



Neuronal Nicotinic Receptors as Targets for Enhancing Cognition in Schizophrenia

Samantha L. Rushforth

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Neuronal Nicotinic Receptors as Targets for Enhancing Cognition in Schizophrenia

Samantha L. Rushforth Newcastle University, UK

Supervisors:

Dr Mohammed Shoaib, PhD

Institute of Neuroscience
Newcastle University
Framlington Place
Newcastle upon Tyne
NE2 4HH
United Kingdom

Dr Fiona LeBeau, PhD

Institute of Neuroscience
Newcastle University
Framlington Place
Newcastle upon Tyne
NE2 4HH
United Kingdom

Dr Thomas Steckler, PhD

Johnson and Johnson PRD
Neuroscience
Turnhoutseweg 30
B-2340 Beerse
Belgium

Candidate's Declaration

I certify that this thesis is my own work and has not been submitted for any degree other than that of Doctor of Philosophy at the University of Newcastle upon Tyne.

During this process I received help in running the Odour Span Task for the examination of PHA-543613, presented in Chapter 5, from research associate Emma Malcolm. I was involved from start to finish in the decision making process with regards to choice of compound and doses along with experimental protocol, but did not carry out the experiment myself. All data analysis and conclusions are my own.

Data for Chapters 2, 3, 4, 6 and 7 were collected at Newcastle University. The data presented in Chapter 5 was collected at Newcastle University ($\alpha 7$ agonist data) and at Janssen Pharmaceutical ($\alpha 4\beta 2$ agonist data). Local injection data presented in Chapter 8 was collected wholly at Janssen Pharmaceutical.

Samantha Leigh Rushforth

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Abstract

Cognitive deficits are a core disabling feature of schizophrenia, yet remain inadequately treated by current pharmacological or behavioural therapies. The non-competitive NMDAR antagonist ketamine can pharmacologically induce cognitive deficits in both rodents and humans, presenting a novel translational approach for examining mechanisms underlying cognitive deficits associated with schizophrenia (CDS). Nicotine can improve working memory in rodents and in smokers with schizophrenia where heavy tobacco use may reflect self-medication to ameliorate CDS. The roles of the two main subtypes of nAChRs, the $\alpha 7$ and $\alpha 4\beta 2$, in mediating cognitive improvement have yet to be determined. Cohorts of male hooded-Lister rats were trained in the Odour Span Task (OST) until demonstrating asymptotic performance and then exposed to a sub-anaesthetic dose of ketamine or vehicle daily for 5 consecutive days. This sub-chronic regimen produced a replicable, dose-dependent impairment in OST performance that was not restored following anti-psychotic treatment. Nicotine, $\alpha 7$ and $\alpha 4\beta 2$ nAChR-selective agonists improved performance in ketamine-treated animals, with nicotine and one $\alpha 4\beta 2$ agonist also improving the performance of control subjects. These data indicate the $\alpha 4\beta 2$ nAChR as the main receptor subtype mediating the effect of nicotine on the OST in control animals, with a lesser role for the $\alpha 7$ nAChR. The $\alpha 7$ nAChR however was shown to have a role in improving the performance of ketamine-treated animals, as demonstrated by the enhancing effect of allosteric modulator PNU-120596 and Compound T on OST performance; an effect that was blocked by the $\alpha 7$ nAChR antagonist methyllycaconitine. When administered locally into the medial prefrontal cortex (mPFC), nicotine improved, and muscimol impaired OST performance; suggesting the mPFC as the neural site of action in the OST. Complementary data using an *in-vitro* electrophysiological gamma frequency model of network oscillations indicated an enhancing effect of nicotine on normal gamma frequency oscillations in the rat mPFC and is proposed as a potential mechanism behind the behavioural data. Collectively, these results provide further impetus for targeting nAChRs in the treatment of CDS.

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Table of Contents

Table of Contents	vi
List of Tables	xiv
List of Figures.....	xv
List of Abbreviations	xix
<u>Chapter 1: General Introduction.....</u>	1
1.1 An overview of the Schizophrenia Syndrome	2
1.1.1 <i>Clinical symptoms and diagnosis.....</i>	<i>2</i>
1.1.2 <i>Causes of schizophrenia: Genetic or environmental?</i>	<i>4</i>
1.1.3 <i>Treatment</i>	<i>8</i>
1.1.4 <i>The TURNS and MATRICS initiative.....</i>	<i>11</i>
1.2 The prefrontal cortex.....	15
1.2.1 <i>Primate vs Rat PFC: Structure</i>	<i>16</i>
1.2.2 <i>Primate vs Rat PFC: Function.....</i>	<i>17</i>
1.3 Impaired cognition in schizophrenia.....	19
1.3.1 <i>Working memory</i>	<i>20</i>
1.3.2 <i>Causes of working memory deficits in schizophrenia</i>	<i>21</i>
1.3.3 <i>Network oscillations as neural correlates of working memory</i>	<i>23</i>

1.4 Smoking and schizophrenia	25
1.4.1 <i>Nicotine and memory</i>	26
1.4.2 <i>The nicotinic acetylcholine receptors: $\alpha 7$ and $\alpha 4\beta 2$</i>	28
1.5 Assessing working memory in the rat.....	33
1.5.1 <i>Maze tasks: Radial Arm Maze, Morris Water Maze and T-Maze</i>	34
1.5.2 <i>Delayed Match-to-Sample and Non-Match-to-Sample tasks: The N-Back, Paired Associate Learning, Novel Object Recognition and the Odour Span Task</i>	36
1.6 Pharmacological models of cognitive impairment in schizophrenia	39
1.6.1 <i>Dopaminergic models</i>	41
1.6.2 <i>Glutamatergic models</i>	42
1.6.3 <i>Non-pharmacological models of schizophrenia</i>	44
1.7 Aims and Objectives	46
<u>Chapter 2: Materials and Methods</u>	48
2.1 The Odour Span Task.....	49
2.1.1 <i>Animals</i>	50
2.1.2 <i>Equipment</i>	51
2.1.3 <i>Shaping and acquisition of the Non-Matching-to-Sample rule</i>	51
2.1.4 <i>Odour Span Task: Training</i>	52

2.1.5 <i>Probe sessions and scent marking</i>	53
2.1.6 <i>Sub-chronic exposure to ketamine</i>	53
2.1.7 <i>Odour Span Task: Testing</i>	60
2.1.8 <i>Statistical analysis</i>	60
2.1.9 <i>Choice of drugs</i>	61
2.2 <i>Surgery and local injection procedure</i>	65
2.2.1 <i>Implanting bilateral cannulae into the medial prefrontal cortex (mPFC)</i>	65
2.2.2 <i>Local injection procedure</i>	67
2.3 <i>Gamma frequency network oscillations</i>	69
2.3.1 <i>Preparation of the brain slices</i>	69
2.3.2 <i>Initiation of gamma frequency oscillations</i>	69
2.3.3 <i>Measuring and recording gamma frequency oscillations</i>	71
2.3.4 <i>Statistical analysis</i>	71
<u>Chapter 3: The effect of exposure to a sub-chronic, sub-anaesthetic ketamine dosing regimen on OST performance</u>	73
3.1 <i>Introduction</i>	74
3.2 <i>Methods</i>	77
3.3 <i>Results</i>	78

3.3.1 <i>Sub-chronic, sub-anaesthetic ketamine treatment impaired OST performance</i>	78
3.3.2 <i>Ketamine-induced deficits were stable and long-lasting</i>	80
3.4 Discussion	82
3.5 Conclusions	85
<u>Chapter 4: The effect of nicotine, clozapine and LY404039 on ketamine-induced cognitive deficits</u>	86
4.1 Introduction	87
4.2 Methods	90
4.3 Results	91
4.3.1 <i>Nicotine dose-dependently enhanced OST performance</i>	91
4.3.2 <i>Clozapine had no effect on ketamine-induced deficits in the OST and impaired control animals</i>	93
4.3.3 <i>LY404039 had no effect on ketamine-induced deficits in the OST and impaired control animals</i>	95
4.4 Discussion	97
4.5 Conclusions	102

Chapter 5: Examining the effect of $\alpha 7$ and $\alpha 4\beta 2$ nAChR agonists on ketamine-induced deficits in the OST 103

5.1 Introduction104

5.2 Methods106

5.3 Results107

5.3.1 *The $\alpha 7$ nAChR agonist PHA improved OST performance in compromised animals.* 107

5.3.2 *Acute administration of metanicotine improved performance in the OST.....* 109

5.3.3 *Acute administration of 5IA improved performance in the OST* 111

5.4 Discussion113

5.5 Conclusions118

Chapter 6: Allosteric modulators for the $\alpha 7$ nAChR improve OST performance: An effect blocked by $\alpha 7$ antagonist methyllycaconitine .. 119

6.1 Introduction120

6.2 Methods123

6.3 Results124

6.3.1 *Acute administration of $\alpha 7$ allosteric modulator PNU improved OST performance* 124

6.3.2 *The $\alpha 7$ allosteric modulator Compound T improved OST performance* 126

6.3.3 *The $\alpha 7$ antagonist MLA blocked $\alpha 7$ PAM-induced improvements in OST performance and also impaired uncompromised animals* 128

6.4 Discussion 133

6.5 Conclusions 138

Chapter 7: Nicotine enhances gamma frequency oscillations in the PrL region of the PFC: An effect blocked by mecamylamine 139

7.1 Introduction 140

7.2 Methods 143

7.3 Results 144

7.3.1 *Nicotine increases gamma frequency oscillations* 144

7.3.2 *The enhancing effect of nicotine was blocked by mecamylamine* 147

7.3.3 *Neither $\alpha 7$ nor $\alpha 4\beta 2$ nAChR agonists significantly increased gamma frequency oscillations.* 149

7.4 Discussion 152

7.5 Conclusions 156

Chapter 8: Local injection of nicotine into the mPFC enhances OST performance 157

8.1 Introduction 158

8.2 Methods.....	161
8.3 Results.....	162
8.3.1 <i>Local administration of nicotine dose-dependently enhanced OST performance in control subjects and ketamine-treated animals</i>	162
8.3.2 <i>Local administration of muscimol dose-dependently impaired OST performance in control subjects and ketamine-treated animals</i>	164
8.4 Discussion	166
8.5 Conclusions.....	171
<u>Chapter 9: General Discussion</u>	172
9.1 Main Findings	173
9.1.1 <i>Sub-anaesthetic, sub-chronic ketamine in the OST as a model of cognitive deficits.</i>	173
9.1.2 <i>PCP as an alternative to ketamine: Differences pre-clinically in response to antipsychotic treatment</i>	174
9.1.3 <i>Nicotine as a treatment for CDS: Ethical considerations?</i>	175
9.1.4 <i>Enhanced gamma oscillations as a mechanism of cognitive enhancement in the OST</i>	175
9.1.5 <i>The $\alpha 7$ nAChR as a drug target for treatment of CDS</i>	176
9.1.6 <i>The $\alpha 4\beta 2$ nAChR as a drug target for treatment of CDS</i>	177
9.2 How will these results impact on patients?	181

9.3 Limitations of the OST.....	183
9.4 Weaknesses of this research.....	187
9.5 Future research.....	189

Chapter 10: References.....192

Chapter 11: Appendix.....218

11.1 Conferences.....	219
11.1.1 As a speaker.....	219
11.1.2 <i>Posters</i>	219
11.2 Peer-reviewed publications.....	220
11.3 Awards.....	220
11.4 Society Memberships.....	221

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List of Tables

Chapter 1

Table 1.1:	Schneider's first rank symptoms (Lieberman <i>et al.</i> 2006)	5
Table 1.2:	DSM-IV-TR criteria for schizophrenia	6
Table 1.3:	MATRICES assessment battery	13
Table 1.4A:	Secondary measures: To be used in TURNS	14
Table 1.4B:	Identified cognitive domains impaired in schizophrenia.....	14

Chapter 2

Table 2.1:	The three phases of Odour Span Task training	57
Table 2.2:	Odour Span Task: Drugs	62
Table 2.3:	Electrophysiology: Drugs	72

List of Figures

Chapter 1

Figure 1.1:	The $\alpha 4\beta 2$ nicotinic acetylcholine receptor	29
Figure 1.2:	Nicotinic acetylcholine receptor subtypes and their neuroanatomical location	30
Figure 1.3:	Mechanism of ketamine action.....	40

Chapter 2

Figure 2.1:	Basic OST training	54
Figure 2.2:	Half-OST in pictures	55
Figure 2.3:	Diagram of the OST task.....	56
Figure 2.4:	Acquisition of the OST task	58
Figure 2.5:	OST training and testing timeline flow diagram	59
Figure 2.6:	Surgical apparatus	66
Figure 2.7:	Cannulae placement.....	68
Figure 2.8:	Location of extracellular recording	70

Chapter 3

Figure 3.1: Sub-chronic, sub-anaesthetic ketamine treatment impaired OST performance 79

Figure 3.2: Ketamine-induced deficits were stable and long lasting 81

Chapter 4

Figure 4.1: Nicotine dose-dependently enhanced OST performance 92

Figure 4.2: Clozapine had no effect on ketamine-induced deficits in the OST and impaired control animals 94

Figure 4.3: LY404039 had no effect on ketamine-induced deficits in the OST and impaired control animals 96

Chapter 5

Figure 5.1: The $\alpha 7$ nAChR agonist PHA improved OST performance in compromised animals 108

Figure 5.2: Acute administration of metanicotine improved performance in the OST 110

Figure 5.3: Acute administration of 5IA improved performance in the OST 112

Chapter 6

Figure 6.1:	Acute administration of $\alpha 7$ allosteric modulator PNU improved OST performance	125
Figure 6.2:	The $\alpha 7$ allosteric modulator Compound T improved OST performance	127
Figure 6.3:	The $\alpha 7$ antagonist MLA impaired uncompromised animals	130
Figure 6.4:	The $\alpha 7$ antagonist MLA blocked PNU-induced improvements in OST performance	131
Figure 6.5:	The $\alpha 7$ antagonist MLA blocked Compound T-induced improvements in OST performance	132

Chapter 7

Figure 7.1:	Nicotine increases percentage change in size of gamma frequency oscillations	145
Figure 7.2:	Nicotine increases percentage change in power of gamma frequency oscillations	146
Figure 7.3:	The enhancing effect of nicotine was blocked by mecamylamine	148
Figure 7.4:	Neither $\alpha 7$ or $\alpha 4\beta 2$ nAChR agonists significantly increased gamma frequency oscillations	150
Figure 7.5:	Neither $\alpha 7$ or $\alpha 4\beta 2$ nAChR agonists were able to significantly increase gamma frequency oscillations	151

Chapter 8

Figure 8.1: Local administration of nicotine dose-dependently enhanced OST
performance in control subjects and ketamine-treated animals 163

Figure 8.2: Local administration of muscimol dose-dependently impaired OST
performance in control subjects and ketamine-treated animals 165

Chapter 9

Figure 9.1: The downstream effects of $\alpha 7$ nAChR activation 180

Figure 9.2: The automated OST 186

List of Abbreviations

5-CSRTT	5-Choice-Serial-Reaction-Time-Task
5-HT	5-hydroxytryptamine, serotonin
5IA	5-iodo-A-85380
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ACd	Dorsal anterior cingulate
ACh	Acetylcholine
ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
CATIE	Clinical Antipsychotic Trials of Intervention Effectiveness
CBT	Cognitive behavioural therapy

CDS	Cognitive deficits associated with schizophrenia
Cg1	Anterior cingulate cortex
CICR	Calcium-induced-calcium release
CNS	Central nervous system
Compound A	(R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide
DA	Dopamine
DH β E	Dihydro-beta-erythroidine
DLPFC	Dorsolateral prefrontal cortex
DMXBA	3-(2,4-dimethoxybenzylidene) anabaseine
dPAL	Different Paired Associate Learning
DSM I-IV	Diagnostic and Statistical Manual I-IV
EEG	Electro-encephalography
EPS	Extra-pyramidal side effects
ERK	Extracellular signal-regulated kinase
FDA	U.S Food and Drug Administration
FGA	First generation antipsychotic
fMRI	Functional magnetic resonance imaging
Fr2	Frontal region 2
GABA	Gamma-aminobutyric acid

GAD67	Glutamic acid decarboxylase 67
i.c.v.	Intracerebroventricular
i.m.	Intramuscular
i.o.	Intraoral
i.p.	Intraperitoneal
IL	Infralimbic
IL-6	Interleukin-6
ING	Interneuron gamma
IPSP	Inhibitory postsynaptic potential
IQ	Intelligence quotient
KO	Knockout
LTP	Long term potentiation
mAChR	Muscarinic acetylcholine receptor
MATRICES	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MCCB	MATRICES Consensus Cognitive Battery
MD	Mediodorsal thalamic nucleus
MEG	Magneto-encephalography
mGluR	Metabotropic glutamate receptor

mIPSC	Mini inhibitory postsynaptic current
MLA	Methyllycaconitine
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
MWM	Morris Water Maze
n.s.	Not significant
nAChR	Nicotinic acetylcholine receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NC3R	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NMDAR	N-methyl-D-aspartate receptor
NMTS	Non-matching-to-sample
NOR	Novel Object Recognition
OST	Odour Span Task
PAL	Paired Associate Learning
PAM	Positive allosteric modulator
PCP	Phencyclidine
PET	Positron emission topography
PFC	Prefrontal cortex

PHA	PHA-543613
PING	Pyramidal interneuron gamma
PNU	PNU-120596
PrL	Prelimbic
PV	Parvalbumin
RAM	Radial Arm Maze
s.c.	Subcutaneous
sEPSC	Spontaneous excitatory postsynaptic current
SGA	Second generation antipsychotic
sPAL	Same Paired Associate Learning
STDP	Spike-timing dependent potentiation
TCA	Tricyclic antipsychotic
TURNS	Treatment Units for Research in Neurocognition in Schizophrenia
VTA	Ventral tegmental area

Chapter 1

General Introduction

1.1 An overview of the Schizophrenia Syndrome

Schizophrenia is a debilitating disorder affecting approximately 1% of the population. It is a multi-faceted disease with wide ranging symptoms that comprise positive (auditory hallucinations, disorganised and deluded thoughts), negative (flattening of affect, apathy and anhedonia), and cognitive (attention and memory) (Diagram adapted from American Psychiatric Association, 2000). Significant and disruptive to everyday living, these deficits result in many patients being unable to cope in the community or hold down a long-term job, despite available anti-psychotic medication. This has an impact socially and economically for patient and wider community alike; the cognitive problems in particular demonstrate a very serious unmet clinical need. The real problem is that schizophrenia is not one disorder but rather an umbrella term for a whole syndrome of disorders that rarely present in exactly the same way in any two patients. This means that the task of finding safe, efficacious medication to treat all symptoms in all patients is a challenge akin to finding the proverbial needle in the largest of haystacks.

1.1.1 *Clinical symptoms and diagnosis*

In the late 19th and early 20th century, German psychiatrist Emil Kraepelin challenged the traditional methods of diagnosis for mental illnesses (For review see Kendler and Jablensky, 2010). He called for a novel classification according to a common pattern of symptoms; a syndrome, rather than diagnosis through similarities in major symptoms (Kraepelin, 1893, 1919). Kraepelin recognised that elements of mental illness such as mania, depression or cognitive deficits could be present in various different psychiatric disorders, but that it was the pattern in which they occur that allows their distinction into separate syndromes (Kraepelin, 1893, 1919). Through this method, Kraepelin divided the varied classes of functional psychoses into two groups. The first, ‘manic depressive insanity’, was described as a long-term disorder in which the patient had many episodes of

illness but was also fully functional for significant periods of time (Kraepelin, 1893, 1919). The second, 'dementia praecox' was a term initially coined by Morel, and in contrast included hebephrenia, as described by Hecker in 1871, catatonia, as described by Kahlbaum, and dementia paranoides. These classifications formed the early diagnostic criteria for schizophrenia and indeed the basis of modern psychiatric diagnosis as we know it today (Hecker, 1871; Kahlbaum, 1863; Kraepelin, 1893, 1919; Morel, 1860). In contrast to Kraepelin, who very much believed psychoses to be an organic disease of the brain, Bleuler in 1911 described 'the schizophrenias' in psychological terms as much as in neuropathological terms. He coined the now widely used term 'schizophrenia', meaning 'split mind', to describe loosened associations between various brain functions leading to impaired cognitive, emotional and volitional processes (Bleuler, 1911, 1950). His diagnostic criteria focused upon negative symptoms such as thought disorder, autism and ambivalence, and to some extent ignored the positive criteria set out by Kraepelin which included hallucinations and delusions. When translated into English in 1950, Bleuler's ideas significantly influenced the first two editions of the Diagnostic and Statistical Manual (DSMI and DSMII) (Andreasen, 1989). This changed with the development of DSMIII with the introduction of Kurt Schneiders' 'first rank symptoms' (table 1.1). He outlined specific symptoms such as the idea that thoughts were being broadcast or inserted from a third party. He proposed that the nature of such symptoms offered a direct, accurate diagnosis of schizophrenia because they were unique to the disorder. This has now been shown to be incorrect as many of the described symptoms are present in other neuropsychiatric disorders. Despite this, Schneiderian symptoms have formed the core of diagnostic criteria from the DSMIII to present day (table 1.2). Until clear knowledge of what causes schizophrenia or any reliable biomarkers are available, diagnosis will remain a significant challenge.

1.1.2 Causes of schizophrenia: Genetic or environmental?

The spectrum of disorders that make up the schizophrenia syndrome are thought to be the result of both genetic and environmental factors. The neurodevelopmental hypothesis is the predominant theory explaining the cause of schizophrenia. This hypothesis stipulates that a person with a genetic predisposition to schizophrenia who is additionally exposed to environmental insults in-utero or during early childhood may have neurodevelopmental errors. This may result in altered neuronal development and expression of a schizophrenic phenotype (Fatemi and Folsom, 2009).

Support for this comes from a longitudinal Finnish adoption study by Wynne *et al* (2006) where both mothers with a psychiatric diagnosis and control mothers, who had given up a child for adoption, were recruited. The respective adoptees and their adoptive families were also recruited. The psychiatric history of the biological mother was taken as a measure of genetic risk for development of a schizophrenia spectrum disorder. Based upon an independent psychiatric assessment, high risk adoptees were further split between risk of a broad schizophrenia spectrum disorder diagnosis and a narrow diagnosis of schizophrenia. Adoptive families were observed and assessed for several days using the Global Family Rating scales. Adoptees were followed up at 12 and 21 years for evidence of psychiatric disorder development. It emerged that adoptees that had low genetic risk and a normal family environment were least likely to develop a schizophrenia spectrum disorder (2.9%). Less likely was the discovery that, when either reared in a severely deviant environment or categorized as having high genetic risk, adoptees were only moderately more likely to develop a psychiatric disorder (9.3% and 6.4% respectively). However, when a high genetic risk was combined with a deviant environment this risk greatly increased to 40% (Wynne *et al*, 2006).

Table 1.1: *Schneider's first rank symptoms (Lieberman, 2006)*

Symptoms	Description
Three special forms of auditory hallucinations	<ol style="list-style-type: none">1. Hearing one's thoughts spoken aloud.2. Hearing voices referring to himself/herself, made in the third person.3. Auditory hallucinations in the form of a running commentary on one's activity.
Thought withdrawal, insertion, and interruption	
Thought broadcasting	One's thoughts are broadcast to others.
Somatic hallucinations	Somatic passivity, in which the patient is the passive and reluctant recipient of externally-imposed bodily functions.
Delusional perception	A normal perception followed by a delusional and highly personalized interpretation.
Ideas of passivity	Feelings, impulses, or motor activity are experienced, influenced, or controlled by external agents.

Table 1.2: DSM-IV-TR criteria for schizophrenia (American Psychiatric Association, 2000)

Diagnosis of schizophrenia

- A. Two or more of the following symptoms present for one month:
 - 1. *Delusions.*
 - 2. *Hallucinations.*
 - 3. *Disorganized speech.*
 - 4. *Grossly disorganized behaviour or catatonic behaviour.*
 - 5. *Negative symptoms (i.e., affective flattening, alogia, avolition).*
 - B. Decline in social and/or occupational functioning since the onset of the illness.
 - C. Continuous signs of illness for at least six months with at least one month of active symptoms.
-

Criteria for subtypes of schizophrenia

- A. Paranoid type schizophrenia
 - 1. *Characterized by a preoccupation with one or more delusions or frequent auditory hallucinations.*
 - 2. *Paranoid type schizophrenia is characterized by the absence of prominent disorganization of speech, disorganized or catatonic behaviour, and flat or inappropriate affect.*
-
- B. Disorganized type schizophrenia
 - 1. *Prominent disorganized speech, disorganized behaviour, and flat or inappropriate affect.*
-
- C. Catatonic type schizophrenia is characterized by at least two of the following:
 - 1. *Motoric immobility.*
 - 2. *Excessive motor activity.*
 - 3. *Extreme negativism or mutism.*
 - 4. *Peculiar voluntary movements such as bizarre posturing.*
 - 5. *Echolalia or echopraxia.*
-
- D. Undifferentiated type schizophrenia
 - 1. *Meets criteria for schizophrenia, but it cannot be characterized as paranoid, disorganized, or catatonic type.*
-
- E. Residual type schizophrenia
 - 1. *Characterized by the absence of prominent delusions, disorganized speech and grossly disorganized or catatonic behaviour and continued negative symptoms or two or more attenuated positive symptoms.*
-

Logically, this would demonstrate an active contribution of environment to the development of schizophrenia: In line with this, the scientific community are now exploring the idea that what triggers schizophrenia in one individual over another may be the result of epigenetic modifications that ‘switch on’ a relevant gene in a susceptible individual in response to environmental experiences. This may also explain why it is unusual for there to be a 100% concordance rate for diagnosis of schizophrenia in monozygotic twins and also why so many candidate genes have been associated with an increased risk of schizophrenia (Harrison and Weinberger, 2005; Walsh *et al*, 2008).

DISC1 (Disrupted In Schizophrenia 1), located on chromosome 1, is one such gene. Expressed in the brain, DISC1 is likely to play a role in neurite outgrowth, neuronal migration, synaptogenesis, and glutamatergic neurotransmission. Single nucleotide polymorphisms (SNPs) in DISC1 have been associated with increased likelihood of developing both schizophrenia and severe affective disorder (Hodgkinson *et al*, 2004). Other susceptibility genes for schizophrenia include neuregulin1 (NRG1) and (ErbB4) which, like DISC1, are involved in various aspects of pre- and postnatal neurodevelopment (Jaaro-Peled *et al*, 2009). Another promising candidate gene for schizophrenia susceptibility is dystobrevin-binding protein 1 (DTNBP1) which encodes dysbindin, a synaptic protein involved in excitatory synaptic neurotransmission (Allen *et al*, 2008; Karlsgodt *et al*, 2011). The gene encoding reelin is also associated with schizophrenia but instead of being involved in direct neurotransmission, this protein is involved in synaptic formation and CNS plasticity and is reduced in the brains of patients with schizophrenia. Alternatively, catechol-o methyl transferase (COMT) is an enzyme associated with the breakdown of catecholamine neurotransmitters including dopamine (Glatt *et al*, 2003). COMT polymorphisms at the Val158Met locus have been associated with an increased risk of susceptibility to schizophrenia but there is significant variation in the literature as to whether this is a true susceptibility gene (For review see Harrison *et al*, 2005). Other genes have also been linked to an increased susceptibility to schizophrenia, with the general consensus being that it is highly unlikely that we will ever find a single genetic ‘cause’ of

schizophrenia. Instead it is likely that research will continue to uncover further susceptibility genes which serve to increase the likelihood of developing schizophrenia.

1.1.3 Treatment

As a result of the hugely varied nature of the schizophrenia syndrome and the relative scarcity of knowledge we have on its cause, treatment is still, as it was in the 1950's, focused on resolving symptoms. The development of chlorpromazine in the 1950's revolutionised the treatment of schizophrenia when doctors noted its calming yet non-sedating properties. Through blockade of dopamine (DA) D2 receptors, chlorpromazine allowed control of the disabling positive symptoms in approximately 70% of patients, enabling the large majority of patients previously requiring long-term hospital care to return to the community (Delay *et al*, 1952)

Despite this, not all of the outcomes of chlorpromazine and other 'typical' first generation antipsychotic (FGA) treatments, such as haloperidol, were beneficial; severe extrapyramidal side effects (EPS) and tardive dyskinesia were common and on occasion, could be as disabling as the original symptoms. This, in addition to the fact that FGA treatment had little or no effect on the negative and cognitive symptoms, meant the search continued for more efficacious medication with fewer side effects. Progress was hampered by the belief that signs of catalepsy (indicative of EPS) signified antipsychotic properties. It was not until 1989 with the discovery of tricyclic antipsychotic (TCA) clozapine, that this myth was dispelled: Clozapine has higher efficacy than any of its predecessors, especially in treatment resistant patients, and almost no evidence of EPS (Bonham and Abbott, 2008; Hippus, 1989; Kane *et al*, 1988). This is thought to be as a result of lower occupancy of, and fast dissociation from, DA D2 receptors in the basal ganglia; an area responsible for control of motor function (Bonham *et al*, 2008; Kapur and Seeman, 2001). Other side effects however soon became apparent, with severe agranulocytosis (depletion of white

blood cells) leading to the deaths of eight people during the early 1970s and resulting in clozapine being removed from the U.S Food and Drug Administration (FDA) approved drugs list for a number of years (Crilly, 2007). Further ‘atypical’ second generation antipsychotics (SGAs) such as olanzapine, quetiapine and risperidone were developed and prescribed subsequent to the initial fall-out in favour of clozapine. These SGAs effectively reduced positive symptoms but did not induce EPS. This was found to be the result of fast dissociation from D2 receptors and lower receptor occupancy in the striatal regions (~60%) with SGAs as opposed to over 80% with FGAs (Kapur *et al*, 2001). Some SGAs such as risperidone and olanzapine are modestly more effective in treating negative symptoms (Leucht *et al*, 1999). However, although this may be a primary effect through activation of 5-hydroxytryptamine (serotonin, 5-HT)₂ and D4 receptors; it could also be because SGAs offer a significantly reduced likelihood of EPS which may reduce any related dysphoria. Indeed, Kapur *et al* (2001) demonstrated that the atypical antipsychotic effect of SGAs is dependent upon appropriate D2 receptor occupancy and not, for example, on the level of 5-HT receptor occupancy. Additionally, it is also the dissociation from the D2 receptor and not the 5-HT₂ or D4 receptor which most accurately predicts atypicality of SGAs (Kapur *et al*, 2001).

Whilst an improvement on typical FGAs, SGAs are not a cure for schizophrenia and there is still a significant need for more efficacious medication which can treat all aspects of this disorder. This is the case despite more than two decades of research since the arrival of SGAs.

There have been some promising starts such as the Eli Lilly metabotropic glutamate receptor (mGluR)_{2/3} agonist LY404039. LY404039 was found to be as effective as olanzapine in the first Phase II clinical trial, leading to widespread excitement in the field (Patil *et al*, 2007b). This would have been the first antipsychotic agent to work on a pathway that did not involve direct blockade of DA D2 receptors. Unfortunately, the

second trial failed as placebo was found to be as effective as the active compound and thus enthusiasm surrounding this project has since waned (Kinon *et al*, 2011).

As pharmacotherapy alone does not currently provide adequate treatment, it is often combined with psychological therapies. Psychosocial education is one of these and aims to better inform patients and their families about schizophrenia and the various treatments and drugs which are available. This has advantages in that it can improve compliance; if a patient understands why a medication might not work for several weeks, they may persevere in taking it for the full time-course despite side effects. This may account for the correlation of this therapy with decreased relapse and reducing hospital re-admission rates (Mari and Streiner, 1994; Pilling *et al*, 2002). Cognitive behavioural therapy (CBT) and cognitive remediation courses are also used with patients: Cognitive remediation leads to some short term improvements in various impaired cognitive functions (d'Amato *et al*, 2011; Demily and Franck, 2008; Grynszpan *et al*, 2011; Pfammatter *et al*, 2006). This is important as scores for attention, working memory and social cognition are approximately one standard deviation below the mean score for healthy controls, and are currently considered the most debilitating aspect of this disorder (Pfammatter *et al*, 2006). CBT is designed to help patients rationally analyse their disease symptoms and examine different ways in which they can respond to them. This can help to reduce the severity of symptoms and help with coping strategies (Pilling *et al*, 2002; Turkington *et al*, 2008). Both CBT and cognitive remediation have been shown to have some efficacy in combination with pharmacotherapy for the treatment of positive symptoms, though there is significant variability within the literature (For review see Demily and Franck, 2008; Pfammatter *et al*, 2006; Rector and Beck, 2001). Indeed, Wykes *et al* (2011) carried out a meta-analysis encompassing over 100 different studies and over 2000 patients. They found that although there was a significant effect of CBT and cognitive remediation, these effects were small and transient, disappearing upon follow-up assessment. This highlights the idea that

although there is some evidence that these techniques are useful, care must be taken when interpreting their ‘real-life’ value (Wykes *et al*, 2011).

The question that therefore remains is whether the search for a single so-called ‘super-drug’ which aims to cater for all aspects of the disorder is hampering vital research in this field, and whether we should instead be exploring symptom based treatment? Some strategies such as concurrently prescribing anti-depressants to combat negative symptoms and antipsychotics to combat positive symptoms are being employed (Siris *et al*, 2001). However, this strategy is not fully effective as it doesn’t provide any relief for the cognitive deficits which remain a significant unmet clinical need within this disorder.

1.1.4 The *TURNS* and *MATRICES* initiative

The **MATRICES** (**M**easurement and **T**reatment **R**esearch to **I**mprove **C**ognition in **S**chizophrenia) initiative was born of the need to develop a common method to assess the efficacy of novel pharmacological and behavioural treatments for cognitive deficits in schizophrenia (www.MATRICES.ucla.edu ; Green and Nuechterlein, 2004; Marder and Fenton, 2004). This involved the identification of the most important areas of cognitive impairment in schizophrenia, the best way of measuring these features and the current best pharmacological treatment targets. **MATRICES** also aimed to determine the best experimental design and in order to satisfy the FDA requirements, additionally aimed to identify secondary measures of improvement. The resulting **MATRICES Consensus Cognitive Battery (MCCB)** (table 1.3) was the collaborative effort between the National Institute of Mental Health, academia, the pharmaceutical industry and various regulatory bodies (Harvey, 2006; Nuechterlein *et al*, 2008).

The **TURNS** (**T**reatment **U**nits for **R**esearch in **N**eurocognition in **S**chizophrenia) project aims to utilise the **MCCB** in a collection of treatment trials to investigate the seven areas of cognition which are impaired in patients with schizophrenia (www.TURNS.ucla.edu, table

1.4a and 1.4b). All of these studies will employ randomised double-blind testing, concurrent treatment with anti-psychotics and longer term treatment with compounds that are usually employed in cognition clinical trials. These trials will also utilise the whole, 90 minute long, MATRICS test battery (Harvey, 2006).

Table 1.3: *MATRICES* assessment battery

Verbal memory	Hopkins Verbal Learning – revised
Visual memory	Brief Visual Memory Test – revised
Working memory	University of Maryland letter-number sequencing; Wechsler Memory Scale, Third Edition, spatial working memory
Processing speed	Trail-Making Test, part A; Brief Assessment of Cognition in Schizophrenia, symbol coding; animal naming
Problem solving	Neuropsychological Assessment Battery Mazes
Vigilance	Continuous Performance Test, identical pairs version
Social cognition	MSCEIT Subtests

Table 1.4A: *Secondary measures: To be used in TURNS*

Performance-based everyday living skills	UCSD performance-based skills assessment
Self/observer-rated neuropsychological ability	Schizophrenia Cognition Rating Scale

Table 1.4B: *Identified cognitive domains impaired in schizophrenia (Nuechterlein et al, 2004)*

1	Working memory
2	Attention
3	Visual learning and memory
4	Verbal learning and memory
5	Speed of processing
6	Social cognition
7	Reasoning and problem solving

1.2 The prefrontal cortex

The prefrontal cortex (PFC) is of great interest with respect to schizophrenia, as there is demonstrable evidence that a malfunction in this region is responsible for CDS.

The mammalian PFC is divided into 3 main sub-regions; the orbital, medial, and lateral. In primates it is emotional behaviours which are guided by the orbital and medial regions, while the lateral region provides executive control of behaviour and reasoning as well as speech. This structure-function relationship is different in rats, with the functioning of the medial prefrontal cortex (mPFC) in rats thought to be analogous to the dorsolateral prefrontal cortex (DLPFC) in primates (Vertes, 2004, 2006).

Initially defined by cytoarchitectonic features alone, the PFC was originally characterised as an area that is granulated. PFC definition has since evolved to include definition by connection to the mediodorsal thalamic nucleus (MD) and reciprocity of these connections as well as input from the ventral mesencephalon via dopaminergic fibres (Goldman-Rakic and Porrino, 1985; Gorelova and Yang, 1997; K Brodmann, 1909; Ray and Price, 1992; Rose and Woolsey, 1948; Uylings and van Eden, 1990; Williams and Goldman-Rakic, 1998). These evolving definitions resulted in a continuing debate as to whether the rat has a comparable PFC to humans and ultimately whether rodents represent a translational model. It is now however largely accepted that although structurally different, so-called class-common functions are largely the same and therefore reasonably representative of each other, and thus rodents are suitable test models.

1.2.1 *Primate vs Rat PFC: Structure*

Rats have a frontal area that is earlier along the evolutionary path, and is less differentiated and segregated than is found in primates. Therefore whether rats have a PFC and particularly a region within the brain that is consistent with that of the DLPFC in primates has been questioned. Through analysis of cytoarchitectonic characteristics, it was initially thought that the PFC was unique to humans and primates, as only they possess a DLPFC with a well developed internal granular layer IV.

Revolutionary work by Rose, Wooley and Akert instead demonstrated dense innervations to anterior and ventral parts of the rat brain from the MD; suggestive of an agranular, non-primate, functional homologue of the primate PFC in the rat (Akert and Hartmann-von Monakow, 1980; Rose *et al*, 1948). However, Rose and Wooley's original assumption that the MD was the only nucleus with thalamocortical projections to the PFC has since been shown to be incorrect: The PFC also connects to other thalamic nuclei such as the intralaminar, midline and anterior thalamic nuclei (Berendse and Groenewegen, 1991; Dermon and Barbas, 1994; Shibata and Kato, 1993). This meant that defining the rodent PFC through thalamic connections alone was no longer accurate. Uylings and Van Eden (1990) therefore employed a system of using strong *reciprocal* connections from the MD to define the PFC. This allowed inclusion of the frontal region 2 (Fr2) and dorsal anterior cingulate (ACd); regions previously classified as premotor regions and disputed by some as not strongly enough connected to the MD to warrant inclusion (Uylings *et al*, 1990). This dispute is critical as exclusion of these regions would mean there are no cortical areas in rats that are comparable to the primate DLPFC. However, as there are more prefrontal than pre-motor connections, these regions are now generally accepted as PFC regions and the pattern of connectivity of the MD in the rat is considered comparable to that in non-human primates. Supporting this is the parallel basal ganglia-thalamocortical circuitry present in both primates and rats. Many regions project to the basal ganglia but very few receive

reciprocal connections. The PFC receives the majority of input from the basal ganglia mediated mainly by the medial, laminar and anterior thalamic nuclei. The resulting functionally segregated, basal ganglia-thalamocortical circuits are paralleled in both primates and rat, leading towards a conclusion that rats have a homologous PFC to primates.

1.2.2 Primate vs Rat PFC: Function

Rats and humans will never of course be described as the same, but in many functional respects their behaviours are similar: They have the capacity to take in sensory information and act upon it; they can adapt to survive in their respective environments and are able to complete complex tasks. For example, both rats and primates use sensory information to identify an object. For the human this is through excellent tri-chromatic vision, supported by the touch and feel of the object. The rat however has detailed tactile sensory information through use of whiskers and support from reasonably limited visual information. In both cases, the ability of the PFC to receive this information and produce appropriate corresponding behaviour is crucial.

One of the most significant functions of the PFC is the initiation and organisation of movement. In this respect both primates and rodents are surprisingly similar. Despite clear differences in their abilities to manipulate objects, both species have comparable motor maps whereby the size of the motor representation reflects the motor ability of a body region (Whishaw *et al*, 1992a, b). However, perhaps the most significant function of the PFC is that described earlier; the ability to act as a control centre for managing sensory input and guiding resultant behaviour. This can be current on-line information or information from short term working memory (such as the memory of oncoming traffic when crossing a road). Actions mediated by the PFC are also influenced by long term stored memory of previous actions or events (such as almost being run over by a speeding

car and so being extra vigilant in future). The PFC acts to select and coordinate this information to generate an appropriate behaviour. This behaviour may be completely novel or be similar to previous behaviours (i.e. crossing the road is a very similar process each time, but each time is slightly different). This novel information functionally connects the PFC with other cortices until it becomes routine or automatic. At this point the connection with the PFC is reduced and taken over by other brain structures. Fuster (1998) suggests that this function is common to both primates and rodents, and indeed all mammalian species (Fuster, 1998).

One way to investigate function is to lesion the areas in question and examine which behaviours are lost. A comparison of prefrontal lesions in rodents and primates in the 1970s demonstrated that lesions of the medial and lateral orbital regions of the rat and the dorsolateral and orbitofrontal regions of primates produce similar behaviours (Kolb, 1984). For example, dorsolateral lesions in primates produce working memory and attentional deficits; this is also seen in rats with lesions to the mPFC, in particular the prelimbic (PrL) region, (Birrell and Brown, 2000; Granon *et al*, 2000; Muir *et al*, 1996). This supports the theory that these regions are analogous; crucial knowledge for the development of reliable animal models of CDS.

1.3 Impaired cognition in schizophrenia

Spearman in 1928 was the first to distinguish between general and specialised cognition (Spearman, 1928). Patients with schizophrenia usually have impaired general intellectual functioning alongside specific cognitive disabilities in attention, executive function, working memory and processing speed, among others. However, the differences in severity in these specific abilities vary greatly, perhaps determined by the severity of general intellectual deficits.

Patients with schizophrenia perform consistently poorer in tests of general cognition when compared to healthy controls with performance being approximately one standard deviation below normal (Fioravanti *et al*, 2005; Laws, 1999; Mesholam-Gately *et al*, 2009). Cognitive abnormalities are however present before the onset of the disorder as demonstrated by Crow and Done (1995) in their landmark study. Beginning in 1958, they followed 15000 children into adulthood. Those who developed schizophrenia during adulthood had shown notable differences to their peers during childhood including behavioural problems and impaired reading ability at age 7. At age 11, IQ was 5 to 10 points lower than expected and these children were also delayed in establishing hemisphere dominance, thought to be crucial to the full development of language (Crow *et al*, 1995, 1996). The degree of cognitive impairment in patients with schizophrenia is central to the disorder and is a key predictor of functional outcome (For review see Harvey, 2009)

There are also physical abnormalities present in patients with schizophrenia. These include neurological soft signs such as poor sensory integration and motor coordination along with ocular abnormalities: Patients with schizophrenia can blink up to 80 times per minute compared to the average adult blinking rate of 12 times per minute (Varambally *et al*, 2012). Patients also show brain structure differences consisting of small changes in

ventricular size and reduced cortical grey matter volume (De Peri *et al*, 2012; Lawrie and Abukmeil, 1998; Selemon *et al*, 2002).

1.3.1 Working memory

Working memory is a form of short term memory that allows the use of currently relevant information to make a decision but that is forgotten once that information is no longer relevant. In the context of experimental research Dudchenko defines working memory in the rat as '*short term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions*' (Dudchenko, 2004). Working or 'on-line' memory has long since been described as an 'executive process' as it enables selection of appropriate responses and behaviour, and their subsequent coordination. This process also allows the brain to discard information which is no longer relevant, such as how many cars were coming down the road when you crossed it last week.

In order to test spatial working memory in animals, Olten and Samuelson developed the radial arm maze (RAM) in 1976. This task and several variations are still among the most widely used for testing working memory today. The original RAM had 8 radial arms, some of which contained food pellets. Rats were able to retrieve food pellets without revisiting a previously visited arm (Olton and Samuelson, 1976). This demonstrates an 'on-line' memory for which arms had been investigated and the pellet retrieved. However, on a session the next day, this information is no longer relevant as different arms are baited; in fact if animals did not 'forget' the information from the previous session, their performance may well be hampered on the current session. It is this transience of information that distinguishes working memory from the reference memory which is used to remember the rules of the task.

There is evidence that patients with schizophrenia perform poorly in working memory tasks when compared to healthy controls: Forbes *et al* (2009) carried out a meta-analysis of 187

studies examining working memory in patients with schizophrenia. This revealed that patients had significant deficits in working memory in comparison to healthy subjects which could not be explained by discrepancies in IQ of test subjects (Forbes *et al*, 2009). Even prodromally, patients exhibit performance at approximately one standard deviation below normal in standard working memory tests (Simon *et al*, 2007). These deficits alone may constitute a poorer outcome because patients are less able to cope with their varying symptoms. However, patients may also have more difficulty manipulating information in a way that a healthy person can, such that they have difficulty in basic concentration, learning and reasoning along with everyday situations such as remembering the list of ingredients on a shopping list. This means that working memory deficits within schizophrenia represent a serious, unmet clinical need which significantly affects the daily lives of patients.

1.3.2 Causes of working memory deficits in schizophrenia

There have been a multitude of studies examining working memory in schizophrenia but the exact nature or causes of these deficits are unknown. However, there is evidence that patients with schizophrenia have significant deficits in working memory and imaging studies have shown that these deficits correlate with functional changes in the DLPFC (MacDonald *et al*, 2005; Menon *et al*, 2001; Weinberger and Gallhofer, 1997). The way in which DLPFC functioning deviates in patients depends upon the nature of the task used for testing: Low working memory load induces increased DLPFC activity in patients compared to control subjects with no poorer performance seen in patients versus controls. Conversely, high working memory load is associated with lowered DLPFC activity in comparison to controls, and patients demonstrate impaired performance on the task (Tan *et al*, 2007).

In addition, studies of patients with schizophrenia have demonstrated a reduction in prefrontal grey matter volume and a reduction in N-acetylaspartate, a metabolite indicating volume and viability of neurons, in prefrontal regions (Gur *et al*, 2000; Hirayasu *et al*, 2001; Keshavan *et al*, 2000).

On a molecular level, one of the most significant findings in patients with schizophrenia is a reduction in PFC messenger ribonucleic acid (mRNA) for the 67 kDa isoform of the gamma-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD67) (Volk *et al*, 2000). A reduction in GAD67 expression in parvalbumin (PV)+ve interneurons, either through N-methyl-D-aspartate receptor (NMDAR) hypofunction or a central deficit in GAD67 mRNA, may lead to a reduction in GABA production (Lewis *et al*, 2005; Moghaddam, 2004). Reduced GABA could then cause disinhibition of DLPFC pyramidal neurons, in turn inducing excess glutamate at non-NMDARs and thus disrupting cortical circuit function (Moghaddam, 2004). This is supported by evidence that normal DLPFC functioning has been shown to be essential to working memory: Rao *et al* (2000) found that application of GABA antagonist bicuculline to pyramidal cells and interneurons in the DLPFC of monkeys impaired their performance on a working memory task (Rao *et al*, 2000). This indicates an essential role for GABA and PV+ve interneurons in working memory processes. PV+ve interneurons are additionally instrumental in the generation of gamma frequency oscillations, which are central to working memory processes: The size and the synchronicity of gamma frequency oscillations have been shown to correlate with working memory load (Gonzalez-Burgos and Lewis, 2008; Howard *et al*, 2003) In patients with schizophrenia, Cho *et al* (2006) have shown that gamma frequency activity increased with task difficulty in control subjects but no changes were observed in patients with schizophrenia (Cho *et al*, 2006). This supports the idea that gamma frequency oscillations play a significant role in working memory and that this function is impaired in patients with schizophrenia compared to healthy controls.

Therefore, although the causes of working memory deficits in schizophrenia have yet to be fully delineated, there is likely to be significant involvement of aberrant gamma frequency

oscillations arising from an impairment in GABA-mediated regulation of pyramidal cell networks.

1.3.3 Network oscillations as neural correlates of working memory

Neural oscillations allow communication of information between different brain regions. Oscillations exist in different frequency bands such as beta (15-30 Hz), gamma (30-80hz), theta (4-12hz) and delta (1-4hz) (Whittington *et al*, 2000). These different frequency band oscillations act as parallel communication channels for different networks, coordinating different but sometimes related, information (Benchenane *et al*, 2011). Fast oscillations such as gamma and beta function to enhance the neuronal representation of sensory stimuli, resulting in enhanced activity of neuronal groups communicating behaviourally relevant information (Womelsdorf and Fries, 2007). Beta has been implicated in long-range synchronisation with long transmission delays where as there is evidence for gamma oscillations in shorter range synchronisation (Kopell *et al*, 2000). Theta frequency network oscillations also have been shown to be essential to spatial working memory tasks and can function to coordinate communication between the hippocampus and the prefrontal cortex (Jones and Wilson, 2005).

Mechanistically, it is widely accepted that inhibitory interneurons have a significant role in the generation of gamma frequency oscillations, with the firing ability of pyramidal neurons being strictly controlled by the level of inhibitory input (Cardin *et al*, 2009; Sohal *et al*, 2009). This means that only in a state of synchronous inhibition, there exists a window of low inhibitory conductance which allows the cell to fire. The active contribution of pyramidal cells to this process has not as yet been fully delineated: Gamma frequency oscillations can be generated with the passive involvement of pyramidal cells, the interneuron gamma (ING) mechanism, or by the pyramidal interneuron gamma (PING) mechanism (Traub and Whittington, 2010; Whittington *et al*, 2000). In the case of ING, inhibitory interneurons are activated through the binding of glutamate to mGluRs. This

initiates firing of localised interneurons which become more synchronised, which in turn entrains more interneurons. This ultimately results in the generation of inhibitory postsynaptic potentials (IPSPs) which allows synchronous firing to occur following release from inhibition (Cobb *et al*, 1995; Whittington *et al*, 2000). In contrast, the PING mechanism dictates that pyramidal neurons drive the oscillation through the generation of excitatory post-synaptic potentials (EPSPs) in neighbouring pyramidal cells but also interneurons: Activation of sufficient inhibitory interneurons initiates widespread inhibition, thus coordinating the synchronous firing of pyramidal cells following cessation of inhibition (Traub *et al*, 2010; Whittington *et al*, 2000).

Gamma frequency oscillations can be examined *in-vivo*, *in-vitro* and clinically through electro-encephalography (EEG) and magneto-encephalography (MEG). This translatable way of examining neural networks means that findings pre-clinically are more likely to translate to meaningful results for patients and ultimately increase the likelihood of developing novel efficacious compounds for the treatment of CDS.

1.4 Smoking and schizophrenia

The prevalence of smoking in patients with schizophrenia far outweighs that in the normal population at a ratio of approximately 80:20% (Morisano *et al*, 2009). This, in addition to reports that cessation rates in patients are approximately half that of the normal population, led researchers to conclude that patients may be self-medicating with the psychoactive ingredient in tobacco, nicotine (Lasser *et al*, 2000). Nicotine may be used as self-medication as it has been shown to increase hepatic clearance of antipsychotic medications through the induction of metabolising enzyme CYP1A2 (Andrade, 2012; Bozikas *et al*, 2004). By smoking, patients may therefore be able to reduce side effects such as neuroleptic-induced parkinsonism (Chambers, 2009). In addition, nicotine has been shown to improve cognition and sensory gating in patients with schizophrenia (Barr *et al*, 2008; Chen *et al*, 2011; Jacobsen *et al*, 2004). On a molecular level, the self-medication hypothesis is supported by studies showing that patients have reduced nicotinic acetylcholine receptor (nAChR) expression and that nicotine administration paradoxically increases nAChR presence (Durany *et al*, 2000; Leonard *et al*, 2000). Sallette *et al* (2005) propose that this is because nicotine can act intracellularly in a similar way to a chaperone protein, promoting proper folding and assembly of nAChR subunits. This means that the fraction of functional nAChRs on the cell surface is increased (Sallette *et al*, 2005)

Contrary to the self-medication hypothesis, the addiction vulnerability hypothesis proposes that patients with schizophrenia have inherent genetic factors and brain reward pathway abnormalities which predispose them to smoking. This means that patients compulsively smoke despite the negative consequences associated with smoking tobacco. This is not to say that there are not some positive effects associated with smoking, but this hypothesis proposes that they are secondary to a nicotine addiction (Chambers, 2009).

1.4.1 Nicotine and memory

Nicotine has been shown to improve working memory in both compromised and non-compromised animals. Levin *et al* (1997) trained rats in the 16 arm RAM whereby the aim is for the rat to collect food rewards from baited arms without revisiting a previously sampled arm. Rats that were administered nicotine prior to the task demonstrated significantly improved working memory performance. In addition, a low dose of nicotinic antagonist mecamylamine impaired performance in the task, which was attenuated by co-administration of nicotine (Levin *et al*, 1997). Normal, uncompromised animals also demonstrate improved performance following nicotine administration in the Odour Span Task (OST), a non-spatial working memory task where animals are required to select a novel odour from a series of previously sampled odours. As with the study by Levin and colleagues, animals also demonstrated significantly impaired performance following mecamylamine administration (Rushforth *et al*, 2010).

Clinically, the vast majority of patients with schizophrenia smoke and thus many of the studies examining the effect of nicotine on memory, are in patients who have abstained from smoking: Wing *et al* (2011) examined smoking abstinent patients and found their level of working memory impairment correlated with a decrease in circulating levels of nicotine (Wing *et al*, 2011). In addition, Smith *et al* (2006) examined the effect of nicotine on attention, verbal memory, and visual-spatial memory in abstinent smokers. They found that nicotine improved attention and visual-spatial memory: This may provide evidence of a role for nicotine in memory (Smith *et al*, 2006). However, these results are not conclusive as treatment with nicotine may in fact be negating the effects of withdrawal as opposed to enhancing cognition. Mechanistically, Tsukeda *et al* (2005) used positron emission topography (PET) to demonstrate that nicotine is able to reduce the abnormally increased level of prefrontal DA D1 receptor binding in monkeys sub-chronically treated with

NMDAR antagonist MK-801. They correlate these findings with results demonstrating that nicotine was also able to restore working memory performance following MK-801-induced impairment (Tsukada *et al*, 2005). Couey *et al* (2007) put forward that nicotine administration increases GABA output onto pyramidal neurons, which increases the threshold at which they fire. If there is a lot of activity, i.e. under the strain of a particularly demanding task, then this action could function to improve the signal to noise ratio, thus enhancing performance. However, this could also lead to poorer performance if the threshold of neuron firing was increased but the level of input remained the same. This could account for why nicotine can cause impairments in control subjects where nicotine enhances performance in impaired individuals (Couey *et al*, 2007). This mechanism is supported by Newhouse *et al* (2004) who propose that nicotine is only effective in a situation where a task is highly demanding or the subject is impaired. In the case of normal functioning or a simple task, nicotine may cause impairments (Newhouse *et al*, 2004b).

Therefore, whether patients with schizophrenia smoke as a result of vulnerability to addiction or to improve cognition or both, it is likely that there is a role for nAChRs in mediating cognition and thus nAChRs represent a viable target for the development of novel compounds for the treatment of CDS.

1.4.2 The nicotinic acetylcholine receptors: $\alpha 7$ and $\alpha 4\beta 2$

Nicotinic acetylcholine receptors are part of the cholinergic receptor system which also incorporates the muscarinic acetylcholine receptors (mAChRs). The mAChRs are G-protein coupled and found in both the central and parasympathetic nervous systems (Eglen, 2006). The nAChRs are ionotropic, binding both endogenous acetylcholine and exogenous ligands such as nicotine which results in the opening of the ion channel; allowing the flow of cations through the receptor. Structurally, nAChRs are pentameric, consisting of five transmembrane subunits surrounding a central ionic pore. There are 17 currently known subunits that make up the receptor which may be homomeric or heteromeric and mostly formed of α and β subunits (Figure 1.1). By far the most common nAChRs present in the brain are the homomeric $\alpha 7$ nAChR, consisting of five $\alpha 7$ subunits, and the heteromeric $\alpha 4\beta 2$ nAChR consisting of $\alpha 4_{(2/3)}$ and $\beta 2_{(3/2)}$ subunits (Figure 1.2). (Albuquerque *et al*, 2009).

The $\alpha 4\beta 2$ nAChRs are the most widely distributed nAChRs in the brain and have been shown to have a significant role in modulating neurotransmitter output (Albuquerque *et al*, 2009). They exist in two forms consisting of either two α subunits and three β subunits or three α subunits and two β subunits. The $\alpha 4_{(2)}\beta 2_{(3)}$ form has been shown to have a very high affinity for nicotine although $\alpha 4$ containing nAChRs are all classed as high affinity receptors (Wu and Lukas, 2011). Mechanistically, ligand binding to the $\alpha 4\beta 2$ nAChR leads to the influx of sodium ions and low levels of calcium ions along with potassium ion efflux, causing depolarisation. This induces calcium ion influx through voltage gated calcium channels which then triggers vesicular neurotransmitter release (Tsuneki *et al*, 2000).

In contrast, the $\alpha 7$ nAChR is a low affinity nAChR which is highly permeable to calcium. These nAChRs are preferentially localised to elicit direct calcium-induced-calcium release (CICR) from intracellular stores: $\alpha 7$ nAChRs are predominantly located on ryanodine-sensitive nerve terminals where ligands binding to the $\alpha 7$ nAChR induces CICR. This in

Figure 1.1 *The $\alpha 4\beta 2$ nicotinic acetylcholine receptor*

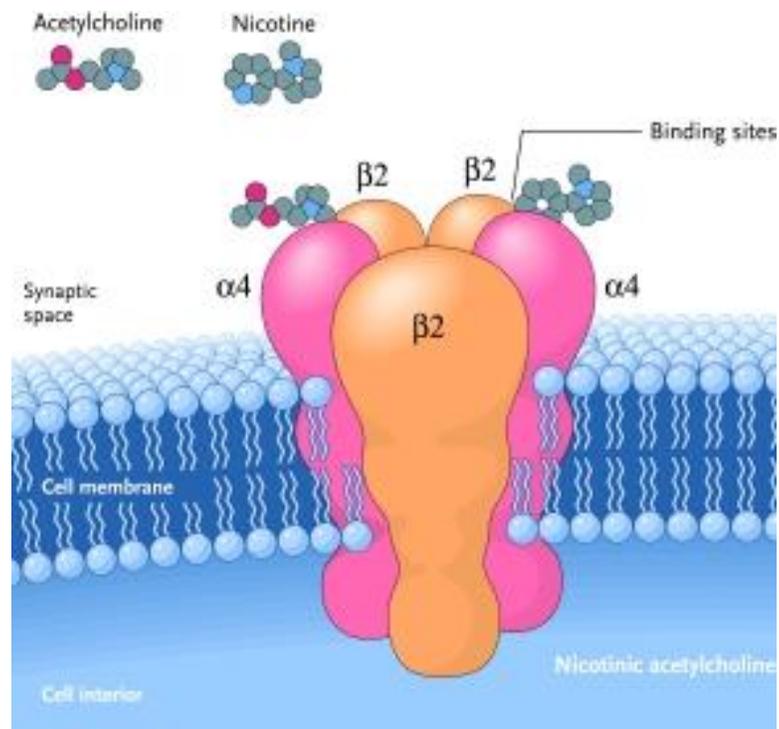


Figure 1.1 Nicotinic acetylcholine receptors are pentameric structures consisting of 5 subunits. These receptors can be heteromeric, consisting of two or more different subunits as depicted above. Alternatively, they may be homomeric, consisting of 5 of the same subunits as in the case of the $\alpha 7$ nAChR (Adapted from Mroziewicz and Tyndale, 2010)

Figure 1.2 Nicotinic acetylcholine receptor subtypes and their neuroanatomical location.

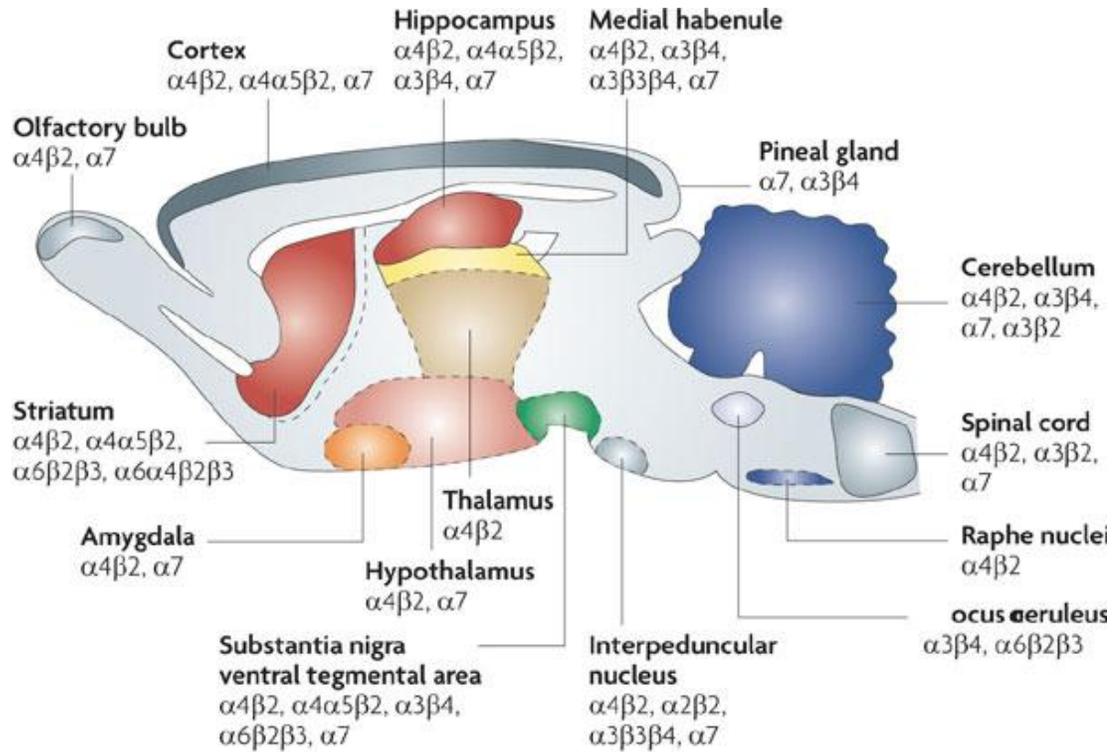


Figure 1.2: Nicotinic acetylcholine receptors are present throughout the brain. The most ubiquitous receptors are the $\alpha 4\beta 2$ nAChR and the $\alpha 7$ nAChR which have also been implicated as having a role in cognition (Adapted from Taly *et al.*, 2009)

turn activates extracellular signal-regulated kinase-1/2 (ERK1/2)-dependent pathways, phosphorylating synapsin-1 which induces vesicle binding to the synaptic membrane and neurotransmitter release (Dickinson *et al*, 2008). Like $\alpha 4\beta 2$ nAChRs, $\alpha 7$ nAChRs are also present in most regions of the central nervous system (CNS), but where as $\alpha 4\beta 2$ nAChRs are localised in the PFC, $\alpha 7$ nAChRs predominate in the hippocampus; located on cholinergic and non-cholinergic presynaptic terminals (Albuquerque *et al*, 1997; Alkondon *et al*, 1999).

Both $\alpha 7$ and $\alpha 4\beta 2$ nAChRs have been shown to improve working memory: Pre-clinically, Lippiello *et al* have shown that $\alpha 4\beta 2$ agonist metanicotine was able to improve passive avoidance retention following scopolamine-induced amnesia as well as improving working and reference memory in the 8-arm RAM (Lippiello *et al*, 1996). Chan *et al* (2007) examined both $\alpha 4\beta 2$ and $\alpha 7$ agonists on the RAM and found that both were able to improve working memory function and that both $\alpha 7$ antagonist methyllycaconitine and $\alpha 4\beta 2$ antagonist dihydro-beta-erythroidine (DH β E) impaired working memory (Chan *et al*, 2007). Further support for nicotine improving working memory comes from Castner *et al* (2007) who found that AZD0328, which is a selective $\alpha 7$ nAChR agonist, significantly improved working memory performance in rhesus macaques following acute administration (Castner *et al*, 2011). The role of $\alpha 7$ nAChRs in working memory is supported by Young *et al* (2007) who also found that $\alpha 7$ knockout (KO) mice were impaired in the OST; a measure of non-spatial working memory (Young *et al*, 2007b). Finally, Buccafusco and Terry (2009) demonstrated that partial $\alpha 7$ nAChR agonist 3-(2,4-dimethoxybenzylidene) anabaseine (DMXBA) was able to fully reverse a ketamine-induced deficit in a computer assisted-delayed response task in monkeys (Buccafusco and Terry, 2009). Clinically, DMXBA has been shown to improve working memory in a Phase II clinical trial (Freedman *et al*, 2008). In addition, Dunbar *et al* (2011) found that selective $\alpha 4\beta 2$ nAChR agonist TC-1734 improved attention and memory in older subjects with age-

associated memory impairment (Dunbar *et al*, 2011). This demonstrates a key role for nAChRs in working memory and indicates that they are likely to have a significant role in mediating the effect of nicotine on cognition.

1.5 Assessing working memory in the rat

Working memory is distinct from other forms of memory in that it requires active maintenance, is subject to interference and with respect to working memory tasks in rodents, must be forgotten between trials in order to prevent interference with future trails. Working memory provides a representation of available stimuli, be that arms visited in the RAM or odours previously sampled in the OST, that can be used to guide behaviour. Dudchenko (2004) defines working memory in a rodent as '*a short term memory for an object, stimulus or location that is used within a testing session, but not typically between sessions*' (Dudchenko, 2004).

Access to translatable cognitive preclinical tasks that can be used in conjunction with disease models is essential in the development of novel compounds to treat CDS. This poses various problems in that several of the tasks used to measure human memory are difficult to replicate in the rat: Verbal working memory tasks being the most obvious but complex tasks such as the n-back task as carried out in humans are also difficult to replicate in rats. These tasks must therefore be adapted to be species relevant. However in some cases, researchers have done the reverse and adapted a rodent task for humans: There is now a computerised virtual reality version of the RAM, which has been used to demonstrate that patients with schizophrenia exhibit working memory errors consistent with studies examining RAM performance in animal models of schizophrenia (Spieker *et al*, 2012).

The most commonly used tasks in rodents involve maze-based spatial working memory or delayed alternation tasks, although the use of non-spatial tasks such as the OST are becoming more popular.

1.5.1 Maze tasks: Radial Arm Maze, Morris Water Maze and T-Maze

The RAM was developed by Olten and Samuelson in 1979 and consists of a central platform with 8 arms radiating from it. Olten and colleagues found that rats placed on the central platform would rapidly learn to retrieve a food reward from the end of each arm without revisiting a previously sampled arm (Olton *et al*, 1976). Since then there have been many variations on the task including increasing the number of arms to 12 or even as many as 24 (Buresova and Bures, 1982; Cook *et al*, 1985). Another factor introduced was delay: Bolhuis *et al* (1986) demonstrated that it took a delay of 60 minutes between trials to significantly impair performance on the RAM and a 120 minute delay for animals to perform at chance levels (Bolhuis *et al*, 1986). The Morris Water Maze (MWM) requires rats to find a submerged platform in a pool of water. In the working memory version of the task, which may also include delays between the first and second trial, the platform location remains the same for trials on the same day but is different between days (Steele and Morris, 1999).

Although both the RAM and MWM are spatial working memory tasks, the behaviours observed on each may be quite different: Ormerod *et al* (2002) tested rats in both tasks and found that rats took almost twice as many trials to learn the RAM versus MWM, and that scopolamine administration impaired working memory only in the RAM (Ormerod and Beninger, 2002). This is potentially because the motivating factors for completing the tasks are different; in the MWM, rats are averse to water and must find the platform to escape where as in the RAM, unless very hungry, the possibility of missing a food reward is not especially aversive. Additionally, there are few strategic options available in the MWM: Rats must use extra-maze spatial cues to determine orientation. Conversely in the RAM, rats may ‘microsample’ entryways to each platform or scent-mark areas previously investigated meaning that the cognitive map and brain regions employed by rats to solve

each task may be significantly different. This must be carefully considered before inferring structure-function relationships (Hodges, 1996).

Hodges *et al* (1991) exemplify this, demonstrating that animals with damaged connections to the hippocampus and cortex were impaired in both the MWM and RAM compared to control subjects: This is in contrast to hippocampal lesions which impaired performance in the MWM to a greater extent than in the RAM (Hodges *et al*, 1991; Jarrard, 1993). Buresova *et al* (1982) provide a solution to this problem with the development of a submerged RAM. This required rats to swim to the end of a maze arm for the opportunity to sit on a submerged platform for 20 seconds. After 20 seconds, this platform would collapse requiring the rat to swim to a new arm for relief. In the same way as the RAM, rats were measured on their ability to remember which arm had been visited (Buresova *et al*, 1985). This task prevents potential confound such as ‘microsamples’ and scent-marking meaning task navigation is achieved as a result of a visuo-spatial construct. However, the anxiogenic and aversive nature of the task may have its own confounding effects on working memory.

Delayed alternation using the T-maze encompasses the natural curiosity of the rat for novelty. The rat is placed in the long arm of the ‘T’ and must make a decision about which arm to enter. On the next trial, following a varying delay, the animal is rewarded for entering the alternate arm to one previously explored. This task is very sensitive to hippocampal lesions; animals perform at chance levels with only a 15 second delay (Dudchenko *et al*, 2000). Again, this illustrates how different each spatial memory task is and that knowledge of both strategy and corresponding neural correlates are vital for the interpretation of findings.

Ultimately however, these studies illustrate that findings in one spatial working memory task may not extrapolate to others, as the motivation and strategies employed to complete the task may be very different. Thus, each task may provide useful but different information regarding brain structure-function relationships which must be considered with initial task selection.

1.5.2 Delayed Match-to-Sample and Non-Match-to-Sample Tasks: The N-Back, Paired Associate Learning, Novel Object Recognition and the Odour Span Task

The n-back task is commonly used in humans, not least because it can be combined with imaging to provide a more accurate indication of structure-function relationships. In the human version, subjects are asked to respond to a letter when it matches the presented letter (0-back), the previous letter (1-back) or the penultimate letter (2-back). In rats, a series of lever presentations are used in place of letters and rats must respond to the previously presented lever or the penultimate lever from a random sequence of lever presses. Ko *et al* (2009) have shown that this version of the task is sensitive to impairments by both amphetamine and MK-801, but that performance was not restored by SKF-38393 (D1, D5 partial agonist) or nicotine. The authors put forward that this may be as a result of interference from the continuing pattern of lever presentations and that nicotine may in fact improve attending to each novel presentation, causing previous presentations to be forgotten more quickly (Ko and Evenden, 2009). However, the logarithmic dose range used (0.1, 0.3 and 1mg/kg) may miss out a therapeutic dose between the 0.3 and 1mg/kg dose which has been shown to improve performance in other non-spatial working memory tasks (Rushforth *et al*, 2010).

Paired Associate Learning (PAL) in humans requires subjects to examine five boxes on a computer screen and remember the contents; one of these sample objects is then displayed and the subject must match it to the correct box (Sahakian *et al*, 1988). In the rodent version of the task, rats are trained to respond to an object displayed at a correct response

location in the presence of another object at an incorrect location on a touchscreen. The objects displayed may be the same (same paired associate learning, sPAL) i.e. two flowers located at space one and three, where only the flower at space one is rewarded, or different (different paired associate learning, dPAL), such as a correct spider at space one, an incorrect aeroplane at space two followed by a correct flower at space two (Talpos *et al*, 2009). Talpos *et al* (2009) demonstrated that hippocampal impairments, using local administration of compounds such as MK-801 could be induced in the dPAL task only, attributing this factor to the more difficult nature of the task. The significant advantage of this task is that it is fully automated meaning that multiple animals can be tested in parallel and the measurable outcomes are fully objective. In addition, this is similar to the PAL used in humans and may therefore prove useful in the development of novel compounds which are efficacious in man.

The OST is a complex task that involves increasing amounts of information which must remain active in working memory in order to successfully complete the task. Developed by Dudchenko *et al* (2000), the OST works by initially presenting a bowl of woodchip containing a food reward, scented by a household spice such as coriander (A+); this bowl is presented at a numbered location where the rat must dig in the bowl and retrieve the food reward. On the next trial, a second baited bowl, containing a different odour such as garlic, is introduced at another numbered location (B+) and the, now unbaited, first bowl (A-) is also moved to a new location: The animal must investigate both bowls and dig in only the bowl with the novel scent. This continues with additional bowls for each trial until the animal makes a mistake or reaches 15 bowls. When the animal makes a mistake, it is removed from the board and the number of correct bowls identified minus the first (which has no memory requirement) gives a span score (figure 2.3). A spatial version of the OST can also be employed where the location of the bowl is instead the relevant factor. In the paper first describing this task by Dudchenko (2000), one experiment induced neurotoxic

hippocampal lesions in rats previously trained in the OST. These animals demonstrated no impairment on the normal OST task but were substantially impaired on the spatial version of the task. This indicates a role for the hippocampus in spatial working memory but also alludes to the involvement of other brain regions in mediating this task (Dudchenko *et al*, 2000). Since then, various groups have picked up this task, demonstrating that performance can be enhanced with nicotinic agonists and that it is sensitive to impairment with NMDAR antagonists such as MK-801 (Galizio *et al*, 2012; Rushforth *et al*, 2010).

1.6 Pharmacological models of cognitive impairment in schizophrenia

The aim for any animal model of a disorder is to have face, construct and predictive validity. This means the model should induce similar symptoms, have similar molecular pathology and respond in the same way to treatment as the original disease, respectively. The best way to do this is to replicate the underlying cause of the original disease but this becomes difficult when the origin of the disorder is unknown. This is particularly the case with syndromes such as schizophrenia where there are numerous disorders under the umbrella term of schizophrenia which often present slightly and sometimes significantly different from patient to patient. As a result, current models do not serve as the complete animal equivalent but are instead largely fragmented; examining only for certain aspects of the disorder. The main pharmacological models of schizophrenia have arisen from the two main hypotheses of schizophrenia; the DA hypothesis and the glutamate hypothesis. The first, hypothesising abnormal dopaminergic tone, is evidenced by studies showing that psychotic symptoms can be induced by amphetamine in healthy subjects, that patients release more DA following amphetamine than controls and that all successful antipsychotic drugs antagonise the D2 receptor (Abi-Dargham *et al*, 2009; Breier *et al*, 1997; Carlsson and Lindqvist, 1963; Morland, 2000). However, whilst amphetamine administration models are able to model positive symptoms and antipsychotics can treat them, the negative and cognitive aspects of the disorder remain unaccounted for. Conversely, treatment with NMDAR antagonists such as ketamine, MK-801 or phencyclidine (PCP) models, not only positive symptoms but negative and cognitive symptoms as well (Figure 1.3). In healthy subjects, NMDAR antagonists induce a syndrome indistinguishable from a psychotic episode as well as significant cognitive impairments. Additionally, ketamine has been shown to exacerbate psychosis and cognitive impairments in patients with schizophrenia (Krystal *et al*, 1994b).

Figure 1.3 *Mechanism of ketamine action.*

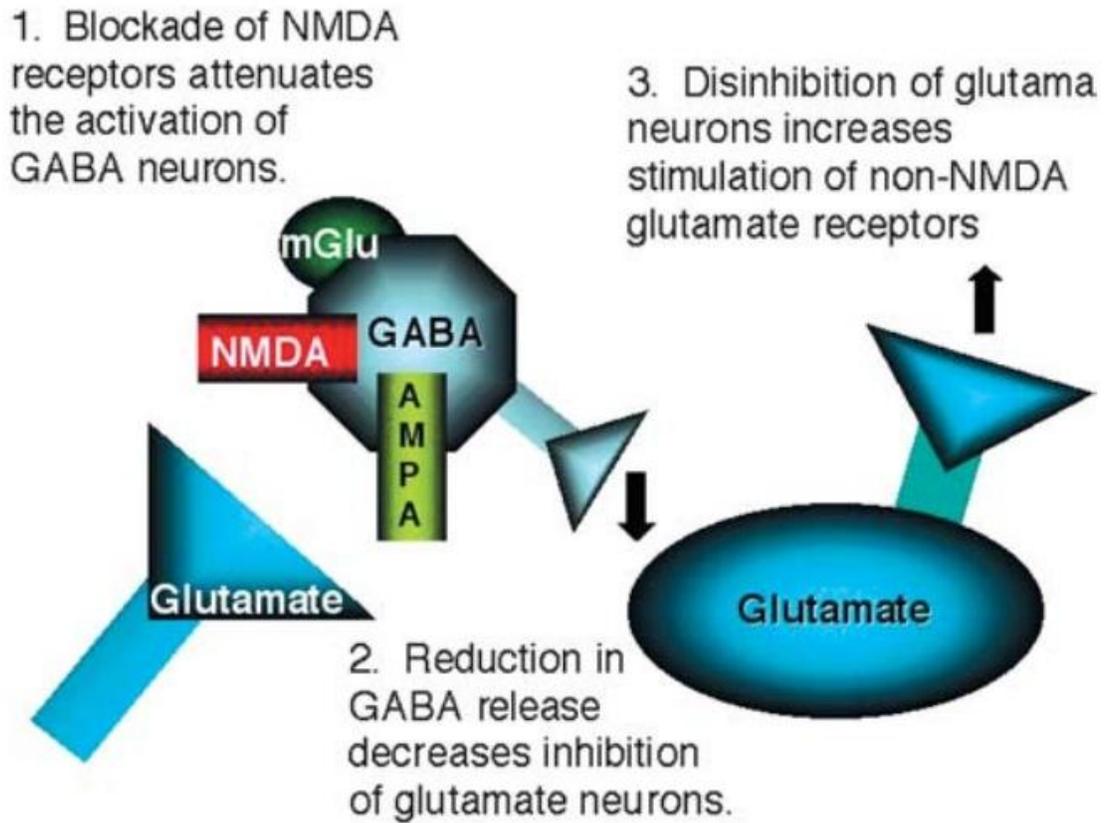


Figure 1.3: Hypofunction or blockade of NMDA receptors on GABAergic interneurons reduces the output of GABA onto pyramidal neurons. This in turn causes disinhibition of pyramidal cells, effectively enhancing glutamate output onto non-NMDA receptors such as AMPA and kainate (Krystal *et al*, 2003).

1.6.1 Dopaminergic models

The modified DA hypothesis indicates that there is hyperactivity of the mesolimbic DA neurons which is reported to account for the positive symptoms of schizophrenia and a hypoactivity of frontal mesocortical DA neurons that accounts for negative symptoms of schizophrenia (Dworkin and Opler, 1992; Seeman, 1987). Support for this comes from current treatments: All current successful antipsychotics antagonise the D2 receptor and their efficacy correlates with the degree of antagonism at this receptor (Sharp *et al*, 1987). In addition, animals given DA agonist amphetamine show increased striatal and nucleus accumbens DA levels, behaviorally exhibiting hyperlocomotion and stereotypy; an effect that can be blocked with antipsychotics (Leite *et al*, 2008; Sharp *et al*, 1987). Clinically, Abi-Dargham *et al* (2009) have shown that patients with schizophrenia have increased baseline and amphetamine-induced DA release in comparison to healthy controls (Abi-Dargham *et al*, 2009). Amphetamine has also been shown to exacerbate psychotic symptoms in patients with schizophrenia with the severity of that response being proportional to the degree of DA release (Laruelle *et al*, 1999). Furthermore, in healthy controls, acute amphetamine can also induce psychotic symptoms (Curran *et al*, 2004). However, a review of DA receptor agonist apomorphine by Depatie and Lal (2001) reveals that this compound does not induce psychotic symptoms in healthy subjects and fails to exacerbate symptoms in patients with schizophrenia.

Manipulation of DA can emulate psychotic symptoms and can induce some negative and cognitive symptoms such as impaired attention (Fletcher *et al*, 2005). However, this model does not effectively replicate the significant cognitive deficits which are a mainstay of this disorder; in particular working memory where amphetamine has been shown to have no effect on task performance (Shoblock *et al*, 2003; Stefani and Moghaddam, 2002). This indicates that models which manipulate DA have significant flaws as not all symptoms are reliably induced: This is likely to mean that the fundamental mechanisms by which these

deficits are induced are not likely to be the cause of schizophrenia. Despite this, models based on the DA hypothesis appear to be useful in terms of examining positive and some negative and cognitive symptoms, but also have significant shortcomings meaning other pharmacological manipulations may be more appropriate.

1.6.2 *Glutamatergic models*

In contrast to treatment with amphetamine, administration of non-competitive NMDAR antagonists such as PCP, ketamine and MK-801 to humans can induce the broad range of symptoms associated with schizophrenia (Jentsch and Roth, 1999; Krystal *et al*, 1994b). NMDAR antagonists are also able to induce psychosis in patients with schizophrenia currently in remission (Malhotra *et al*, 1997b; Malhotra *et al*, 1996). These observations lead to the postulation that schizophrenia may occur as a result of NDMAR hypofunction or the ‘hypoglutamatergic hypothesis of schizophrenia’ (Coyle, 1996). This is supported by evidence that patients with chronic schizophrenia demonstrate significantly lower levels of glutamate in the DLPFC compared to healthy controls (Ohrmann *et al*, 2005).

NMDAR antagonists have been shown to model the symptoms of schizophrenia pre-clinically (Neill *et al*, 2010). Acute administration of PCP prevented acquisition of the MWM in addition to disrupting performance on the spatial alternation task when given as a challenge dose following chronic PCP treatment (Stefani *et al*, 2002; Wass *et al*, 2006). Sub-chronic PCP also impairs cognition in the form of working memory as well as social interaction in rats (Seillier and Giuffrida, 2009). This effect on cognition is supported by McLean *et al* (2008) and Grayson *et al* (2007) who found that sub-chronic PCP was able to impair performance in the both the attentional set-shifting task and the NOR task. Both studies additionally found that this effect could be reversed by atypical anti-psychotic treatment (Grayson *et al*, 2007; McLean *et al*, 2008). In non-human primates repeated PCP administration has been shown to cause enduring working memory impairments correlating with enhanced DA release in the PFC; these effects were also ameliorated by atypical

antipsychotic clozapine (Jentsch *et al*, 1997). Acute ketamine has been shown to cause disruption in a battery of cognitive tasks in a study by Taffe and colleagues (2002) who administered a range of doses acutely before testing in the tasks (Taffe *et al*, 2002). Additionally, Featherstone *et al* (2012) have demonstrated that sub-chronic ketamine can induce significant cognitive deficits in mice (Featherstone *et al*, 2012a). Becker *et al* (2003) support this finding, having also shown that sub-chronic administration of ketamine induces cognitive deficits in rats which are still present 4 weeks following final drug exposure (Becker *et al*, 2003a). Furthermore, in another study, Becker and Grecksch (2004) demonstrated that sub-chronic ketamine could induce changes in the social interaction test that were normalised by treatment with atypical antipsychotics (Becker and Grecksch, 2004). These findings suggest that treatment with NMDAR antagonists may provide a model of schizophrenia with a level of face validity for most features of the disorder; the main discrepancy being that acute doses of antipsychotics are able to reverse cognitive deficits and control positive symptoms after a single dose whereas this is not the case in the clinic. This means in terms of predictive validity, these models may be inclined to produce ‘false positive’ results when translated into humans.

Neurobiologically, chronic administration of NMDAR antagonists decreases striatal D1 receptor expression and reduces GABA_A receptors in the frontal cortex, hippocampus and striatum, in addition to a more widespread reduction in NMDAR binding (Beninger *et al*, 2010; Choi *et al*, 2009). Following chronic PCP administration, basal levels of PFC DA are reduced but are hyper-responsive to amphetamine; replicating human findings (Jentsch *et al*, 1998). Basal PFC glutamate release is also reduced in rats treated with chronic PCP (Fattorini *et al*, 2008).

One of the most striking changes observed in post-mortem brains of patients with schizophrenia is the reduction of PV⁺ neurons: Bernstein *et al* (2007) report a 50% reduction in comparison to healthy controls (Bernstein *et al*, 2007). This finding is

replicated in non-human primates treated with sub-chronic PCP (Morrow *et al*, 2007). McKibben *et al* (2010) also demonstrate that this treatment regimen in rats reduced performance on a NOR task which correlates with reduced PFC PV interneurons (McKibben *et al*, 2010). This is supported by Abdul-Monim *et al* (2007) have shown that sub-chronic PCP can induce deficits in reversal learning which correlates with reduced density of PV+ve neurons in several regions including the PFC and hippocampus (Abdul-Monim *et al*, 2007). Damgaard *et al* (2011) have also shown that sub-chronic PCP induced deficits in memory can be reversed by modulating extrasynaptic GABA_A receptors (Damgaard *et al*, 2011). Behrens *et al* (2007) show that repeated ketamine exposure also leads to loss of PV expression and Braun *et al* (2007) demonstrate this finding in hippocampal PV interneurons following MK-801 exposure (Behrens *et al*, 2007; Braun *et al*, 2007). In this case however, no reduction was seen in PFC PV+ve neurons which may indicate that MK-801 administration may not have the same level of construct validity as PCP or ketamine administration.

Taken together, these studies illustrate that NMDAR antagonist models of schizophrenia are the clear choice of current pharmacological model, although further characterisation of both the MK-801 and ketamine models is needed.

1.6.3 Non-pharmacological models of schizophrenia

Developmental animal models of schizophrenia induce abnormalities in CNS development through environmental or pharmacological manipulation during the sensitive perinatal period (Fone and Porkess, 2008). Rats are very social animals with a hierarchical social structure that is critical to their development. Social isolation of rodents by housing them individually from the age of weaning causes developmental abnormalities as well as behavioural deficits in adulthood. These include neophobia, impaired sensorimotor gating, social withdrawal and cognitive inflexibility which are also affected in patients with

schizophrenia (Lapiz *et al*, 2003). These behavioural changes are in addition to neuroanatomical and neurochemical changes such as reductions in PFC volume and hippocampal synaptic plasticity, hyperfunction of mesolimbic dopaminergic systems and hypofunction of mesocortical DA which are also present in patients with schizophrenia (Fone *et al*, 2008).

Methylazoxymethanol (MAM) is a selective anti-mitotic compound that disrupts CNS neuroblast proliferation (Cattabeni and Di Luca, 1997). When given to a pregnant dam during gestation, the rat pups present behavioural abnormalities such as deficits in prepulse inhibition to startle, hyperlocomotion and working memory deficits (Moore *et al*, 2006). These rats also present neurological changes such as reduced glutamatergic transmission (Hradetzky *et al*, 2012).

Maternal immune activation, induced by either a virus or a synthetic compound, is also able to induce abnormal behaviour in the adult offspring (Zuckerman and Weiner, 2005). This behaviour can be alleviated by antipsychotic treatments and exacerbated by NMDA antagonist MK-801. This model is however less well characterised and has been used less than either the isolation rearing or MAM developmental models.

Neonatal ventral hippocampal (vHip) lesions, involves using the local injection of a neurotoxic compound such as ibotenic acid or electrolytic lesioning (Lipska, 2004). These lesions lead to post-puberty behavioural abnormalities including deficits in social interaction, hyperlocomotion and sensitivity to pharmacological challenges (Naert *et al*, 2013).

These neurodevelopmental models replicate many of the core symptoms of schizophrenia, many of which are seen post-puberty which is in line with the clinical timeline of this disorder. However, there are also alternative methods of modelling schizophrenia including genetic manipulations. These models modify or knock out key genes such as DISC1 and

COMT (as discussed on page 7), which have been linked to the development of schizophrenia.

1.7 Aims and Objectives

This body of work ultimately aims to establish whether neuronal nicotinic receptors represent viable targets for the treatment of CDS. This encompasses several objectives, with the initial task being the investigation of ketamine as a model of CDS in the context of the OST. This will involve establishing a suitable dosing regimen that will induce deficits in the OST using a sub-chronic, sub-anaesthetic dose of ketamine. Furthermore, it will be established whether those deficits are transient or long-lasting. Another objective is to examine if currently used antipsychotic clozapine can reverse these deficits and how this compares to a novel antipsychotic LY404039.

Secondly, the self-medication hypothesis will be considered; examining whether there is a role for nicotine as a cognitive enhancer. Nicotine will be administered in a range of doses to see whether nicotine can reverse ketamine-induced deficits. We will also examine whether nicotine administration in this task can replicate earlier data showing that nicotine administration enhances OST performance in uncompromised animals (Rushforth *et al*, 2010). Furthermore, the degree of improvement will be observed and the hypotheses that nicotine is reversing the deficit induced by sub-chronic ketamine exposure directly, or simply compensating for these deficits will be considered.

Where appropriate, the nAChRs mediating this process will also be investigated through the use of subtype selective agonists for two of the most common nAChRs; the $\alpha 7$ nAChR and the $\alpha 4\beta 2$ nAChR. The ability of both Type I and Type II $\alpha 7$ nAChR PAMs to enhance OST performance will additionally be assessed to elucidate whether reducing the activation point of the $\alpha 7$ nAChR is as effective as a direct $\alpha 7$ nAChR agonist. Whether these effects

can be blocked with a selective $\alpha 7$ nAChR antagonist will also be assessed. The contribution of the $\alpha 7$ nAChR in mediating the OST will be examined by assessing performance in the task following administration of the $\alpha 7$ nAChR antagonist alone.

A neural correlate of working memory will also be established, with the aim of providing mechanistic insight into the behavioural effects of nicotine. This will be achieved by inducing network rhythms, which are thought to underlie working memory, in rodent PFC slices and examining the effect of nicotine on cortical oscillatory activity. The effect of both $\alpha 4\beta 2$ and $\alpha 7$ nAChR agonists will also be examined in order to provide comparison points between behavioural and *in-vitro* data.

Examining oscillatory activity in rodent PFC slices may further our understanding of the brain regions mediating the effects of nicotine: In order to examine the contribution of the PFC behaviourally, nicotine and GABA antagonist muscimol will be administered locally into the mPFC, with subsequent testing on the OST providing evidence for or against the involvement of this region, as these compounds should enhance and impair performance, respectively.

Chapter 2

Materials and Methods

2.1 The Odour Span Task

The protocol for the OST used in this task is based on the original procedures developed by Dudchenko et al (2000) and the further four publications using the OST in existence prior to the start of these experiments (Dudchenko *et al*, 2000; Turchi and Sarter, 2000; Young *et al*, 2007a; Young *et al*, 2007b; Young *et al*, 2009). The papers published following Dudchenko et al (2000) largely used the same training methods, number of scents and protocol with some minor changes, including use of mice instead of rats in the studies by Young et al (2007b,c and 2009) (Young *et al*, 2007a; Young *et al*, 2007b; Young *et al*, 2009). Young et al (2007b,c and 2009) also removed the scents cumin and garlic as the mice found them aversive. Dudchenko (2000) established that the OST in rats was not dependent on the hippocampus as hippocampectomised rats could still complete the OST (Dudchenko *et al*, 2000). Turchi and Sarter (2000) used the OST to demonstrate in rats that a significant role for frontal cholinergic innervations as a 60-80% reduction of AChE positive fibres in frontal cortical regions resulted in significant OST impairments (Turchi *et al*, 2000). Young and colleagues (2007b,c) further developed the OST in mice, demonstrating that mice overexpressing human caspase c, an apoptogenic protein, demonstrated significant impairments in the OST (Young *et al*, 2007b). Additionally, knock-out mice also demonstrated impairments in the OST but not the 5-CSRTT in comparison to wild-types, indicating a significant role for the $\alpha 7$ nAChR in mediating performance in the OST (Young *et al*, 2007a). Additionally, Young (2009) also established a model of Alzheimer's disease using Tg2576 mice which over express human amyloid. These mice were found to be impaired on the OST and thus provide a model from which novel compounds for treatment of Alzheimer's disease can be treated (Young *et al*, 2009). These studies demonstrate that the OST provides a reasonable window within which to examine working memory from both an impairing and enhancing perspective.

This task was therefore chosen for use in these studies as it was a reasonably well established complex task which had the potential to reflect both positive and negative effects of pharmacological compounds on cognition. The use of this task therefore provided an opportunity to develop a non-spatial working memory task for preclinical use. Also, as lesions of the hippocampus have previously been shown not to impair performance in the OST, there was potential for this task to be mediated by the PFC (Dudchenko *et al*, 2000). This means that the OST had the potential for greater translation to human subjects with regard to schizophrenia as impairments in working memory have been linked to this region (Menon *et al*, 2001). This task had demonstrated previous success within the lab in measuring the enhancing effects of nicotine on cognition in uncompromised subjects.

2.1.1 *Animals*

A total number of 132 male hooded Lister rats (Harlan, UK) were used, trained in cohorts of 12 or 24. Rats were aged 8 weeks and weighed 150-200g at the beginning of training. Animals were group housed under standard conditions (a temperature regulated room with a 12 hour light/dark cycle, lights on at 0700h). Rats were food restricted to 90% of free feeding bodyweight at approximately 8 weeks of age, for the duration of the study. Weight was monitored daily and amount of food was gradually increased throughout the experiment to allow for natural growth. Under this schedule no animals showed a weight of less than 85% *ad libitum* body weight. *Ad libitum* body weight was determined using a Harlan, UK growth chart for freely-feeding animals. Animals were permitted free access to water in the home cage and all testing was conducted in the light phase of the 12 hour light/dark cycle. All experiment was carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, or the guidelines set out by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) in Belgium.

2.1.2 *Equipment*

All training and testing took place on a 93cm square platform with 5cm raised border that was elevated 83cm from the floor. The platform was made of wood, covered in black vinyl, 93cm square with 5cm raised border and elevated 83cm from the floor by placing on a table (studies in Newcastle University) or a purpose built black plastic platform 95 cm square with a 5cm raised border and raised 85 cm from the floor (studies in Janssen). The bowl locations were evenly spaced around the platform. Once training began, the position of the platform and table was kept the same throughout. Opaque ceramic dishes were used to house the scented woodchip used for the task. The 24 following odours were used in the task: rosemary, mint, onion powder, oregano, cinnamon, thyme, mixed spice, Chinese-five spice, paprika, fenugreek, nutmeg, garlic powder, caraway seed, celery salt, tea leaves, ginger, cocoa powder, cumin, coffee powder, coriander, parsley, sage, dill, lemon tea. Odours were supermarket own brand or Schwartz™. Three grams of each odour was mixed with 100g of clean animal bedding and nine powdered Nestlé® Cheerio's (added to prevent the rat digging in the correct bowl using scent of the food reward). Odours were replenished at least every two weeks to ensure the odours did not fade.

2.1.3 *Shaping and acquisition of the Non-Matching-to-Sample rule*

Rats were handled for approximately 5 minutes daily during the week prior to the start of training. The initial training sessions took place in a cage on top of the platform. Rats were trained to dig in unscented woodchip for a food reward (half a Nestlé® Cheerio). A 'dig' was counted if the animal displaced any of the woodchip with a foreleg or nose (figure 2.1). Once reliably digging in the woodchip for the food reward, scented bowls were introduced. The animal dug in the first bowl, retrieved the food reward and was then removed from the platform. The first bowl was relocated and a second differently-scented bowl was added. The rat had to smell both odours and then dig in the bowl containing the novel odour. This

was the only bowl baited. Each rat took part in up to 10 trials per session for 3 sessions (time limited to 15 minutes), until they had learned the non-matching-to-sample (NMTS) rule. The odours used were chosen randomly each day from the 24 mentioned above using a random letter list. All animals were exposed to all 24 scents within the 3 sessions. In all sessions, the time at which they were trained or tested was pseudo-randomised with the first cage tested being rotated on a daily basis and then tested in cage number order i.e. cages 4,5,6 then 1,2,3. The first animal tested in each cage was chosen randomly.

2.1.4 Odour Span Task: Training

In this phase, rats were introduced to the experimental platform where they underwent one further day of NMTS training with only two bowls at a time. From then on, the Odour Span Task (OST) proceeded as the NMTS task but after a correct choice was made on the second bowl, an additional bowl containing a novel scent was added. This meant the animal had to assess all three bowls and choose the third bowl. This was on the basis of scent alone as all bowls were relocated to prevent spatial cues aiding the choice, with bowl location determined through random number generation. This procedure was repeated until 10 bowls were present or the rat had spent 15 minutes on the platform. This was the case despite any errors in sample choice. If a mistake was made, the animal was removed from the platform, the bowls relocated and the animal reintroduced. Once a correct choice was made, the rat was permitted to carry on to the next level. At this point, bowls were relocated but kept in within 3 locations of each other (a half-OST, figure 2.2). This was done so that the animals learned more quickly (approximately 4 sessions) that they must sniff all odours before making a choice, rather than attempting to dig in every bowl. Following four half-OST sessions, animals took part in the full OST, in which bowls could be located in any of the 24 spaces and the task was ended once a mistake was made (figure 2.3). This continued for a further 9-10 sessions, until the animal's demonstrated asymptotic performance (table 2.1, figure 2.4). The main parameter measured in this task was the 'span'. This was

determined as the number of correct, consecutively chosen bowls minus 1 (as the first bowl generates no memory load). Asymptotic performance was determined as achieving a span of over 5 in at least 2 consecutive sessions and fluctuating within a minimum of 3 spans over four consecutive sessions. For example, 3-5-5-4, 5-7-5-6, and 8-8-6-8 were all acceptable span patterns for four sessions.

2.1.5 Probe sessions and scent marking

At random points during the training sessions, the reward for a correct choice was dropped into the bowl *after* a correct choice was made. In addition, trials were also carried out where every bowl contained a food reward. A correct choice indicated the animals were responding to the olfactory cues provided and not to the scent of the reward. Occasionally bowls and scented woodchip were replaced during the trial to ensure animals were not scent-marking and using this to identify the novel bowl. If a rat was visibly marking the bowls, these bowls were removed and replaced with a fresh bowl containing the same odour.

2.1.6 Sub-chronic exposure to ketamine

Once exhibiting stable performance, ketamine or vehicle treatment was given once daily for 5 consecutive days. This dosing regimen is based upon that by Becker et al (2003,2004) who were able to demonstrate that this regimen caused impairment in the NOR, as well as inducing a 25% reduction in prefrontal glutamate that was present 4 weeks post ketamine treatment. After acute injection, animals were placed in an incubator until behaviour returned to normal which was usually 15-20 minutes. Behavioural effects observed following acute ketamine exposure at all doses include head weaving, motor impairment and impaired co-ordination. Animals were given two days washout before testing baseline and then tested once each day until demonstration of a stable performance.

Figure 2.1: *Basic OST training*

A: Rat exploring the board prior to digging.



B: Example dig 1



C: Example dig 2



Figure 2.2: *Half-OST in pictures*

A: Rat moving to investigate the bowls



B: Investigating, but not digging in, bowl 1 and 3.



C: Investigation of bowl 2



D: Investigation of bowl 4, followed by digging.



E: Retrieval of food reward



Figure 2.3: Diagram of the OST task

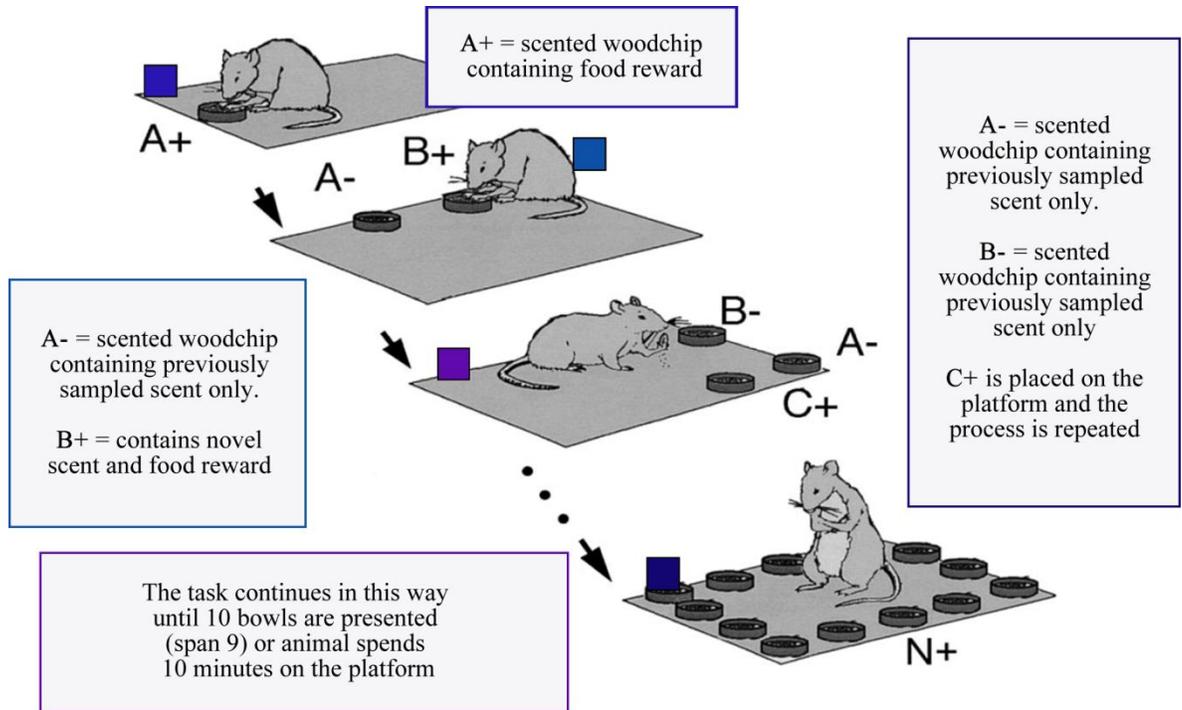


Figure 2.3: Initially, the animal is presented with a bowl, scented with a household spice and baited with a food reward (A+). The animals must dig in the bowl and retrieve the food reward. He is removed from the board, the first bowl now with no food reward, A-, is relocated and a second bowl is added in a novel location: B+ is scented with a different odour and is baited. The animal must then smell both bowls and only dig in the novel-scented bowl. Once retrieving the second food reward, C+ is now added to A- and B- with all bowls relocated. The animal must now dig only in C+ using the novel odour as a guide. This continues until the animal spends 10 minutes on the platform, reaches 10 or 15 bowls (training vs. testing), or the animal makes a mistake. At any of these points the animal is removed from the board and returned to the home cage (Turchi and Sarter, 2000).

Table 2.1: *The three phases of Odour Span Task training*

Training Session	Training Stages
1-4	Learning the non-matching-to-sample rule.
5-8	The half-OST: An easier version of the OST in which odours are presented in close proximity and relocated when a mistake is made.
9-18	The full-OST: Bowls can now be located in any one of 24 locations on a 93cm square platform. Locations are chosen using a random number generator.

Figure 2.4: *Acquisition of the OST task*

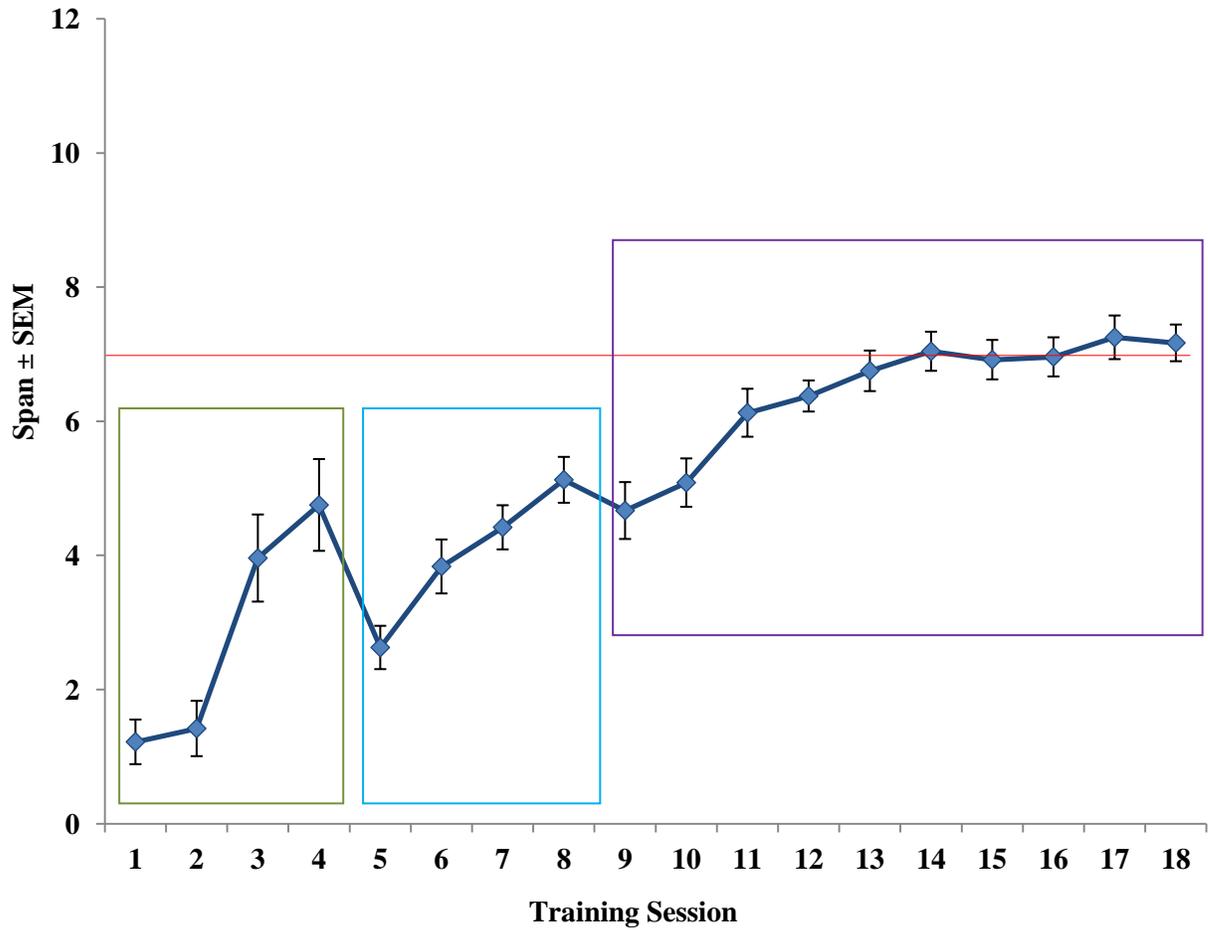


Figure 2.4: This graph illustrates group acquisition of the OST in the 3 task phases. Session 1 to 4 demonstrates learning the NMTS rule (green box). Sessions 5 to 8 is performance on the half-OST (blue box) and sessions 8 to 18 shows performance on the full OST task (purple box). The red line indicates the point at which animals demonstrate asymptotic performance and are ready for the next phase.

Figure 2.5: OST training and testing timeline flow diagram.



Figure 2.5: This diagram illustrates an example timeline of training and testing in the OST with a 5 day ketamine dosing regimen and testing 5 doses of compound plus vehicle.

2.1.7 Odour Span Task: Testing

Compounds were tested in a crossover design whereby each animal received all compound doses, pseudo-randomised across test days. Following a drug testing day, the rats were rested to allow for ‘washout’ followed by retraining on the OST prior to the next test. The experimenter was blinded to all drug treatments except the $\alpha 7$ nicotinic acetylcholine receptor (nAChR) positive allosteric modulator (PAM) compounds as these drugs were tested at the same time but were visibly in solution at the highest doses. Blinding was carried out by a third party who labelled identical vials of the compound A, B, C, D and only revealed the code following the end of the experiments.

2.1.8 Statistical analysis

The odour span from each group of rats was analysed using a two-way, repeated measures analysis of variance (ANOVA) with dose of novel drug as the within-subject factor, and ketamine treatment as the between-subjects factor. A two way, repeated measures ANOVA was used in these cases as it allowed assessment of effect of multiple drug doses as well as then allowing an assessment of any interaction between this effect and prior treatment with ketamine or saline.

One way ANOVAs were also carried out on individual datasets, differentiating on both ketamine treated and control vehicles to assess the effects on these individual groups. This was done so that the significance of individual doses of drug could be assessed on each group. This also revealed the most effective drug dose for each group of animals.

Post- hoc analysis was carried out using Bonferroni correction for number of comparisons made. All data was also subject to Mauchly’s test of sphericity with a significant result meaning that the Greenhouse-Geisser corrected p-value was used. Both the Bonferroni and Greenhouse-Geisser corrections were used as they both produce conservative values which

means a false-positive result in terms of significance is very unlikely. Significant was defined at $p < 0.05$. All analyses were performed using SPSS for Windows (SPSS Inc., V19.0).

2.1.9 Choice of drugs

Ketamine was chosen for use in the odour span task as ketamine can be used in the clinic and thus may provide a translational model where other NMDA antagonists such as PCP and MK-801 cannot. Krystal et al (1994) also state that ketamine reproduces the symptoms of schizophrenia more accurately than any other compound (Krystal *et al*, 1994). It has also been suggested that whilst acute ketamine treatment may most accurately reproduce the psychotic symptoms of schizophrenia, sub-chronic treatment may be more representative of cognitive deficits associated with this disorder (Morgan *et al*, 2009). This is supported by Morgan et al 2009) who have shown that long-term ketamine abuse is associated with impairments in working memory, episodic memory and executive function (Morgan *et al*, 2009). Additionally, as few studies have been carried out with this compound, further validation was necessary. In terms of treatment regimen, Becker et al (2003) have shown that 30mg/kg dose of ketamine, given once daily for 5 days can impair latent inhibition 4 weeks after the final ketamine treatment (Becker *et al*, 2003). Keilhoff et al (2004) have also shown that this regimen induces changes in parvalbumin, neuronal nitric oxide synthase and cFOS similar to that found in patients with schizophrenia (Keilhoff *et al*, 2004). This regimen was therefore also used for the OST along with a log-smaller dose of 10mg/kg to see whether this could induce deficits in OST performance.

Nicotine is a broad spectrum nAChR agonist and as the subject of this these was whether nicotinic receptors are valid targets for the treatment of cognitive deficits, it was the ideal

Table 2.2: Odour Span Task: Drugs

Drug	Doses	Route	Vehicle	Notes	Dose source
Ketamine	10, 30 mg/kg	i.p.	0.9% w/v NaCl		(Becker <i>et al.</i> , 2003b)
Nicotine (systemic)	0.025, 0.05, 0.1 mg/kg	i.p.	0.9% w/v NaCl	pH 5-5.5	(Rushforth <i>et al.</i> , 2010)
Nicotine (local)	1, 2, 4 µg/side	i.c.	ACSF		(Hahn <i>et al.</i> , 2003b)
Clozapine	1, 3, 10 mg/kg	i.p.	0.9% w/v NaCl	Dissolved in 0.1M HCl. Final pH 5.7	Dose advised from JNJ as a result of in house PK data
LY404039	0.3, 1, 3, 10 mg/kg	s.c.	0.9% w/v NaCl		Dose advised from JNJ as a result of in house PK data
PHA-543613	0.3, 1, 3 mg/kg	i.p.	0.9% w/v NaCl		(Wishka <i>et al.</i> , 2006)
Metanicotine	0.03, 0.1, 0.3 mg/kg	i.p.	0.9% w/v NaCl		(Rushforth <i>et al.</i> , 2010)
5-iodo-A-85380	1, 3, 6µg/kg	i.p.	0.9% w/v NaCl		Unpublished in-house data (Doses effective in ASST)
PNU-120596	0.03, 0.1, 0.3, 1, 3 mg/kg	s.c.	5% DMSO + 5% solutol + PBS	Dosing = 3ml/kg Sonicated 1,3 mg/kg in suspension	Dose advised from JNJ as a result of in house PK data
Compound T	0.1, 0.3, 1, 3, 9 mg/kg	i.p.	10% cyclodextrin in sterile H ₂ O + NaCl	Dosing = 5ml/kg Sonicated	Dose advised from JNJ as a result of in house PK data
Methyllycaconitine	2, 6 mg/kg	i.p.	0.9% w/v NaCl		Dose advised from JNJ as a result of in house PK data
Muscimol	1, 2 µg/side	i.c.	ACSF		(Stackman <i>et al.</i> , 2012)

Dosing was 1ml/kg unless otherwise stated and all values are expressed as those of the base. All drugs were supplied by Sigma Aldrich, UK; Tocris, UK or Johnson and Johnson, Belgium and pH balanced to 7 unless otherwise stated

compound for use in the OST. In addition, it had previously been shown in this lab to improve OST performance in uncompromised animals (Rushforth *et al*, 2010).

Clozapine was chosen as this compound has been reported as the most effective antipsychotic for the treatment of cognitive deficits. In addition other pharmacological models of schizophrenia using NMDA antagonist such as PCP, where cognitive deficits have been induced, clozapine can reverse these impairments (Grayson *et al*, 2007). It was therefore important to examine whether this would be the same in a ketamine-based model.

LY404039 was chosen as it had recently been shown in a Phase II clinical trial to be as effective as olanzapine in the treatment of the positive symptoms of schizophrenia. The effects of this compound on cognition were not reported so examining the effect of LY404039 on the OST aimed to further knowledge on this compound.

PHA-543613 was chosen to investigate the $\alpha 7$ nAChR as it is selective for the $\alpha 7$ nAChR with good brain penetration and reported *in-vitro* and *in-vivo* activity (Acker *et al*, 2008; Wishka *et al*, 2006). 5-Iodo-A-85380 (5IA) and metanicotine were also chosen for their selectivity, but for the $\alpha 4\beta 2$ nAChR (Lippiello *et al*, 1996). 5IA has also been shown to elicit dopamine release in the PFC and metanicotine has previously been demonstrated to improve OST performance in uncompromised animals (Livingstone *et al*, 2009).

Choice of $\alpha 7$ allosteric modulator was done in conjunction with Janssen Pharmaceutical companies of Johnson and Johnson (JNJ). In order to get an indication of mechanism both Type I and Type II allosteric modulators were chosen. The first, PNU-120596 is the best characterised Type II PAM and has been shown to have cognitive-enhancing properties *in-vivo* (Hurst *et al*, 2005; Timmermann *et al*, 2007). Compound T is type I PAM in development by JNJ and advised as a suitable compound for testing in the OST. JNJ conducted PK studies to ensure doses of both drugs were brain penetrant

MLA is a well characterised nAChR antagonist that has been shown to be selective for the $\alpha 7$ nAChR (Ward *et al*, 1990). Turek *et al* (1995) demonstrated doses of MLA which are brain penetrant and also confirmed that pharmacologically relevant concentrations of MLA are present in the brain following systemic administration (Turek *et al*, 1995). In addition, Schilstrom *et al* (2007) have shown that administration of MLA attenuated galantamine-induced DA release (Schilstrom *et al*, 2007). MLA has also been shown by Tucci *et al* (2003) to block nicotine-induced behaviour (Tucci *et al*, 2003). This compound was therefore chosen in order to block the $\alpha 7$ nAChR in order to assess the contribution of this receptor to performance in the OST.

Muscimol is a well characterised potent GABAA antagonist with partial antagonist activity at the GABAC receptor (Akhondzadeh and Stone, 1995; Johnston, 1996). Application of muscimol can inactivate specific brain regions when given locally and has been shown by Stackman *et al* (2012) to cause impairments in working memory (Stackman *et al*, 2012). Muscimol was therefore chosen for use in the OST in order to inactivate the PFC to ascertain whether this would impair OST performance, which would indicate a contribution of this brain region in mediating this task.

2.2 Surgery and local injection procedure

2.2.1 *Implanting bilateral cannulae into the medial prefrontal cortex (mPFC)*

Rats were anaesthetised and maintained under anaesthesia using isoflurane and their eyes coated with ophthalmic ointment. They were given meloxicam (1mg/kg s.c.) before being mounted into a stereotaxic frame (Kopf Instruments, CA, USA) with the incisor bar set 5mm above the interaural line. The head area was shaved, wiped with alcohol and then iodine solution was applied. A central incision to expose the skull was made. Topical local anaesthetic was applied, rubbed in using a cotton bud and left for 30 seconds to act. Following this, 5 small crew holes were made (two anterior, three posterior with the central middle posterior being the furthest back) and screws attached by 1.5 turns. Two small holes were drilled bilaterally using coordinates relative to bregma (AP +2.8 mm, L \pm 0.5 mm, V 4.0 mm). The guide cannulae (22 gauge; Plastics One, Roanoke, Va., USA) were lowered to 1mm above the target sites and fixed in place using dental screws and light-fixing dental cement. Three sutures were put in place to secure (one anterior, two posterior). Following surgery, rats were given additional local anaesthetic along with 1ml saline (s.c.) to assist rehydration and placed in a 27°C recovery chamber with *ad libitum* access to water and food pellets soaked in warm water. Animals were then pair housed and left to recover for 10 days with further doses of meloxicam given for the first 5 days. Animals were weighed throughout and checked daily for any sign of infection or pain which was treated accordingly (meloxicam (Metacam) 1.5mg/kg i.o. for pain relief and enrofloxacin (Baytril) 10mg/kg s.c. for infection prevention).

During the experimental process, the caps used to protect the cannulae were occasionally chewed by the cohabiting rat but these were easy to replace and posed no significant problem. This improved animal welfare in comparison to usual procedures (single housing) and prevented any effects that social isolation may have had on OST performance.

Figure 2.6: *Surgical apparatus*

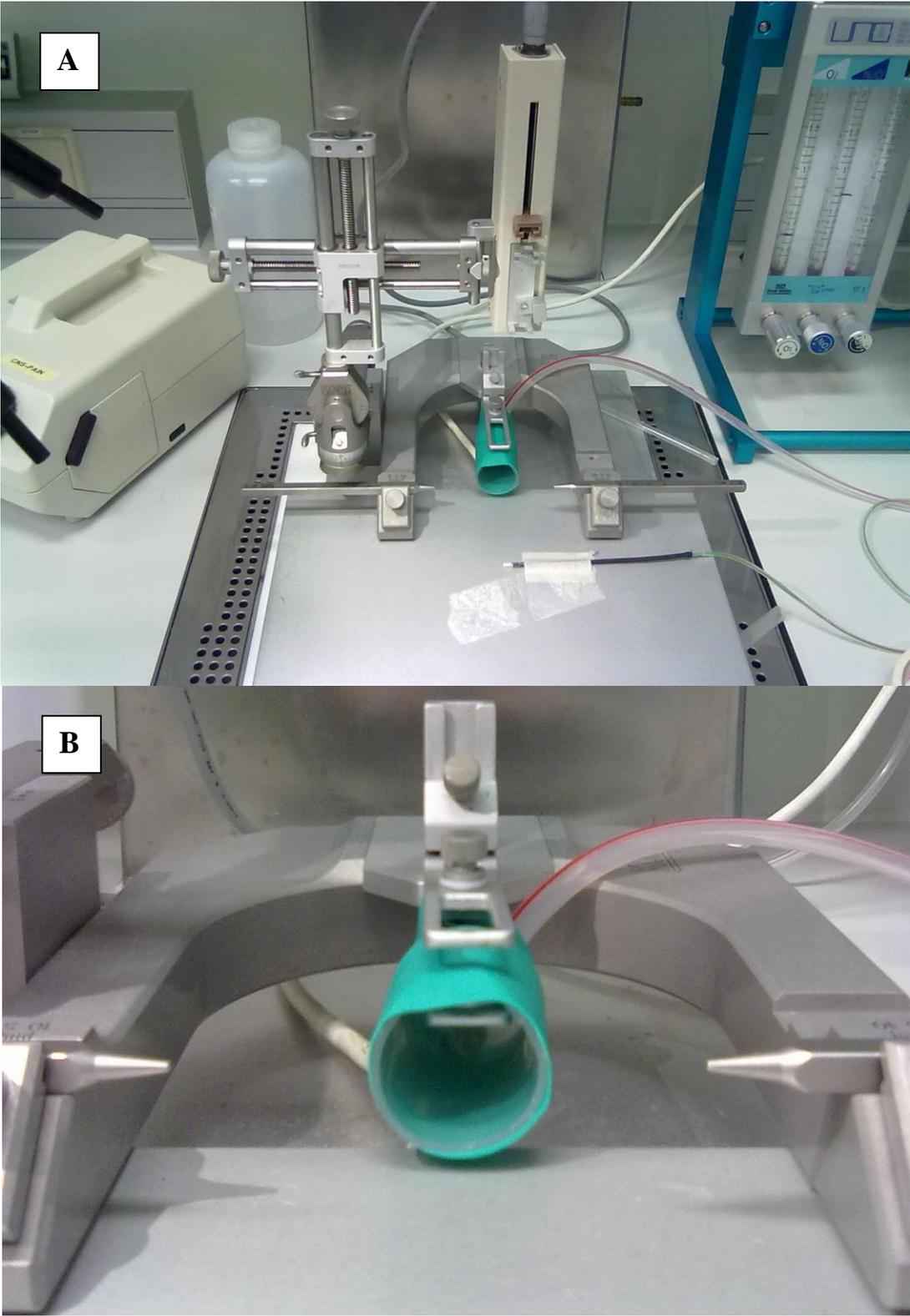


Figure 2.6: (A) Depicts the surgical apparatus used to implant bilateral cannulae into the mPFC of rats trained in the OST. (B) Provides further detail on the stereotaxic frame including ear bars, incisor bar and anaesthesia mask.

Following testing on the OST, animals were locally administered methylene blue and immediately culled by decapitation. Brains were removed and frozen and then sliced using a cryostat to verify correct cannulae placement with placements located within the PrL/ACg region. Four animals were removed from data analysis, two animals died before data collection was complete and their cannulae placements were not collected. Two animals had cannulae placements outside of the PrL, IL or ACg regions and were therefore removed from data analysis. Methylene blue distribution is representative of the distribution of drug approximately 5 minutes into the OST task. This means that at the start of the task, drug would be more localised and additionally distributed further upon completion of that task. This is also assuming that the distribution of methylene blue and test compounds are comparable and so it is possible that this may not be exactly the same as test compounds. In addition, only coronal slices and not horizontal slices were taken which means that the basis for inclusion or elimination here are as a result of vertical diffusion only.

All animals were treated in accordance with procedures outlined by the AAALAC with additional advice from the in-house animal welfare and veterinary team.

2.2.2 Local injection procedure

Following the recovery period, animals were retrained and then tested on the OST to assess baseline. Prior to the first intracranial injection, rats were subjected to a sham handling procedure prior to a training session. Animals were lightly restrained and subject to a bilateral microinjection of saline in a volume of 0.5 μ l over a 27 second period, after which the injection cannulae were left in place for further 60 seconds before training on the OST. Delivery was from a Hamilton syringe mounted in an infusion pump connected to the injection cannulae by polycarbon tubing. The tips of the injection cannulae (28 gauge; Plastics One) extended beyond the guide cannulae by 1mm.

Figure 2.7: Cannulae placement

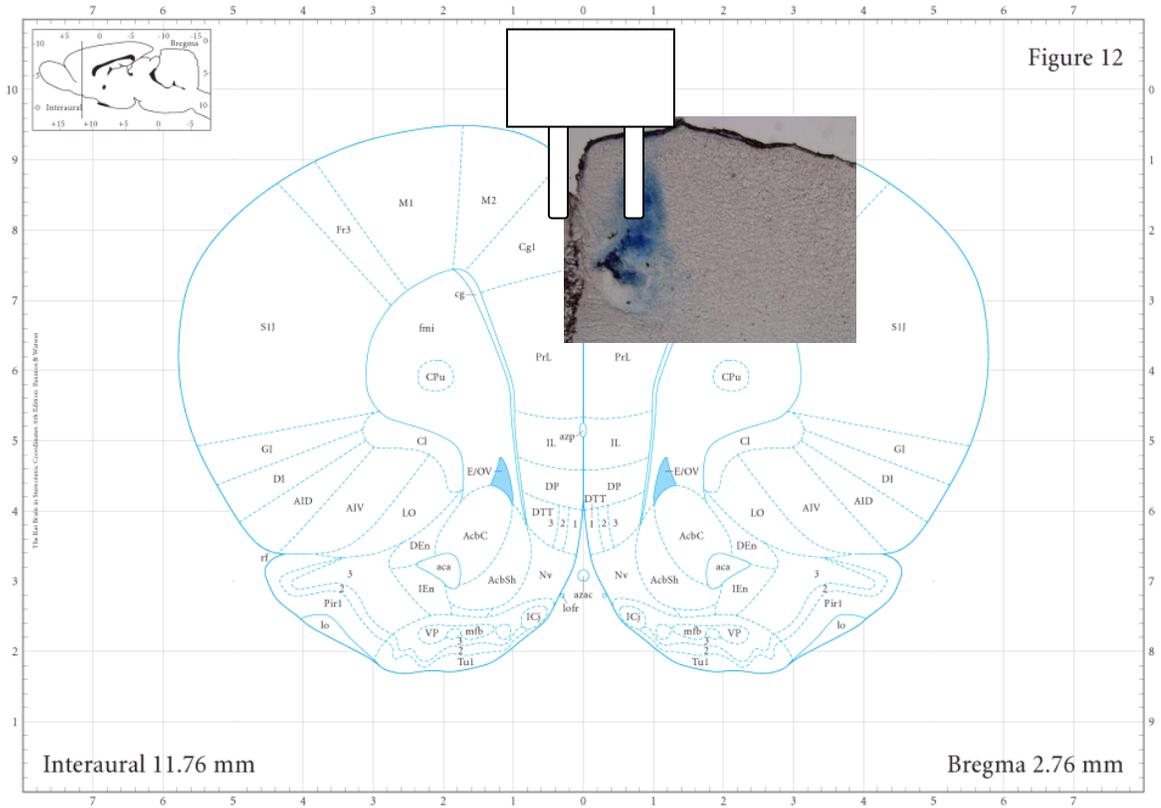


Figure 2.7: This shows a coronal section of the rat brain that has been adapted to demonstrate the location of the implanted cannulae. The overlaid section with methylene blue dye demonstrates the area of the mPFC reached by local injection.

2.3 Gamma frequency network oscillations

2.3.1 Preparation of the brain slices

Male hooded Lister rats weighing 200-300g were anaesthetised with inhaled isoflurane and then given ketamine and xylazine (100mg/kg + 10mg/kg i.m.). Upon termination of respiration, animals were intracardially perfused with 50ml of modified artificial cerebral spinal fluid (in mM: 252 sucrose, 3 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 2 CaCl₂, 10 glucose and 2 MgSO₄). All animals were sacrificed in accordance with the UK Animals (Scientific Procedures) Act 1986.

The brain was then removed and submerged in ACSF at a temperature of 4–5°C. 450µm thick coronal prefrontal cortex (PFC) slices were cut using a Leica vibroslice. Slices were held in a holding chamber and maintained at room temperature in oxygenated (95% O₂, 5% CO₂) ACSF. After one hour, slices were placed into an interface recording chamber and maintained at 31-32°C with a continuous 1.2ml/minute stream of oxygenated, humidified ACSF (in mM: 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.6 CaCl₂, 10 glucose).

2.3.2 Initiation of gamma frequency oscillations

After a further 30 minutes in the recording chamber, gamma frequency oscillations were generated by simultaneous bath application of the cholinergic agonist carbachol (10µM) and the ionotropic glutamatergic agonist kainate (200nM). Gamma frequency activity was recorded extracellularly in the prelimbic (PrL) regions of the PFC (figure 2.7). Power, peak frequency and amplitude values of persistent gamma oscillations were taken from power spectra generated from a 60 second recording of PFC activity using Fourier analysis in the Axograph software package (Axon Instruments, Foster City, CA). Power was defined as the area under the peak in the power spectra between 15 and 48 Hz for

Figure 2.8: *Location of extracellular recording*

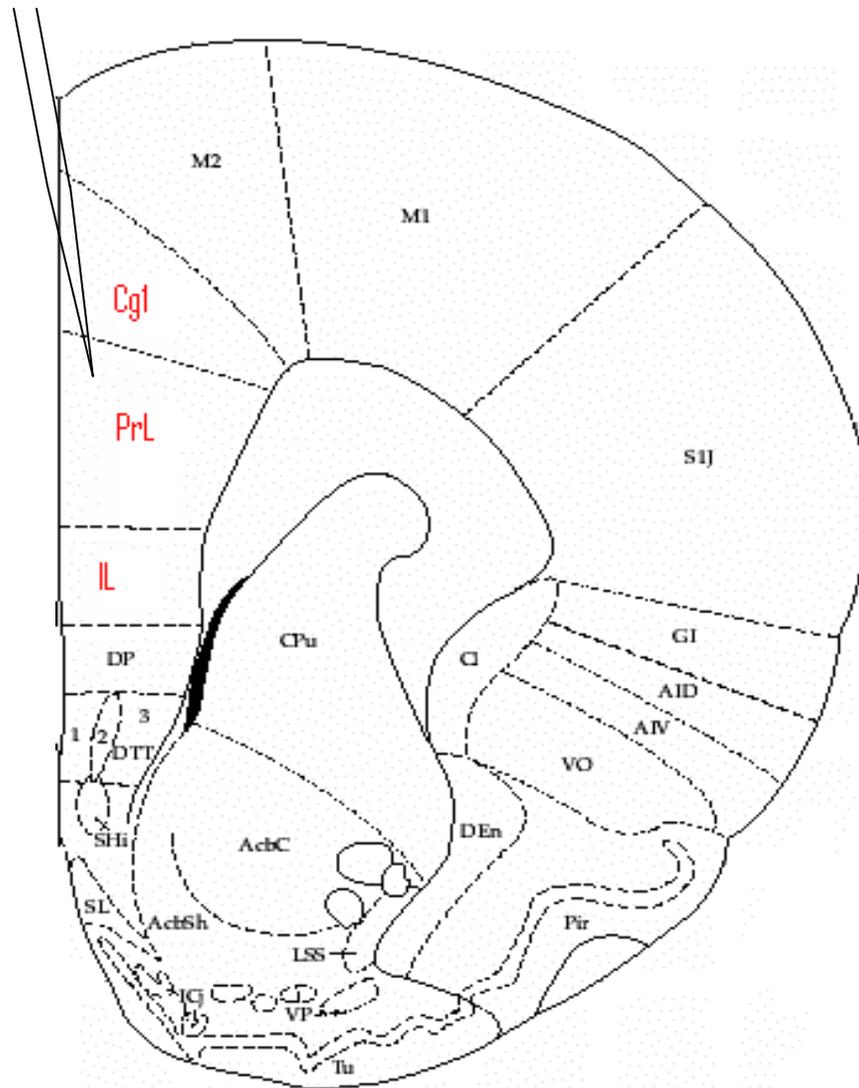


Figure 2.8: Coronal schematic representation of the rat PFC showing subregions: Cg1 - anterior cingulate cortex; PrL - prelimbic cortex; IL - infralimbic cortex (Paxinos and Watson, 1998).

gamma frequency oscillations. Following application of carbachol and kainate, gamma oscillations increase in size; stabilising after 2 to 3 hours. Upon establishing a stable baseline, defined as three consecutive area readings with no more than 10% change, test compounds were delivered via the circulating bath artificial cerebrospinal fluid (ACSF).

2.3.3 Measuring and recording gamma frequency oscillations

Extracellular recording electrodes were pulled from borosilicate glass (Harvard Apparatus Ltd., Kent, UK) and filled with ACSF with resistance in the range of 2–5M Ω . Gamma frequency activity was recorded extracellularly in the PrL regions of the PFC. Power, peak frequency and amplitude values of persistent gamma oscillations were taken from power spectra generated from a 60 second recording of PFC activity using Fourier analysis in the Axograph software package (Axon Instruments, Foster City, CA). Power was defined as the area under the peak in the power spectra between 15 and 48 Hz for gamma frequency oscillations.

2.3.4 Statistical analysis

Analysis was carried out using Sigmaplot 11 software. Different statistical tests were used according to the dataset in question. This included both one way and two way ANOVA where appropriate, for normally distributed data with Holm-sidak post hoc analysis. Paired t-tests in the form of Wilcoxon Signed Rank Tests were used for data which was not normally distributed. For clarity, the type of analysis used is stated within each experimental dataset for Chapter 7.

Table 2.3: Electrophysiology: Drugs

Drug	Concentration	Supplier
Isoflurane		Abbott Laboratories Ltd., Kent, UK
Ketamine	100mg/kg	Ketaset, Fort Dodge Animal Health Ltd., UK
Xylazine	10mg/kg	Millpledge Veterinary, Retford, UK
Carbachol	10 μ M	Sigma-Aldrich, UK
Kainate	200nM	Tocris, UK
Nicotine	1, 10 μ M	Sigma-Aldrich, UK
Mecamylamine	10 μ M	Sigma-Aldrich, UK
PHA-543613	10 μ M	Sigma-Aldrich, UK
5-iodo-A-85380	3 μ M	Sigma-Aldrich, UK

ACSF Ingredients (BHD-Merck UK and Sigma Aldrich, UK)

126mM NaCl,

3mM KCl

1.25mM NaH₂PO₄

24mM NaHCO₃

1.6mM CaCl₂

10mM glucose

Chapter 3

The effect of exposure to a sub-chronic, sub-anaesthetic ketamine dosing regimen on OST performance.

3.1 Introduction

N-methyl-D-aspartate receptor (NMDAR) antagonism has long since been used to model cognitive deficits associated with schizophrenia (CDS). MK-801, also known as dizocilpine, is a non-competitive NMDAR antagonist widely used to model the symptoms of schizophrenia in animals that has been shown to induce psychosis and impair cognition (Andine *et al*, 1999; de Lima *et al*, 2005; Mandillo *et al*, 2003). Translation of models using MK-801 is however, complex as it is not licensed for clinical use due to long lasting effects and the ability of this compound to induce neuronal necrosis (Allen and Iversen, 1990). Another NMDAR antagonist commonly used to model symptoms of schizophrenia is phencyclidine (PCP). Luby *et al* (1959) first administered sub-anaesthetic doses of PCP to healthy volunteers in 1959: PCP induced wide-ranging symptoms similar to those present in patients with schizophrenia, including negative symptoms which were not adequately reproduced in the main pharmacological model of the time using amphetamine (Luby *et al*, 1959). Luby *et al* (1959) thus proposed PCP as a more viable pharmacological model of schizophrenia and, pre-clinically, it is still widely used today (For review see Neill *et al*, 2010). However, like MK-801, PCP is not approved for clinical use due the severity and longevity of its effects: PCP induces long-lasting psychosis, exacerbates symptoms in patients with schizophrenia and has high abuse liability (Krystal *et al*, 1994b).

Ketamine is a less potent analogue of PCP which is approved for clinical use. This is largely because the psychotomimetic effects seen with acute, sub-anaesthetic doses of ketamine are transient and reversible. This means ketamine can be used to induce cognitive deficits in humans with a reduced risk of serious adverse psychological events and shorter lasting effects than PCP (Anis *et al*, 1983; For review see Morgan and Curran, 2006).

Krystal *et al* (1994) demonstrated that ketamine (0.1 and 0.05mg/kg) could dose-dependently induce positive, negative and cognitive symptoms associated with schizophrenia in healthy subjects and proposed that these sub-anaesthetic doses of ketamine could induce the range of symptoms present in schizophrenia more effectively than any other compound (Krystal *et al*, 1994a). A double blind, placebo controlled study carried out by Malhotra *et al* (1997) supported this finding: Thirteen neuroleptic-free patients were treated acutely with sub-anaesthetic ketamine and demonstrated significantly exacerbated psychotic symptoms and cognitive impairment (Malhotra *et al*, 1997a). This suggests that ketamine administration has significant face validity as a model of schizophrenia and was therefore chosen for use in this task.

The ability to use compounds both pre-clinically as well as in human subjects is useful in the development of novel targets for the treatment of CDS. Pre-clinically, Taffe *et al* (2002) demonstrated that sub-anaesthetic doses of ketamine could impair cognition in rhesus monkeys. Seven monkeys were trained in a neuropsychological battery including delayed match-to-sample and self-ordered-spatial search tasks. Treatment with ketamine induced impairments in both of these tasks suggesting impaired visual recognition and working memory. Impairments were both dose and task-difficulty dependent whereby impaired performance was evident following lower doses of ketamine in more difficult tasks (Taffe *et al*, 2002).

In rats, Verma and Moghaddam (1996) have demonstrated that acute ketamine administration impairs performance in a spatial delayed alternation task (Verma and Moghaddam, 1996). Smith *et al* (2011) similarly found that animals treated with acute sub-anaesthetic doses of ketamine demonstrated reduced accuracy on both the 5-Choice-Serial-

Reaction-Time-Task (5-CSRTT) and the delayed-match-to-position task. However, the authors also comment that difficulties were experienced at higher ketamine doses as few animals completed the designated minimum number of trials (Smith *et al*, 2011). This is because ketamine can produce significant motor disturbances when given acutely, which can affect the ability of animals to complete a task. In addition to singular doses of ketamine producing only transient impairments in cognitive performance; it has been proposed that sub-chronic, sub-anaesthetic ketamine exposure may produce a more representative model of CDS.

Sub-chronic, sub-anaesthetic treatment would reduce the confounding acute, non-cognitive effects of ketamine on cognitive task performance. Morgan *et al* (2009) also provide evidence that this regimen could translate into humans, demonstrating that long-term ketamine abuse is associated with impairments in working memory, episodic memory and executive function (Morgan *et al*, 2009). There has been limited use of sub-chronic ketamine in animal models but Becker *et al* (2003) used a daily ketamine (30mg/kg) regimen for 5 days that induced significant behavioural and neurochemical impairments that were present 4 weeks post-treatment (Becker *et al*, 2003a).

The present study therefore aimed to assess whether sub-chronic, sub-anaesthetic exposure to ketamine could induce significant deficits in the Odour Span Task (OST) and which dose of ketamine would prove most effective going forward.

3.2 Methods

Male hooded-Lister rats (125-150g; Harlan UK, n=24) were trained in the OST until demonstration of asymptotic performance. Once trained, animals were pseudo-randomly assigned to treatment groups and treated with ketamine (10 or 30mg/kg i.p.) or vehicle daily for 5 consecutive days. Following a two day washout, animals were tested on the OST task to assess deficits. Initially, tests were carried out daily until performance in ketamine-treated animals was stable. Following evidence of a consistent impairment, assessment of baseline performance was carried out every three days to examine the longevity of these effects. Animals were treated according to procedures outlined in the UK Animals (Scientific Procedures) Act 1986.

3.3 Results

3.3.1 Sub-chronic, sub-anaesthetic ketamine treatment impaired OST performance

A one way analysis of variance (ANOVA) revealed a significant effect of ketamine ($F(2,23)=19.92$, $p<0.001$) on OST task performance. Bonferonni post-hoc analysis revealed that administration of both the 10 and 30mg/kg doses of ketamine significantly impaired OST performance ($p<0.01$; $p<0.001$, respectively; Figure 1).

Figure 3.1: *Sub-chronic, sub-anaesthetic administration of NMDA antagonist ketamine impaired OST performance*

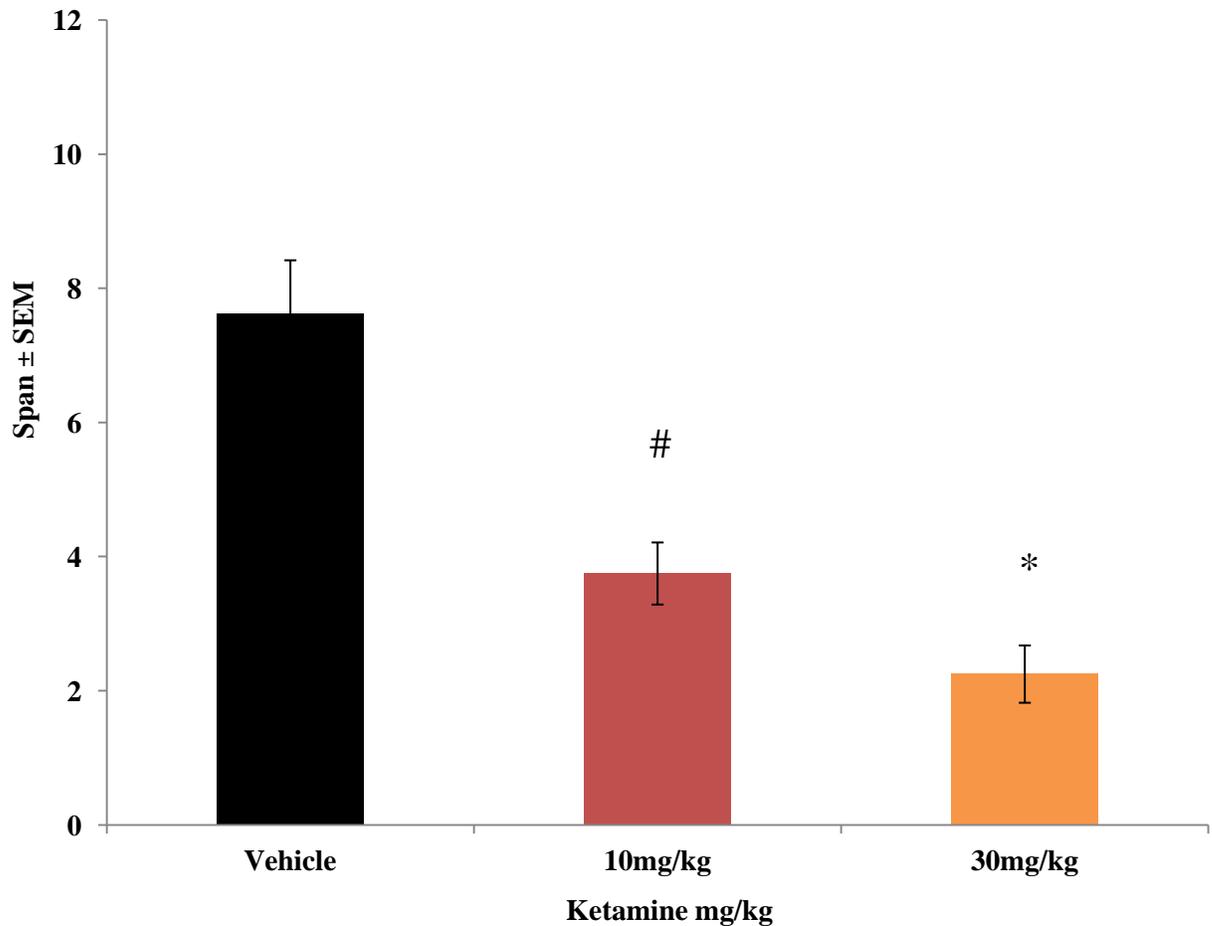


Figure 3.1: This figure demonstrates the effect of two doses of ketamine (10 and 30 mg/kg) on span length in the OST. Both groups of animals (n=8) treated with ketamine exhibited significantly decreased span length when compared with the performance of control animals. Data shown is from the first day of testing following 2 days of ketamine treatment washout and is representative of the deficits present throughout the compound testing phase. # denotes statistical significance from vehicle treatment ($p < 0.01$) * denotes statistical significance from vehicle treatment ($p < 0.001$).

3.3.2 Ketamine-induced deficits were stable and long lasting

A two-way ANOVA revealed a significant effect of ketamine treatment over the 5 testing dates ($F(2,70)=58.07$, $p<0.001$). There was no significant interaction between treatment and day of testing, suggesting that a statistically similar effect of treatment was seen on each day ($F(8,70)=0.28$, $p=0.97$ n.s.). Further analysis was performed using separate one-way ANOVAs, examining each group of ketamine-treated animals per dose. This analysis revealed that both groups of animals treated with ketamine at 10 or 30mg/kg demonstrated impaired OST performance when compared to vehicle-treated controls, ($F(1,79) = 78.98$, $P<0.001$, $F(1,79) = 93.68$, $P<0.001$), respectively (figure 3.2).

Figure 3.2: *The deficits induced by NMDA antagonist ketamine were stable and long lasting*

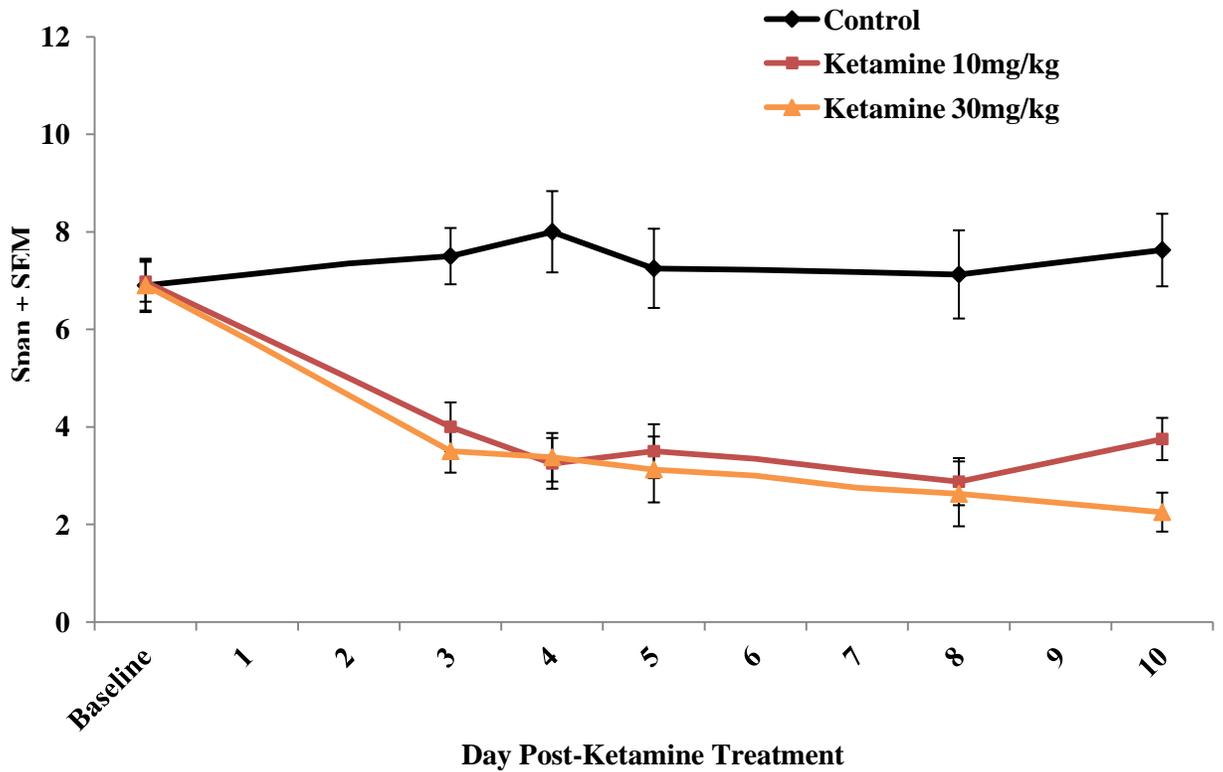


Figure 3.2: Treatment with sub-chronic ketamine at both 10 and 30 mg/kg (n=8) induced significant deficits in OST task performance that were still present at day 10 following the final ketamine exposure. * denotes statistical significance of the 10mg/kg ketamine-treated group from the vehicle-treated group ($p < 0.001$). # denotes statistical significance of the 30mg/kg ketamine-treated group from the vehicle-treated group ($p < 0.001$).

3.4 Discussion

This study demonstrates that sub-chronic exposure to ketamine at both 10 and 30mg/kg is effective in causing significant and lasting working memory deficits in the OST (For publication see Rushforth *et al*, 2011). This supports previous findings by Vanancio *et al* (2011) who administered ketamine (5mg/kg) for 14 days and observed impairments in the Novel Object Recognition (NOR) task (Venancio *et al*, 2011). Interestingly, in the same study, no effect was seen at the higher 10mg/kg dose. This is different to the findings in this study where the higher 30mg/kg dose was statistically no more effective than the 10mg/kg dose; raising the possibility that lower doses of ketamine may still produce a significant impairment in the OST task. Becker *et al* (2003) however, demonstrated that rats exposed to sub-chronic, sub-anaesthetic ketamine at 30mg/kg daily for 5 days demonstrated impaired cognitive performance 4 weeks post last exposure (Becker *et al*, 2003a). Additionally, this study also found that neurochemically, glutamate binding in the prefrontal cortex was decreased by 25%, potentially as a result of a reduction in PV+ve interneurons in the PFC (Becker *et al*, 2003; Behrens *et al*, 2007). This may explain the long-lasting effects of treatment with ketamine in this study and supports the theory that changes in OST performance may be due to a hypoglutamatergic state in the prefrontal cortex (PFC).

Behrens *et al* (2008) have shown that repetitive exposure to ketamine increases levels of the inflammatory cytokine interleukin-6 (IL-6). IL-6 has been shown to increase cortical nicotinamide adenine dinucleotide phosphate- (NADPH-) oxidase levels in the PFC, in turn significantly enhancing brain superoxide levels. This oxidative stress has been shown by Behrens to cause dysfunction in parvalbumin-containing GABAergic interneurons (Behrens *et al*, 2008). Spike timing in pyramidal neurons is controlled by GABAergic activity and any changes in GABA could therefore affect the synchronised firing underlying gamma

frequency oscillations (Fisahn *et al*, 1998). These oscillations are known to have a role in information processing, encoding and retrieval and so ketamine-induced damage to GABAergic interneurons could affect the functioning of gamma frequency oscillations and ultimately lead to cognitive impairment (Cunningham *et al*, 2006).

Zhang *et al* (2008) have shown that a reduction in GABA results in reduced power of gamma frequency oscillations. Animals were treated with ketamine (30mg/kg) for two days and sacrificed on the following day. Electrophysiological analysis of brain slices taken from these animals revealed a 20% reduction in GABA receptor-mediated mini inhibitory postsynaptic currents (mIPSCs) in comparison to vehicle-treated controls. This group also found a 31% reduction in the presence of glutamic acid decarboxylase 67 (GAD67), a key enzyme for GABA synthesis, in parvalbumin interneurons (Zhang *et al*, 2008). This means that aberrant GABA activity may be part of the mechanism surrounding cognitive deficits in schizophrenia. Support for this comes from post-mortem studies of patients with schizophrenia who show a significant reduction in GAD67 expression in GABAergic interneurons (Benes *et al*, 2007; Volk *et al*, 2000). In addition, Lee *et al* (2003) found that gamma frequency oscillations are reduced in schizophrenia and the degree to which this reduction occurs, correlates with the negative symptoms of the disease (Lee *et al*, 2003).

Clinically, Morgan *et al* (2009) carried out a cross-sectional study examining ketamine abuse and cognition. Five groups were examined consisting of frequent ketamine users, infrequent ketamine users, abstinent users, poly-drug controls and non-users of illicit drugs. Subjects were examined on a variety of cognitive tasks including working memory, pattern recognition memory and verbal fluency. They found frequent ketamine use was associated with impairments in working memory, episodic memory and executive function as well as decreased psychological wellbeing. In contrast, infrequent or ex-users did not have

significant cognitive impairments but did have increased delusional and dissociative symptoms (Morgan *et al*, 2009). This study was followed up a year later, re-examining 80% of the original subjects in the same battery of tasks. Again, cognitive deficits were observed mainly in frequent ketamine users whereby increases in ketamine use over the previous year was associated with a decline in working memory and pattern recognition memory (Morgan *et al*, 2010). This supports the use of a sub-chronic ketamine regimen in rats to model cognitive deficits but does raise the possibility that these effects may not be as long-lasting in human subjects. It is possible that the dose used in our study (10mg/kg, i.p.) may be higher than that those used by recreational users (0.6-2.2mg/kg, given orally) and if this was the case, could account for the lasting effects seen here (Shram *et al*, 2011). However, the route of administration and data collection methods are different in the study by Shram *et al* (2011) and so this cannot be stated conclusively.

An additional finding by Morgan and colleagues (2010) was that frequent and abstinent ketamine users were found to have increased depression scores over the 12 months between the two assessments (Morgan *et al*, 2010). Although further work would be needed to confirm this effect, it is particularly interesting given the recent interest in the use of ketamine to treat depression (For review see Murrough, 2012).

3.5 Conclusions

Administration of ketamine causes significant deficits in OST performance and may prove to be a clinically relevant model of cognitive deficits associated with schizophrenia. However, further work must be carried out to assess whether these deficits can be reversed as well as examining the effect of current, clinically effective antipsychotics. Further work also needs to be undertaken to understand the neurobiological and neurochemical mechanisms mediating the effects of sub-chronic, sub-anaesthetic doses of ketamine on cognition.

For future work, the lower dose of ketamine (10mg/kg) will be used as this dose produced a significant and long-lasting deficit. This follows the National Centre for the Replacement, Refinement and Reduction of Animals in Research's (NC3R's) guidelines to reduce, refine and replace wherever possible in experimental work with animals. In this study the 10mg/kg dose was observed to cause less behavioural distress than the 30mg/kg dose as well having a shorter recovery time. It may also be possible that a lower dose will produce a deficit which is more sensitive to cognitive restoration. The next chapter examines the reversal of these ketamine induced deficits with nicotine and antipsychotics.

Chapter 4

The effect of nicotine,
clozapine and LY404039
on ketamine-induced
cognitive deficits

4.1 Introduction

Tobacco smoking is undertaken by approximately 30% of the general population, a figure that rises to approximately 80% when you consider patients with schizophrenia alone. As the vast majority of patients smoke, smoking has been proposed as a form of self-medication to ameliorate deficits in cognition (de Leon *et al*, 1995).

Nicotine, the primary psychoactive agent in tobacco smoke, is well known for its cognitive-enhancing effects. Studies with tobacco smokers have demonstrated that cognitive deficits induced by smoking abstinence can be restored by nicotine administration in various memory and attention tasks (Atzori. *et al*, 2008). Nicotine administration has also been shown to improve cognition in non-smokers but these results are not as conclusive with variation between studies: Foulds *et al* (1996) tested abstaining smokers and non-smokers in a 9-task cognitive battery and found that nicotine improved reaction time in both groups, but only enhanced performance in abstaining smokers (Foulds *et al*, 1996). Jubelt *et al* (2008) also tested the effect of nicotine on non-smoking patients with schizophrenia and non-smoking controls. In this study, nicotine treatment improved accuracy in recognising novel items and response speed in both groups, but a more pronounced effect was found in non-smokers with schizophrenia (Jubelt *et al*, 2008).

Pre-clinically, Socci *et al* (1995) has shown that systemic nicotine improves performance in the Morris Water Maze (MWM) in both young and aged rats (Socci *et al*, 1995). Hahn *et al* (2003) have also demonstrated that local administration of nicotine into the prefrontal cortex (PFC) was effective in enhancing accuracy in the 5-Choice-Serial-Reaction-Time-Task (5-CSRTT) in rats (Hahn *et al*, 2003a). Furthermore, previous work in our lab has

also demonstrated that nicotinic agonists can enhance Odour Span Task (OST) performance in normal animals. Uncompromised animals treated with nicotine or specific $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptor (nAChR) agonists demonstrated significantly enhanced performance in the OST when compared to vehicle-treated controls (Rushforth *et al*, 2010). However, it remains to be seen whether activation of nAChRs can restore ketamine-induced deficits in the OST.

It is also unknown whether current antipsychotic treatments can reverse ketamine-induced deficits in the OST. The large-scale Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) trial showed that atypical antipsychotics such as olanzapine, risperidone and quetiapine modestly improved cognition in patients with schizophrenia (Keefe *et al*, 2007). Clozapine has also been widely studied in clinical trials focusing on cognitive performance and has been shown to improve attention, learning and memory more effectively than other atypical agents. However, these effects are modest at best and performance is still within the impaired range (For review see Weiss *et al*, 2002). As a result, cognitive deficits still represent a significant unmet clinical need for patients with schizophrenia.

Despite the successful use of N-methyl-D-aspartate receptor (NMDAR) antagonists to model symptoms of schizophrenia, it is only recently that a glutamatergic drug has been tested with any success. LY404039 is a highly selective agonist for metabotropic glutamate receptors 2/3 (mGluR2/3), developed by Eli Lilly and currently in Phase II clinical trials for treatment of schizophrenia. Activation of mGluR2/3 functions to normalise the excessive glutamatergic tone characteristically found in schizophrenic patients. Patil *et al* (2007) carried out a double-blind, placebo controlled study with LY2140023 (an oral pro-drug of

LY404039) in patients with schizophrenia, using olanzapine as an active control. Patients treated with LY2140023 or olanzapine showed statistically significant improvement in both positive and negative symptoms when compared to placebo (Patil *et al*, 2007a). These results are encouraging but the failure of the subsequent trial due to an effect of placebo means that further research will be necessary to elucidate the full role for mGluR2/3 in terms of treatment for cognitive deficits (Kinon *et al*, 2011).

The present experiments aim to assess whether nicotine and novel mGluR2/3 agonist LY404039 are effective in reversing ketamine-induced deficits in OST performance. This study will also examine the effect of clozapine on OST performance as a point of reference to other studies and measures of cognition.

4.2 Methods

On establishing stable performance, the first cohort of hooded Lister rats (n=24) were pseudo-randomised into 3 groups (n=8) and treated with vehicle or ketamine (Sigma Aldrich, U.K. 10 or 30mg/kg i.p.) for 5 consecutive days. They were given 2 days wash out, then tested on the OST for 3 consecutive days and periodically after this to assess the stability of the ketamine-induced deficits in performance. Following this, rats were treated with vehicle or acute nicotine (Sigma Aldrich, U.K. 0.025, 0.05, and 0.1 mg/kg i.p., (n=8)) 10 minutes before testing in a within-subjects, repeated measures design. Here, each rat received each dose in a pseudo-randomised fashion with a day washout between each test day.

The second cohort of rats (n=24) were trained in the OST until demonstrating asymptotic performance and then pseudo-randomised into two groups (n=12/group) and treated with ketamine (10mg/kg) or vehicle for 5 consecutive days after. Once deficits were evident and stable, the two groups were each split in half (n=6/group) and assigned drug treatment. Drug treatment was with either the atypical antipsychotic clozapine (1, 3 and 10 mg/kg i.p.) 45 minutes prior to testing on the OST, or novel mGluR2/3 agonist LY404039 (0.3, 1, 3, 10 mg/kg s.c.) 30 minutes prior to testing.

Both experiments were designed in a within subjects, repeated measures format in which each animal served as his own control, receiving all doses over a period of 3 weeks. Doses were randomised to ensure no effect of day. Testing occurred in a 3 day cycle examining baseline OST performance on day one, compounds were tested on day 2 and day 3 was a wash-out day with no testing or training.

4.3 Results

4.3.1 Nicotine dose-dependently enhanced OST performance

A repeated measures analysis of variance (ANOVA) revealed an overall effect of nicotine on OST performance ($F(2,30)=14.54$, $p<0.001$). No significant interaction between pre-treatment and nicotine was found ($F(4,30)=0.90$, $p=0.48$ n.s.) indicating that nicotine had the same effect regardless of whether subjects had previously received ketamine or vehicle treatment (figure 4.1).

To examine the most effective dose on each pre-treatment group, a one way ANOVA was carried out on each individual dataset. Each ANOVA demonstrated a significant effect of nicotine on saline, ketamine 10mg/kg and ketamine 30mg/kg pre-treatment ($F(3,23)=3.42$, $p<0.05$), ($F(3,23)=8.89$, $p<0.05$) and ($F(3,23)=10.47$, $p<0.01$) respectively. Bonferroni post-hoc analysis indicated that the 0.05mg/kg dose was only individually statistically significant dose in saline-pre-treated animals ($p<0.05$). In both of the ketamine pre-treatment groups, both the 0.05 and 0.1mg/kg doses had a significant effect with the 0.1mg/kg dose producing the most significant enhancement in OST performance ($p<0.05$) (figure 4.1).

Figure 4.1: Broad spectrum nAChR agonist nicotine dose-dependently enhanced OST performance

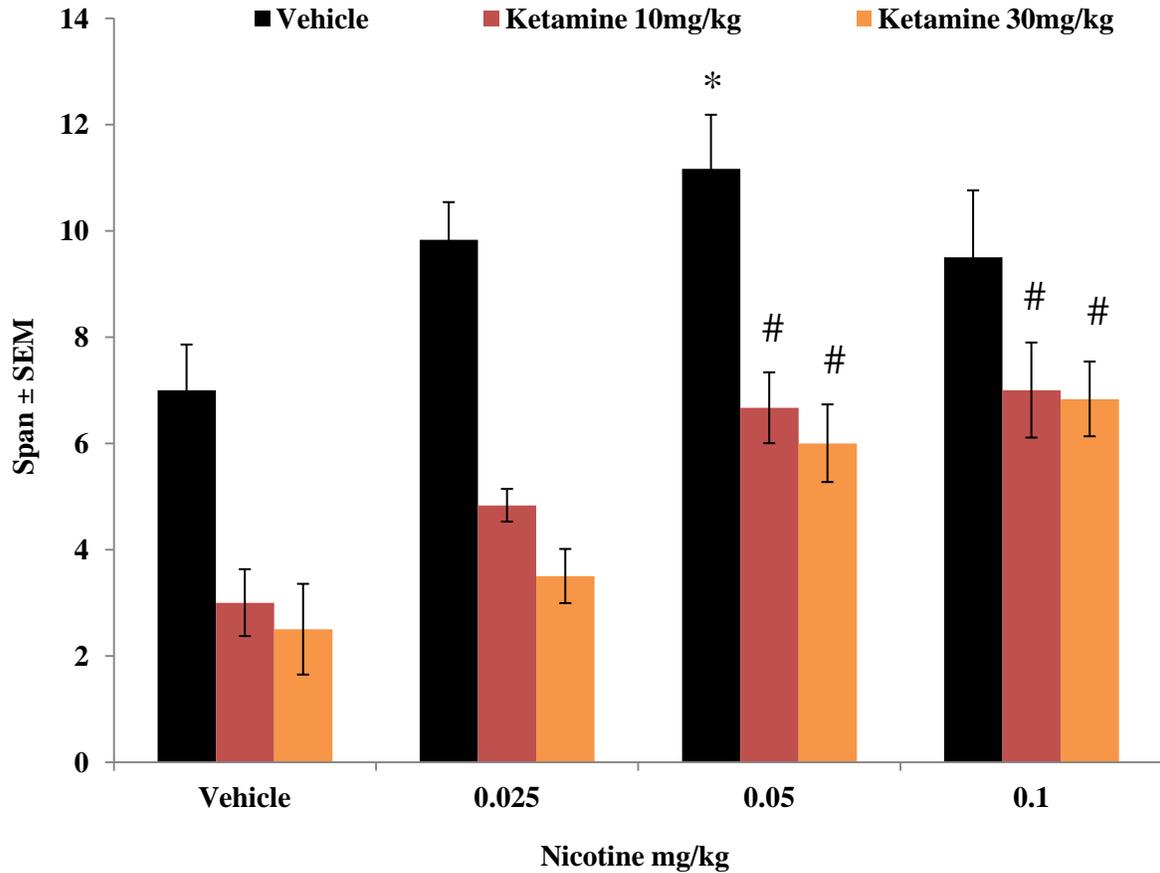


Figure 4.1: Administration of nicotine (n=8) enhanced OST performance in both control and ketamine treated animals in a dose-dependent manner. The 0.1mg/kg nicotine dose achieved full reversal of ketamine-induced deficits to baseline and the 0.05mg/kg nicotine dose improved performance in control animals. * denotes statistical significance from vehicle-treated control animals ($p < 0.05$). # denotes statistical significance from vehicle treated animals with ketamine-induced deficits ($p < 0.05$)

4.3.2. Clozapine had no effect on ketamine-induced deficits in the OST and impaired control animals

A two-way repeated measures ANOVA revealed clozapine had an overall significant effect on OST performance, ($F(3,30)=6.20$, $p<0.01$), along with a significant interaction with ketamine ($F(3,30)=4.55$, $p<0.05$). When further analysed with a one way ANOVA, clozapine had no significant effect on OST performance in animals previously exposed to ketamine, ($F(3,23)=0.10$, $p=0.96$ n.s.), but caused a dose dependent impairment in performance of control animals, ($F(3,23)=10.91$, $p<0.001$) (figure 4.2).

Figure 4.2: Atypical antipsychotic clozapine had no effect on ketamine-induced deficits in the OST and impaired control animals

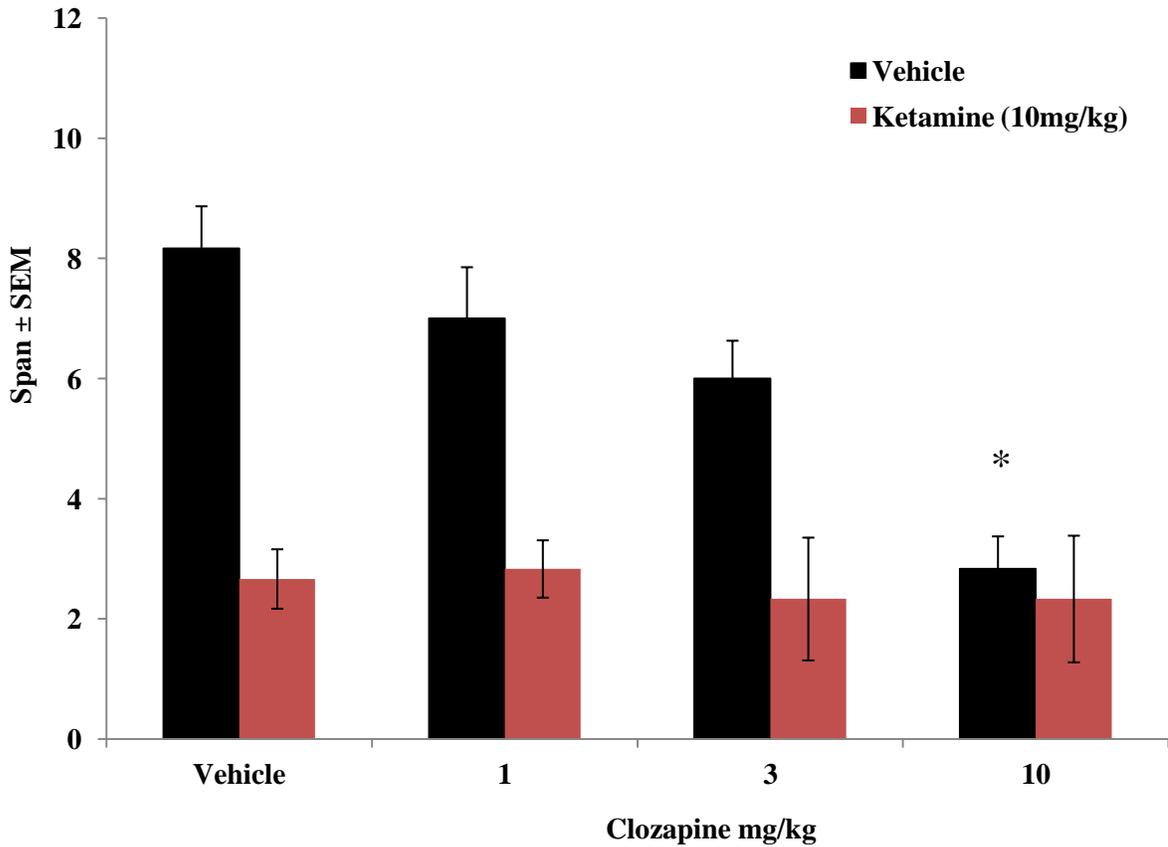


Figure 4.2: Clozapine had an overall effect on OST performance with a significant interaction between dose of clozapine and ketamine pre-treatment (n=6) ($p < 0.01$; $p < 0.05$, respectively). Further analysis determined that acute administration of clozapine had no effect on ketamine-treated animals, but caused a dose dependent impairment in performance of control animals ($p = 0.96$, $p < 0.001$ respectively). *denotes statistical significance from vehicle treated animal where $p < 0.001$.

4.3.3 LY404039 had no effect on ketamine-induced deficits in the OST and impaired control animals

A two-way repeated measures ANOVA revealed LY404039 had an overall significant effect on OST performance, ($F(4,40)=12.28$, $p<0.001$), along with a significant interaction with ketamine ($F(4,40)=3.52$, $p<0.01$). Further analysis using a one way ANOVA has shown LY404039 impaired in performance of control animals ($F(4,29)=5.55$, $p<0.01$), and had a small but significant overall effect in animals treated with ketamine ($F(4,29)=3.76$, $p<0.05$). However, Bonferroni post-hoc analysis did not reveal any individual dose of LY404039 to have a significant effect on OST performance in ketamine treated animals when compared to vehicle treatment (figure 4.3).

Figure 4.3: *Novel mGlu2/3 agonist LY404039 had no effect on ketamine-induced deficits in the OST and impaired control animals*

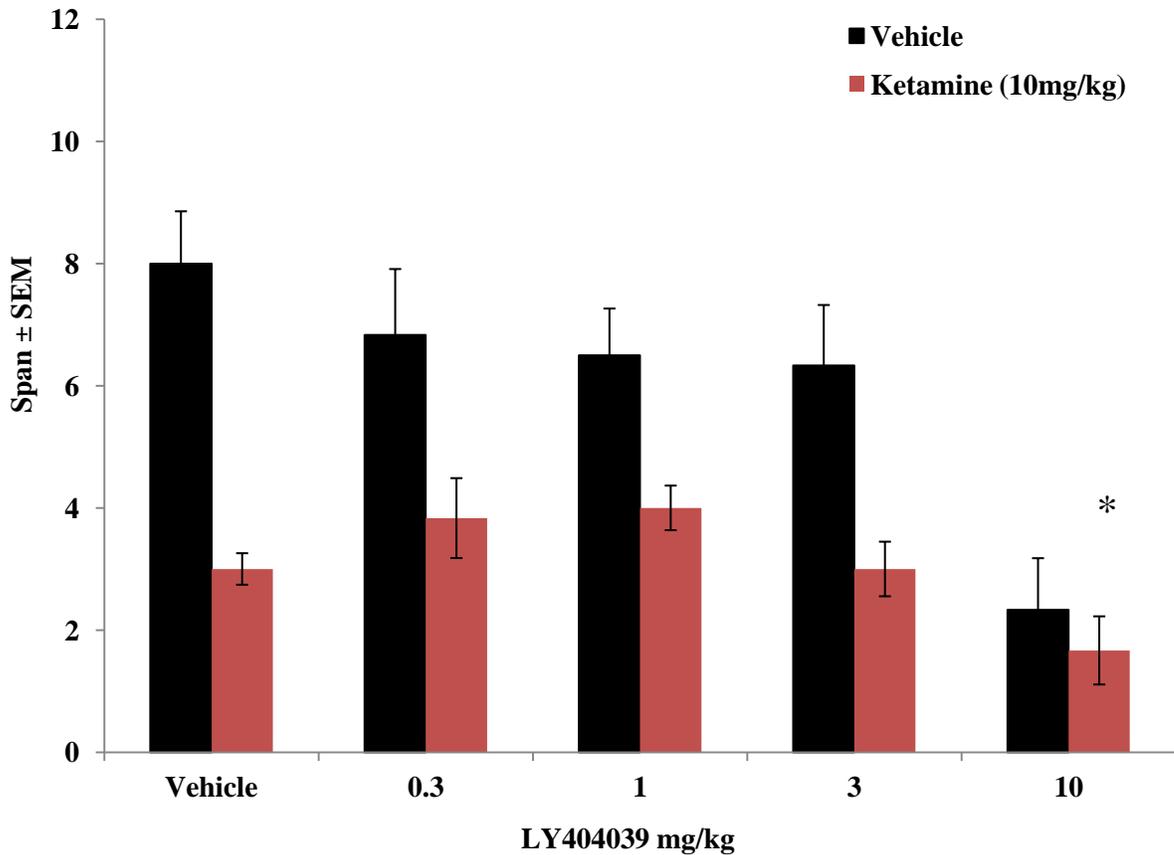


Figure 4.3: LY404039 had an overall effect on OST performance with a significant interaction between dose of LY404039 and ketamine pre-treatment (n=6) ($p < 0.001$: $p < 0.01$, respectively). Further analysis determined that acute administration of LY404039, like clozapine, caused an impairment in control animals ($p < 0.01$). LY404039 also had an overall significant effect on ketamine-treated animals ($p < 0.05$) but post-hoc analysis revealed that no individual dose was able to produce a statistically significant improvement. *denotes statistical significance from vehicle treated animal where $p < 0.01$

4.4 Discussion

Administration of clozapine had no effect on the performance of ketamine treated animals in the OST whilst, at the highest dose, also causing a significant impairment in the performance of vehicle-treated animals. This lack of improvement in impaired animals, is in contrast to studies such as that by Grayson *et al* (2007) who demonstrated that clozapine can reverse a phencyclidine- (PCP-) induced deficit in the novel object recognition (NOR) task (Grayson *et al*, 2007). However, Levin *et al* (2006) support the findings of this study by demonstrating that clozapine potentiated hippocampal lesion-induced deficits the radial arm maze (RAM) (Levin and Christopher, 2006a). Clinical data also supports these findings: McGurk *et al* (2005) examined the effect of clozapine on spatial working memory in patients with schizophrenia using a computerised delayed response test whereby treatment with clozapine lead to a significant impairment in performance on the task (McGurk *et al*, 2005). Melzer and McGurk (1999) also reviewed 12 studies investigating the effect of clozapine on cognition. They concluded that whilst clozapine was effective in measures of attention and verbal fluency, results were inconclusive when considering outcome in tasks involving working memory (Meltzer and McGurk, 1999). The findings of the current study are in line with clinical data but there are alternative explanations for these results: The dose of ketamine used in these studies may maximally reduce cognitive performance to a point where any subtle effects of antipsychotic treatment are lost. This theory is supported by the fact that both 10 and 30mg/kg doses reduced OST task performance to a similar degree (Chapter 3). Clinically, clozapine is also given repeatedly and takes several weeks before showing significant effects on cognitive performance, it may be that chronic or sub-chronic administration is necessary in order to see significant

results. A study carried out by Gray *et al* (2009) however, does not support this theory; mGluR5 knockout (KO) mice were treated with 5mg/kg clozapine (i.p.) daily for 8 weeks but still failed to show any improvement in Y-maze performance (Gray *et al*, 2009). Whilst the efficacy of clozapine in restoring compromised animals is not conclusive, the impairing effect on the performance of control animals is a well documented effect (Addy and Levin, 2002; Levin and Rezvani, 2007; Pocivavsek *et al*, 2006). This effect has been replicated in various studies and has been attributed to the potent anti-muscarinic activity of this compound (McGurk *et al*, 2005). Overall, these data provide a meaningful point of reference when considering novel compounds in the OST task for the treatment of ketamine-induced deficits.

LY404039 (10mg/kg) impaired control animal performance, possibly as a result of significant motor effects observed at this dose. This could prevent normal completion of the OST task and the poor performance may not therefore reflect impairments in cognition *per se*. At lower doses, where no motor disturbance was observed, LY404039 has no effect in control animals and was ineffective in restoring performance in animals with ketamine-induced working memory deficits, although a significant overall effect of LY404039 was found in ketamine-treated animals.

mGluR2/3 are primarily localised presynaptically where they are negatively coupled via G_i proteins to adenylate cyclase. LY404039 acts as an agonist at the mGluR2/3 which functions to inhibit glutamate release, reducing postsynaptic excitability (Manzoni *et al*, 1997). This, in theory, should function to normalise the excessive glutamatergic tone found in patients with schizophrenia and thus improve associated deficits. In this study, LY404029 showed an overall trend towards improvement but no singular dose of this

compound significantly enhanced OST performance. As with clozapine, the effect of ketamine treatment on the OST may be so marked that it may be masking a significant effect of LY404039. In addition, chronic treatment may be more beneficial in reversing these deficits: The 2007 clinical trial by Patil and colleagues report a significant effect of LY404039 in treating positive symptoms at week four of treatment, suggesting acute doses may be insufficient to see significant changes although the effects of this compound specifically on cognition are not discussed in their report (Patil *et al*, 2007a). In addition further clinical trials have since been carried out which did not find any significant effect of LY404039 in patients due to an overwhelming effect of placebo (Kinon *et al*, 2011). Despite this, it is possible that this compound may prove useful with further investigation but too few clinical or pre-clinical studies on the effects of this compound on cognition exist to make full conclusions at this stage.

In contrast, nicotine has been well characterised, showing a significant, and in most cases enhancing, effect on cognition in smokers, non-smokers, laboratory animals previously exposed to nicotine, and nicotine naïve animals (Levin *et al*, 2006b). This supports the finding in this study that nicotine (0.025, 0.05 and 0.1mg/kg) dose-dependently enhances OST working memory performance in both control and ketamine treated animals in comparison to vehicle-treated controls. This is in addition to previous findings from our laboratory indicating nicotine improved task performance in normal animals. Uncompromised male hooded Lister rats treated acutely with nicotine (0.05 or 0.1mg/kg) or vehicle 10 minutes prior to testing on the OST task demonstrated enhanced performance in comparison to vehicle-treated controls (Rushforth *et al*, 2010)

Ketamine-treated animals improved significantly following nicotine treatment but did not perform as well as control animals given the same dose of nicotine. Newhouse *et al* (2004) proposed that nicotine has differential effects, depending on both the state of the subject undergoing tests and also the nature of the task used. If a subject is cognitively impaired and thus exhibiting sub-optimal performance, performance is enhanced by nicotine administration. However, if the subject is performing optimally, nicotine will have no effect or may even impair performance. Similarly, if the subject has no impairment but the task is particularly demanding, this effectively puts the subject in a sub-optimal state and therefore nicotine administration in this situation will also have beneficial effects (Newhouse *et al*, 2004a). This explains the data observed in our research on several levels: Firstly, because the OST is a highly demanding task, nicotine is beneficial in normal uncompromised rats and dose-dependently improves performance. Secondly, because rats treated sub-chronically with ketamine perform sub-optimally, nicotine also enhances performance here. Furthermore, rats treated with ketamine only return to control baseline performance upon nicotine administration. Thus, it is proposed that nicotine can only compensate for either the impairment *or* the high demands of the task and thus nicotine will only restore impaired individuals to a normal level of performance.

The nAChR is the primary target responsible for mediating the diverse actions of nicotine on behaviour (Wonnacott *et al*, 2005). Nicotine elicits the release of a multitude of neurotransmitters crucial to cognition, including acetylcholine (ACh), dopamine (DA), glutamate, serotonin (5-HT, 5-hydroxytryptamine) and gamma-aminobutyric acid (GABA) (Decker and McGaugh, 1991; ED Levin and BB Simon, 1998; Wonnacott *et al*, 1989). The exact mechanism by which nicotine mediates improved cognition is still unknown but is it

likely that the $\alpha 7$ and $\alpha 4\beta 2$ nAChR subtypes have a significant role in this process. This is supported by Young *et al* (2007) who demonstrated that $\alpha 7$ KO mice demonstrated impaired performance in comparison to wild-type controls (Young *et al*, 2007a). Moreover, previous work in our laboratory using the OST has shown that both $\alpha 7$ specific agonist (R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide (Compound A) and $\alpha 4\beta 2$ specific agonist metanicotine could significantly improve OST performance in normal animals (Rushforth *et al*, 2010). In the next study, the effect of selective nAChR antagonists on OST performance in ketamine-treated animals was therefore examined. This allowed further insight into relevant nAChR targets for the development of novel compounds for treating cognitive deficits but also furthered our mechanistic understanding of nicotine's effect on cognition.

4.5 Conclusions

Neither clozapine nor LY404039 restored performance in ketamine treated rats tested on the OST. This replicates data found in the literature and serves as a comparison point to other cognitive tasks. In contrast, nicotine administration fully reversed ketamine-induced OST deficits as well as improving performance in uncompromised subjects. However, future work must be carried out to elucidate the nAChR subtypes which mediate this effect. Taken together, these data support the use of ketamine in the OST to model cognitive deficits in schizophrenia and indicate that nAChRs may be useful targets for the development of novel targets for their treatment.

Chapter 5

Examining the effect of $\alpha 7$ and $\alpha 4\beta 2$ nAChR agonists on ketamine-induced deficits in the OST

5.1 Introduction

Acute administration of nicotine improves the performance of control animals in the Odour Span Task (OST) in addition to restoring impairments in rats following ketamine-treatment. The nicotinic acetylcholine receptor (nAChR) subtype mediating this process is yet to be fully elucidated as nicotine is known to activate multiple nAChR subtypes. There is however likely to be a role for $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in working memory tasks as rats treated with $\alpha 4\beta 2$ and $\alpha 7$ nAChR selective agonists show improved performance (Buccafusco *et al*, 2007; Chan *et al*, 2007; Rushforth *et al*, 2010). In addition, administration of selective nAChR antagonists impairs performance in working memory tasks and transgenic knockout (KO) mice lacking either the $\beta 2$ or $\alpha 7$ nAChR subunits also exhibit impaired performance on the radial arm maze (RAM); a well-established measure of spatial working memory in the rat (Chan *et al*, 2007; Levin *et al*, 2009).

The $\alpha 4\beta 2$ nAChR agonist metanicotine is highly selective for the $\alpha 4\beta 2$ nAChR and has been shown to improve performance in cognitive tasks (For review see: Bencherif *et al*, 1996; Lippiello *et al*, 1996). Lippiello *et al* (1996) demonstrated that metanicotine improved performance in rats showing cognitive deficits in the RAM following ibotenic acid lesions to the nucleus basalis and medial septal area (Lippiello *et al*, 1996). Rushforth *et al* (2010) have also shown that metanicotine can enhance performance in the OST in uncompromised animals, but whether this compound is effective in improving OST performance in compromised animals is currently unknown. 5-Iodo-A-85380 (5IA) is also a selective agonist for the $\alpha 4\beta 2$ nAChR with additional secondary affinity for the $\alpha 6\beta 2$ nAChR, and is thus considered as a $\beta 2^*$ agonist. This compound has yet to be fully

examined behaviourally, but Livingstone *et al* (2009) have demonstrated that 5IA is able to elicit dopamine (DA) overflow in the prefrontal cortex (PFC), which may represent a mechanism by which $\alpha4\beta2$ nAChR activation is able to improve performance in cognitive tasks (Livingstone *et al*, 2009). 5IA also exhibits rapid blood-brain barrier penetration, low toxicity and a high degree of specificity for $\beta2^*$ nAChRs versus the $\alpha7$ nAChR (Mukhin *et al*, 2000). PHA-543613 (PHA), in contrast, is highly selective for the $\alpha7$ nAChR. Like 5IA, PHA has demonstrated efficacy *in-vitro*, but this compound is also effective *in-vivo*: Wishka *et al* (2006) found that PHA ameliorated sensory gating deficits induced by acute exposure to amphetamine as well as enhancing cognitive performance in the Novel Object Recognition (NOR) task (Wishka *et al*, 2006). This suggests there may be a significant contribution from both the $\alpha4\beta2$ and/or $\alpha7$ nAChRs to memory processes and that either or both of these receptors may contribute to the enhancing effect of nicotine in the OST.

This study therefore aims to assess the effects of $\alpha4\beta2$ and $\alpha7$ nAChR selective agonists on OST performance in normal and compromised rats to elucidate the contribution of these nAChR subtypes to the cognitive enhancing effects of nicotine in the OST task.

5.2 Methods

Two cohorts (n=12, n=24 respectively) of male hooded Lister rats (125-150g; Harlan UK) were trained in the OST until demonstration of asymptotic performance. Cohort one was trained and tested in Newcastle University by research associate Emma Malcolm (Newcastle Upon Tyne, UK). I trained and tested cohort two in Janssen Pharmaceutical Companies of Johnson and Johnson (Beerse, Belgium). Once trained, animals were pseudo-randomly assigned to treatment groups and treated with ketamine (10mg/kg, i.p.) or vehicle daily for 5 consecutive days. Following a two day washout, animals were tested on the OST task to assess deficits. Once the ketamine-induced deficits were stable, animals were pseudo-randomly allocated to treatment groups (n=6/group). Animals were then tested in a two day testing, one day washout routine whereby baseline was measured on day one, on day two animals were treated with either $\alpha 7$ agonist PHA (cohort one; 0.3, 1 and 3mg/kg i.p.), $\alpha 4\beta 2$ agonists metanicotine (0.03, 0.1 and 0.3mg/kg i.p.) or 5IA (1, 3 and 6 μ g/kg i.p.) (Cohort two), or vehicle and then tested on the OST. Day three was a washout day with no testing. In this design, each animal received all doses of the respective compounds or vehicle and thus served as his own control. Animals in cohort one were treated according to procedures outlined in the UK Animals (Scientific Procedures) Act 1986 and in cohort two according to procedures outlined in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

5.3 Results

5.3.1 *The $\alpha 7$ nAChR agonist PHA improved OST performance in compromised animals*

Acute administration of PHA at doses of 0.3, 1 and 3mg/kg produced contrasting effects in normal and compromised animals. This was evident from the significant interaction obtained between sub-chronic ketamine treatment and dose ($F(3,21) = 5.40, p < 0.01$), suggesting that PHA treatment caused differing effects in control and ketamine-treated rats. Further analysis was carried out in the form of separate one-way ANOVAs, examining vehicle-treated and ketamine-treated animals as separate groups. This analysis revealed that PHA had no effect on uncompromised animals ($F(3,3) = 18.11, p = 0.61$ n.s.) when compared to vehicle-treated controls. Conversely, PHA improved overall performance in ketamine-treated animals when compared to the vehicle-treated control subjects ($F(3,20) = 10.46, p < 0.001$). Bonferroni post-hoc comparisons revealed that the 3mg/kg doses to be the most effective ($p < 0.05$). Pairwise comparisons revealed the performance of ketamine-treated animals following the 3mg/kg dose of PHA was no different from vehicle-treated controls, suggesting a full restoration ($p = 0.54$ n.s.) (figure 5.1).

Figure 5.1: *The $\alpha 7$ nAChR agonist PHA improved OST performance in compromised animals*

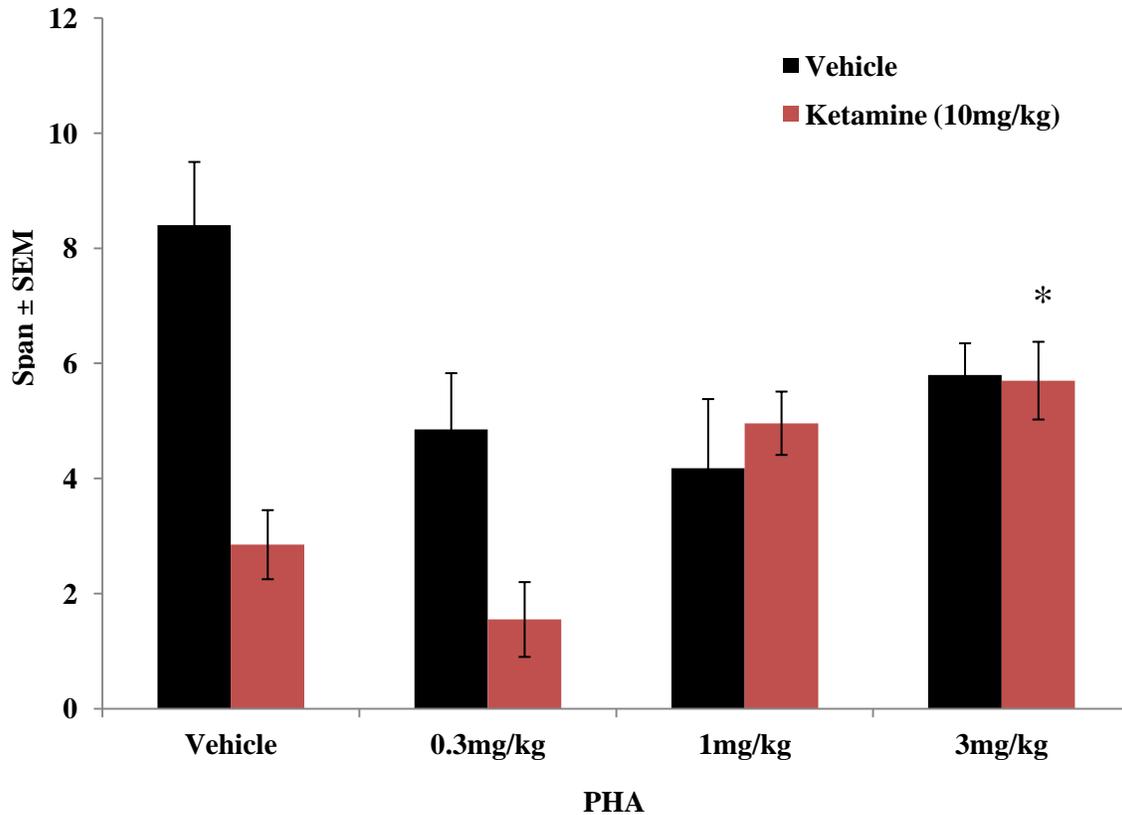


Figure 5.1: This figure demonstrates that the group of animals with ketamine-induced impairments in OST performance (n=6) were significantly improved following PHA administration to a point where performance of ketamine-treated and vehicle treated animals was insignificantly different ($p < 0.01$; $p = 0.54$ ns, respectively). The seeming impairment caused by PHA in control animals was not statistically significant ($P = 0.061$ n.s). * denotes statistical significance from vehicle treatment ($p < 0.05$).

5.3.2 *Acute administration of metanicotine improved performance in the OST*

Acute administration of metanicotine dose-dependently enhanced OST task performance ($F(3,23) = 9.70$ $p < 0.01$). There was no interaction between the two factors ($F(3,18) =$, $p = 0.69$, n.s.), suggesting that metanicotine was able to enhance OST performance irrespective of ketamine pre-treatment. One way ANOVAs conducted for each group revealed that metanicotine treatment significantly enhanced performance in both vehicle-treated and ketamine-treated animals ($F(3,14) = 6.54$ $p < 0.01$, $F(3,11) = 4.75$ $p < 0.05$). Bonferroni post-hoc analysis revealed the 0.1mg/kg dose of metanicotine to be the most effective dose in both groups (figure 5.2) ($p < 0.05$).

Figure 5.2: *Acute administration of $\alpha 4\beta 2$ agonist metanicotine improved performance in the OST*

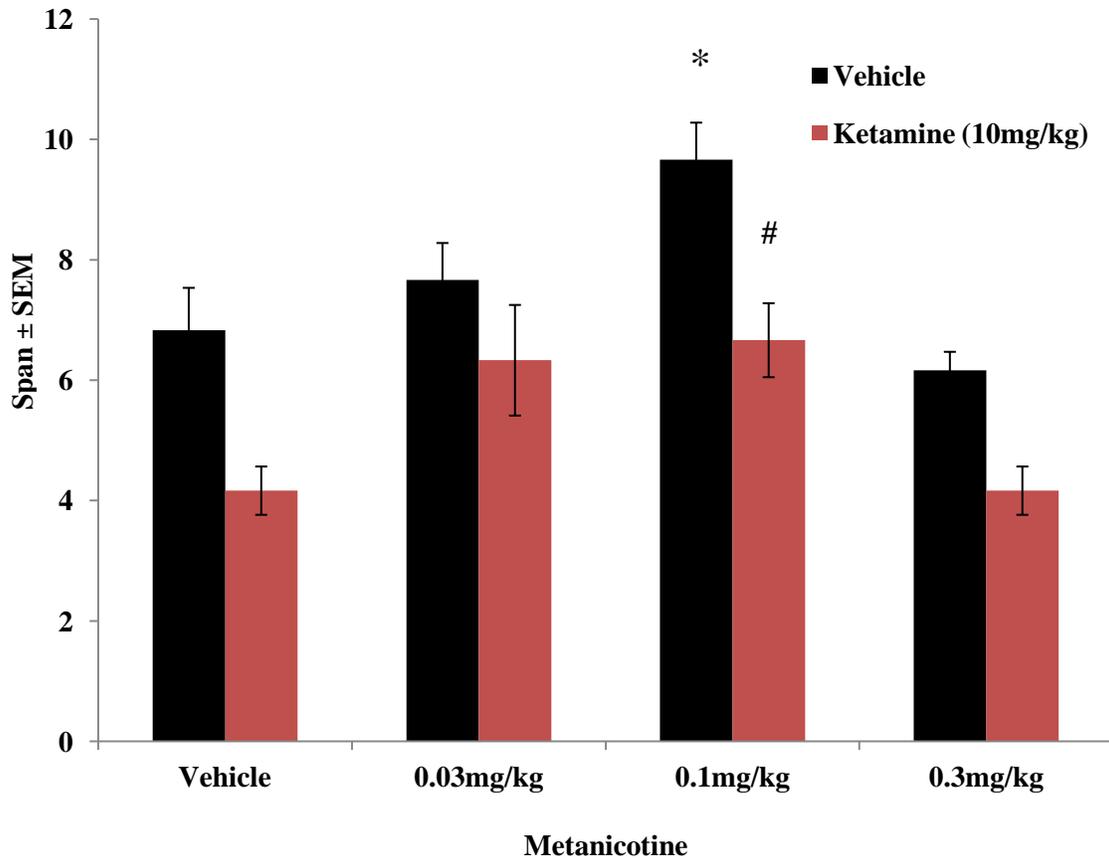


Figure 5.2: Animals with ketamine-induced impairments in OST performance (n=6) were significantly improved following metanicotine (p<0.05). Metanicotine also significantly enhanced performance in control subjects (p<0.01). * denotes statistical significance from vehicle treatment where p<0.05 in control subjects and # denotes statistical significance from vehicle treatment where p<0.05 in ketamine-treated subjects.

5.3.3 *Acute administration of 5IA improved performance in the OST*

A two way ANOVA revealed a significant overall effect of 5IA in the OST ($F(3, 20) = 9.16, p < 0.01$). There was however, no interaction between pre-treatment and 5IA indicating that the effects were similar in both groups ($F(3,3) = 2.32, p = 0.29, n.s.$). Individual one way ANOVAs for each group, separated by pre-treatment, revealed that administration of 5IA produced a dose-dependent increase in task performance in ketamine-treated animals ($F(3,19) = 8.5, p < 0.01$), with Bonferroni post-hoc analysis showing the $6\mu\text{g}/\text{kg}$ dose to be most effective ($p < 0.001$). This was not observed in control subjects with no overall effect of treatment in this group alone ($F(3, 4) = 1.5, p = 0.25, n.s.$). Pairwise comparisons within ketamine-treated and vehicle-treated groups given 5IA at $6\mu\text{g}/\text{kg}$ demonstrated full restoration of OST performance as there was no significant difference between these two groups ($p = 0.25, n.s.$).

Figure 5.3: *Acute administration of $\alpha 4\beta 2$ agonist 5IA improved performance in the OST*

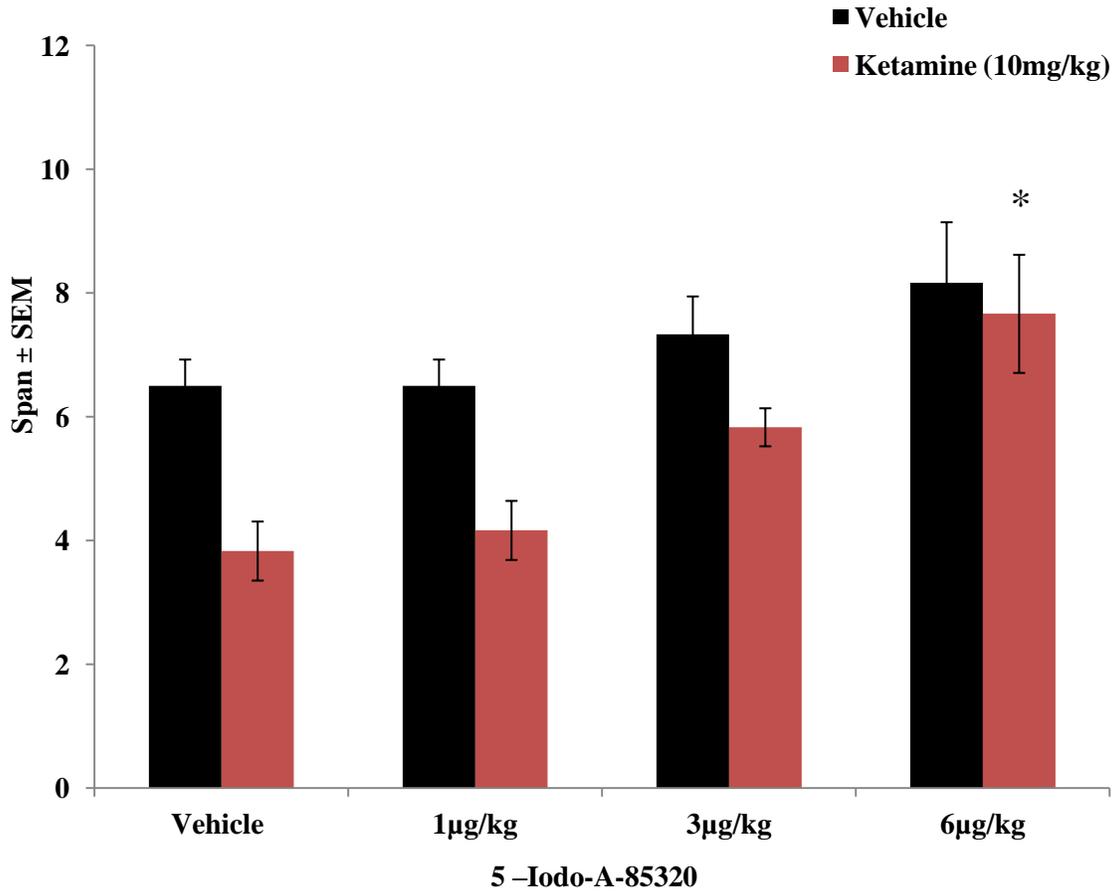


Figure 5.3: Animals with ketamine-induced impairments in OST performance (n=6) were improved following 5IA ($p < 0.01$) to a point where full restoration was seen and performance of control animals and ketamine-treated animals were insignificantly different ($p = 0.25$, n.s.). 5IA had no statistically significant effect on control subjects ($p = 0.25$, n.s.). * denotes statistical significance from vehicle treatment where $p < 0.01$.

5.4 Discussion

The $\alpha 7$ nAChR selective agonist PHA dose-dependently enhanced OST performance in ketamine-treated rats, suggesting $\alpha 7$ nAChRs may mediate the improvements observed with nicotine in the OST task (Chapter 3). However, in contrast to the effect seen with systemic nicotine treatment, performance was not improved to baseline control animal level by PHA administration. Additionally, although a small impairment seems evident, PHA had no statistically significant effect on the performance of control animals on the OST.

This result was unexpected because PHA has previously been shown to improve performance in the NOR task (Wishka *et al*, 2006) and other agonists selective for the $\alpha 7$ nAChR have also been shown to improve cognition. Pichat *et al* (2007) showed that a selective $\alpha 7$ nAChR agonist SSR180711 was able to reverse impairments in the Morris and Linear maze (Pichat *et al*, 2007). Meyer *et al* (1997) also used the Morris maze to demonstrate that rats impaired by means of bilateral ibotenic acid lesions of the nucleus basalis made fewer errors following acute administration of the $\alpha 7$ agonist 3-(2,4-dimethoxybenzylidene) anabaseine (DMXBA) (Meyer *et al*, 1997). In addition, previous work in the present laboratory has shown another $\alpha 7$ nAChR agonist, Compound A, to be effective in enhancing the performance of uncompromised animals in the OST task (Rushforth *et al*, 2010).

Mechanistically, activation of $\alpha 7$ nAChRs located on glutamatergic terminals facilitates the release of glutamate and subsequent activation of ionotropic glutamate receptors in various neuronal networks (Livingstone *et al*, 2010). Gamma-aminobutyric acid (GABA)ergic activity is influenced by metabotropic glutamate receptor (mGluR) activation, subsequently

mediating the synchrony, power and duration of gamma frequency oscillations which are central to working memory processes (Traub *et al*, 2004; Whittington *et al*, 1995). Howard *et al* (2003) provide clinical evidence; they demonstrated that gamma frequency oscillations increased linearly with cognitive load (Howard *et al*, 2003). Tallon-Baudry *et al* (1998) also observed that gamma frequency oscillations were induced during the delay period of the delayed-matching-to-sample task where, in order to complete the task successfully, the memory of the original stimulus must be maintained. Conversely, this induction was not found when memory maintenance was not required, suggesting the involvement of gamma frequency oscillations in the maintenance of information in working memory (Tallon-Baudry *et al*, 1998).

This suggests that $\alpha 7$ nAChR activation is able to modulate working memory through regulation of gamma frequency oscillations. However, glutamate release induced by $\alpha 7$ nAChR activation also activates mGluRs on dopaminergic neurons, facilitating DA release (Livingstone *et al*, 2010). This can also shape working memory processes by influencing the fidelity of synaptic transmission and facilitating long term potentiation (LTP). The $\alpha 7$ nAChR is central to these processes as demonstrated by Ondrejcek *et al* (2012) who found that $\alpha 7$ nAChR agonist Compound A was able to persistently enhance synaptic transmission in the hippocampus and that this effect was blocked by $\alpha 7$ antagonist methyllycaconitine (MLA) (Ondrejcek *et al*, 2012). Welsby *et al* (2006) was also able to show that MLA prevented nicotine-induced enhancement of LTP induction in the rat dentate gyrus, providing further evidence of a role for the $\alpha 7$ nAChR in these processes (Welsby *et al*, 2006).

This overwhelming evidence of $\alpha 7$ nAChR involvement in memory processes, along with previous work demonstrating that selective $\alpha 7$ nAChR agonist Compound A is able to improve OST performance in control animals, suggests that the impairment induced by PHA administration is not necessarily representative of $\alpha 7$ nAChR agonism in this task (Rushforth *et al*, 2010). The results seen here may instead be as a result of unknown non-specific effects of PHA, particularly as this compound has not been tested extensively *in-vivo*. However, it is also possible that PHA rapidly desensitises the $\alpha 7$ nAChR, functioning to reduce transmission in the relevant neuronal networks and thus causing cognitive impairments. Support for this theory comes from Seipel and Yakel (2010) who demonstrate that PHA is able to cause inhibition of dopaminergic neurons through desensitisation of $\alpha 7$ nAChRs following high frequency stimulation (Seipel and Yakel, 2010). They also show that this effect is similar to that produced by selective antagonism of the $\alpha 7$ nAChR with MLA. Rapid desensitisation of the $\alpha 7$ nAChR following PHA administration may explain why PHA treatment did not restore ketamine-treated animals to their baseline level of performance. It is however unlikely that the complete $\alpha 7$ nAChR population were desensitised as control animals were still able to perform the task to a higher standard than compromised animals, and PHA treatment was also able to enhance OST performance in ketamine-treated animals. It may be that ketamine-treated animals present such a low baseline that even minimal activation of $\alpha 7$ nAChRs has a beneficial effect, especially if this results in modulation of aberrant gamma frequency oscillations, which may not be present in animals without ketamine-induced deficits.

In contrast to treatment with $\alpha 7$ agonists, both $\alpha 4\beta 2$ agonists were effective in fully restoring OST performance in ketamine-treated animals. This supports findings by

Lippiello *et al* (1996) who profiled metanicotine in comparison to nicotine using the RAM. They found that metanicotine administration reduced the number of errors made by compromised animals but that metanicotine was not as efficacious as nicotine in improving task performance (Lippiello *et al*, 1996). Metanicotine at a dose of 0.3 μ mol/kg was also effective in improving performance of control animals in the cued RAM, which supports our earlier data with metanicotine in the OST (Rushforth *et al*, 2010). The β 2* nAChR agonist 5IA has not been tested extensively *in-vivo* but the results in this study complement neurochemical findings: Livingstone *et al* (2009) demonstrated that 5IA was able to elicit dopamine release in the rat PFC; an effect that was blocked by α 4 β 2 antagonist dihydro-beta-erythroidine (DH β E) (Livingstone *et al*, 2009). This may explain how α 4 β 2 nAChRs mediate the enhanced OST performance seen in this study, although glutamate has also been implicated in this process: Lambe *et al* (2003) demonstrated that nicotine was able to induce glutamate release from thalamocortical axons onto layer V pyramidal neurons in the PFC, increasing spontaneous excitatory postsynaptic currents (sEPSCs). Induction of sEPSCs can strengthen thalamocortical connections which are essential to working memory processes. They determined the involvement of α 4 β 2 nAChRs by demonstrating that both β 2 KO mice and animals treated with α 4 β 2 antagonist DH β E did not demonstrate this increase in sEPSCs. Conversely, an agonist of the α 7 nAChR did not cause any induction of sEPSCs (Lambe *et al*, 2003). This is a potential mechanism by which activation of α 4 β 2 nAChRs may enhance PFC activity. This activity strengthens cortical networks which are weakened in patients with schizophrenia (Krystal *et al*, 2003; Pinault, 1995).

Activation of the α 4 β 2 nAChR has also been shown to enhance GABAergic inhibition in pyramidal neurons; more so than through activation of α 7 nAChRs (Alkondon and

Albuquerque, 2004; Aracri *et al*, 2010). It is this disinhibition of GABA signalling that is thought to be responsible for the cognitive deficits induced by N-methyl-D-aspartate receptor (NMDAR) antagonists such as ketamine that model those deficits seen in patients with schizophrenia (Alkondon *et al*, 2004; Krystal *et al*, 1994b; Moghaddam *et al*, 1997). It may be that normalising GABA signalling compensates for the disruption following ketamine treatment, which could explain the greater efficacy of both metanicotine and 5IA in comparison to PHA in enhancing OST performance in compromised animals.

Despite these improvements neither $\alpha 7$ nor $\alpha 4\beta 2$ agonist treatment was as effective as systemic nicotine treatment in enhancing OST performance (Rushforth *et al*, 2010). Since nicotine acts at several nAChRs, this may indicate a potential synergistic effect of activating more than one nAChR subtype is necessary for the full agonist effect of activating nAChRs to be realised.

5.5 Conclusions

$\alpha 4\beta 2$ nAChR agonists metanicotine and 5IA are more effective in enhancing OST task performance in both compromised and control animals than $\alpha 7$ agonist PHA. This may suggest that the $\alpha 4\beta 2$ nAChR is the prominent receptor mediating the effect of nicotine on OST performance. However, since the maximal effect of nicotine OST performance could not be fully replicated by $\alpha 4\beta 2$ nAChR agonists alone, optimal performance is more likely to be achieved by co-activation of this receptor along with the $\alpha 7$ nAChR subtype. An alternative explanation is that the $\alpha 7$ nAChR is being rapidly desensitised with an agonist present and so the next chapter examines the effects of $\alpha 7$ positive allosteric modulators which should increase the activity of the $\alpha 7$ nAChR as well as negating any desensitisation effects.

Chapter 6

Allosteric modulators for the $\alpha 7$
nAChR improve OST performance:
An effect blocked by $\alpha 7$ antagonist
methyllycaconitine

6.1 Introduction

Nicotine fully reverses working memory deficits in the Odour Span Task (OST) caused by sub-chronic ketamine exposure, as well as improving performance in uncompromised animals (See chapter 3, 4, and Rushforth *et al*, 2011). The selective $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR) agonists 5-Iodo-A-85380 (5IA) and metanicotine were also able to improve OST performance. This effect was not however, observed with administration of $\alpha 7$ nAChR agonist PHA-543613 (PHA). PHA was only able to modestly enhance performance in compromised animals and impaired performance of control animals. The author proposed this was as a result of the $\alpha 7$ nAChRs becoming desensitised, thus ultimately reducing nAChR-induced neurotransmitter output (Chapter 5). Treatment with $\alpha 7$ allosteric modulators may present a viable alternative to treatment with $\alpha 7$ nAChR agonists as they can reduce desensitisation and convert already desensitised receptors to an active state, as well as lowering the threshold at which the receptor is stable in an open conformation (Hurst *et al*, 2005). More specifically, receptors exist in multiple states and allosteric modulators are able to change the overall properties of the receptor by modifying the isomerisation coefficients between these states. Positive allosteric modulators (PAMs) lower the energy barrier between resting and active states, increasing the effect produced by an agonist or endogenous ligand (Monod *et al*, 1965). There are two types of PAM: Type I, which increase the peak current across the current and Type II, which act by both increasing the peak current as well as changing the point at which the receptor becomes desensitised.

Steroids, such as 17- β -estradiol, have been shown to act allosterically at the $\alpha 4\beta 2$ nAChR but in some cases demonstrate differing actions in rat and human: Paradiso *et al* (2001) demonstrated that 17- β -estradiol was unable to increase acetylcholine (ACh) currents in rodent $\alpha 4\beta 2$ nAChRs but it was able to do so in human $\alpha 4\beta 2$ nAChRs (Paradiso *et al*, 2001). The metallic chemical element zinc is also able to modulate the $\alpha 4\beta 2$ nAChR, increasing ACh-induced currents at $\alpha 4\beta 2$ receptors. It is however, non-selective; also acting positively at $\alpha 7$ nAChRs, negatively at $\alpha 3\beta 2$ nAChRs as well as having activity at the 5-hydroxytryptamine (serotonin, 5-HT)_{1A} receptor (Barrondo and Salles, 2009; Hsiao *et al*, 2001). As a result of limited development of selective $\alpha 4\beta 2$ nAChR PAMs, most are non-selective; providing few useful pharmacological tools for allosteric modulation of the $\alpha 4\beta 2$ nAChR. The only current compound of interest which has been reported is $\alpha 4\beta 2$ PAM NS9283, which was recently shown by Timmermann and colleagues (2012) to improve performance in the 5-Choice-Serial-Reaction-Time-Test (5-CSRTT) and the Morris Water Maze (MWM) (Timmermann *et al*, 2012). This is the first study to examine the effect of NS9283 on cognition and so further work is necessary.

In contrast, development of PAMs for the $\alpha 7$ nAChR has been more favourable: Ivermectin, an antihelminthic agent and a Type I PAM, was identified as the first selective $\alpha 7$ nAChR PAM. Krause *et al* (1998) demonstrated that ivermectin was able to enhance ACh-evoked currents in both human and chick $\alpha 7$ nAChRs (Krause *et al*, 1998). NS-1738 also belongs to the Type 1 PAM class and has been shown to enhance the potency of ACh *in-vitro*. In addition, NS-1738 improves recognition memory and is able to reverse scopolamine-induced impairment in the MWM (Timmermann *et al*, 2007). Compound T is

a novel Type I PAM in development by Janssen Pharmaceutical Companies of Johnson and Johnson that is yet to be examined in a working memory task.

Of the Type II class of $\alpha 7$ nAChR PAMs, the best characterised is PNU-120596 (PNU). Hurst *et al* (2005) demonstrated that this compound was able to increase ACh-induced signals and reduce the level of receptor desensitisation as well as improving cognitive processes. PNU therefore may be useful in assessing the whether the $\alpha 7$ nAChR is involved in mediating improvements in OST performance (Hurst *et al*, 2005).

One of the most frequently used compounds to assess the contribution of the $\alpha 7$ nAChR in a given task is the selective $\alpha 7$ nAChR antagonist methyllycaconitine (MLA). MLA has been shown to impair performance in the radial arm maze (RAM), a test of spatial working memory, where the involvement of the $\alpha 7$ nAChR has been well characterised (Felix and Levin, 1997). However, it is yet to be determined whether MLA causes impairment in the OST or prevents any $\alpha 7$ nAChR PAM-induced enhancements in OST performance.

This study will assess the effect of Type I and Type II PAMs compound T and PNU on improving OST performance in both control animals and those with ketamine-induced deficits, as well as whether MLA is able to block these effects. This should further our knowledge on the contribution of the $\alpha 7$ nAChR to performance in the OST and contribute to the development of novel compounds for the treatment of cognitive deficits associated with schizophrenia (CDS).

6.2 Methods

Male hooded Lister rats (n=24) initially weighing 125-150g (Harlan UK) were trained in the OST until demonstration of asymptotic performance. They were then pseudo-randomly assigned to treatment groups and administered ketamine (10mg/kg i.p.) or vehicle daily for five consecutive days. Following a two day washout, animals were then tested to assess deficits. Once deficits were evident and stable, animals were pseudo-randomly allocated to one of four treatment groups (n=6/group). Animals were then tested in a two day testing, one day washout routine whereby day one measured baseline OST performance and day two measured OST performance following treatment with either $\alpha 7$ nAChR PAM PNU (0.03, 0.1, 0.3, 1 and 3mg/kg), compound T (0.1, 0.3, 1, 3 and 9mg/kg) or vehicle. Day three was a washout day with no testing or training. In this design, each animal received all doses of the respective compounds as well as vehicle and thus served as his own control. The difficulty of getting PNU into solution should be noted: All doses of this compound were sonicated to assist solubility but the 1 and 3mg/kg doses of this compound remained in suspension which was taken into consideration when interpreting results.

Following the conclusion of this experiment, all animals were given maintenance training every other day for a period of one week to ensure a stable baseline. They were then tested on the OST task following administration of the most effective dose of PNU (0.3mg/kg) or compound T (1mg/kg) in conjunction with MLA (2, 6mg/kg). Animals were treated according to procedures outlined in the UK Animals (Scientific Procedures) Act 1986.

6.3 Results

6.3.1 *Acute administration of $\alpha 7$ allosteric modulator PNU improved OST performance*

A two way repeated measures analysis of variance (ANOVA) revealed a significant overall effect of PNU administration ($F(3,29) = 5.99, p < 0.01$). There was also a significant interaction between PNU treatment and ketamine pre-treatment suggesting that PNU had differing effects dependent upon ketamine or vehicle pre-treatment ($F(3,29) = 3.84, p < 0.05$). One-way ANOVA analysis examining vehicle-treated and ketamine-treated animals as separate groups revealed that acute administration of PNU had no significant effect on OST performance of control animals ($F(5,35) = 0.94, p = 0.47$ n.s.). In contrast, ketamine-treated animals displayed a dose-dependent improvement in performance ($F(5,35) = 8.86, p < 0.01$), with Bonferroni post-hoc tests confirming that the 0.1 and 0.3mg/kg PNU were the most effective doses when compared to vehicle treated controls ($p < 0.05, p < 0.01$ respectively). At this dose, pairwise comparisons revealed that the performance of ketamine-treated animals was not significantly different from vehicle-treated controls ($p = 0.33$ n.s.) (figure 6.1).

Figure 6.1: *Acute administration of $\alpha 7$ allosteric modulator PNU improved OST performance*

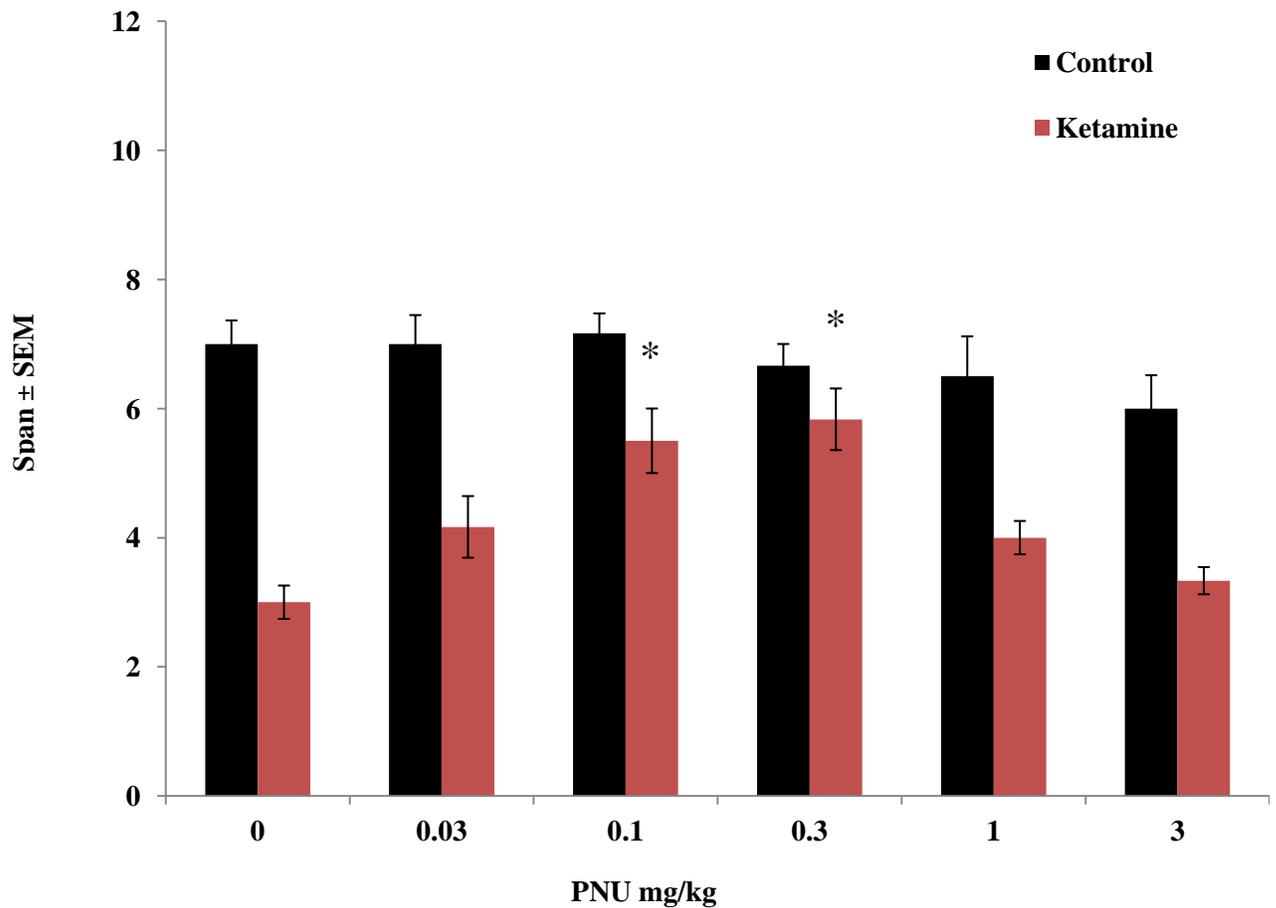


Figure 6.1: PNU administration improves performance in ketamine-treated animals (n=6) with both 0.1 and 0.3mg/kg doses being most effective (p<0.05, p<0.05 respectively). At the 0.3mg/kg dose, the performance of ketamine treated animals and control animals in the OST task was insignificantly different (p=0.33). PNU also had no discernible effect on the OST performance of control animals (p=0.47). * denotes statistical significance from vehicle treatment (p<0.05)

6.3.2: *The $\alpha 7$ allosteric modulator Compound T improved OST performance*

A two way repeated measures ANOVA demonstrated that Compound T had a significant overall effect on OST performance ($F(3,26)=5.85$, $p<0.01$). A significant interaction between Compound T administration and pre-treatment was also observed ($F(3,26)=3.68$, $p<0.05$), suggesting that Compound T had differing effects dependent upon ketamine or vehicle pre-treatment.

Further analysis using a one-way ANOVA that examined vehicle-treated and ketamine-treated groups individually revealed that acute administration of Compound T had no significant effect on OST performance of control animals ($F(5,35) = 0.35$, $p=0.88$ n.s.). However, the performance of ketamine-treated animals was significantly improved following compound T administration ($F(5,35) = 6.13$, $p<0.01$), with Bonferroni post-hoc analysis indicating the 1, 3 and 9mg/kg doses to be most effective ($p<0.05$, $p<0.01$ $p<0.01$ respectively).

Figure 6.2: *The $\alpha 7$ allosteric modulator Compound T improved OST performance*

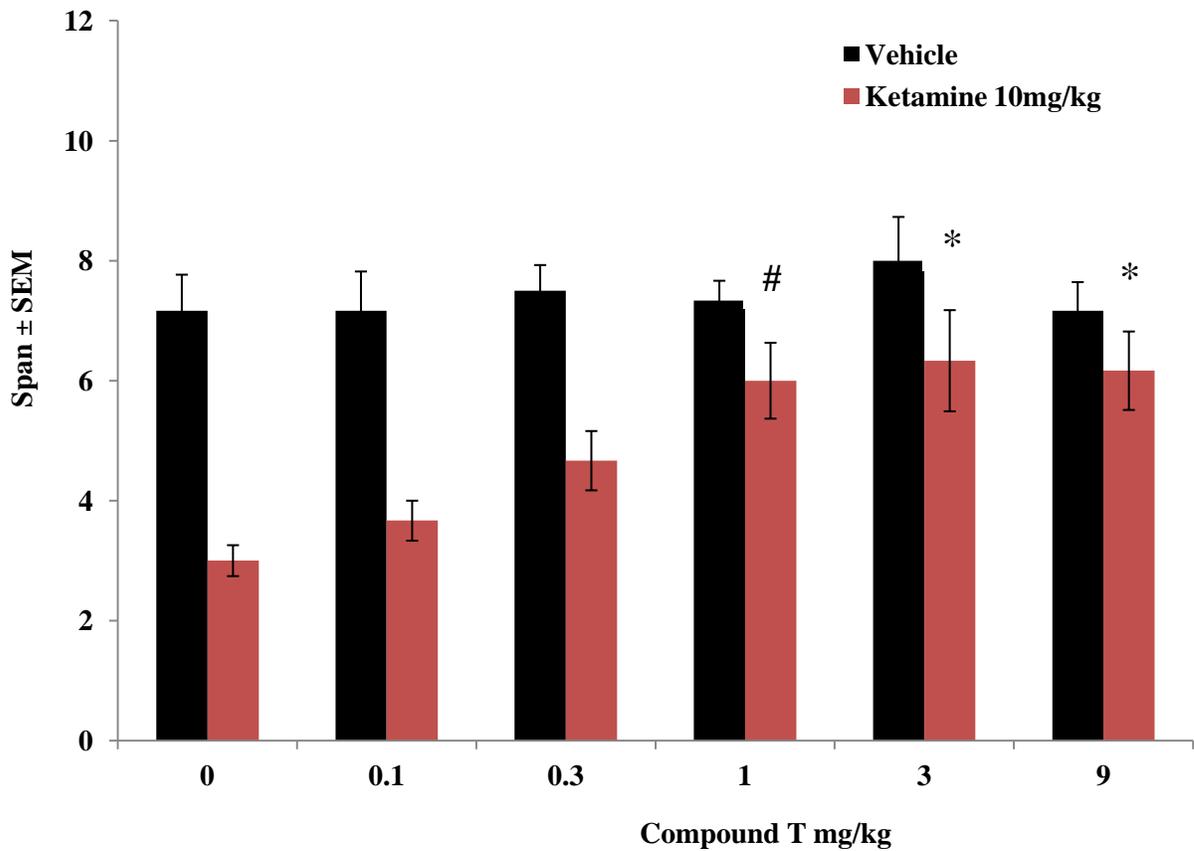


Figure 6.2: Administration of Compound T was able to improve performance in ketamine-treated animals (n=6) with the 1, 3 and 9mg/kg doses being effective ($p < 0.05$, $p < 0.01$ $p < 0.01$ respectively). Compound T had no discernible effect on the OST performance of control animals ($p = 0.88$). # denotes statistical significance from vehicle treatment ($p < 0.05$) and * denotes statistical significance from vehicle treatment ($p < 0.01$)

6.3.3: The $\alpha 7$ antagonist MLA blocked $\alpha 7$ PAM-induced improvements in OST performance and also impaired uncompromised animals

A one way ANOVA demonstrated that, in control subjects, a significant impairment of OST performance was induced by MLA administration ($F(2,17) = 15.14, p < 0.001$). Bonferroni post-hoc tests revealed this impairment in response to both the 2mg/kg and 6mg/kg doses of MLA ($p < 0.01, p < 0.001$) (figure 6.3).

Prior to treatment with MLA, baseline OST performance following the most effective dose of PNU (0.3mg/kg) was re-examined in both control and ketamine treated animals. When the same animals were then given this dose of PNU in conjunction with prior MLA, an overall significant effect of MLA was observed ($F(2,20) = 10.18, p < 0.01$). There were no interaction effects between the two factors ($F(2,20) = 2.54, p = 0.10$ n.s.) suggesting that treatment had the same effect irrespective of ketamine or vehicle pre-treatment. Further analysis using one way ANOVAs in both control and ketamine groups demonstrated that MLA treatment had no significant effect on the performance of control animals ($F(2,17) = 2.72, p = 0.12$ n.s.). However, MLA was able to block the enhancing effects of PNU 0.3mg/kg on ketamine-treated animals: Following administration of MLA at 2 or 6mg/kg, no significant difference seen between when those given vehicle and those given PNU 0.3mg/kg ($F(2,17) = 2.72, p = 0.12$ n.s.) (figure 6.4).

As before, prior to treatment with MLA, the baseline performance and OST performance following the most effective dose of Compound T (1mg/kg) in both control and ketamine-treated animals was re-examined.

When MLA was administered prior to Compound T treatment, no main effect of MLA was noted ($F(2,20) = 2.21, p=0.14$). One way ANOVAs were carried out on both ketamine and control groups. In control subjects, Compound T administration in conjunction with MLA had no significant effect on OST performance ($F(2,17) = 3.24, p=0.07$ n.s.). In ketamine-treated animals, MLA blocked a Compound T-induced improvement in performance so that these subjects were no different from controls ($F(2,17) = 3.61, p=0.05$ n.s.) (figure 6.5).

Figure 6.3: *The $\alpha 7$ antagonist MLA impaired uncompromised animals*

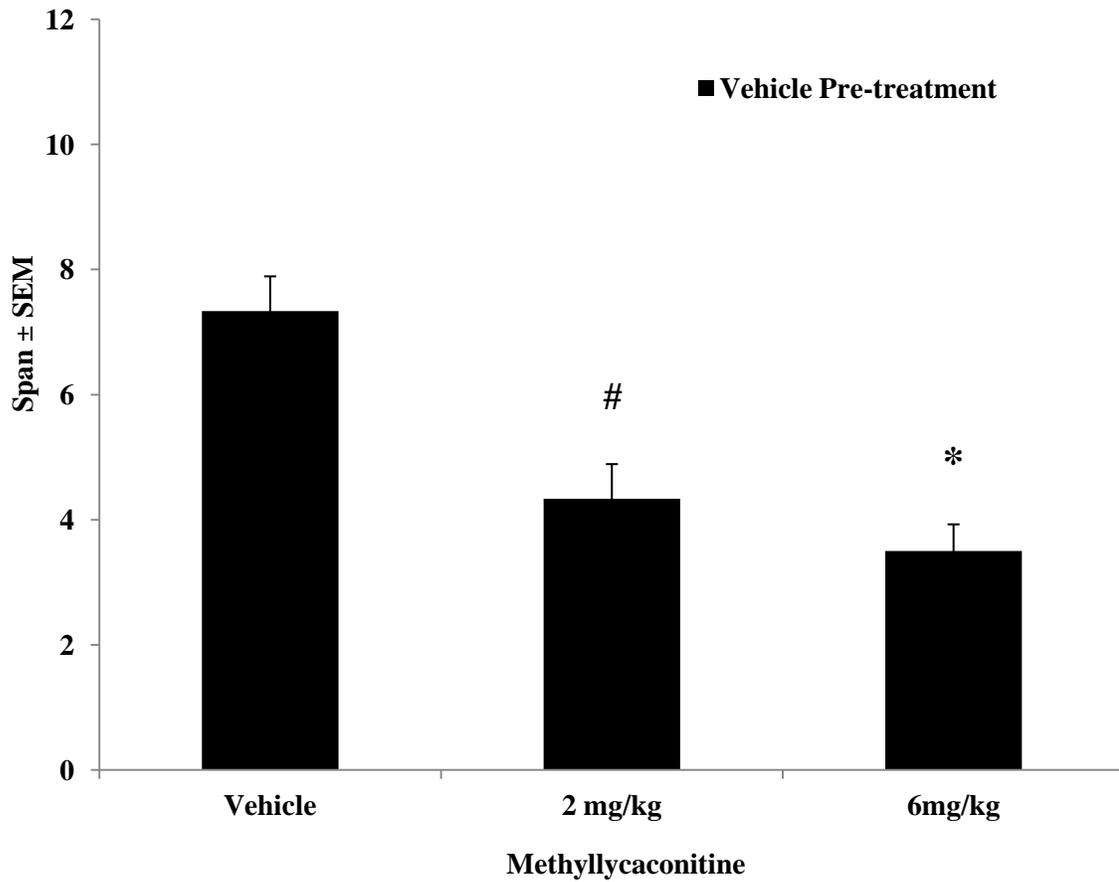


Figure 6.3: Administration of MLA was able to impair the performance of control animals (n=6) at both 2mg/kg and 6mg/kg ($p < 0.01$, $p < 0.001$) # denotes statistical significance from vehicle treatment ($p < 0.01$) and * denotes statistical significance from vehicle treatment ($p < 0.001$)

Figure 6.4: *The $\alpha 7$ antagonist MLA blocked PNU-induced improvements in OST performance*

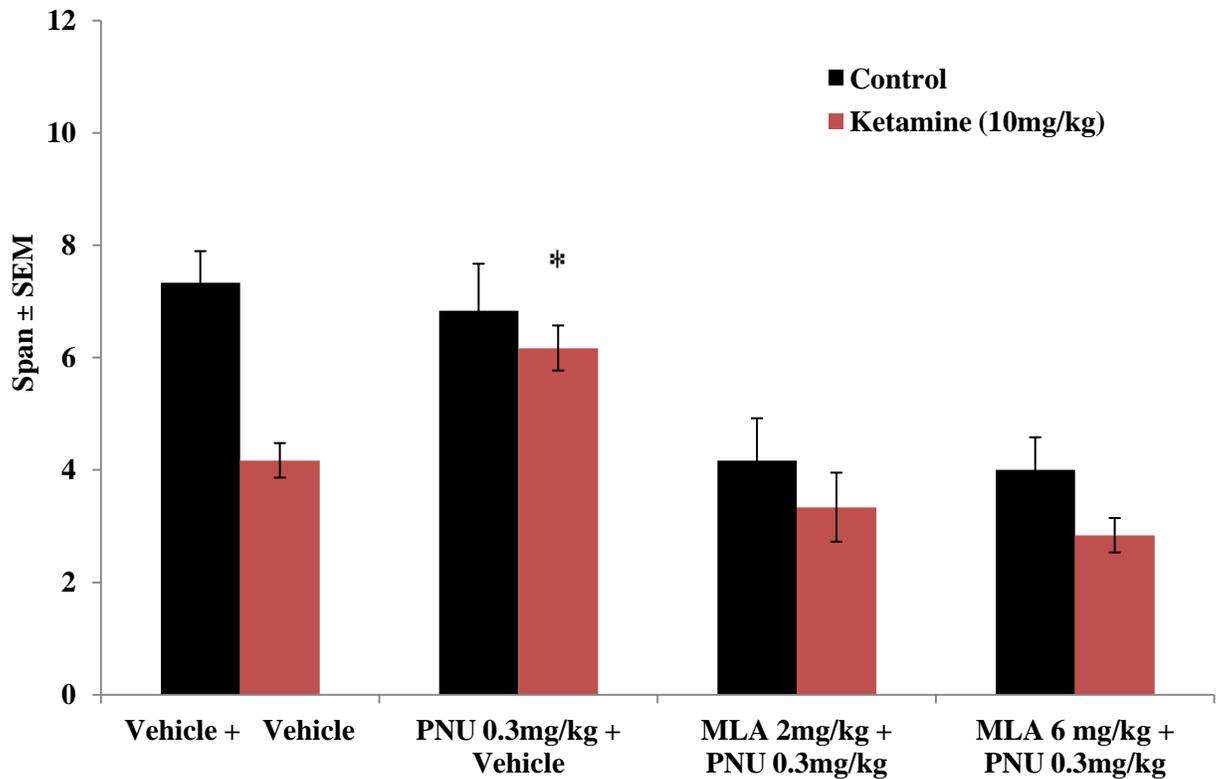


Figure 6.4: Administration of MLA was able to block the PNU-induced improvement in the OST performance of ketamine treated animals (n=6), so that performance was no different to controls (p<0.01, p=0.12, respectively). PNU administration also failed to prevent an MLA induced impairment in OST performance. * denotes statistical significance from vehicle treatment (p<0.01)

Figure 6.5: *The $\alpha 7$ antagonist MLA blocked Compound T-induced improvements in OST performance*

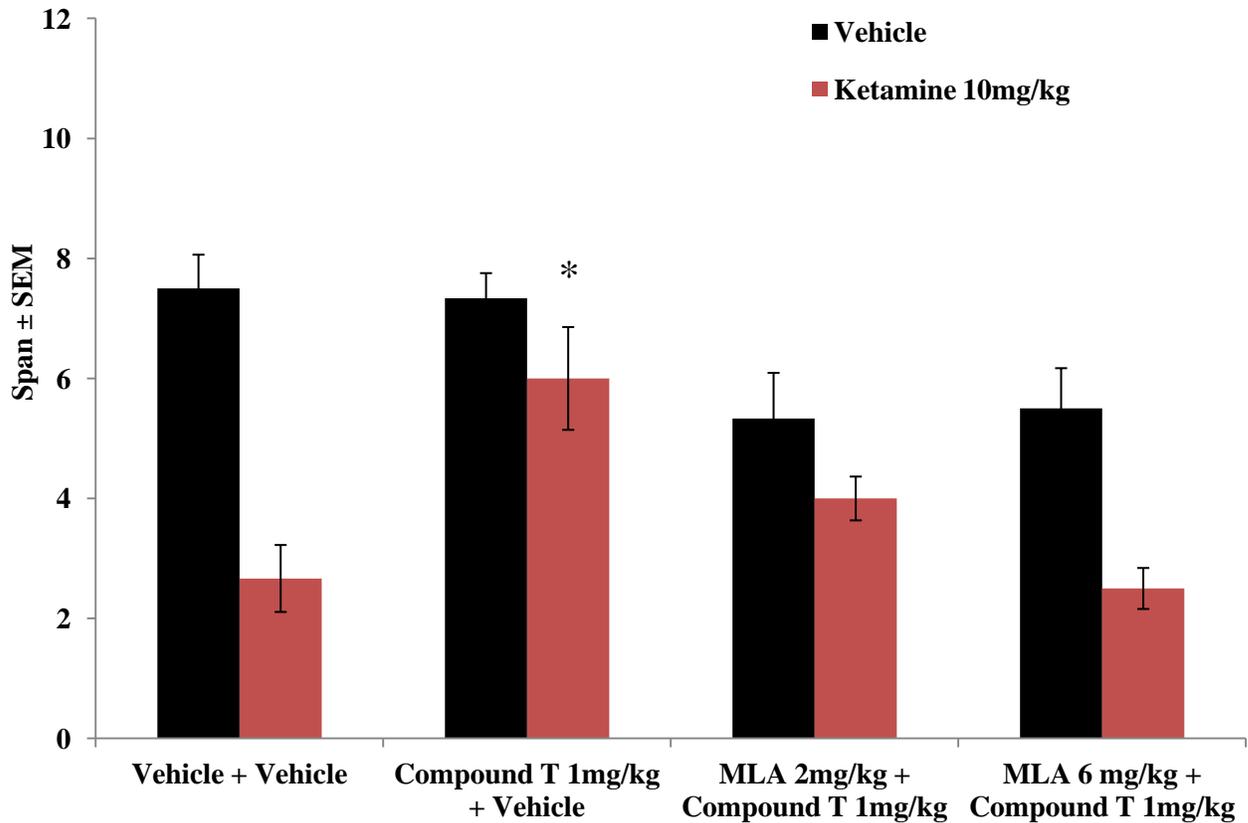


Figure 6.5: Administration of MLA was unable to block the Compound T-induced improvement in the OST performance of control animals(n=6) ($p=0.14$). Compound T administration also failed, in control animals, to prevent an MLA induced impairment in OST performance ($p=0.07$). MLA blocked the enhancing effect of Compound T on OST performance in ketamine-treated animals ($p<0.05$) * denotes statistical significance from vehicle treatment ($p<0.01$)

6.4 Discussion

Both allosteric modulators were able to improve the performance of ketamine-treated animals in a dose-dependent manner. However, in contrast to results seen with nicotine treatment, neither PNU nor Compound T was able to enhance the performance of control animals in the OST task.

Allosteric modulation of the $\alpha 7$ nAChR using PNU has been shown to enhance transmission at the $\alpha 7$ nAChR without causing desensitisation (Hajos *et al*, 2005). Sitzia *et al* (2011) have also shown that PNU is able to reduce the likelihood of nAChR desensitisation as well as being able to recover nAChR from a desensitised to active state even in the continued presence of an agonist (Sitzia *et al*, 2011). Despite this enhanced transmission and lack of desensitisation, acute PNU treatment did not enhance performance in control animals. This is in contrast to the effect of acute nicotine administration which did improve the performance of control subjects in the OST task (Chapter 4). This implies a lesser role for the $\alpha 7$ nAChR in mediating the enhancing effects of nicotine in control animals tested with the OST. Similar effects of PNU treatment have been seen before, as shown by Thomsen *et al* (2011) using the social discrimination test as a measure of working memory. This study demonstrated that uncompromised rats did not show any improvement in performance following acute or chronic PNU treatment (Thomsen *et al*, 2011). An alternative explanation to a lack of $\alpha 7$ nAChR involvement is that there is a strong correlation between temperature and the ability of PNU to enhance ACh-induced transmission: Using a patch-clamp measure of ACh current, Sitzia *et al* (2011) have been able to demonstrate that PNU is most effective at room temperature and that this effect is significantly reduced as the temperature is increased to near-physiological levels (Sitzia *et*

al, 2011). This means the effect of PNU *in-vivo* may be much less than previously indicated by *in-vitro* studies, although it is unlikely that physiological temperature completely attenuated the effects of PNU as there was significant recovery observed in ketamine-treated animals in the OST task. It may instead be that modest enhancements in ACh transmission at the $\alpha 7$ nAChR did not confer any phenotypic advantage in control animals or perhaps that this improvement was beyond the sensitivity of the OST task.

However, this study also provides evidence *for* the involvement of the $\alpha 7$ nAChR, as PNU was able to restore OST performance in animals with ketamine-induced deficits. PNU increases the probability of the $\alpha 7$ nAChRs being in an active state whilst also preventing desensitisation of these nAChRs in addition to de-desensitising any $\alpha 7$ nAChRs that are currently in the desensitised state. This results in increased ACh transmission both pre and post-synaptically. Post-synaptic $\alpha 7$ nAChRs are present on interneurons and their activation mediates fast cholinergic excitatory synaptic transmission which in turn facilitates cortical strengthening and long term potentiation (LTP) (Alkondon *et al*, 1998; Frazier *et al*, 1998). Presynaptic activation of $\alpha 7$ nAChRs on glutamatergic neurons increases intraterminal Ca^{2+} levels, facilitating glutamate release (Gray *et al*, 1996). Becker *et al* (2003) have shown that rats exposed to a sub-chronic ketamine regime have been shown to have 25% less glutamate binding in the prefrontal cortex (PFC) when compared to vehicle treated controls (Becker *et al*, 2003b). Presynaptic activation of $\alpha 7$ nAChRs on glutamatergic neurons may therefore serve to compensate for the reduced level of glutamate found following exposure to sub-chronic ketamine treatment and restore normal functioning. In addition, this release of glutamate has follow-on effects: Livingstone *et al* (2010) have shown that glutamate acts at metabotropic glutamate receptors (mGluRs) on dopaminergic terminals, facilitating

dopamine (DA) release. Increased DA has been shown to increase the efficiency of processing within cortical networks, ultimately resulting in a short term enhancement of synaptic plasticity (Gonzalez-Burgos *et al*, 2005; Kroener *et al*, 2009). DA is also able to modulate GABAergic input which is involved in the regulation, synchrony and amplitude of gamma frequency oscillations that are known to be essential to the normal functioning of working memory (Seamans *et al*, 2001). Further evidence for this mechanism as an explanation for the effects of PNU in this study comes from an *in-vivo* microdialysis study by Livingstone *et al* (2010). They demonstrated that reverse dialysis of PNU was able to augment DA release in the medial PFC (mPFC) *in-vivo* in the presence of $\alpha 7$ agonist (R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide (Compound A) (Livingstone *et al*, 2010).

Relatively little is known about Compound T, as it is a novel compound in development by Janssen Pharmaceutical Companies of Johnson and Johnson. It is classed as a Type I PAM which means that Compound T is able to increase peak amplitude at the $\alpha 7$ nAChR but has little or no effect on desensitisation. Both the Type I and Type II PAMs used in this study demonstrate similar effects on OST performance. As PNU is a Type II PAM which increases peak amplitude but also reduces desensitisation, it may be expected to improve OST performance to a greater degree than a Type I PAM which improves peak amplitude alone. This is because $\alpha 7$ nAChRs rapidly desensitise and so Type II allosteric modulation would be expected to confer greater transmission in comparison to Type I allosteric modulation. It may be that the full effect of PNU was somewhat attenuated as a result of *in-vivo* physiological temperature which Sitzia *et al* (2011) demonstrate reduced the effect of PNU (Sitzia *et al*, 2011). Alternatively, it may be that the improvement in performance

seen in ketamine-treated animals is as a result of an increase in peak amplitude and not as a result of any reduction in desensitisation.

These data serve to demonstrate an involvement of the $\alpha 7$ nAChR in enhancing the performance of ketamine-treated animals but does not necessarily implicate these receptors as being involved in mediating the performance of control animals in the OST task. Neither PNU nor Compound T were able to confer any protection against an MLA-induced impairment in OST performance when administered prior to treatment with selective $\alpha 7$ nAChR antagonist MLA in control animals, although a trend towards significance was observed in animals pre-treated with Compound T prior to MLA administration. In ketamine-treated animals, MLA prevented both Compound T and PNU-induced improvements in performance. This is similar to results seen with $\alpha 7$ nAChR selective PAM JNJ-1930942 in a study by Dinklo *et al* (2011), who demonstrated that this compound was able to reverse a deficit in sensory gating and that this effect was blocked by MLA treatment (Dinklo *et al*, 2011). MLA has been long since been shown to antagonise the $\alpha 7$ nAChR (Macallan *et al*, 1988; Ward *et al*, 1990). Mechanistically, MLA competitively binds to $\alpha 7$ nAChRs and preferentially stabilises the receptor in a closed state. This prevents activation by endogenous ligands such as ACh, reducing neurotransmission and thus no enhancing effect is seen. A study by Livingstone *et al* (2010) provides further evidence for this mechanism as MLA was able to prevent $\alpha 7$ nAChR agonist-induced DA overflow in the PFC (Livingstone *et al*, 2010).

In control animals, MLA treatment was also able to impair performance on the OST task. This is supported by Levin *et al* (2002) who demonstrated that MLA was able to impair working memory in both the 8 and 16-arm RAM (Levin *et al*, 2002).

6.5 Conclusions

These data suggest a role for the $\alpha 7$ nAChR in mediating the restoration of baseline performance in ketamine-compromised subjects. It is also evident that normal functioning of the $\alpha 7$ nAChR is required for uncompromised subjects to complete the task but that increasing transmission at this receptor when no deficit is present confers no advantage in the task. As nicotine is able to elicit improvements in OST performance of uncompromised animals, it is likely that the $\alpha 7$ nAChR does not mediate the full effect of nicotine on this task. This suggests involvement of other nAChRs such as the $\alpha 4\beta 2$ nAChR in mediating the effects of nicotine on OST performance of control animals. To further investigate potential mechanisms by which these receptors may mediate the effects of nicotine, the next chapter examines the effect to nicotine and nicotinic agonists on gamma frequency network oscillations in the PFC.

Chapter 7

Nicotine enhances gamma frequency oscillations in the PrL region of the PFC: An effect blocked by mecamylamine

7.1 Introduction

Fast network oscillations such as beta (15-30 Hz) and gamma (30-80 Hz) are essential for higher cognitive processes such as attention and working memory, where synchronous oscillations correlate with improvement in cognitive tasks (Jensen *et al*, 2007). Roux *et al* (2012) have demonstrated that gamma frequency oscillations in the prefrontal cortex (PFC) contribute significantly to the maintenance of behaviourally relevant information during working memory (Roux *et al*, 2012). Mainy *et al* (2007) further support this, demonstrating that human subjects tasked with remembering a series of letters, exhibited higher gamma frequency activity in several brain regions including the PFC and hippocampus (Mainy *et al*, 2007).

Gamma oscillations are initiated by excitation and firing of pyramidal neurons and controlled by parvalbumin-(PV)-positive interneurons through gamma-aminobutyric acid-(GABA)-induced inhibition (Mann *et al*, 2005; Whittington *et al*, 2001; Whittington *et al*, 2000). Following decay of this inhibition, pyramidal cells have a window within which they can fire, initiating the next oscillation. Pyramidal cells involved in the same set of neural codes become synchronised and the stronger the input, the more cells are entrained into the same oscillation (Bartos *et al*, 2007). Therefore, when working memory load is increased, gamma frequency oscillations increase in power and synchrony in order to maintain information (Howard *et al*, 2003; Tallon-Baudry *et al*, 1998). Cho *et al* (2006) have shown that this ability to increase induced gamma band activity is aberrant in patients with schizophrenia and may therefore explain the presence working memory deficits in this disorder (Cho *et al*, 2006). This abnormality in gamma frequency oscillations is also evidenced by Lee *et al* (2003) who replicated the finding that gamma frequency oscillations

are reduced in schizophrenia and furthermore, demonstrated that the degree to which this reduction occurs correlates to negative symptoms of the disease (Lee *et al*, 2003).

Mechanistically, several studies have confirmed a reduction in PV-expression and glutamic acid decarboxylase 67 (GAD67), the enzyme which synthesises GABA, in patients with schizophrenia (Volman *et al*, 2011). GABA is inextricably linked to gamma frequency oscillations as the timescale of inhibition generated by GABA_A receptor activation matches the 40Hz gamma frequency oscillations (Tiesinga *et al*, 2004). Thus, a potential mechanism for disrupted cognition in schizophrenia is a malfunction of PV-positive interneurons, which results in impaired function of these interneurons and therefore impaired generation of gamma frequency activity (For review see Lewis and Moghaddam, 2006; Lisman *et al*, 2008).

Nicotine improves performance in cognitive tasks in control subjects and subjects with schizophrenia and has also been shown to enhance gamma frequency oscillations in the hippocampus (Featherstone *et al*, 2012b; Song *et al*, 2005). In the hippocampus, application of nicotine depolarises interneurons as well as inhibiting the inhibitory synapses onto interneurons (Wanaverbecq *et al*, 2007). This leads to excitation of interneurons and enhanced GABA release thus increasing inhibitory output (Hulo and Muller, 2001; Wanaverbecq *et al*, 2007). The $\alpha 7$ nicotinic acetylcholine receptor (nAChR) subtype has been implicated in this process in the hippocampus as $\alpha 7$ nAChRs have been shown to be expressed in interneurons in this region (Hulo *et al*, 2001; Wanaverbecq *et al*, 2007). However, the contribution of the $\alpha 4\beta 2$ nAChR to inhibitory function and gamma frequency oscillations in the hippocampus is yet to be elucidated.

Few studies have examined the effect of nicotine in the PFC, and it is possible that different nAChR subtypes mediate the actions of nicotine to those in the hippocampus. This is

pertinent with regard to the PFC as there are significantly more $\alpha 4\beta 2$ nAChRs in the PFC than in the hippocampus and also significantly more $\alpha 4\beta 2$ nAChRs than $\alpha 7$ nAChRs in the PFC (Poorthuis *et al*, 2013). Despite this, very little work exists in the literature examining the effect of nicotine on gamma frequency oscillations in the PFC and the different nAChRs which may mediate nicotine's effect. This means that the overall notion of whether nicotine can enhance gamma frequency network oscillations in the PFC is still unknown.

This investigation therefore aims to address whether nicotine is able to increase gamma frequency oscillations in the prelimbic regions of the PFC *in-vitro* and whether there is any involvement of the $\alpha 7$ or $\alpha 4\beta 2$ nAChR in mediating the effect of nicotine.

7.2 Methods

Slices were prepared as described in Chapter 2. Persistent gamma oscillations were generated by co-application of carbachol (10 μ M) and kainate (200nM). Following application of carbachol and kainate, gamma frequency oscillations increase in size; stabilising after 2 to 3 hours. Upon establishing a stable baseline, defined as three consecutive area readings with no more than 10% change in area power, test compounds were delivered via the circulating bath artificial cerebrospinal fluid (ACSF). The peak effect of agonist was measured at 15 minutes post- application or 15 minutes post-washout. Nicotine (1 and 10 μ M) were examined in individual slices (n=6, n=8 respectively) from different rats. The effect of the nicotinic antagonist mecamylamine was also investigated alone and in combination with nicotine (n=7). Following these results, α 7 and α 4 β 2 nAChR selective agonists PHA-543613 (PHA) and 5-iodo-A-85380 (5IA), respectively, were examined individually, (n=7, n=2) and in combination (n=4).

7.3 Results

7.3.1. *Nicotine increases gamma frequency oscillations*

Extracellular field recordings from the PrL region of the mPFC demonstrate that bath application of a 1 μ M concentration of nicotine did not increase the power of gamma frequency oscillations in this region (figure 7.1, A (trace) and B (power spectra)). However, when the concentration of nicotine was increased to 10 μ M, the power of gamma frequency oscillations in the PrL region was enhanced (figure 7.1, A (trace) and B (power spectra)), increasing in size by approximately 70% (figure 7.2).

One way analysis of variance (ANOVA) on group data revealed a significant overall effect of nicotine on the percentage increase in the size of gamma network oscillations ($p < 0.001$). Holm-Sidak post-hoc analysis further demonstrated that nicotine at a dose of 10 μ M but not 1 μ M was able to significantly enhance the area power of gamma network oscillations ($p < 0.05$) (figure 7.2).

Figure 7.1: Nicotine increased percentage change in size of gamma frequency oscillations

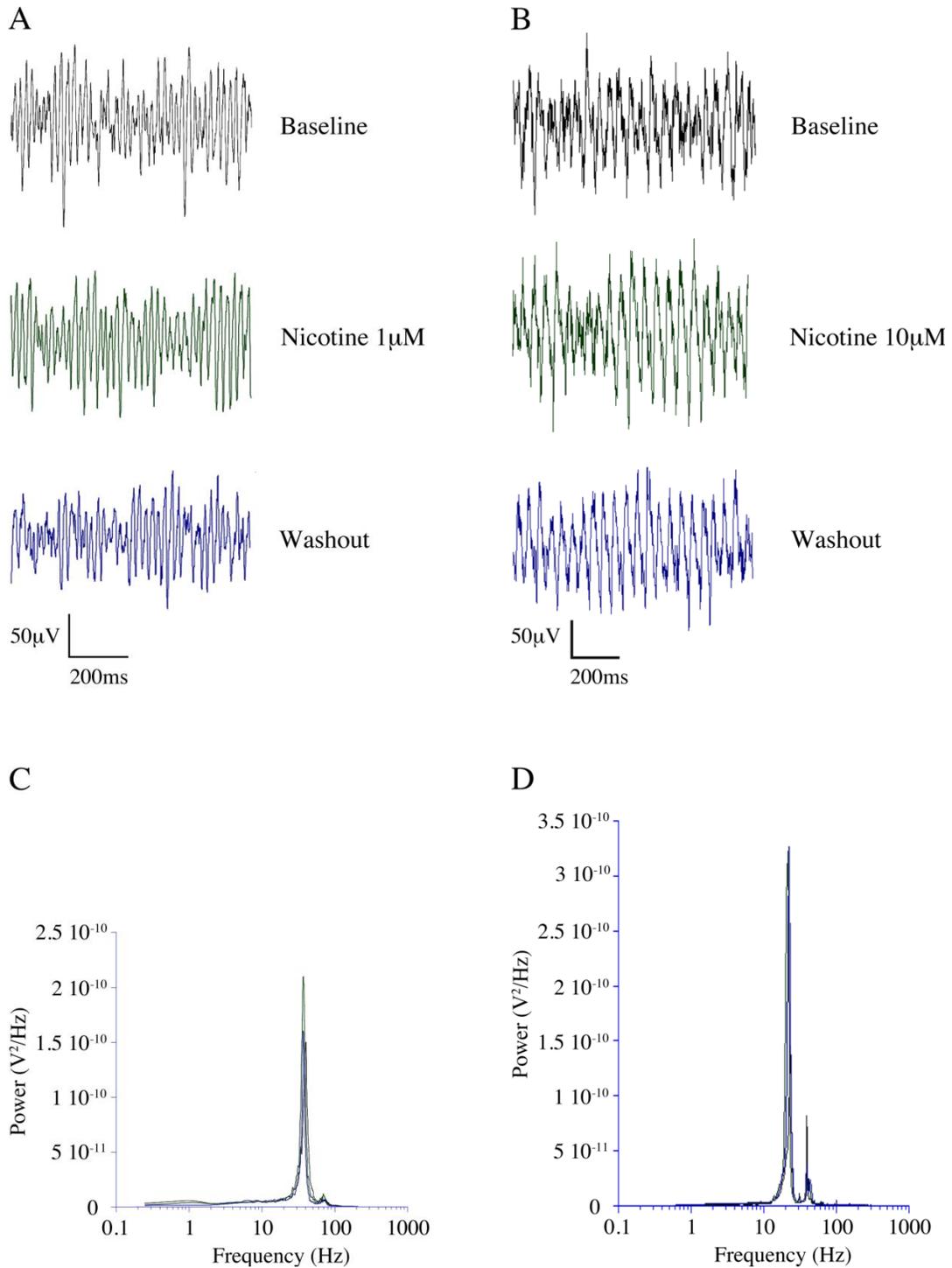


Figure 7.1: This figure shows an example of extracellular field recordings of PrL region gamma frequency oscillations at baseline (black), following bath application of nicotine 1 μM (n=6) (A) and 10 μM (n=8) (B) (green) and following washout (blue): With increasing time into bath application of nicotine, the power of the oscillation is significantly increased following application of the 10 μM nicotine concentration (D) but not the 1 μM nicotine concentration (C). These readings are taken 15 minutes after drug application and represent the peak response.

Figure 7.2: Nicotine increased percentage change in power of gamma frequency oscillations.

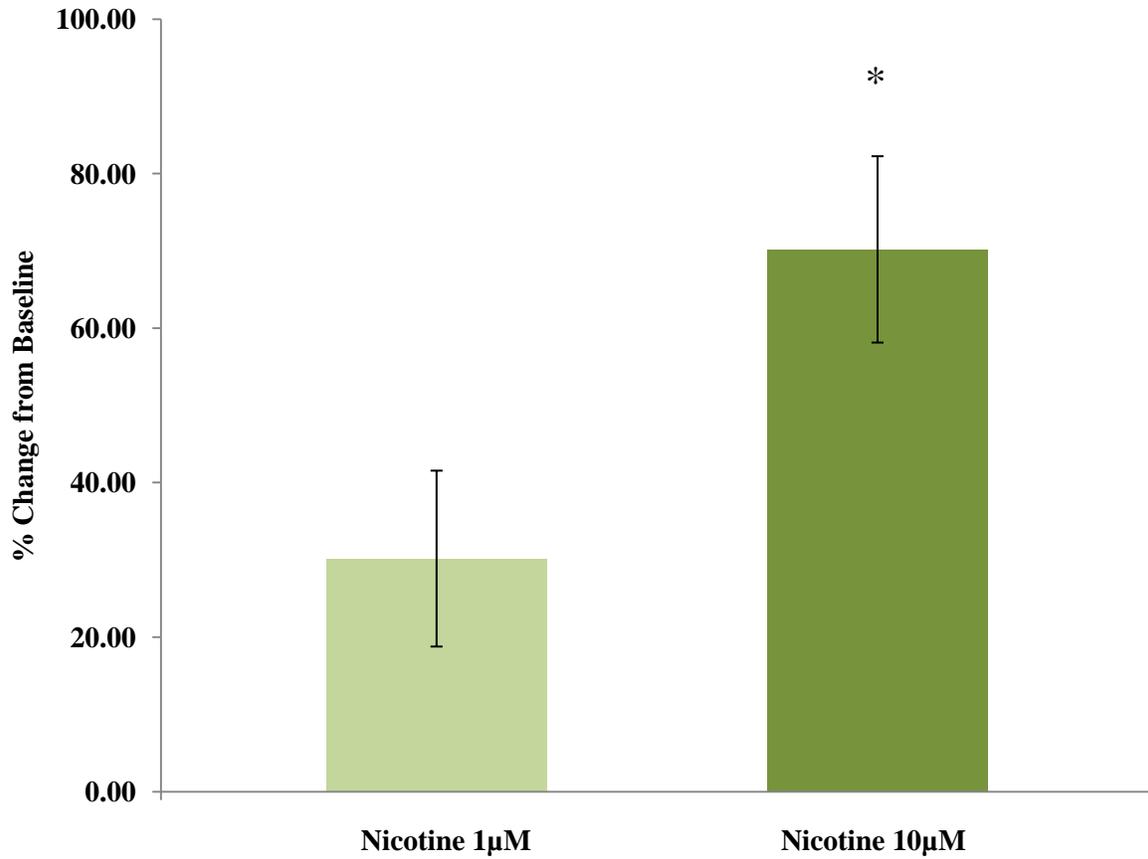


Figure 7.2: Group data (n=6) show that the 1µM nicotine did not significantly enhance the power of gamma frequency oscillations. However, the 10µM (n=8) concentration of nicotine produced a significant increased in power (p<0.05). These readings are taken 15 minutes after drug application and represent the peak response. * denotes significance where p<0.05.

7.3.2. The enhancing effect of nicotine was blocked by mecamylamine

Extracellular field recordings from the PrL region of the mPFC demonstrate that bath application of a 10 μ M concentration of mecamylamine did not increase the power of gamma frequency oscillations in this region. Bath application of nicotine at a concentration of nicotine 10 μ M following one hour exposure to mecamylamine caused a significantly attenuated response of gamma frequency oscillations in comparison to bath application of nicotine 10 μ M alone (figure 7.1, A (trace) and B (power spectra)).

One way ANOVA on group data revealed a significant overall effect of drug treatment on gamma frequency oscillations ($p < 0.05$). Paired T-tests examining the effect of mecamylamine alone and mecamylamine plus nicotine in comparison to their respective baselines however revealed no significant difference at either point ($p = 0.16$, $p = 0.71$, respectively) (figure 7.3, C).

Figure 7.3: The enhancing effect of nicotine was blocked by broad spectrum nicotinic antagonist mecamlamine

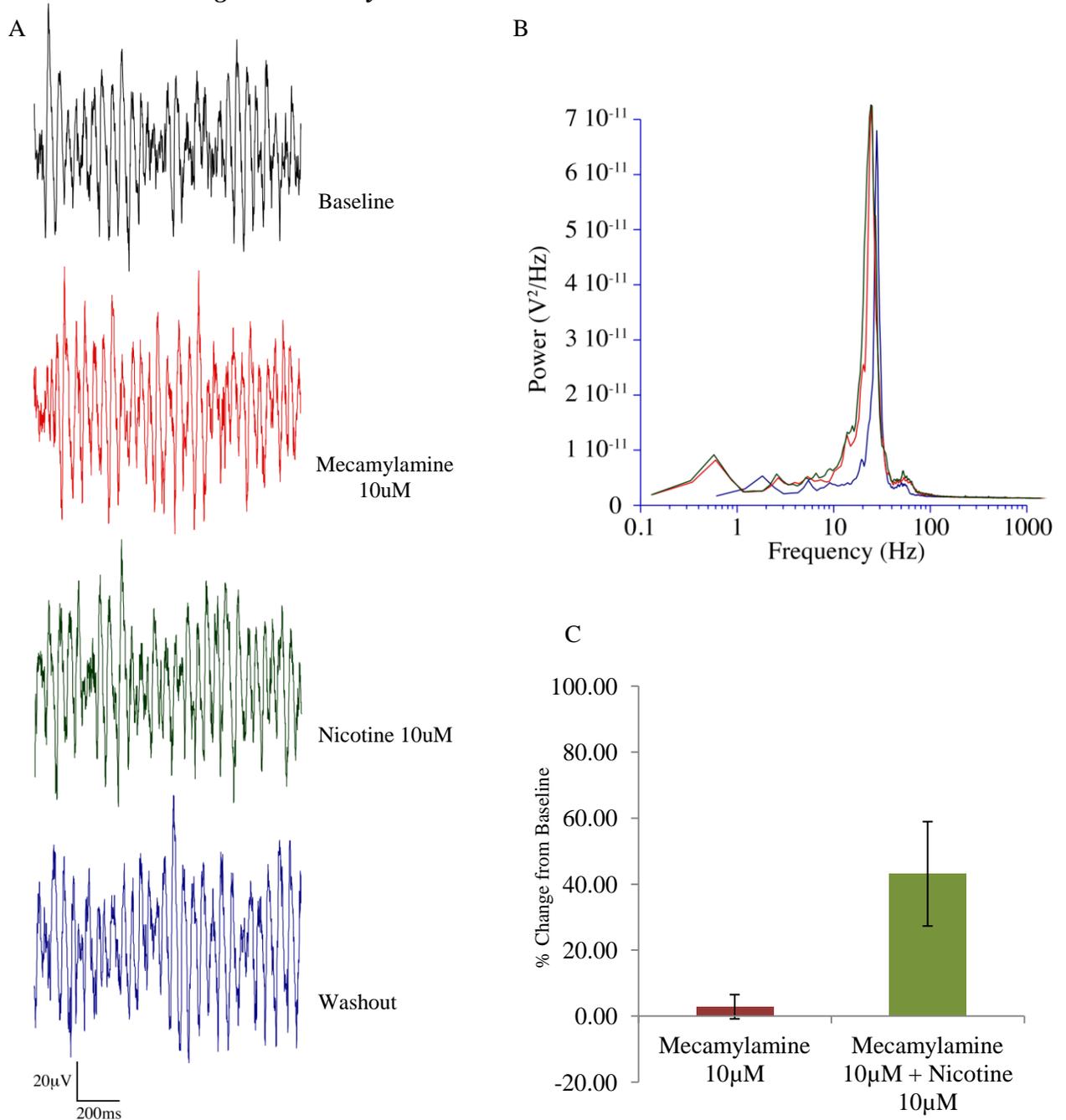


Figure 7.3: (A) This figure shows an example of extracellular field recordings of PrL region gamma frequency oscillations at baseline (black), following bath application of mecamlamine (red), following bath application of mecamlamine plus nicotine (10 µM) (green) and following washout (blue). (B) Power spectra demonstrating mecamlamine (10µM) application for one hour had no effect alone and was also able to prevent any effect from the subsequent application of nicotine (10µM) on gamma frequency oscillations. (C) Group data (n=7) show that the 10µM mecamlamine has no significant effect on the power of gamma frequency oscillations ($p=0.16$). Application of 10µM mecamlamine also prevented the 10µM concentration of nicotine from producing a significant increased in gamma oscillation power ($p=0.71$). These readings are taken 15 minutes after drug application and represent the peak response.

7.3.3. Neither $\alpha 7$ or $\alpha 4\beta 2$ nAChR agonists significantly increased gamma frequency oscillations

Extracellular field recordings from the PrL region of the mPFC demonstrate that bath application of neither 5IA at a concentration of $3\mu\text{M}$ (figure 7.1, A (trace) and C (power spectra)) or PHA at a concentration of $10\mu\text{M}$ (figure 7.1, B (trace) and D (power spectra)) were able to increase the power of gamma frequency oscillations in this region.

Group data was not normally distributed so a Wilcoxon Signed Rank Test was carried out to examine the effect of PHA on baseline gamma frequency oscillations, revealing no significant effect of dose at $10\mu\text{M}$ ($p=0.94$). Similarly, 5IA demonstrated no significant effect on gamma frequency oscillations ($p=0.33$). As nicotine acts at many nAChRs, co-application of both PHA and 5IA was also examined for any synergistic effects on gamma frequency oscillations: This also had no effect on gamma frequency oscillations ($p<0.85$).

Figure 7.4: *Neither $\alpha 7$ or $\alpha 4\beta 2$ nAChR agonists significantly increased gamma frequency oscillations*

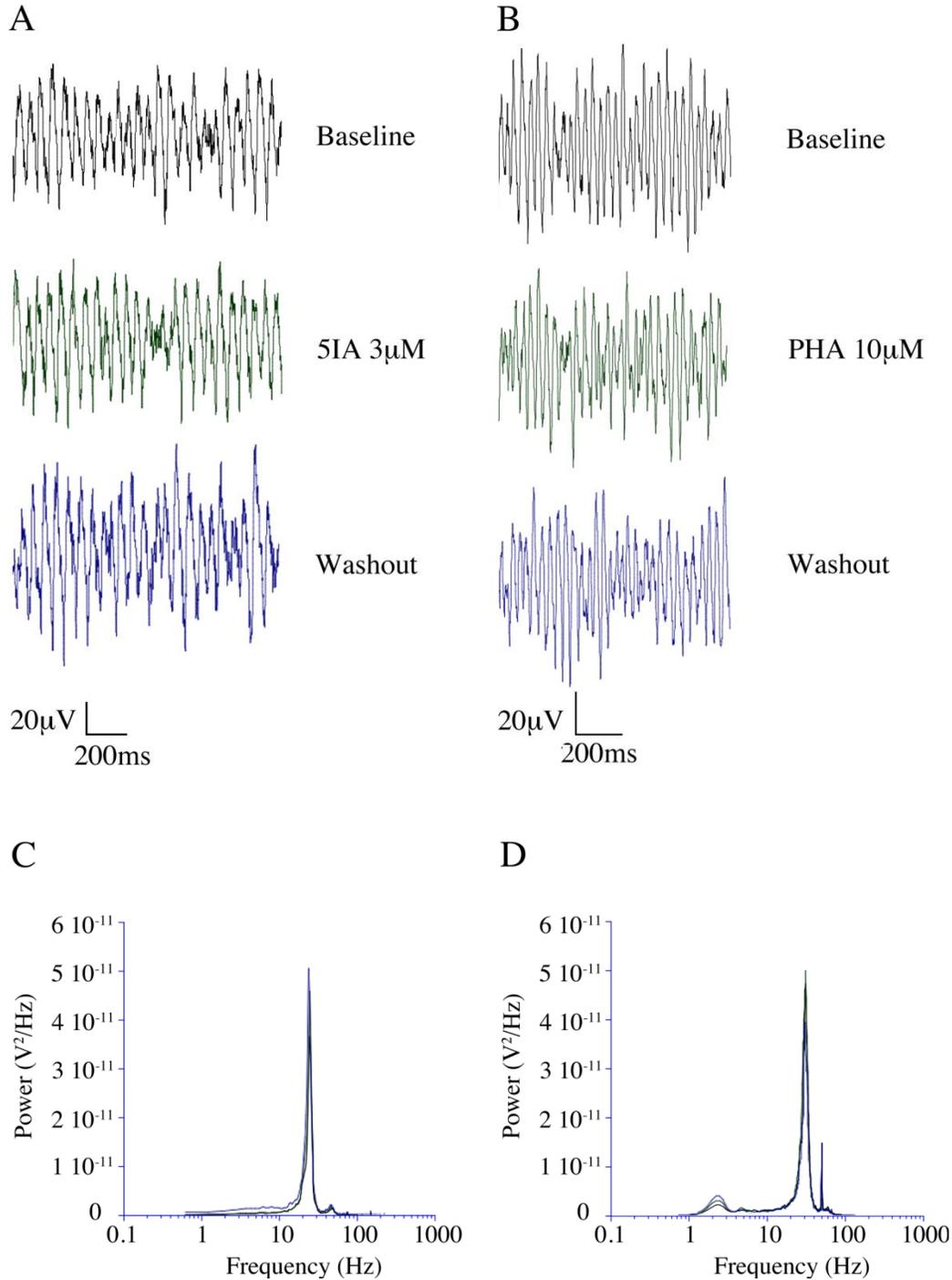


Figure 7.4: This figure shows an example of extracellular field recordings of PrL region gamma frequency oscillations at baseline (black), following bath application of $\alpha 4\beta 2$ nAChR agonist 5IA 3 μ M (n=2) (A) and $\alpha 7$ nAChR agonist PHA 10 μ M (n=7) (B) (green) and following washout (blue). Neither 5IA 3 μ M or PHA 10 μ M significantly increased the power of gamma frequency oscillations. These readings are taken 15 minutes after drug application and represent the peak response.

Figure 7.5: *Neither $\alpha 7$ or $\alpha 4\beta 2$ nAChR agonists were able to significantly increase gamma frequency oscillations*

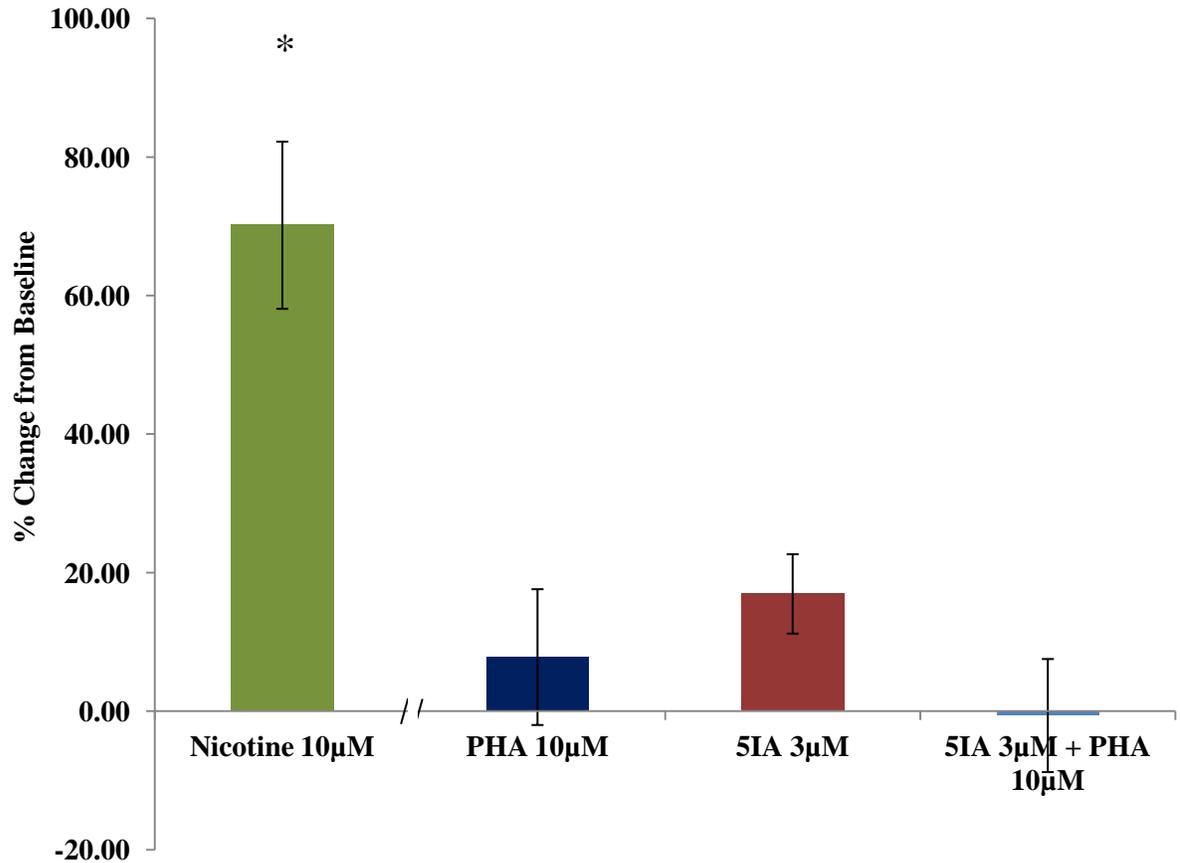


Figure 7.5: Group data show that the 10µM PHA (n=7) had no significant effect on gamma frequency oscillations (p=0.938). No significant increased in gamma frequency oscillation power was also found with 3µM 5IA (n=2) (p=0.33). Combination of 5IA and PHA (n=4) also failed to have any significant effect on the power of gamma frequency oscillations (p<0.85). Nicotine alone at 10µM, from the previous experiment is provided for reference. * denotes significance at the level of p<0.05. These readings are taken 15 minutes after drug application and represent the peak response.

7.4 Discussion

Acute bath application of nicotine enhanced gamma frequency oscillations in the prelimbic (PrL) region of the PFC when examined in an *in-vitro* brain slice preparation. This enhancing effect of nicotine was blocked by pre-application of broad spectrum nAChR antagonist mecamylamine. This indicates that the enhancing effect of nicotine is likely to be as a result of action at the nAChRs as opposed to non-specific effects.

These results correlate with findings from behavioural studies where nicotine was able to enhance performance in the Odour Span Task (OST) (Chapter 4) and may therefore provide a mechanism behind the nicotine-induced enhanced OST performance observed in control subjects. However, the enhancing effect of nicotine on gamma frequency oscillations is in contrast to the findings of Mansvelder *et al* (2005) who showed that application of nicotine in the PrL region of the PFC in an *in-vitro* brain slice preparation completely attenuated the carbachol-induced oscillatory state (Mansvelder *et al*, 2006). Two main explanations exist for this dichotomy: The rats used in Mansvelder's study may not have had fully developed inhibitory interneuron networks as the rats were only three weeks old (Mansvelder *et al*, 2006). Significant development of inhibitory networks occurs during adolescence in rats between 28 and 55 days of age (Spear, 2000). As the mechanism by which nicotine increases gamma frequency oscillations is proposed to be via modulation of inhibitory interneurons, these effects may not be seen in immature brain network. Aracri *et al* (2010) examined the effect of nicotine on oscillatory activity and demonstrated that the level of glutamatergic tone determines whether nicotine will inhibit GABAergic neurons or excite them (Aracri *et al*, 2010). Application of nicotine following blockade of ionotropic glutamate receptors in the PFC inhibited inhibitory postsynaptic potentials (IPSPs). This is in contrast to nicotine alone which increased IPSP output (Aracri *et al*, 2010). This means that the lack of effect seen in the study by Mansvelder and colleagues could be as a result of reduced glutamatergic tone, which would cause nicotine to inhibit inhibitory neurons and

thus reduce IPSPs; resulting in a state whereby oscillatory drive is impaired. In the current study, gamma frequency oscillations are induced through co-application of carbachol and kainate, as opposed to carbachol alone as used by Mansvelder *et al* (2005). Thus, in our experimental conditions, with kainate receptor activation, we may have a greater glutamatergic tone that could enable nicotine to elicit increased GABA release.

Aracri *et al* (2010) further demonstrated that the $\alpha 7$ nAChR specific antagonist methyllycaconitine (MLA) and $\alpha 4\beta 2$ nAChR specific antagonist dihydro-beta-erythroidine (DH β E) could reverse the effect of ionotropic glutamate receptor blockade on nicotine induced IPSP reduction (Aracri *et al*, 2010). This indicates that both the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs are involved in mediating both excitation and inhibition of interneurons depending on the overall glutamatergic drive although the involvement of these receptors was not confirmed by in the current study: Application of either $\alpha 7$ nAChR agonist PHA, or $\alpha 4\beta 2$ nAChR agonist 5IA did not enhance gamma frequency network oscillations either alone or in combination. Co-application of both PHA and 5IA was not examined in the OST, but the application of these agonists alone also replicates what is seen behaviourally whereby 5IA and PHA were only able to enhance performance in ketamine-treated animals (Chapter 5). These findings indicate that another receptor subtype may be responsible for nicotine's enhancing effect on the OST in normal animals or that synergistic activation of an alternative combination of nAChR subtypes mediates the effect of nicotine in normal subjects.

However, it must also be noted than $\alpha 4\beta 2$ nAChR selective agonist metanicotine did improve performance in control subjects behaviourally and that studies such as that by Featherstone *et al* (2012) have shown that $\alpha 4\beta 2$ nAChR subtype-selective agonist AZD3480 can enhance gamma frequency oscillatory activity in the hippocampus (Featherstone *et al*, 2012b). The $\alpha 7$ nAChR subtype has historically been implicated in this process in the hippocampus as $\alpha 7$ nAChRs are expressed in interneurons in this region

(Hulo *et al*, 2001; Wanaverbecq *et al*, 2007). However, Featherstone *et al* (2012) demonstrate that the enhancing effect of nicotine on hippocampal oscillations is blocked by $\alpha 4\beta 2$ antagonist DH β E but not $\alpha 7$ nAChR antagonist MLA, indicating a selective effect of nicotine at $\alpha 4\beta 2$ nAChR in the hippocampus (Featherstone *et al*, 2012b). Aracri (2010) also support a role for the $\alpha 4\beta 2$ nAChR over the $\alpha 7$ nAChR in the PFC as they have shown that it is mainly $\alpha 4\beta 2$ nAChRs which are present on GABAergic interneurons in this region, although there is little direct evidence for selective $\alpha 4\beta 2$ nAChR involvement at this stage (Aracri *et al*, 2010). This is further supported by Tsutsui-Kimura *et al* (2010) who demonstrate that nicotine stimulates $\alpha 4\beta 2$ nAChRs in the infralimbic (IL) region but not the PrL region of the PFC. This suggests that whilst the current *in-vitro* findings correlate with behavioural work, it may be other brain medial prefrontocortical (mPFC) brain regions, such as the IL or anterior cingulate cortex (Cg1) which mediate the effect of nicotine on the OST (Tsutsui-Kimura *et al*, 2010). These inconsistencies mean that further work is needed to elucidate the role for $\alpha 4\beta 2$ nAChRs in mediating the effect of nicotine on gamma frequency oscillations and that conclusions from the current work must be drawn cautiously at this stage.

In the hippocampus, application of nicotine exerts its enhancing effects on gamma frequency oscillations through depolarising interneurons as well as by inhibiting the inhibitory synapses onto interneurons; enhancing GABA release and thus enhancing excitation of interneurons and increasing inhibitory output (Hulo *et al*, 2001; Wanaverbecq *et al*, 2007). Aracri *et al* (2010) have also shown that nicotine (1-100 μ M) is able to increase GABAergic IPSPs onto pyramidal neurons in layer V of the PFC (Aracri *et al*, 2010). This increase in IPSPs is due to a nAChR-dependent stimulation of glutamate release from thalamocortical afferents acting at PV-positive interneurons (Aracri *et al*, 2010; Gioanni *et al*, 1999; Kruglikov and Rudy, 2008; Lambe *et al*, 2003). This means that the processes mediating a nicotine-induced increase in network oscillations may be similar

in the hippocampus and PFC, but the nAChRs involved in this process in each region need to be examined further.

Although further work is needed, these initial findings provide a proof of concept that the OST may be frontally mediated, as nicotine was able to improve gamma frequency activity.

7.5 Conclusions

A mechanism for nicotine's positive effects on control animals in the OST may be as a result of enhanced gamma frequency oscillations, as these are increased upon application of nicotine in the PrL region of the PFC. It is possible that the PrL region specifically may not mediate OST response but these data indicate there is a potential role for this region in mediating the enhancing effects of nicotine. Further work is needed to address the contribution of specific nAChRs involved as well as examining the effect on nicotine in a network model of cognitive impairment. This work also indicates that it will be useful to understand the contribution of the mPFC to the OST task and whether local application of nicotine replicates systemic findings; this will be discussed in the following chapter.

Chapter 8

Local injection of nicotine into the
mPFC enhances OST performance

8.1 Introduction

Zola-Morgan and colleagues (1986) identified the hippocampus as having an individual role in memory when studying Patient R.B. who had developed anterograde amnesia following an ischemic episode. The bilateral lesion caused by the ischemia was limited to the CA1 field of the hippocampus and demonstrated for the first time that damage to the hippocampus alone could cause impairments in memory (Zola-Morgan *et al*, 1986).

Pre-clinically there have been many studies examining the role of the hippocampus in working memory using tasks such as the Radial Arm Maze (RAM) and Morris Water Maze (MWM). These studies indicate that the hippocampus has a significant role in spatial working memory (For review see Jarrard, 1993). Spatial working memory is disordered in patients with schizophrenia: Piskulic and colleagues (2007) conducted meta-analyses on 33 studies examining spatial working memory deficits in these patients which revealed a consistently greater spatial working memory deficit in patients with schizophrenia in comparison to control subjects (Piskulic *et al*, 2007).

However, Perlstein *et al* (2003) have shown that patients with schizophrenia also have significant impairments in non-spatial working memory. The n-back task is a sequential letter memory task with varying memory load conditions, requiring a subject to match a singular letter (0-back), or a letter identical to that presented in the one or two trials preceding (1-back, 2-back). Perlstein and colleagues (2003) found that patients with schizophrenia made significantly more errors in the high memory load condition than control subjects. In addition, fMRI analysis also demonstrated that when completing this task, patients with schizophrenia showed a significant reduction in the activation of the

dorsolateral prefrontal cortex (DLPFC) in comparison to control subjects (Perlstein *et al*, 2003). Barch *et al* (2003) also found that patients with schizophrenia did not show activation of the DLPFC in response to the n-back task in contrast to both control subjects and patients with major depression who both demonstrated activation of this area (Barch *et al*, 2003). This demonstrates a significant role for the PFC in the working memory deficits present in schizophrenia. This is supported by lesion work in both monkeys and humans which demonstrates a role for the PFC in memory (Mishkin and Pribram, 1955; Sakurai and Sugimoto, 1985).

The brain regions involved in the OST are yet to be fully elucidated: Dudchenko *et al* (2000) have demonstrated that hippocampectomised rats are still able to complete the OST indicating that it is unlikely that the hippocampus has a significant role in mediating the OST (Dudchenko *et al*, 2000). As the literature indicates a significant role for the DLPFC in human working memory, it may be that in rats the OST task is mediated by the mPFC, the rodent functional homologue of the human DLPFC region (Seamans *et al*, 2008; Uylings *et al*, 2003). Nicotine has been shown to be effective in the OST when given systemically (Chapter 4). Local administration of nicotine to the mPFC is therefore likely to improve OST performance if there is a significant contribution of the PFC in mediating this task.

To confirm the contribution of the PFC, the effect of local administration of GABA antagonist muscimol will also be examined in the OST. Local injection of muscimol into the nucleus basalis has been shown to impair working memory on the double Y maze (Beninger *et al*, 1992). Additionally, intracerebroventricular (ICV) administration of muscimol also impairs rodent working memory in a matching-to-position task (Ramirez *et*

al, 2005). Sawaguchi and Iba (2001) have also examined the performance of monkeys on a visual working memory task following local administration of muscimol to the DLPFC, demonstrating significant muscimol-induced impairments in performance (Sawaguchi and Iba, 2001). The effect on OST performance following nicotine or muscimol administration should give a good indication of the involvement of the PFC in this task.

4.2 Methods

Twenty-four male hooded Lister rats were trained in the OST. On establishing stable performance, animals were pseudo-randomised into two treatment groups (n=12) and administered vehicle or ketamine (10mg/kg i.p.) for 5 consecutive days. They were given 2 days wash out, then tested on the OST to establish baseline performance. Following stable performance, all 24 animals underwent stereotactic surgery to implant bilateral cannulae into the medial prefrontal cortex (for details of exact methodology see Chapter 2).

Following recovery from surgery and familiarisation with handling for local injections, as well as a preliminary baseline saline injection, rats were given local injection of nicotine (1, 2 or 4 µg/side) or vehicle (ACSF) in the procedure as outlined in chapter two and immediately tested on the OST. As with previous studies, this was a within-subjects, repeated measures design where each rat received each dose in a pseudo-randomised fashion with a day washout and a drug-free baseline testing day between doses.

All animals were treated in accordance with procedures outlined in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) with additional advice from the in-house animal welfare and veterinary team.

8.3 Results

8.3.1 Local administration of nicotine dose-dependently enhanced OST performance in control subjects and ketamine-treated animals

A repeated measures ANOVA revealed an overall effect of local nicotine administration on OST performance ($F(3,54)=9.40$, $p<0.001$). No significant interaction between ketamine pre-treatment and nicotine was found ($F(3,54)=0.25$ $p=0.86$ n.s.), indicating that nicotine had the same effect regardless of whether subjects had previously received ketamine or vehicle treatment. Pairwise comparisons showed that local administration of $2\mu\text{g}$ nicotine was the most effective dose in enhancing overall OST performance when compared to vehicle-treated controls ($p<0.001$).

One way ANOVAs were carried out examining control animals and ketamine-treated animals individually. Nicotine had an overall significant effect on OST performance of control animals ($F(3,35)=3.15$, $p<0.05$) with Bonferroni post-hoc analysis revealing the $2\mu\text{g}$ dose significantly enhanced performance when compared to vehicle treated controls. Ketamine-treated animals also improved overall following local administration of nicotine ($F(3,43)=23.49$, $p<0.001$): In this case, both the 2 and $4\mu\text{g}$ doses significantly improved OST performance in comparison to vehicle-treated controls ($p<0.001$, $p<0.001$).

Figure 8.1: *Local administration of broad spectrum nAChR agonist nicotine dose-dependently enhanced OST performance in control subjects and ketamine-treated animals*

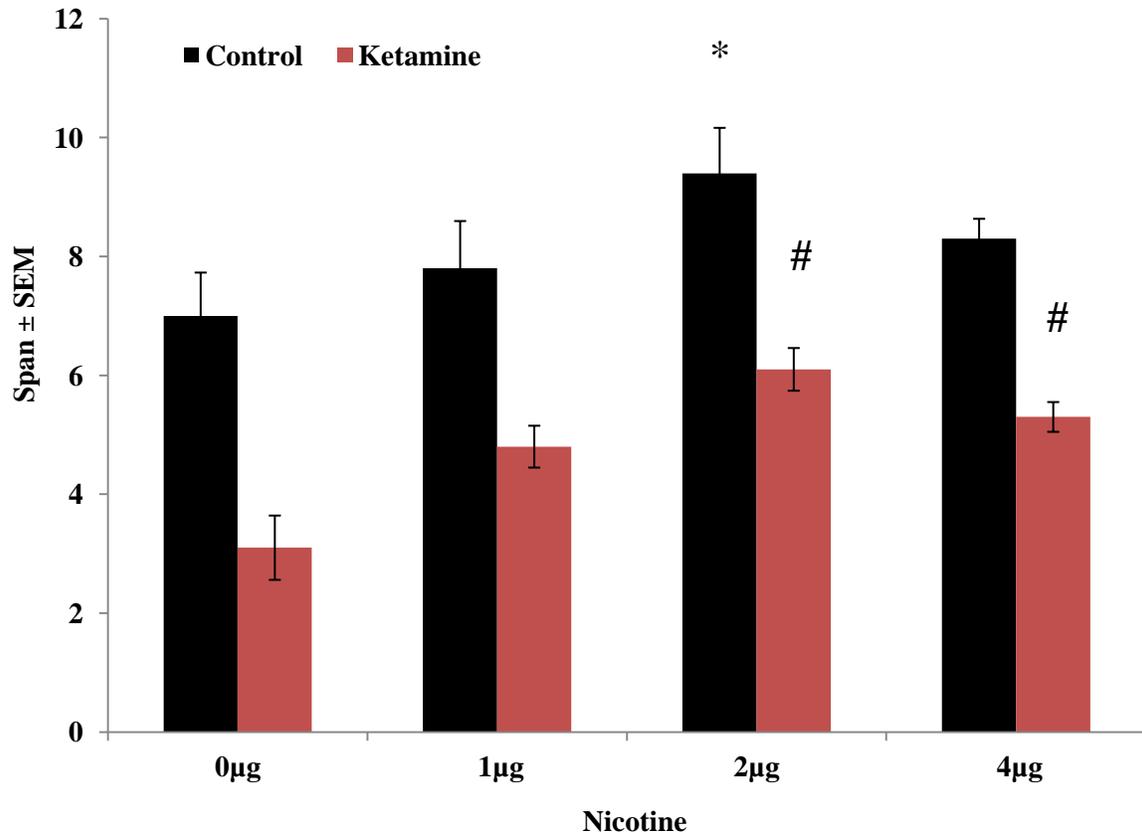


Figure 8.1: Local administration of nicotine was able to enhance the performance of ketamine treated animals (n=11) in the OST at both 2µg and 4µg doses. The 2µg dose also improved the performance of control animals (n=9). * denotes statistical significance from vehicle treatment (p<0.001) in control animals. # denotes statistical significance from vehicle treatment (p<0.001) in ketamine-treated animals.

8.3.2: Local administration of muscimol dose-dependently impaired OST performance in control subjects but not ketamine-treated animals

A repeated measures ANOVA revealed an overall effect of local muscimol administration on OST performance ($F(2,36)=7.48$, $p<0.01$). No significant interaction between pre-treatment and muscimol treatment was found ($F(2,36)=1.57$, $p=0.22$ n.s) indicating that muscimol had the same effect regardless of whether subjects had previously received ketamine or vehicle treatment.

One way ANOVA on control animals alone determined that there was an overall significant effect of muscimol on OST performance ($F(2, 26)=6.00$, $p<0.01$) with Bonferroni post-hoc analysis showing the $2\mu\text{g}$ dose to be most effective in impairing performance in control animals ($p>0.01$). The overall effect of muscimol on ketamine-treated animals, as shown by one way ANOVA, was not significantly different to vehicle-treated subjects ($F(2,32) = 1.53$, $p=0.23$ n.s.).

Figure 8.2: Local administration of GABA antagonist muscimol dose-dependently impaired OST performance in control subjects but not ketamine-treated animals

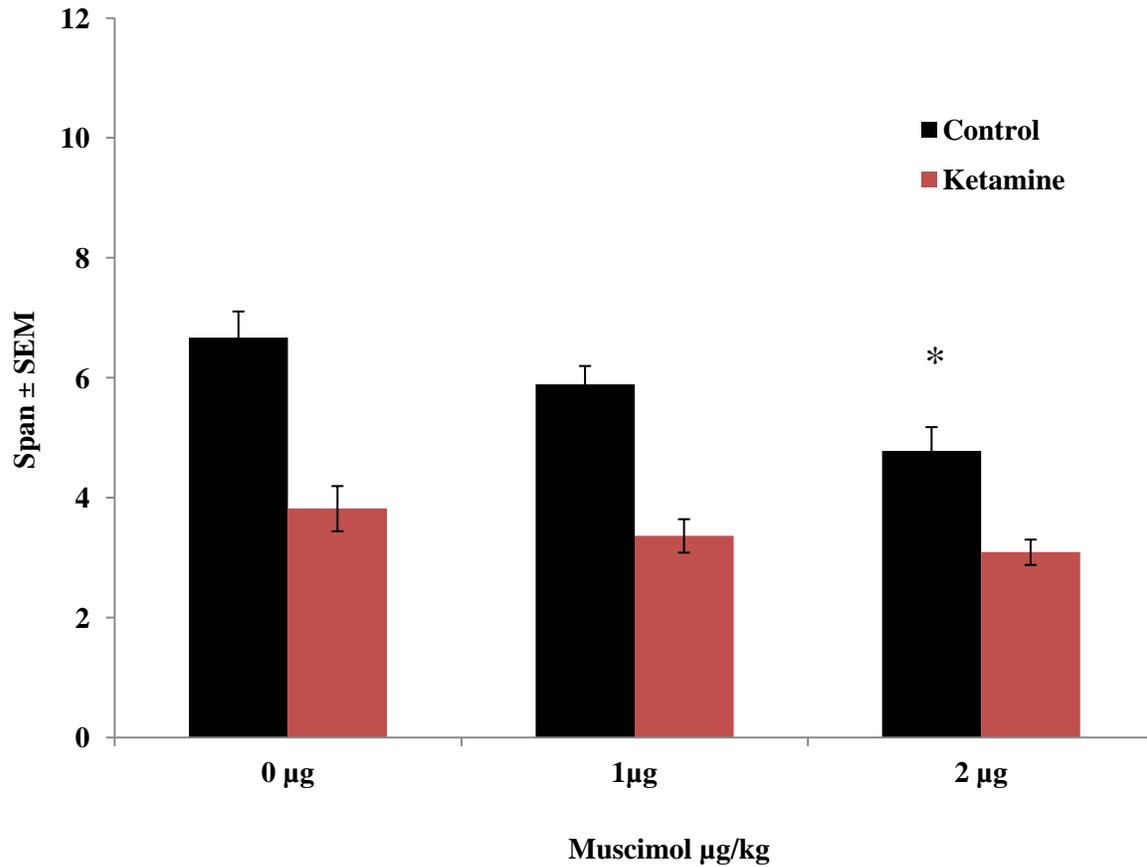


Figure 8.2: Local administration of 2µg muscimol impaired the performance of control animals (n=9) ($p > 0.01$). Muscimol had no effect of the performance of ketamine-treated animals at any of the doses tested (n=11) ($p = 0.23$). * denotes statistical significance from vehicle treatment ($p < 0.01$) in control animals.

8.4 Discussion

Local administration of nicotine into rat mPFC enhances performance in both control and ketamine-treated animals. This suggests a significant contribution of the mPFC to the OST task and is supported by data from Dade *et al* (2001) who used fMRI to determine the brain regions activated in human subject during an olfactory working memory task. They found that regions involved in this task include the polar frontal cortex, the DLPFC and the ventrolateral frontal cortex (Dade *et al*, 2001). As the DLPFC in humans is thought to be functionally homologous to the rodent mPFC, the finding of Dade and colleagues supports the findings in this study implicating the rodent mPFC in working memory (Uylings *et al*, 2003). Tsukada *et al* (2005) provide further support, demonstrating that chronic systemic MK-801 administration to non-human primates reduced D1 receptor binding in the PFC. This effect was normalised following intravenous nicotine administration which was additionally able to elicit a significant increase in DA release into PFC extracellular fluid. These findings also correlated with improved performance following MK-801 induced deficits in a working memory task (Tsukada *et al*, 2005).

The findings of Tsukada *et al* (2005) suggests that nAChR mediated activation of dopaminergic neurons may have a role in mediating improvements in working memory. This is supported by Marshall *et al* (1997) who demonstrated, using microdialysis, that nicotine elicits DA release in the PFC of freely moving rats (Marshall *et al*, 1997). In terms of receptor subtype, Livingstone *et al* (1997) demonstrate that the ability of nicotine to elicit DA in the PFC can be blocked by administration of $\alpha 4\beta 2$ nAChR antagonist dihydro-beta-erythroidine (DH β E). In future studies, it may therefore be useful to examine whether local administration of selective $\alpha 4\beta 2$ nAChR agonists into the PFC also enhances OST

performance. This may help to elucidate the mechanism by which nicotine elicits an effect on the OST.

The consequences of increased DA are yet to be fully elucidated, however Kolomiets *et al* (2009) demonstrate *in-vitro* that prefrontal DA functions to increase ERK phosphorylation, increasing neurotransmitter release and in turn inducing LTP. This may provide a mechanism by which nicotine elicits improvements in working memory (Kolomiets *et al*, 2009). Lambe *et al* (2003) have also shown that nicotine induces glutamate release onto layer V pyramidal neurons of the PFC via thalamo-cortical afferents (Lambe *et al*, 2003). This may also increase LTP in the PFC and thus improve cognitive performance. In contrast, work by Couey *et al* (2007) describes a mechanism for the action of nicotine on cognition whereby nAChR activation increases GABA_A output onto layer V pyramidal neurons in mouse PFC and thus increases the threshold for spike-timing dependent potentiation (STDP). This in turn decreases LTP and may account for why, in some tasks, nicotine can cause cognitive impairment. However, as discussed by Newhouse *et al* (2004), the baseline activity and the nature of the task can determine the ability of nicotine to enhance performance: Thus, it may be that an increased threshold for STDP allows the signal to noise ratio to be improved, enhancing task performance (Newhouse *et al*, 2004c).

The effects seen with local administration of nicotine are not dissimilar to that seen with systemic nicotine administration (Chapter 4; Rushforth *et al*, 2010; Rushforth *et al*, 2011). This suggests that the PFC is also likely to play a significant role in mediating the cognitive-enhancing effects of systemic nicotine on OST performance. However, ketamine-treated animals were not fully restored to baseline performance following local nicotine administration as was found with systemic nicotine. There could be further contribution

from other brain regions, although this is difficult to interpret for this data as the brain concentration of systemically dosed nicotine is unknown so a conclusive comparison cannot be made. However, if there was a contribution from another brain region, the first likely candidate would be the hippocampus given that this brain region has well documented involvement in working memory. However, Dudchenko *et al* have shown that hippocampectomised rats can still complete the OST task and recent data from our laboratory also supports this finding. Rats implanted with bilateral cannulae into the hippocampus were examined on the OST: Local administration of nicotine (2, 4 or 8µg) failed to improve OST performance in both control and ketamine-treated animals. Performance was, however, improved with systemic nicotine administration in the same subjects, suggesting that there is a lack of hippocampal contribution to mediating the OST and that the systemic effect of nicotine on OST performance is unlikely to be mediated by this region (Mitchelmore *et al*, 2012, unpublished data). This does not rule out any contribution of the hippocampus but indicates that it is unlikely to be the main brain region mediating this effect.

Evidence that the PFC may instead play a significant role comes from Enomoto *et al* (2009). They examined the effect of sub-chronic ketamine treatment on working memory performance using the delayed spatial win-shift radial arm maze which is dependent on a neural circuit containing the mPFC and hippocampus along with dopaminergic inputs. They found that 10-day ketamine treatment impaired performance on the PFC-dependent task but not a hippocampally dependent random foraging task (Enomoto and Floresco, 2009). These findings suggests a contribution of the PFC to non-spatial working memory and supports the outcomes of the current study: Data presented here demonstrates that local

administration of GABA_A antagonist muscimol could impair the OST performance of control subjects. However, Horst *et al* (2009) also discovered that local muscimol into the PFC can impair spatial working memory. As Dudckenko *et al* (2000) have shown that activation of the hippocampus is needed in order to complete a spatial version of the OST, it may be that there is a combined role for both the hippocampus and PFC in spatial working memory tasks. This is supported by Yoon *et al* (2008) who examined the effect of both dorsal-hippocampal and mPFC inactivation, using muscimol, on the ability of rats to successfully complete a delayed alternation task. This was a figure 8 maze requiring the animals to alternate arms for a food reward. They found that inactivation of either brain region resulted in task impairments but that the nature of these impairments was different: Inactivation of the mPFC caused impairment of working memory but not reference memory compared to dorso-hippocampal inactivation which impaired both memory functions. This demonstrates that both regions are involved in working memory and indicates a synergistic effect of having both regions intact (Yoon *et al*, 2008).

The fact that the hippocampus and mPFC contribute to differing memory functions may serve to explain the effects of local muscimol administration to the mPFC of ketamine-treated subjects: These animals did not show any significant deficits in OST performance indicating that basal performance of the OST for animals treated with ketamine is not PFC dependent. Therefore, another brain region, such as the hippocampus, may be mediating low level working memory performance. However, Mitchelmore (2012) demonstrate that rats with ketamine-induced deficits that were administered muscimol directly into the hippocampus, did not display any reduction in OST performance (Mitchelmore *et al*, 2012).

This indicates that other brain regions may be involved or that local muscimol administration may not have fully inactivated the PFC, leaving basal function intact.

8.5 Conclusions

The data presented in this chapter indicate a significant role for the PFC in mediating OST performance. There are significant deficits in PFC-dependent memory in patients with schizophrenia and MRI studies also demonstrate that patients have reduced PFC volume (Volpe *et al*, 2012): The OST in rodents may therefore provide a translational model for the development of novel compounds to treat neuropsychiatric disorders such as schizophrenia.

Chapter 9

General Discussion

9.1 Main Findings

The overarching outcome of this research indicates that nAChRs do represent viable targets for the treatment of cognitive deficits in schizophrenia with a potential role for both the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in reversing impaired cognitive performance. The use of the sub-chronic, sub-anaesthetic 10mg/kg ketamine dosing regimen has repeatedly been shown to induce stable and long-lasting deficits in the OST. These cognitive deficits translate to those seen in patients with schizophrenia, as they are not ameliorated following treatment with currently used or novel antipsychotics. Acute local injection of nicotine has demonstrated that the nAChRs in the mPFC mediate improvements in OST performance. Contribution of the mPFC region was further supported by local administration of muscimol to the mPFC which induced impairment in the OST. A neural correlate model of fast network oscillations was developed, providing a potential mechanism behind the behavioural data: Activation of nAChRs in the PrL region of the mPFC mediated enhancement of gamma frequency oscillations. Compounds which target nAChRs in the PFC may therefore prove to be a useful adjunct therapy in combination with current treatments, providing more targeted relief for the heterogeneous symptoms seen in this disorder.

9.1.1 Sub-anaesthetic, sub-chronic ketamine in the OST as a model of cognitive deficits

This body of work has demonstrated that a sub-anaesthetic, sub-chronic ketamine dosing regimen in the OST may be a useful tool for examining novel compounds for the treatment of CDS. The ketamine-induced deficits were dose-dependent and the 10mg/kg dose was chosen since it produced a marked deficit and thus provided a window to observe cognitive enhancement by psychoactive drugs.

The ketamine dosing regimen described in this body of research may prove to be a valuable alternative to the commonly used PCP treatment. The use of ketamine is advantageous in that it only needs to be given once daily for 5 days in comparison to the twice daily for 7 days regimen commonly used in sub-chronic PCP dosing. From an animal welfare perspective, the ketamine regimen presents more than a 60% reduction in the number of injections the animals receive: This aligns well with the aims of the 3 Rs to reduce the suffering of animals used in experimental procedures, without compromising on research. In addition, the 5 day regimen logistically fits in better to the normal working week, and working day.

9.1.2 PCP as an alternative to ketamine: Differences pre-clinically in response to antipsychotic treatment.

The PCP model has been repeatedly shown to induce cognitive deficits in a wide range of tasks (For review see Neill *et al*, 2010). In order to assess whether the sub-chronic, sub-anaesthetic 10mg/kg ketamine regimen is more reliable in producing deficits than the sub-chronic PCP regimen, it would be necessary to examine the PCP regimen in the OST. Comparison of the sub-chronic ketamine and PCP regimens on the OST would be useful as although one of the significant advantages of using ketamine pre-clinically is that it is also used in the clinic, the 5 day regimen may be more similar to studies examining people who abuse ketamine rather than the single acute dose which is licensed for use in clinical experiments with healthy subjects. However, (Javitt *et al* 2012) report that whilst acute ketamine administration does not induce auditory hallucinations, behaviours associated with such phenomena in monkeys, including increased scanning behaviour, were observed during sub-chronic but not acute ketamine administration (Javitt *et al*, 2012). In addition, the sub-chronic, sub-anaesthetic ketamine regimen has been shown to induce deficits in the OST that are not reversed with either acute clozapine or LY404039 treatment. This is in line with clinical findings where several weeks of treatment are needed before any changes

are noted, but in contrast to other preclinical models where atypical antipsychotics are often effective after a single dose: Atypical antipsychotics such as clozapine and olanzapine have been shown to be effective in reversing cognitive deficits in models using PCP which is also in contrast to the clinic where patients often gain little improvement after a single dose (Abdul-Monim *et al*, 2006; Dunn and Killcross, 2006; Grayson *et al*, 2007). This suggests that the sub-chronic ketamine regimen may provide improved predictive validity for the development of novel compounds in that false positives are less likely. This being said only clozapine and LY404039 have been examined to date and thus further examination of other typical and atypical antipsychotics is necessary for this finding to be confirmed. In addition, it would be useful to determine whether the effect of chronic exposure to antipsychotic treatment also follows typical clinical outcomes in the OST.

9.1.3 Nicotine as a treatment for CDS: Ethical considerations?

The finding that systemic and local administration of nicotine directly into the mPFC was able to reverse the deficits induced by ketamine is indicative of nAChRs as viable targets for the treatment of CDS. As nicotine has such a profound effect, it could be argued that an effective and low-cost option could be to prescribe nicotine patches or gum as an adjunct therapy for the treatment of CDS. However, nicotine has dependence-producing effects and additionally is able to improve the performance of control subjects: **Both of these factors raise ethical considerations, although restrictions over use could control the latter.** This means that the development of a non-addictive compound, which targets the specific nAChRs involved in mediating enhanced cognition, is more favourable.

9.1.4 Enhanced gamma oscillations as a mechanism of cognitive enhancement in the OST

Local administration of nicotine to the mPFC induced similar levels of enhanced OST performance to systemic administration, indicating that there is a significant contribution of

nAChRs in this region. This was further confirmed by the fact that local administration of muscimol into the mPFC impaired task performance. When nicotine was applied to the PrL region of the PFC in an *in-vitro* brain slice preparation, gamma frequency oscillations were significantly increased. Gamma frequency oscillations increase with increased working memory load and are impaired in patients with schizophrenia. Therefore it may be that the mechanism by which nicotine is mediating improved performance in the OST is through enhancing gamma frequency oscillations, although this has only been examined in normal subjects thus far. The fact that application to the PrL region improved gamma oscillations also correlates with the behavioural findings that the PFC is heavily involved in the mediation of the OST task.

9.1.5 The $\alpha 7$ nAChR as a drug target for treatment of CDS

It is likely that there is a role for the $\alpha 7$ nAChR in mediating the effect of nicotine on OST performance as the $\alpha 7$ agonist PHA and allosteric modulators PNU and Compound T were able to improve performance in the OST. The $\alpha 7$ nAChR antagonist MLA also impaired performance in ketamine treated animals. This would indicate that the $\alpha 7$ nAChR is a viable target in terms of developing novel compounds for CDS. Both type I and type II allosteric modulators were able to restore performance to baseline levels where as PHA did improve OST performance but did not fully restore to baseline.

This suggests that in terms of drug development, allosteric modulation which incorporates lowering the activation threshold of the $\alpha 7$ nAChR, may be more effective than direct agonism. This is supported by McLean and colleagues (2011, 2012) who demonstrate that PNU-282297 and PNU-120506 can reverse deficits in cognitive tasks such as reversal learning, novel object recognition and the attentional set shifting task (McLean *et al*, 2011; McLean *et al*, 2012). However, it would be useful to examine other $\alpha 7$ agonists in the OST to confirm the findings with PHA120596. In terms of mechanism, $\alpha 7$ nAChR activation by

endogenous or exogenous agonists causes calcium influx and in turn the induction of calcium induced calcium release (CICR). This in turn activates the ERK1/2-dependent pathways, leading to the phosphorylation and activation of synapsin-1, a key protein in the synaptic neurotransmitter vesicle release machinery (figure 9.1) (Dickinson *et al*, 2008).

The $\alpha 7$ nAChR does not appear to mediate the enhancing effect of nicotine on control subjects as neither systemic administration of agonists or allosteric modulators for this receptor improved performance in the OST. In addition, bath application of $\alpha 7$ nAChR agonist PHA was also unable to enhance gamma frequency oscillations. However, there is clearly some contribution of the $\alpha 7$ nAChR as MLA was able to induce deficits in control animals. Ethically, it may be beneficial that compounds targeting this receptor are unlikely to enhance cognition in control subjects as there is significant controversy surrounding the widespread use of cognitive enhancers. For example, does a student who revises and takes an exam with a cognitive enhancer on board have an unfair advantage over a student who does not? Similarly, will employees feel under pressure to take such compounds if they were available over the counter, in order to keep ahead of their peers or competitors? If compounds for CDS were only effective in those with deficits, this problem would be avoided. **Having said that, if a drug which has cognitive enhancing effects in the normal population has appropriate restrictions, this should not be a problem.** Ultimately, compounds which target the $\alpha 7$ nAChR may prove to be a useful adjunct therapy for CDS, as well as other disorders which feature cognitive deficits.

9.1.5 The $\alpha 4\beta 2$ nAChR as a drug target for treatment of CDS

Restoration of ketamine-induced deficits on the OST by nicotine is also likely to be mediated by the $\alpha 4\beta 2$ nAChR, as the $\alpha 4\beta 2$ nAChR agonist's metanicotine and 5IA were both able to fully reverse ketamine-induced deficits. As these agonists were able to fully restore baseline performance, it may be that the $\alpha 4\beta 2$ nAChRs are more involved than $\alpha 7$

nAChR, or this may simply be because there are higher numbers of $\alpha 4\beta 2$ nAChRs in the PFC than $\alpha 7$ nAChRs in the PFC: The region where local injection work indicates the OST is likely to be mediated. The $\alpha 4\beta 2$ nAChR is therefore another viable drug development target for the treatment of CDS. However, the $\alpha 4\beta 2$ nAChR may also have a role in mediating the enhancing effect of nicotine in control animals as in contrast to agonists for the $\alpha 7$ nAChR, the $\alpha 4\beta 2$ nAChR agonist metanicotine was able to improve the performance in control animals. This means that there could be ethical concerns in the development of cognition-enhancing compounds which target this receptor as they could improve performance in those with no clinical deficit. Despite this, further clarification is needed, as another $\alpha 4\beta 2$ nAChR-specific agonist, 5IA, failed to enhance performance of control subjects. In addition, application of 5IA to the PrL region of the rat mPFC did not improve gamma frequency oscillations in an *in-vitro* brain slice model, which may be explained by Tsutsui-Kimura *et al* (2010) who found that nicotine stimulates $\alpha 4\beta 2$ nAChRs in the IL region but not the PrL region of the PFC. This further suggests that whilst the current *in-vitro* findings correlate with behavioural work, it may be other brain mPFC brain regions, such as the IL or Cg1 which mediate the effect of nicotine on the OST (Tsutsui-Kimura *et al*, 2010).

Mechanistically, the $\alpha 7$ nAChR and the $\alpha 4\beta 2$ nAChR elicit their effects through differing processes: Although the exact mechanism for $\alpha 4\beta 2$ -induced neurotransmitter release is not yet known, the $\alpha 4\beta 2$ nAChR has low calcium ion permeability in comparison to the $\alpha 7$ nAChR. In addition, Dickinson *et al* (2008) have shown that activation of $\alpha 4\beta 2$ nAChRs by 5IA does not induce neurotransmitter release through CICR and activation of the ERK1/2-synapsin-1 pathway as is the case for $\alpha 7$ nAChRs (figure 9.1). Despite these clear mechanistic differences, activation of both the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs has been shown to enhance GABAergic inhibition onto pyramidal neurons, as well as increasing DA release in

the PFC. This means that the net effects of activation at both of these receptors are similar, which may give further insight into the mechanisms behind improving cognition.

Taken together the research undertaken for this thesis indicates that sub-chronic, sub-anaesthetic administration of ketamine in the OST is a useful model of CDS in a prefrontally-driven task. Additionally, the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs are both viable targets for the development of novel compounds to treat CDS and may elicit their enhancing effects through enhanced gamma frequency network oscillations.

Figure 9.1: The downstream effects of $\alpha 7$ nAChR activation

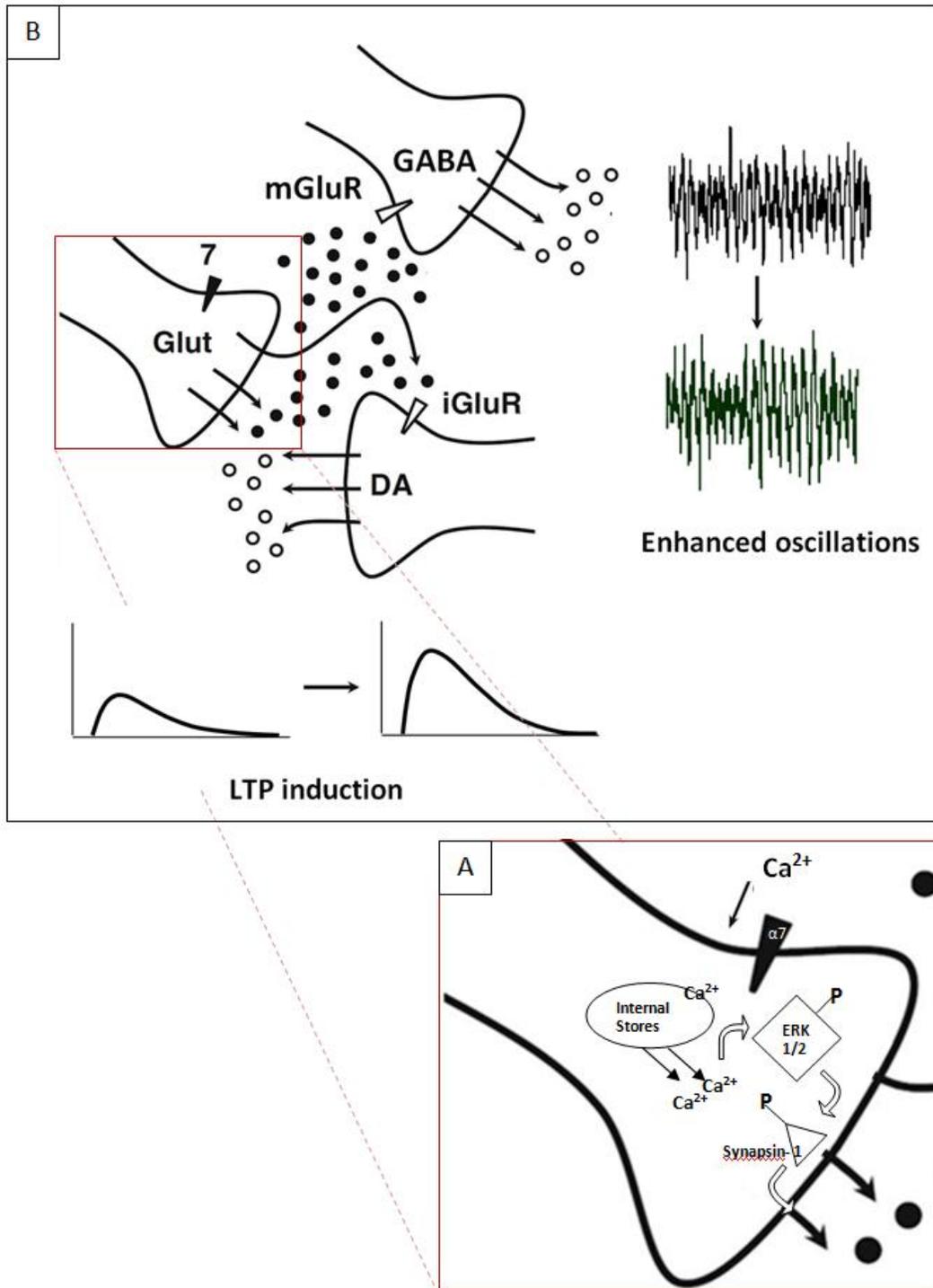


Figure 9.1 A: $\alpha 7$ nAChR activation by endogenous or exogenous agonists causes calcium influx and in turn the induction of calcium induced calcium release (CICR). This in turn activates the ERK1/2 pathways, phosphorylating synapsin 1 which induces vesicle binding to the synaptic membrane and neurotransmitter release. B: Glutamate acts at ionotropic receptors on DA neurons, increasing DA release which can shape working memory processes by influencing the fidelity of synaptic transmission and facilitating long term potentiation. In addition, activation of metabotropic receptors on GABAergic interneurons facilitates GABA release, increasing the synchrony and size of gamma oscillations which are also central to working memory

9.2 How will these results impact on patients?

This body of research demonstrates that nicotine is able to improve cognition in animals that were impaired on a prefrontally-driven cognitive task. This supports findings with patients who have schizophrenia who are proposed to treat their cognitive deficits by tobacco smoking. This research found that $\alpha 7$ nAChRs are likely to be involved in mediating positive effects of nicotine on and are thus a viable target for the development of novel compounds. Partial $\alpha 7$ nAChR agonist DMXB-A has been clinically tested in patients with schizophrenia by Olincy *et al* (2006). They found that on the Repeatable Battery for the Assessment of Neuropsychological Status total scale score, patients treated with DMXB-A demonstrated significant neurocognitive improvement in comparison to placebo treatment (Olincy *et al*, 2006).

However, the study by Olincy and colleagues (2006) was in a small group of non-smoking patients (Olincy *et al*, 2006). Freedman *et al* (2008) examined DMXB-A in a larger group of patients using the MATRICS test battery. Patients were tested over 3 treatment arms. Overall, DMXB-A was not found to have any significant effect on cognitive measures but this was, in part, attributed to strong effects of test repetition where it was observed that subjects improved markedly in their performance over 4 months. When the initial treatment arm was examined individually, a significant improvement in both attention/vigilance and working memory was seen with DMXB-A treatment compared to placebo (Freedman *et al*, 2008). However, other cognitive measures still failed to demonstrate any improvement. In the current work, PHA did improve cognitive performance of ketamine subjects but this was not as significant as when treated with nicotine (Chapter 5). In contrast, both $\alpha 7$ nAChR PAMs, PNU-120596 and Compound T, produced robust improvements when examined in the OST (Chapter 6). Thus, it may be useful to examine the effect of $\alpha 7$ nAChR PAM in a clinical setting, as this may provide a superior outcome in comparison to $\alpha 7$ nAChR agonism.

This study also found that $\alpha 4\beta 2$ nAChRs might prove to be valuable targets for the treatment of CDS. The results presented here demonstrated the enhancing effects of $\alpha 4\beta 2$ nAChR agonists but promising work has recently been published by Timmerman and colleagues (2012) using NS9283, an $\alpha 4\beta 2$ nAChR PAM. They found that NS9283 improved social recognition as well as performance in the 5CSRTT and MWM (Timmermann *et al*, 2012). These positive findings regarding the $\alpha 4\beta 2$ nAChR means that preclinical as well as clinical studies are warranted in order to develop new treatments for CDS which target this receptor.

9.3 Limitations of the OST

Whilst the OST may have been successfully used for the purposes of this research, there are several shortcomings which could be addressed. The main concern is that measurement of performance in this task is subjective: It is the decision of the investigator as to what constitutes a ‘dig’ and what is simply sampling. This concern was overcome in these experiments by experimenter blinding, as well as strictly defining a dig as the displacement of digging media. However, although these measures go a significant way to ensuring that the results of these experiments are consistent, it is likely that there will be slight differences in scoring between different laboratories. There is also the issue of training methods: As with people, different rats learn the task at different rates and how these rats are trained may also differ slightly from person to person and laboratory to laboratory. In our laboratory, rats consistently plateau at a span score between 6 and 8, but other laboratories train animals to a point where they can achieve span scores of 20 and above. When our animals were trained to this level by an undergraduate student, it was found that ketamine did not impair their performance on the task. This illustrates that training technique and individual judgement are significant factors in this task and make extensive handover training essential for any new person running the task in order for results to be comparable.

Although every care was taken to prevent scent marking, this is still a mechanism by which rats could successfully discriminate between novel bowls, and those previously sampled. This was controlled for by changing bowls for new ones with the same odour at random points during training as well as replacing any bowls that were obviously marked.

Animals were exposed to all 24 scents in a short period of time, the order of which was pseudo-randomised for each testing and training session using a random letter chart: However even though a ratio of 3g of scent to every 100g of woodchip was used, there was

no easy way to control for the individual strength or appeal of each scent. For example, 3g of cumin has a significantly stronger odour than 3g of cocoa powder. Despite this, each animal received the odours in the same order within each trial and so any effect this may have had would be consistent across the group, though scores may have been affected from day to day.

The OST is a very time consuming task: It takes between 4 and 5 months to complete the training of one cohort of 24 animals and test one set of compounds. Each animal receives several doses of the test compound as well as vehicle. Repeated testing with several compounds in a single animal was avoided to prevent previous treatment from confounding findings. This approach is therefore costly in both time and money.

The OST is also still a relatively new task and with each laboratory training their animals in a slightly different way, time frame and even apparatus, significant further research using this task is needed in order to refine the protocol.

In order to improve the efficiency of the OST, an automated OST could be developed. An initial prototype could be built using three nose-poke walls from a standard skinner box (figure 9.2). Animals would be presented with increasing set of odours as in the OST. However, these odours could be sampled through a hole in the test wall, similar to a nose-poke space. Odours behind each slot could be in moveable, cylindrical holding pots with small holes on the side so the spice can be scented by the rat. Instead of digging in bowls for a food reward, animals therefore learn to nose poke to indicate a choice. If this choice is correct, a pellet is dispensed on the other side of the arena. Retrieval of the pellet then triggers the start of the next trial. During pellet collection and consumption, a pick and place robot could move the various odours to new slots. This ensures that, as with the manual OST, the automated task is not reliant upon spatial working memory.

There are a number of benefits to automating the OST, the main benefit being that this test would then be standardised between laboratories. Animals would also be less able to scent mark and as the experimenter is only required to place, or remove animals from the experimental apparatus; one experimenter could essentially train as many animals as apparatus was available. The development of the automated OST would increase the level of throughput which means that this task would be a more viable option for the pharmaceutical industry. If the OST can be used in the industrial as well as academic environment, the yield in the development of novel compounds for CDS is likely to be significantly higher.

Figure 9.2: *The automated OST*

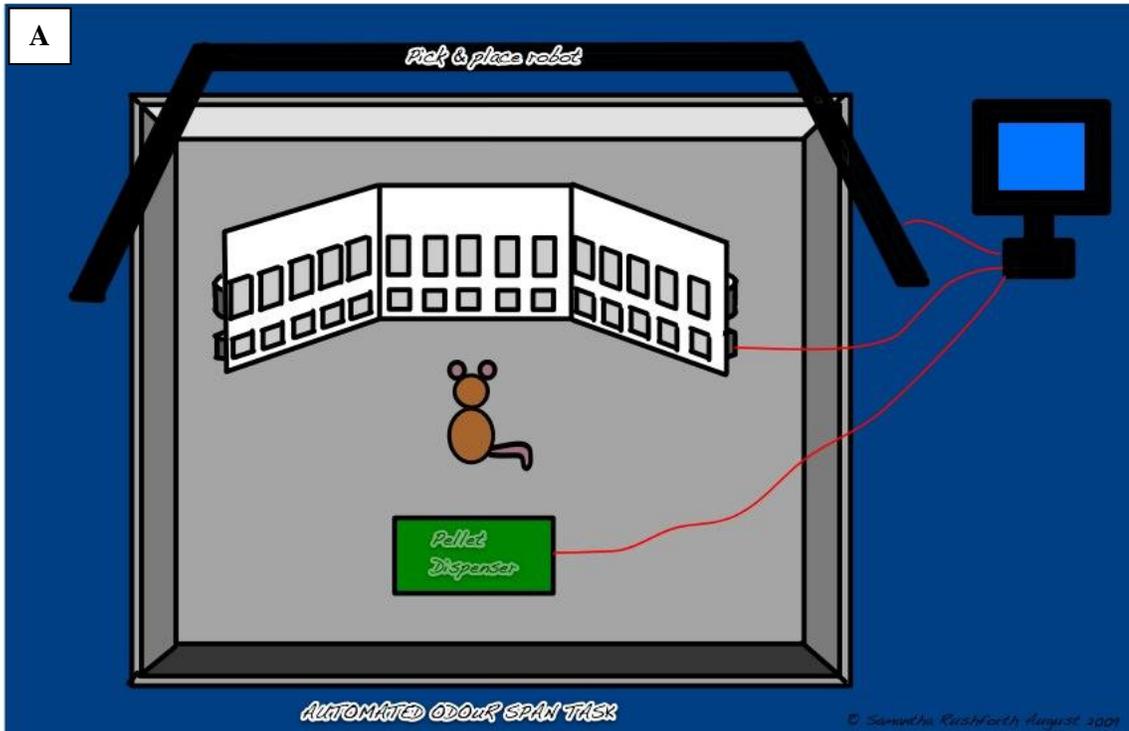


Figure 9.2 A: A diagrammatic representation of the automated OST. The animals would sample the odours from the top hole and nose poke in the bottom hole to indicate a choice. An incorrect choice would signify the end of the test, or retrial, and a correct choice would trigger a food pellet reward. Retrieval of the reward would then trigger the start of the next trial. Automating the OST would provide a way of both standardising OST training and testing between laboratories as well as increasing the efficiency of the task with regard to testing novel compounds.

9.4 Weaknesses of this research

In the body of this research, the aim has been to draw the most logical conclusions based on what is presented within the current literature. However, there are other possibilities and more abstract explanations for these findings. For example, there is the possibility that ketamine could alter blood/brain barrier permeability or have a direct impact on the mechanism by which nicotinic agonists work, although we are yet to find evidence for such explanations. Additionally, there is the possibility that ketamine and indeed nicotine could simply be affecting smell. If ketamine impaired smell and nicotine enhanced smell, this could explain the findings that we are interpreting as impaired and enhanced OST performance. However, the finding that local administration of nicotine into the PFC (which should leave olfaction unaffected) improves OST performance supports the theory that it is memory specifically which is being affected. Also, if ketamine treated animals are given a trial of many sets of just two odours to choose from, they can achieve a normal span score. It is when this is increased to more than two that errors are made. In addition, the sub-chronic ketamine regimen used in this task does not affect the simple discrimination or compound discrimination of set shifting (Shoaib, 2013). This supports the idea that both of these compounds are affecting cognitive processes as opposed to olfaction.

Local injection of nicotine cannot be directly compared to the systemic administration of nicotine as the brain concentration of the systemic nicotine is unclear. In addition, the intracerebral distribution over time is unknown as one dose of methylene blue was given and brains taken at approximately 5 minutes. This means that it is possible that the OST was carried out at a point where the drug was still very local or widely distributed and affecting other brain regions. One way to overcome this would be to give several groups of rats methylene blue and cull at differing time point to assess brain distribution over time.

This could then be correlated with the time course for completion of the OST which would give a more accurate picture of drug distribution.

Another weakness is that only one behavioural test was used as a result of the time consuming nature of the OST. In addition the OST has only one outcome measure in the testing phase. This is because any error in performance indicates the end of the task. Other factors which could be measured such as search strategies would be useful and could be accomplished perhaps by using a camera and behavioural tracking software such as that used in the open field test. It would be prudent in future to directly test for non-specific effects such as motor function. This could be achieved by running additional tests alongside the OST such as the open field test, rotarod task and attentional set shifting task. That being said, the OST is very time consuming and so additional tasks would need to be chosen carefully.

The gamma frequency network oscillation data was only carried out on brain tissue from uncompromised animals. A significant strengthening of this data would have come from also carrying out these experiments in animals which had been treated with ketamine. Unfortunately this was not completed as there was a limited amount of time available in the electrophysiology lab. In addition, another student whose complete project surrounded the effects of ketamine on gamma oscillations was due later on to carry out similar experiments and so it was felt that it was inappropriate for them to be carried out twice or for me to complete these studies first.

9.5 Future research

In the PrL region of the PFC, which has been described as the most morphologically similar region to the primate DLPFC, gamma frequency oscillations can be generated in an *in-vitro* brain slice model. McNally *et al* (2011) have shown that in the PrL, the NMDA antagonist ketamine can increase the area power of gamma frequency oscillations. This is a conflicting finding in that NMDA antagonism is often used to model CDS and increasing gamma frequency oscillations is associated with improved cognition. However, it may be that acute NMDA antagonism, which produces many of the positive symptoms of schizophrenia, does not induce cognitive deficits with the drug on board. Instead, it may be that repeated exposure to NMDA antagonists is required to induce a reduction in gamma frequency oscillations. Work by Gilloughley *et al* (2012) however found that acute application of ketamine reduced gamma frequency oscillations in the Cg1 regions of the PFC. It would therefore be a logical next step to examine the effect of the sub-chronic-sub-anaesthetic 10mg/kg ketamine regimen in the *in-vitro* brain slice model. Animals would be treated in the same way as for the OST, including a 6 day wash-out, the point at which deficits are evident behaviourally. If a reduction in gamma oscillations was evident, then nicotine and subtype-selective nAChR agonists could be examined. If these results were found to correlate with behavioural data, it may provide a reasonably high-throughput method for screening novel compounds before testing them in the full behavioural model.

It would also be interesting to assess the effect of nicotine and ketamine treatment on the generation of oscillatory activity in both the anterior cingulate and infralimbic regions: This would give further insight as to whether the mPFC as a whole is involved in mediating the effect of nicotine on gamma oscillations or whether this process is sub-region specific. Knowledge of which brain regions mediate the effects of nicotine may help in the development of novel compounds that target nAChRs. In addition, further examination of the contribution of $\alpha 4\beta 2$ nAChRs in mediating gamma frequency oscillations in all regions

of the mPFC would be useful in order to elucidate their contribution to the effect of nicotine on gamma frequency oscillations and also on OST performance.

In line with this, it would also be important to examine the effect of a sub-chronic, sub-anaesthetic ketamine regimen on the expression of PV positive neurons in the PFC. Carlen *et al* (2012) demonstrate a critical role for NMDA receptors in PV positive interneurons by selectively knocking out these receptors in PV positive neurons alone. Although baseline oscillations were increased, the induction of gamma frequency oscillatory activity was impaired following optogenetic drive onto PV positive neurons, which correlated with animals exhibiting significant cognitive impairments (Carlen *et al*, 2012). McKibben *et al* (2010) have also demonstrated that PV positive interneurons in the PFC are reduced 6 weeks after sub-chronic treatment with NMDA antagonist phencyclidine. This was specifically observed in the PrL region but not the IL or cingulate cortices (McKibben *et al*, 2010). Examination of a sub-chronic, sub-anaesthetic ketamine regimen would compliment these studies and further our understanding of the brain regions involved in mediating the effect of ketamine on the OST. Taken together, these studies may help to determine the sub-region of the mPFC where ketamine is likely to be exerting an effect, along with whether nicotine or nicotinic agonist treatment is mediating restoration through the same, different or a combination of sub-regions. It could then be established whether or not the effect of ketamine and subsequent restoration by nAChR activation correlates to the size and synchrony of gamma frequency oscillations.

As all of the electrophysiological studies so far have examined extracellular field potentials, it would be useful to examine the effect of acute and sub-chronic ketamine, nicotine, selective nAChR agonists, antagonists and allosteric modulators intracellularly. This would help to further determine the cellular processes involved in the changes induced by these compounds which in turn may lead to novel targets for the treatment of CDS.

Behaviourally, one option would be to examine the effect of local administration of selective nAChR agonists and PAMs in the mPFC to see whether this replicates systemic administration. Additionally, as it would also be useful to explore the contribution of other brain regions to the OST through local administration of nAChR agonists or area specific lesions. Recent work by our group has established that local administration of nicotine to the hippocampus has no effect on the OST, which in contrast, significantly improves performance when given systemically to the same subjects. This means that there is unlikely to be a significant contribution of the hippocampus in mediating the OST, but the contribution of other regions with input to the PFC such as the entorhinal cortex or the ventral tegmental area (VTA) is yet to be elucidated.

It would also be valuable to explore the possibility of automating the OST, as described above although this is potentially a PhD thesis project in itself.

Overall, this thesis has discovered that nAChRs are viable targets for the development of novel drugs for the treatment of CDS. These findings may help to progress development of compounds which target these receptors and help to improve the lives of patients suffering with this disorder.

Chapter 10

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Chapter 11

Appendix

11.1 Conferences

11.1.1 As a speaker

S. L. Rushforth, E. L. Malcolm, L. Smith, T. Steckler & M. Shoaib

European College of Neuropsychopharmacology Annual Meeting Young Scientist Award Symposia
Paris, France: September 2011

Targeting nicotinic receptors to restore memory deficits following sub-chronic ketamine exposure

11.1.2: Posters

S. L. Rushforth, E. L. Malcolm, A. S. J. Lesage, F. E. N. LeBeau, T. Steckler & M. Shoaib

Nicotinic Acetylcholine Receptors as Therapeutic Targets: Emerging Frontiers in Basic Research &
Clinical Science, Washington DC November 2011

*Performance in A Working Memory Task Is Enhanced By Nicotine and Impaired By Muscimol When
Administered Locally Into the Prefrontal Cortex*

S. L. Rushforth, E. L. Malcolm, A. S. J. Lesage, F. E. N. LeBeau, T. Steckler & M. Shoaib

Annual Meeting for the Society of Neuroscience, Washington DC 2011 November 2011

*Performance in A Working Memory Task Is Enhanced By Nicotine and Impaired By Muscimol When
Administered Locally Into the Prefrontal Cortex*

S. L. Rushforth, E. L. Malcolm, L. Smith, T. Steckler & M. Shoaib

European College of Neuropsychopharmacology Young Scientist Workshop, Nice: March 2011

Targeting nicotinic receptors to restore memory deficits following sub-chronic ketamine exposure

S. L. Rushforth, E. L. Malcolm, A. S. J. Lesage, F. E. N. LeBeau, T. Steckler & M. Shoaib

Annual Meeting for the Society of Neuroscience, San Diego 2010

*Targeting the alpha7 nicotinic receptor subtype to restore working memory deficits in rats following sub-
chronic ketamine exposure*

S. L. Rushforth, EL Malcolm, T. Steckler, M. Shoaib

British Association for Psychopharmacology Annual Meeting, Harrogate July 2010

*Nicotine but not LY404039 or Clozapine Significantly Reverses Ketamine-Induced Deficits in the Rodent
Odour Span Task*

S. L. Rushforth, T. Steckler, M. Shoaib

European Behavioural Pharmacology Society Biennial Meeting, Rome: September 2009

*Nicotine but not LY404039 or Clozapine Significantly Reverses Ketamine-Induced Deficits in the Rodent
Odour Span Task*

S. L. Rushforth, T. Steckler, M. Shoaib

Society of Biological Psychiatry Annual Meeting, Vancouver: May 2009

*Nicotine Restores Cognitive Impairments Following Sub-Chronic Ketamine Exposure in the Rodent Odour Span
Task.*

S. J. Wonnacott, **S.L. Rushforth**, C. Allison, S. Jayaraman, M. Shoaib
Annual Meeting for the Society of Neuroscience, San Diego, November 2007
Profiling beta2 and alpha7 nicotinic receptor ligands in a cognitive task involving working memory and its relationship to extracellular release of dopamine in the prefrontal cortex of rats.*

S.L. Rushforth, C. Allison and M. Shoaib
British Association for Psychopharmacology Annual Meeting, Harrogate, July 2007
Nicotinic Agonists Enhance Olfactory Working Memory in Normal Rats: A Novel Use of the Odour Span Task.

11.2 Peer-reviewed publications

S. L. Rushforth, C. Allison, S. J. Wonnacott, M. Shoaib (2010)
Nicotinic Agonists Enhance Olfactory Working Memory in Normal Rats: A Novel Use of the Odour Span Task.
Neuroscience Letters **471**(2): 114-118

Rushforth SL, Steckler T, Shoaib M (2011). Nicotine improves working memory span capacity in rats following sub-chronic ketamine exposure. Neuropsychopharmacology **36**(13): 2774-2781.

11.3 Awards

- British Association for Psychopharmacology Robert Kerwin International Bursary 2011: Enabling me to present at the “Nicotinic Acetylcholine Receptors as Therapeutic Targets: Emerging Frontiers in Basic Research & Clinical Science,” satellite and the Annual meeting for the Society for Neuroscience 2011 in Washington DC.
- ECNP Workshop Young Scientist Award 2011: An invitation to speak at the 2011 European College of Neuropsychopharmacology Annual Meeting. Paris, France: September 2011
- Recipient of a fully funded scholarship for the European College of Neuropsychopharmacology Workshop for Young Scientists. Nice, France: March 2011.
- BAP Summer Meeting Poster Prize. Harrogate, UK: July 2010
- BAP Summer Meeting Travel Bursary. Harrogate, UK: July 2010
- BAP Preclinical Certificate Bursary, 2010

- European Behavioural Pharmacology Society Biennial Meeting Poster Prize. Rome, Italy Sept 2009.

11.4 Society Memberships

British Association of Psychopharmacology training member

Schizophrenia International Research Society (SIRS)

Society for Neuroscience