astrocytic neurotrophic factor S100B have also been observed in patient serum (Rothermundt et al. 2004b) and CSF (Rothermundt et al. 2004a). High levels of S100B in the serum correlate closely with negative symptom severity of patients (Rothermundt et al. 2004b), highlighting the importance of studying astrocytic function in schizophrenia. Sub-chronic ketamine (Featherstone et al. 2012) and sub-chronic phencyclidine (5mg/kg i.p. for 7 days, followed by a 7 day washout period, Hajszan et al. 2006) have been shown to increase astrocyte proliferation and process density in male mice and rats respectively, supporting the findings in this chapter showing an increase in GFAP expression. This clinical and preclinical evidence strongly implicates astrocytes in contributing to the pathophysiology observed in NMDA receptor antagonism models. Although the focus of this thesis is on the GABAergic system, the role of potential astrocytic dysfunction in this model should be investigated further due to their intimate neurotransmitter and metabolomic links with neurons, particularly in the glutamatergic system.

3) By what mechanism does sub-chronic PCP cause transcriptional alterations?

Further in vitro experimentation would be required to gain the answer to question 3. A possible mechanism by which sub-chronic PCP affects gene transcription in vivo is suggested in section 6.3 of this thesis, with the evidence from the literature and the studies contained in this thesis hypothesising that calcium plays a role in the transcriptional alterations observed in this model. A primary cell culture model of rat cortical neurons would be the most suitable model in which to study these intracellular mechanisms. The advantages to using a cell based assay rather than an in vivo assay for this mechanistic work are that the extracellular medium conditions can be tightly regulated and it is easier to introduce pharmacological compounds into the cells at more accurate concentrations than if the compound was introduced centrally in vivo. After rats complete the sub-chronic PCP or vehicle administration regime, cortical neurons would be extracted and transfected with a calmodulin based calcium dye such as cameleon. This, combined with real-time fluorescence imaging would allow real-time imaging of the intracellular calcium state of the cells. After the cell cultures have stabilised, the effects of antagonists of calcium channels can be studied with IP₃ receptor, ryanodine receptor, voltage- and store-operated calcium channels and
NMDA receptor antagonists being the most relevant as they are responsible for the majority of calcium influx from intra- and extracellular pools. Following treatment with pharmacological compounds, samples of cells can be easily removed for mRNA analysis to study the effects of calcium signalling on cortical interneuron gene transcription.

4) Does chronic antipsychotic medication play a role in the GABAergic deficits observed in patients and/or the sub-chronic PCP rodent model?

Many of the studies which have shown transcriptional deficits in GABAergic markers (summarised in table 6.4) have contained patients which have been on a variety of antipsychotic and other medications at the time of death (summarised in table 6.4 and appendix table A3), however studies looking at the effects of chronic antipsychotic administration in animal models have not been routinely performed. Amitai and colleagues administered antipsychotics chronically in order to ameliorate sub-chronic PCP-induced deficits, but obtained mixed results. 4mg/kg/day clozapine for 14 days ameliorated the sub-chronic PCP-induced (2mg/kg for 2 days and then 5 days s.c.) cognitive deficits in the 5-CSRTT task (Amitai et al. 2007), however chronic quetiapine (10mg/kg/day for 14 days) was unable to reverse these same deficits in a later study (Amitai and Markou 2009). Clozapine (20mg/kg/day for 28 days), but not haloperidol (1mg/kg/day for 28 days) was also able to reverse chronic PCP-induced (2.58mg/kg on days 1-5, 8, 10, 12, 15, 17, 19, 22, 24, 26) PV mRNA deficits in male rats (Cochran et al. 2003). A study by Grace and colleagues (Gill et al. 2014) looked at the clinically relevant scenario of typical antipsychotic withdrawal and its effects on the efficacy of novel compounds; a novel antipsychotic compound (GABA<sub>A</sub> receptor α5 positive allosteric modulator) was unable to reverse electrophysiological deficits in dopaminergic neurons in the VTA of a developmental model of schizophrenia (MAM model) after chronic haloperidol treatment and withdrawal (21day treatment 0.6mg/kg orally, 7 day withdrawal). This finding suggests that antipsychotics given to patients following withdrawal of their usual antipsychotic medication may have reduced efficacy, which may explain why novel pharmacological compounds have not been as successful in human trials as was expected and in some cases have been known to cause relapse. This study also used a clinically relevant drug administration route (oral), instead of the more common i.p. or s.c. routes, however the data may be
complicated by individual housing of the animals which has been shown to cause deficits in several tests of cognition (McLean et al. 2010a). Investigation into the cognitive and pathological effects of chronic antipsychotic administration and/or withdrawal in the sub-chronic PCP rodent model should also be investigated using the techniques described previously in this chapter to add to the clinical relevance of this model. Therefore it is essential that the effect of chronic antipsychotic administration on gene transcription and protein expression is studied in the sub-chronic PCP model, at a dose and duration regime which is relevant to that which is used by patients.

Answering these four important questions would give a firm indication as to whether the current focus on the GABAergic system is merited, in terms of being a causative factor for the recognition memory deficits that are observed in this model. An advantage to using a well-established animal model is that carrying out this kind of time-course study is not possible in human post-mortem tissue as time- and disease-stage specific tissue is more difficult to obtain than rodent tissue. Another advantage to using well-established animal models is that confounding factors such as antipsychotic medications can cause alterations in cortical and hippocampal pathology, further emphasising the need for pathologically relevant animal models such as the sub-chronic PCP rodent model.

As previously discussed the mechanism by which the sigma-1 receptor agonist PRE-084 and the sigma-1 receptor antagonist NE-100 exert their pro-cognitive effects is thought to involve interaction between the dopaminergic and glutamatergic signalling pathways. Further experimentation using in vivo microdialysis combined with cognitive testing in the NOR task would help to elucidate the role of sigma-1 receptor modulators on these neurotransmitter systems and their role in recognition memory. NOR testing, combined with hippocampal and frontal cortical microdialysis in sub-chronic PCP- and vehicle-treated animals would be essential to study the effects of PRE-084 or NE-100 on neurotransmitter release. Sample collection would begin immediately following central administration of PRE-084 or NE-100 to study the immediate drug effects. Microdialysis samples would be collected throughout the NOR task and divided into two (~10μl per sample) before both would be analysed by high-pressure liquid chromatography (HPLC) with electrochemical detection or mass
spectrometry. One sample would be used to determine alterations in dopamine concentration and the other would be used to analyse glutamate and acetylcholine as previously described (Stanley et al. 2012). This simultaneous recording of 3 neurotransmitters from a single animal would reduce the number of rats used for this experiment. These microdialysis studies could also be combined with in vivo PET scanning after injection of a tracer such as fluorodeoxyglucose to allow for the simultaneous analysis of brain activation and neurotransmitter release in the rodent NOR task following sigma-1 receptor agonism or antagonism. These studies would provide more information about how sigma-1 ligands affect cognitive processes, with a particular focus on frontal cortical dopaminergic and hippocampal glutamatergic changes, as discussed previously in chapter 6.1 of this thesis.

Testing of the sigma-1 agonist PRE-084 and the sigma-1 antagonist NE-100 in more complex cognitive tasks such as attentional set-shifting and reversal learning, which involve the prefrontal cortex and reflect different aspects (problem solving vs recognition memory) of cognition, would add support to the idea that σ1 receptor ligands could be developed to treat multiple aspects of the cognitive deficits observed in schizophrenia, rather than recognition memory alone. These two sets of experiments, if successful, would add to the body of evidence supporting the hypothesis that sigma-1 receptor modulators have the potential to be used as pharmacological treatments for the cognitive deficits observed in schizophrenia.

6.6 Conclusions

Complex neuropsychiatric diseases such as schizophrenia continue to pose problems to researchers, due to the apparent dysfunction of a number of cellular and neurological pathways that is present in these disorders. This complexity has hindered the development of a single valid animal model for schizophrenia. Animal models have instead been utilised to represent certain domains of this disorder, with the sub-chronic PCP model being used by an increasing number of laboratories. The neurobiological data presented in this thesis support the hypothesis that the sub-chronic PCP rodent model of cognitive deficits has considerable construct validity; this dosing regimen in female rats gives rise to cognitive and pathological alterations that are consistently observed in schizophrenia patients. The data in chapter 4 has expanded on the result that sub-chronic PCP administration causes parvalbumin and
GAD1 mRNA reductions, with findings that this model also demonstrates cortical reductions in somatostatin and GAT-1 mRNA levels. These findings mean that this model shows good face validity to clinical findings in patients and therefore is a useful tool to help understand the causative mechanisms of cognitive and social dysfunction in schizophrenia. This model may also be used to help further investigations into potential novel treatments for schizophrenia. The data contained in this thesis demonstrate that sigma-1 receptor agonism and antagonism are both pro-cognitive in two variants of a novel object recognition paradigm, the first study to the authors knowledge to study these compounds in the sub-chronic PCP rodent model. The findings in chapter 5 support the hypothesis that the σ1 receptor is a potential pharmacological treatment for cognitive deficits in schizophrenia, a hypothesis that is supported by small clinical trial data.
Chapter 7

References
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## Appendix I: qRT-PCR

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>Dilution series r² value</th>
<th>Dilution series slope</th>
<th>% efficiency</th>
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<tr>
<td>Parvalbumin</td>
<td>0.9752</td>
<td>-3.634</td>
<td>88.4425</td>
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<tr>
<td>Somatostatin</td>
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<td>Calretinin</td>
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<td>GAD65</td>
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<tr>
<td>GAD67</td>
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<td>103.3581</td>
</tr>
<tr>
<td>GFAP</td>
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<td>104.2555</td>
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<tr>
<td>GAT-1</td>
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<td>GAPDH</td>
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<td>111.965</td>
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**Table A1**: Table showing the percentage efficiency values and the dilution series r² value for each primer reaction used in this thesis.
Appendix II: Autoradiography dilution curves

Figure A1: Protein dilution curves used to calculate $K_D$ and $B_{max}$ for $[^3H]$-flumazenil
Figure A2: Radioligand dilution curves used to calculate $K_D$ and $B_{max}$ for $[^3H]L-655,708$
Appendix III: Autoradiographs for $[^3\text{H}]-\text{flumazenil}$ and $[^3\text{H}]-\text{L-655,708}$

**Figure A3:** Example autoradiographs showing $[^3\text{H}]-\text{flumazenil}$ binding in 20μm sagittal sections of female hooded-Lister rat brain. (a) total binding, (b) – blank (10μM flunitrazepam)

**Figure A4:** Example autoradiographs showing $[^3\text{H}]-\text{L-655,708}$ binding in 20μm sagittal sections of female hooded-Lister rat brain. (a) total binding, (b) – blank (10μM flunitrazepam)
Appendix IV: Western blotting antibody optimisation

![Graphs showing the linear relationship between protein concentration and densitometric values at the recommended antibody concentration for each marker tested in these studies.]

**Figure A5:** Graphs showing the linear relationship between protein concentration and densitometric values at the recommended antibody concentration for each marker tested in these studies.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Linearity (10-40μg protein)</th>
<th>Linearity (20-40μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calretinin</td>
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<tr>
<td>Somatostatin</td>
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<tr>
<td>GAD67</td>
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<td>0.9963</td>
</tr>
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<td>GAT-1</td>
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<td>0.9283</td>
</tr>
<tr>
<td>GAT-3</td>
<td>0.9609</td>
<td>0.9998</td>
</tr>
</tbody>
</table>

**Table A2:** Table showing the linear relationship between frontal cortical protein amount (10-40μg and 20-40μg) and densitometric values in the Western blotting antibody optimisation protocol.
Appendix V: Western blotting images

**Figure A6:** Western blot image for somatostatin in sub-chronic PCP and vehicle female hooded-Lister rat frontal cortex
Expected MW - 13KDa
Observed MW - 12KDa

**Figure A7:** Western blot image for calretinin in sub-chronic PCP and vehicle female hooded-Lister rat frontal cortex
Expected MW - 29KDa
Observed MW - 29KDa

**Figure A8:** Western blot image for GAD$_{67}$ in sub-chronic PCP and vehicle female hooded-Lister rat frontal cortex
Expected MW - 67KDa
Observed MW - 67KDa

**Figure A9:** Western blot image for GAT-1 in sub-chronic PCP and vehicle female hooded-Lister rat frontal cortex
Expected MW - 67KDa
Observed MW - 74KDa
**Figure A10:** Western blot image for GAT-3 in sub-chronic PCP and vehicle female hooded-Lister rat frontal cortex

Expected MW - 74KDa

Observed MW – 72KDa