Title: Neurotransmitter receptor binding in the posterior cingulate cortex in schizophrenia and in the phencyclidine mouse model: an exploration of the NMDA hypofunction hypothesis of schizophrenia

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Neurotransmitter receptor binding in the posterior cingulate cortex in schizophrenia and in the phencyclidine mouse model: an exploration of the NMDA hypofunction hypothesis of schizophrenia

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NEUROTRANSMITTER RECEPTOR BINDING IN THE POSTERIOR CINGULATE CORTEX IN SCHIZOPHRENIA AND IN THE PHENCYCLIDINE MOUSE MODEL

AN EXPLORATION OF THE NMDA HYPOFUNCTION HYPOTHESIS OF SCHIZOPHRENIA

A thesis submitted in fulfillment of the
requirements for the award of the degree

DOCTOR OF PHILOSOPHY

From

SCHOOL OF HEALTH SCIENCES
UNIVERSITY OF WOLLONGONG

By

Kelly Newell

2007

CERTIFICATION

I, Kelly Newell, declare that this thesis, submitted in fulfillment of the requirements for the award of Doctor of Philosophy, in the School of Health Sciences, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. This manuscript has not been submitted for qualifications at any other academic institution.

Kelly Newell

May 2007

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PUBLICATIONS

The following publications and presentations have arisen directly from the work conducted for this thesis.

Publications in Refereed Journals

Newell, K.A., Zavitsanou, K., and Huang, X.F. Short and long-term changes in NMDA receptor binding in mouse brain following chronic phencyclidine treatment. Journal of Neural Transmission, In Press.

Newell, K.A., Zavitsanou, K., and Huang, X.F. Opposing short and long-term effects on muscarinic M1/4 receptor binding following chronic phencyclidine treatment. Journal of Neuroscience Research, 85: 1358-1363, 2007.

Newell, K.A., Zavitsanou, K., Kum-Jew, S. and Huang, X.F. Alterations of muscarinic and GABA receptor binding in the posterior cingulate cortex in schizophrenia. Progress in Neuropsychopharmacology & Biological Psychiatry, 31: 225-233, 2007.

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Newell, K.A., Zavitsanou, K., and Huang, X.F. Ionotropic glutamate receptor binding in the posterior cingulate cortex in schizophrenia patients. NeuroReport, 16(12): 1363-1367, 2005.

Publications in Conference Proceedings

Newell, K.A. Zavitsanou, and Huang X.F. The posterior cingulate cortex: a site of altered neural circuitry in schizophrenia and in NMDA hypofunction. Schizophrenia Bulletin, 33(2): 321, 2007.

Newell, K.A., Zavitsanou, K., and Huang, X.F. NMDA and muscarinic M1/4 receptor binding density is decreased 2 weeks after, but not immediately after chronic PCP treatment. Proceedings of the Australian Neuroscience Society, the 26th Annual Meeting. 17: 105, Sydney 2006.

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Newell, K.A., Klose, B., Zavitsanou, K., Han, M., and Huang, X.F. Effects of clozapine and haloperidol on motor activity in the chronic PCP mouse model. 7th Biennial Australasian Schizophrenia Conference. 7:115, Sydney, 2002.

Additional Publications

The following publications have arisen from other projects I have been involved in throughout my doctoral studies.

Han, M., **Newell, K.A**., Zavitsanou, K., Deng, C., and Huang, X.F. Effects of antipsychotic medication on muscarinic M1 receptor mRNA expression in the rat brain. Journal of Neuroscience Research. Submitted.

Han, M., Deng, C., Burne, T.H.J., **Newell, K.A.,** and Huang, X.F. Olanzapine-induced weight gain is related to a reduction in histamine H1 receptor mRNA expression in the rat hypothalamus. Schizophrenia Research. Submitted.

Deng, C., Han, M., **Newell, K.A.,** and Huang, X.F. No changes in cannabinoid CB1 receptor binding density in the superior temporal gyrus in schizophrenia. Schizophrenia Bulletin, 33(2): 308, 2007.

Han, M., Deng, C., **Newell, K.A.,** and Huang, X.F. Histamine mRNA expression is decreased in the rat hypothalamus following olanzapine treatment. Schizophrenia Bulletin, 33(2): 317, 2007.

Huang, X.F., du Bois, T., Hsu, C., Eftimovska, J., Tan, Y.Y., Zavitsanou, K., **Newell, K.A.,** and Deng, C. NMDA receptor hypofunction during early brain development: relevance to schizophrenia. Schizophrenia Bulletin, 33(2): 317, 2007.

Zavitsanou, K., Nguyen, V., **Newell, K.,** Ballantyne, P., and Huang, X.F. Increased [^{3}H]MK801 binding in the cingulate cortex of the rat after a single injection of phencyclidine. Proceedings of the Australian Neuroscience Society, the 26th Annual Meeting. 17: 102, Sydney, 2006.

Han, M. Zavitsanou, K. **Newell, K.,** and Huang, X.F. Increased CB1 mRNA in the cortical areas of mice prone to diet-induced obesity. Proceedings of the Australian Neuroscience Society, the 24th Annual Meeting. 15:113, Melbourne 2004.

Huang, X.F., Han, M., **Newell, K.,** and Zavitsanou, K. A low level of Y1 and Y5 gene expression may contribute to the prevention of chronic high energy diet-induced obesity. Proceedings of the Australian Neuroscience Society, the 23rd Annual Meeting. 14:228, Adelaide 2003.

Abstract

Schizophrenia is a severe psychiatric disorder with no clear cause. Recent evidence suggests that N-methyl-D-aspartate (NMDA) receptor hypofunction may underlie the pathogenesis of schizophrenia. The posterior cingulate cortex (PCC) has been shown to be the most susceptible brain region to damage caused by NMDA hypofunction in rodents. This suggests that the PCC may play an important role in schizophrenia pathology. However, studies examining neurotransmitter balance in the PCC in schizophrenia have until now been neglected. Furthermore, the long-term consequences of NMDA hypofunction on neurotransmitter balance in animal models have not been studied. The aims of this study were to investigate neurotransmitter receptor binding profiles in the PCC in schizophrenia, while also examining the effects of chronic phencyclidine (PCP; an NMDA antagonist) treatment on neurotransmitter receptor binding in mouse brain in the long-term following treatment. To achieve these aims, the study was divided into two parts.

In experimental part A, PCC sections from 10 schizophrenia and 11 control subjects matched for age, gender and post-mortem interval were obtained from the Tissue Resource Center, Sydney. Using quantitative autoradiography, the density of several neurotransmitter receptors was examined. The results demonstrated specific alterations in neurotransmitter receptors in the PCC in schizophrenia. Specifically, increased NMDA, gamma-aminobutyric acid A ($GABA_A$) and cannabinoid 1 (CB1) receptor density was found in this region, along with reduced muscarinic 1/4 (M1/4) and serotonin 2A (5-hydroxy-tryptamine, $5HT_{2A}$) receptor density. No changes were found in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate or M2/4 receptor density in the PCC in schizophrenia subjects compared to controls. These

results have shown for the first time that there are specific neurotransmitter imbalances in this region, and it is possible that these changes stem from NMDA hypofunction.

In experimental part B, mice were treated chronically (14 days) with PCP. Using quantitative autoradiography the density of several receptors was examined in the short (1hr and 24hr) and long-term (14 days) following chronic PCP treatment. In addition, clozapine and haloperidol were tested for their ability to prevent the PCP-induced alterations in neurotransmitter receptor density. The results showed opposing effects of PCP treatment on neurotransmitter receptor density in the short compared to the long-term. While there were limited increases in NMDA receptor density in the short-term, there were widespread reductions in NMDA receptor density in the long-term following chronic PCP treatment. Muscarinic M1/4 receptor binding, which was increased in the short-term, showed reductions in the long-term in the limbic system, caudate-putamen and cortex, but not in the thalamus in which no change was found. Clozapine and haloperidol treatments were both unable to prevent the PCP-induced long-term changes in receptor density.

In conclusion, this study has provided new information regarding neurotransmitter alterations in the PCC in schizophrenia and in mouse brain in the long-term following chronic PCP treatment. These findings may assist not only in understanding the pathology of schizophrenia, but also for designing new pharmacological treatments for this disease.

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List of Abbreviations

Abbreviations used throughout this thesis are defined below.

Abbreviations	Definition
5-HT	5-hydroxy-tryptamine
Acb	Nucleus accumbens
ACC	Anterior cingulate cortex
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APD	Antipsychotic drug
BSA	Bovine serum albumin
CNS	Central nervous system
CPu	Caudate-putamen
D-AP5	D-2-amino-5-phosphnopentanoate
DSM	Diagnostic and Statistical Manual of Mental Disorders
EDTA	Ethylenediamine teraacetic acid
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GAT 1	GABA transporter 1
HEPES	N-(2-Hydroxyethyl) piperazine-N`-2-ethane sulfonic acid
LSD	Lysergic acid diethylamide
LV	Lateroventral
MRI	Magnetic resonance imaging
NHMRC	National Health and Medical Research Council
NMDA	N-methyl-D-aspartate
PCC	Posterior cingulate cortex
PCP	Phencyclidine
PET	Positron emission tomography
RSC	Retrosplenial cortex
Δ^9-THC	Δ^9-tetrahydrocannabinol

Chapter 1: Schizophrenia - A Review

1.1 What is Schizophrenia?

Schizophrenia is a complex and devastating brain disorder characterized by a combination of positive (additional to normal experience), negative (lacking relative to normal experience), and cognitive symptoms. Positive symptoms typically consist of hallucinations and delusions (often of an auditory, paranoid, and/or religious nature). Negative symptoms include depression, anhedonia (inability to feel pleasure), flattened affect (restricted range and intensity of emotional expression), self-neglect, and social withdrawal. Cognitive dysfunction has also been reported in schizophrenia, including deficits in working and episodic memory, selective attention and general intellectual functioning (Tendolkar et al. 2002; Morris et al. 2005). Schizophrenia is diagnosed, often according to the Diagnostic and Statistical Manual of Mental Disorders (DSM; American Psychiatric Association 1994), based on the presence of the above symptoms.

1.2 What is the Cause of Schizophrenia?

Despite being investigated for over 100 years, schizophrenia has no known cause. Research has established that a combination of genetic and environmental factors cause schizophrenia; however, what factors are involved remains uncertain. Adoption and twin studies have shown a tenfold increase in risk of developing schizophrenia associated with the presence of an affected first-degree family member, while rates of schizophrenia are higher among relatives of patients than in the general population (Mueser and McGurk 2004). The risk of developing schizophrenia is also increased by prenatal events, including maternal influenza, rubella, malnutrition, diabetes mellitus, smoking during pregnancy, and obstetric complications (Mueser and McGurk 2004).

Schizophrenia commonly presents itself during adolescence/early adulthood, suggesting that neurodevelopmental changes in the brain could trigger the onset of schizophrenia (Warner 2004).

1.3 Schizophrenia Hypotheses

It has been proposed that an imbalance of neurotransmitter systems is the underlying cause of schizophrenia (Olney et al. 1999). The classic hypothesis regarding schizophrenia has been the dopamine hypothesis, which suggests that an increase in dopamine activity results in the positive symptoms associated with schizophrenia. This hypothesis was developed based on the discovery that typical antipsychotic drugs (such as haloperidol), which are effective in treating the psychotic symptoms of schizophrenia, are dopamine D2 receptor antagonists. Consistent with this hypothesis, schizophrenia patients have shown increased dopaminergic activity in nucleus accumbens (Bird et al. 1977; Bird et al. 1979; Mackay et al. 1982). However, the dopamine hypothesis does not explain the full spectrum of schizophrenia symptoms, as typical antipsychotic drugs reportedly have no effect on the negative or cognitive symptoms of schizophrenia, which are thought to be the core symptoms of the disease (Blin 1999). In addition, although dopamine agonists such as amphetamine do produce a form of psychosis when administered to healthy controls, this psychosis generally only represents positive symptoms, and in some cases dopamine agonists can actually *improve* negative and cognitive symptoms when administered to schizophrenia patients (Angrist et al. 1982; Kirrane et al. 1996). Therefore, although dopamine does appear to play a role in schizophrenia, it cannot explain the complete schizophrenia pathology.

A more recent hypothesis of schizophrenia arose following the introduction of the atypical antipsychotic clozapine. This drug was considered to be a more effective treatment for schizophrenia than the traditional typical antipsychotics, as in some

patients it treated positive as well as negative symptoms, while also providing somewhat effective treatment to otherwise antipsychotic-unresponsive patients. Clozapine was shown to have low dopamine D2 antagonism, but high serotonin receptor 2 (5-hydroxy-tryptamine2; 5-HT2) antagonism, leading researchers to believe that serotonin could play a major role in the pathophysiology of schizophrenia. In support of this hypothesis, lysergic acid diethylamide (LSD), a serotonin agonist, was shown to produce both positive and negative symptoms in healthy non-schizophrenic humans (Breier 1995). However, a prominent feature of LSD psychosis is visual hallucinations, which are reported to be quite rare in schizophrenia (Jones and Blackburn 2002). Furthermore, LSD does not precipitate symptomatology when administered to schizophrenia patients (Cohen et al. 1962), thereby reducing the validity of this as a schizophrenia model.

1.4 NMDA hypofunction hypothesis of schizophrenia

The advancement in schizophrenia hypotheses came about with the discovery of phencyclidine (PCP). PCP is a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. The NMDA receptor is an ionotropic receptor, an ion channel, which is permeable to Ca^{2+}, Na^{2+}, and K^{+}. PCP binds to a site within the channel pore that is only accessible when the channel is open (Fig. 1.1). In addition to the NMDA receptor, PCP also interacts with the sigma receptor, muscarinic and nicotinic acetylcholine receptors, and the dopamine and noradrenaline transporters (Morris et al. 2005). However, PCP's direct effects on these other systems are less potent than its action on the NMDA receptor, and it has been suggested that PCP's effects on these non-NMDA systems may not to be a major component of its molecular action (Javitt and Zukin 1991). However, actions at these other sites may contribute, at least partially, to the unique psychotogenic properties of PCP.

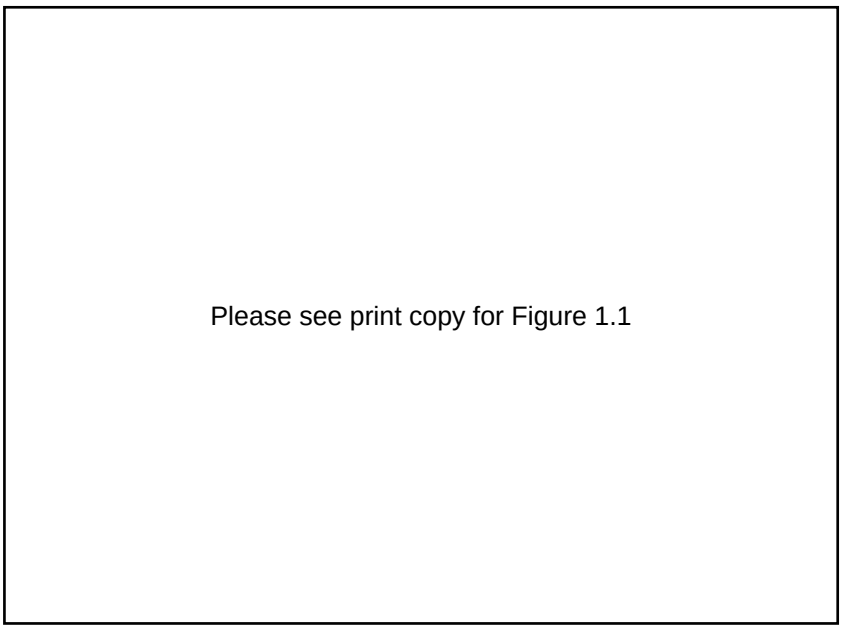

Figure 1.1 Schematic of the NMDA receptor. Adapted from Feldman and Quenzer (1984).

PCP has been shown to mirror the symptomatology of schizophrenia in humans almost incomparably. It induces a form of psychosis that not only mimics the negative and positive symptoms of the disease but also the cognitive deficits. PCP produces not only an acute psychotic reaction in normal humans but also "flashback" recurrences of that same psychosis (Allen and Young 1978). In addition, long lasting cognitive impairments in episodic memory and attention have been reported in recreational users of the PCP analogue ketamine (also a non-competitive NMDA antagonist; Morgan et al. 2004). Furthermore, PCP has been shown to exacerbate symptoms in chronic stabilized schizophrenia patients (Morris et al. 2005). This PCP-induced psychosis can mimic schizophrenia symptoms to the extent that chronic PCP users have previously been misdiagnosed as having schizophrenia. However, it is interesting to note that this PCP-induced psychosis has only been reported in adults. It has been suggested that children exposed to PCP do not develop this psychosis (Farber et al. 1995). Strengthening this suggestion, studies have shown that children do not develop psychosis following

administration of ketamine (White et al. 1982). This is particularly relevant since the onset of schizophrenia is not until late adolescence/early adulthood and suggests that there may be a common mechanism underlying PCP-induced psychosis and schizophrenia.

Ketamine, although not as potent as PCP, does produce schizophrenia-like psychosis in adult humans (Krystal et al. 1994), as do competitive NMDA receptor antagonists (that compete with glutamate by binding to the glutamate binding site on the NMDA receptor), such as CGS 19755 (Grotta et al. 1995). Therefore, it is widely accepted that the psychotomimetic action of PCP is due, at least partly, to the blockade of the NMDA receptor-ion channel complex and not just for agents that activate PCP receptors. However, there are also likely to be effects downstream from the NMDA receptor blockade which may contribute to the schizophrenia-like psychosis.

This discovery of NMDA antagonist-induced psychosis led to the NMDA receptor hypofunction hypothesis of schizophrenia, which suggests that a decrease in function of the NMDA subtype of glutamate receptor may underlie the symptomatology of schizophrenia (Olney and Farber 1995).

It has been suggested that chronic use of PCP and other NMDA antagonists more accurately represents schizophrenia symptomatology than acute use, as it is more likely to lead to a schizophrenia-like psychosis than is a single exposure (Jentsch and Roth 1999). In accordance with this idea, chronic ketamine treatment has been associated with decreased frontal blood flow in humans (Hertzmann et al. 1990), which is reportedly reminiscent of schizophrenia (Andreasen et al. 1992), while acute exposure has been associated with *increased* frontal blood flow (Breier et al. 1997).

Schizophrenia is believed to be caused by a combination of genetic and environmental factors and it has been suggested that complications at birth could be an

environmental participant, as birth complications have been reported in schizophrenia patients (Jacobsen and Kinney 1980; Kotlicka-Antczak et al. 2001). Furthermore, complications at birth such as hypoxia/ischemia and ethanol exposure have been reported to compromise NMDA receptor function, further supporting the NMDA hypofunction hypothesis of schizophrenia. Studies have reported that NMDA receptor binding in humans shows age related increases of greater than 100% in the first 26 weeks of life suggesting that the numbers of NMDA receptors increase during postnatal brain development. Furthermore, morphological studies have shown that this rapid growth rate in early postnatal life is counteracted in adolescence when many of the overproduced synapses are eliminated (Purves and Lichtman 1980; Huttenlocher 1990). Therefore, it is possible that in schizophrenia, NMDA receptors are vulnerable at these crucial stages of development, possibly explaining this delayed onset of schizophrenia.

One of the best confirmations of the glutamate hypothesis of schizophrenia would be to demonstrate that glutamate receptor agonists have antipsychotic properties. However, unphysiologic concentrations of glutamate cause neurotoxicity and neuronal death (Danbolt 2001). Therefore direct glutamate agonists are not acceptable as a treatment for schizophrenia. On the other hand, the use of glycine agonists in order to increase NMDA receptor function, when combined with antipsychotic drugs, has been shown to improve both positive and negative symptoms of schizophrenia (Tsai et al. 1998; Goff et al. 1999). Furthermore, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptor agonists (AMPAkines) have been shown to have therapeutic potential in treating schizophrenia also (Goff et al. 2001).

1.5 Rodent NMDA receptor hypofunction model

Since PCP abuse produces schizophrenia-like psychosis, many researchers have investigated the effects of PCP and other NMDA receptor antagonists in humans and animals, in order to gain insight into this phenomenon. Studies in rats have shown that a single subcutaneous treatment with a relatively low dose of either PCP or dizocilpine (MK-801; a non-competitive NMDA antagonist) caused reversible neuronal injury (intracytoplasmic vacuole formation) confined to pyramidal neurons in the posterior cingulate/retrosplenial cortex (PCC/RSC). In addition, low-dose NMDA antagonist treatment has been shown to trigger abnormal expression of heat shock protein (an indicator of neuronal stress) in PCC/RSC neurons in adult rat brain (Olney et al. 1989; Sharp et al. 1994). High doses of PCP trigger abnormal heat shock protein expression, not only in PCC/RSC, but also in many other corticolimbic brain regions, and neurons in all of these regions are sometimes killed by a high dose or prolonged dosing regimens of PCP (Sharp et al. 1992; Ellison and Switzer 1993; Ellison 1994). Furthermore, it was discovered that PCP and other NMDA receptor antagonists do not produce this injury in juvenile rats (Farber et al. 1995), supporting this as an animal model of schizophrenia.

In rodents, PCP induces a characteristic syndrome of behaviours that are reportedly analogous to the symptoms of schizophrenia as presented in Table 1.1. Several of these behaviours are observed in the short-term following treatment (eg hyperlocomotion), while others are commonly observed in the long-term (eg cognitive deficits). For example, a study by Jentsch et al. (1997b) showed that monkeys treated chronically with PCP developed cognitive deficits that were still present at least 4 weeks after PCP withdrawal, while Noda et al. (1995) showed that increased immobility time in swimming tests were still present 3 weeks after chronic PCP withdrawal.

Table 1.1 Parallels between the behavioural effects of PCP in rodents and schizophrenia symptoms

Please see print copy for Table 1.1

A range of biological effects are also seen in rodents treated with PCP, many of which are reminiscent of those seen in schizophrenia. Among these biological effects are altered neurotransmission (Bowers and Hoffman 1984; Jentsch et al. 1997c), neurotransmitter receptor levels (Gao and Tamminga 1994; Hori et al. 2000), and receptor mRNA expression (Wang et al. 1999). However, many of these biochemical alterations have only been studied in the short-term following treatment, leaving the long-term consequences unknown. In addition, many of these biological effects differ depending on whether the treatment is acute or chronic. Jentsch and Roth (1999) suggest that chronic PCP administration in animals provides a more accurate model of schizophrenia than the acute model since it not only produces more persistent schizophrenia-like symptoms, but also because the biological effects produced in the chronic model tend to be more reminiscent of those reported in schizophrenia. For example, studies have shown that acute PCP administration to rodents increases forebrain dopaminergic transmission (Bowers and Hoffman 1984; Jentsch et al. 1997a), while chronic PCP administration has been shown to *reduce* frontal dopaminergic

transmission in rodents (Jentsch et al. 1997c), in parallel to what has been reported in the cortex in schizophrenia (Dolan et al. 1995; Knable and Weinberger 1997).

In rodents, there are numerous different substances that can prevent PCP-induced neurotoxicity (vacuolization and heat shock protein expression) and psychosis, including clozapine, haloperidol, gamma-aminobutyric acid (GABA) agonists and muscarinic M1 antagonists (Olney et al. 1991; Geyer and Ellenbroek 2003). Therefore, it seems unlikely that the NMDA receptor blockade itself is the only cause of the neurotoxicity and behavioural symptoms. There may be a cascade of events involving numerous different systems. For example, it has been shown that a reduction in NMDA receptor activity, results in a reduction of GABA activity, which may contribute to the neurotoxicity or symptomatology (Olney et al. 1991; Konradi and Heckers 2003).

Based on rodent studies, Olney and Farber (1995) have proposed a disinhibition mechanism underlying NMDA receptor hypofunction-induced changes. They suggest that under normal conditions NMDA receptors located on GABAergic and serotonergic neurons maintain tonic inhibition over excitatory inputs to primary neurons in several corticolimbic brain regions especially the PCC/RSC. These excitatory inputs include glutamate from the anterior thalamus, and acetylcholine from the basal forebrain. Olney and Farber further suggest that NMDA antagonists eliminate this inhibition, resulting in the excessive release of glutamate and acetylcholine. They propose that it is the excessive stimulation of muscarinic as well as glutamatergic receptors on the vulnerable neurons that results in the observed NMDA receptor hypofunction-induced neurotoxicity (Fig. 1.2; Farber et al. 1995; Farber et al. 2003). Therefore, it may not just be NMDA receptor hypofunction that induces schizophrenia-like symptomatology and pathophysiology but NMDA hypofunction coupled with secondary cholinergic and glutamatergic hyperfunction.

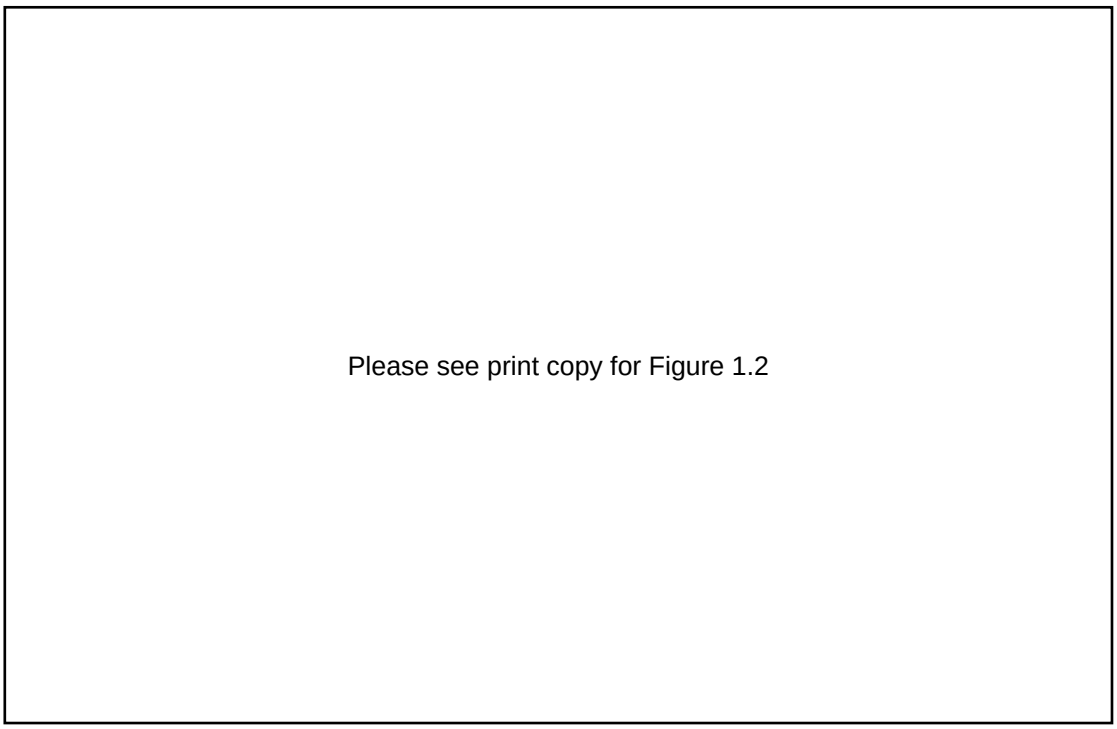

Figure 1.2 Model of NMDA hypofunction in the posterior cingulate cortex (PCC) and its associated psychosis circuit. Modified from Farber (2003). The concept presented here shows that glutamate acting on NMDA receptors located on GABAergic and serotonergic neurons maintains inhibitory control over glutamatergic output from the anterior thalamus, and cholinergic output from the basal forebrain. NMDA hypofunction can abolish this inhibition resulting in excessive output from the anterior thalamus and basal forebrain resulting in overactivity of the corresponding glutamatergic and muscarinic receptors on PCC pyramidal neurons. This is thought to result in excessive glutamate release from the PCC neurons causing neurotoxicity. $5HT_{2A}R$: $5HT_{2A}$ receptor, G_AR: $GABA_A$ receptor, GluR: glutamate receptor, mR: muscarinic receptor, NR: NMDA receptor.

The NMDA receptor hypofunction hypothesis of schizophrenia is one of the most comprehensive schizophrenia hypotheses to date. However, although this hypothesis may explain more symptoms of schizophrenia than traditional hypotheses, it can not and does not rule out multifactorial causes of schizophrenia.

1.6 Why Research Schizophrenia?

Despite over 100 years of research, the aetiology of schizophrenia is still unknown. This disease affects approximately 1% of the worldwide population. Ten per cent of patients commit suicide and a further 30% attempt suicide at least once (NISAD 2004). In Australia, schizophrenia costs approximately $2 billion per year in direct health costs (eg. hospitalization, medication, therapy etc.), and loss of productivity

(NISAD 2004), making it one of the most expensive health issues in Australia. In addition, there are numerous indirect costs associated with this disease. It often leads to long-term disability, unemployment, drug and alcohol abuse, family trauma, homelessness, crime and imprisonment, adding to the enormous cost of this disease. Furthermore, there is still no successful treatment for schizophrenia. Clozapine is considered to be one of the most effective treatments to date, however it is not effective in all patients, and due to its serious side-effects (obesity, agranulocytosis, new-onset diabetes mellitus, increased plasma lipids, and sudden death), patients need to be monitored closely, and may not be able to be treated with this drug for long periods of time (Meltzer 2004). Furthermore, the current antipsychotics provide inadequate treatment of negative and cofnitive schizophrenia symptoms. Therefore, it is important not just scientifically, but also socially and economically, to define the neurobiological substrates of this disease.

Chapter 2: Review of the literature

2.1 General Introduction

Research into schizophrenia in human subjects as well as in animal models has identified specific brain regions that are thought to contribute to the pathology of schizophrenia. One region in particular, the PCC has been implicated in recent years. The PCC is a limbic region involved in memory and spatial orientation, and in rodents is the primary brain region affected by PCP treatment. PCP-induced psychosis and schizophrenia are both thought to produce their symptomatology via abnormal neurotransmission. Several neurotransmitter systems have been implicated including glutamate, GABA, acetylcholine, serotonin, and more recently the endogenous cannabinoid system. Before describing detailed experimental studies of this thesis, it is necessary to review the literature in relation to the PCC, the above neurotransmitters, and their involvement in schizophrenia and NMDA hypofunction. Therefore, the literature related to the following topics will be reviewed and presented in this chapter:

(1) The posterior cingulate cortex

(2) Glutamate

(3) GABA

(4) Acetylcholine

(5) Serotonin

(6) Cannabinoid

2.2 The Posterior Cingulate Cortex

The cingulate cortex, along with the hippocampal and parahippocampal cortices, is defined by Broca as the limbic lobe. It is contained within a gyrus on the medial surface of each hemisphere bordering on the corpus callosum and forms the largest part of the limbic system (Fig. 2.1). The cingulate cortex is a heterogeneous structure with respect to its cytoarchitecture, function, and chemoarchitecture. It can be divided into anterior and posterior regions where the anterior cingulate cortex lacks a granular layer IV and has a prominent layer Va, while the posterior cingulate is granular (Tamminga et al. 2000). Further differences between anterior and posterior divisions are outline in Table 2.1.

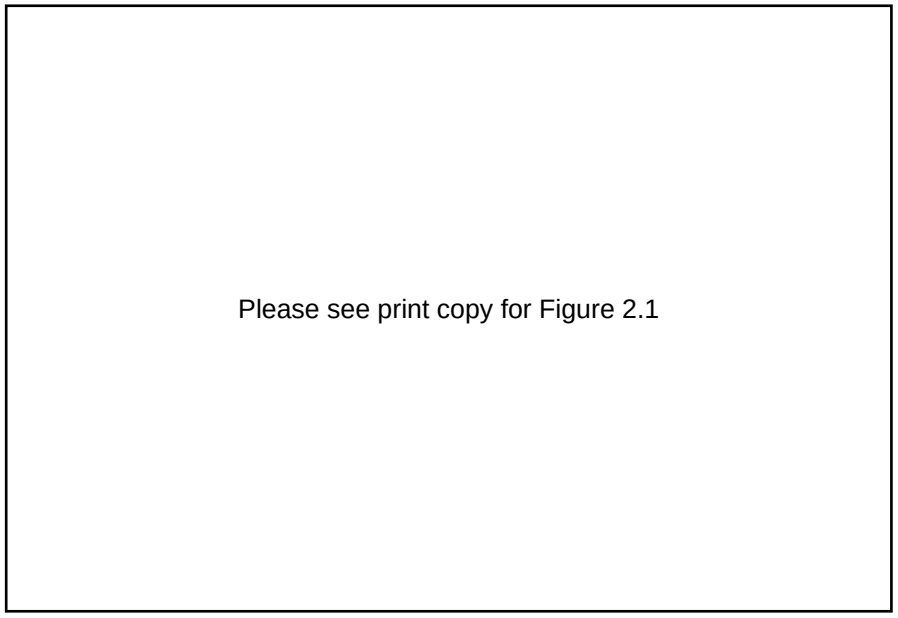

Figure 2.1 A medial view of the human right hemisphere of the brain showing the cingulate gyrus in yellow, including anterior (ACC) and posterior (PCC) divisions. Image adapted from Csernansky (No date).

Table 2.1 A comparison of the anterior cingulate cortex and posterior cingulate cortex

Please see print copy for Table 2.1

2.2.1 Relevance to Schizophrenia

Recent magnetic resonance imaging (MRI) data has consistently shown that the PCC is a site of pathology in schizophrenia, as demonstrated by: (1) a decreased BOLD signal response in the PCC after ketamine administration, during the retrieval of episodic memory, which correlates with positive schizophrenia-like symptoms (Northoff 2004); (2) an inability of schizophrenia patients to produce an increase in blood flow to the PCC in response to a recognition memory test, compared to healthy volunteers (Crespo-Facorro et al. 2001); (3) a reduction in metabolic rate and volume in the PCC of schizophrenia patients compared to controls (Pantelis et al. 2003; Haznedar 2004; Mitelman 2004; Zhou et al. 2005; Shimizu et al. In Press); and (4) impaired PCC functionality in a semantic task in schizophrenia patients, which relates to verbal memory deficits that are frequently observed in schizophrenia patients (Tendolkar et al.

2004). Despite this large body of new imaging data, cellular and molecular studies of the PCC in schizophrenia remain largely unexplored. A recent study by Katsel et al. (2005) which examined gene expression patterns in schizophrenia, found that the PCC in addition to the anterior cingulate cortex and superior temporal gyrus, showed the greatest number of transcripts with altered expression in schizophrenia. The authors suggested that these results indicate that the PCC, as well as the anterior cingulate and superior temporal cortices, are sites of particular vulnerability in schizophrenia, further implicating the PCC in the pathophysiology of this disease.

Studies in rodents demonstrate structural damage, reversible neuronal alteration, neural disinhibition, and heat shock protein expression in the PCC during systemic administration of NMDA receptor antagonists (Sharp et al. 1994), indicating the importance of the PCC in NMDA hypofunction. Further indicating the importance of the PCC in NMDA hypofunction, a recent imaging study demonstrated a relationship between NMDA receptors, the PCC, and positive symptoms in humans (Northoff 2004).

While many people propose that a particular brain region is the primary site of pathology in schizophrenia, Moghaddam (2003) suggests that because of the large range of symptoms experienced by schizophrenia patients, that there must be malfunctions in most, if not all, frontal cortical systems, the limbic system, the basal ganglia and the thalamus. Therefore it is important to examine each of the above regions in order to understand the fundamental changes occurring in the schizophrenia brain. With the unraveling of information regarding those vulnerable regions, it is expected that we would gain a better understanding of the pathology of schizophrenia and how each region is affected.

It is generally accepted that an imbalance of neurotransmission contributes to the pathology of schizophrenia; however it is not known how this imbalance is manifested in individual brain regions. To date, virtually no data is available regarding the status of glutamate, muscarinic, GABA, serotonin, or cannabinoid receptors in the PCC in schizophrenia. It is known however that the PCC does receive a vast array of neurochemical input. It receives glutamatergic fibers primarily from the anterior thalamus (Gonzalo-Ruiz et al. 1997), cholinergic fibers from the nucleus basalis of Meynert (Mesulam 2004), and serotonergic fibers from the raphe nucleus (Kosofsky and Molliver 1987). Studies have shown that receptors for these systems are abundant in the PCC, in addition to receptors for other systems including GABA and cannabinoid (Vogt et al. 1990; Glass et al. 1997). In addition to the lack of data regarding these systems in the PCC in schizophrenia, there is limited information as to the status of these neurotransmitter systems in the long-term following NMDA hypofunction. Therefore, these key neurotransmitter systems will be now be reviewed.

2.3 Glutamate

Glutamate is the major excitatory neurotransmitter in the human brain. Glutamate plays a critical role in neuroplasticity, neurotoxicity and neuronal death (Ozawa et al. 1998). It mediates its excitatory neurotransmission through the actions of two major types of glutamate receptors: the ionotropic receptors and the G-protein coupled metabotropic receptors. The ionotropic glutamate receptors are divided into three subtypes: NMDA, AMPA, and kainate. These receptors are critical in functions such as learning and memory, as well as early brain development including synapse formation, maintenance and plasticity (Hollmann and Heinemann 1994). All three ionotropic receptor channels are permeable to Na^+ and K^+. NMDA receptors however are also highly permeable to Ca^{2+}. While the glutamate receptors are very important for

the normal physiology of brain function, too much activity can cause neuronal death due to excessive Ca^{2+} entry into the cell (Konradi and Heckers 2003).

The ionotropic receptors are composed of 4 or 5 subunits that form ligand-gated ion channels. The NMDA receptor comprises two subunits that are coded by genes designated NR1 and NR2A-D. There is a site for the binding of glutamate and competitive antagonists of the NMDA receptor. A separate glycine-binding site must also be occupied before glutamate can activate the ion channel. There is also a site within the ion channel itself associated with the binding of non-competitive antagonists of the NMDA receptor, such as PCP (Fig. 1.1). Non-NMDA ionotropic glutamate receptor subunits comprise GluR1-4, which are AMPA preferring and GluR5-7, KA1 and KA2, which are kainate preferring (Konradi and Heckers 2003).

At physiological concentrations, NMDA receptor channels are bound with magnesium, which needs to be removed to allow ion influx. AMPA receptor-mediated depolarization of the membrane removes magnesium from the NMDA receptor and allows ion influx through NMDA receptors. Synapses that have only NMDA receptors, but not AMPA receptors, are called 'silent synapses' as they have no electrophysiological response to glutamate under normal resting potentials (Huntley et al. 1994). Thus, NMDA and AMPA receptors are predominantly postsynaptically located and coexist at many synapses. Kainate receptors however are primarily presynaptic and play a role in the regulation of glutamate release. Activation of presynaptic kainate receptors facilitates glutamate release (Konradi and Heckers 2003). Ionotropic glutamate receptors are present extensively throughout the brain with highest densities being reported in the hippocampus (Huntley et al. 1994).

2.3.1 Schizophrenia

Glutamate was first proposed to be involved in schizophrenia in 1980, when a study showed low glutamate in cerebrospinal fluid samples from schizophrenia patients compared to controls (Kim et al. 1980). Although this finding has not been replicated, there is now growing evidence implicating glutamatergic dysfunction in the pathophysiology of schizophrenia. Several post-mortem studies have reported region-specific changes in glutamate receptor protein binding and subunit expression in several cortical and subcortical regions in schizophrenia (Kornhuber et al. 1989; Aparicio-Legarza et al. 1998; Healy et al. 1998; Gao et al. 2000; Ibrahim et al. 2000; Meador-Woodruff and Healy 2000; Dracheva et al. 2001; Zavitsanou et al. 2002). In addition, the suspected involvement of NMDA receptor hypofunction in schizophrenia has also emphasized the potential importance of glutamatergic dysfunction in schizophrenia (see sections 1.4 and 1.5). Recent studies have shown increased activity of the glutamate synthesizing enzyme, phosphate activated glutaminase, in the cortex of schizophrenia patients (Gluck et al. 2002). Consistent with this, Benes et al (1992a) have reported substantially greater glutamate immunoreactivity in anterior cingulate cortex axons in schizophrenia subjects compared to controls

Unfortunately, we cannot treat schizophrenia subjects with direct glutamate agonists because of the potential for injury (and therefore, cannot directly test the glutamate hypothesis in this manner). Current schizophrenia treatments however, have been shown to affect the glutamate system, albeit indirectly (Table 2.2), suggesting that their effects on the glutamate system may contribute to their therapeutic effect. However, the reported effects do vary with treatment drug, dosage and duration.

Table 2.2 The effects of antipsychotic drug treatment on glutamate receptor binding in rodent brain

Please see print copy for Table 2.2

In addition to the above data, chronic haloperidol treatment has been shown to inhibit the glutamate transporter in rat striatum (De Souza et al. 1999), and increase the basal concentration of extracellular glutamate (Yamamoto and Cooperman 1994). Likewise, acute clozapine treatment has been shown to increase the release of glutamate in the rat prefrontal cortex (Daly and Moghaddam 1993) and alter glycine levels in rat striatum (Chapman and See 1996).

Clozapine and haloperidol both have D2 receptor antagonist properties. Mackay et al (1982) suggest that the effectiveness of D2 receptor antagonists in treating schizophrenia may be through their effect on glutamate release. In some brain areas presynaptic D2 receptors act as heteroceptors regulating glutamate release in a negative feedback manner (Wang and Pickel 2002; Hatzipetros and Yamamoto 2006). Therefore D2 receptor antagonists would be expected to increase glutamate release by blocking this negative feedback. In addition, D2 receptors act as autoreceptors regulating dopamine release. Recent research has shown that blocking D2 receptors results in an increase in dopamine relase, thus activating postsynaptic D1 receptors. This activation of D1 receptors has been shown to enhance NMDA receptor mediated currents. Therefore, it has been hypothesized that clozapine and haloperidol may induce their effects on the glutamatergic system via blocking the D2 receptor, and activating the D1 receptor (David et al. 2005). Clozapine is also thought to affect the glutamatergic system via its serotonergic or muscarinic properties. However, studies suggest that these drugs actually have a direct effect on the glutamatetergic system by acting as partial agonists at the glycine site on the NMDA receptor (Fletcher and MacDonald 1993).

2.3.2 NMDA receptor hypofunction

The NMDA receptor hypofunction hypothesis of schizophrenia has attracted significant attention in this research field. Moghaddam et al (1997) showed that administration of ketamine results in increased glutamate release in the rat prefrontal cortex. Studies showing the effects of NMDA receptor antagonists on NMDA glutamate receptor binding primarily focus on the short-term effects following treatment, leaving the long-term effects unknown. Results of these studies are varied depending on the labeling ligand used, the treatment duration, the treatment dose, and the brain regions examined (Table 2.3). It is surprising that although the PCC is the prime target region of

PCP treatment, virtually no study has reported the effects of PCP treatment on NMDA receptor binding in this region.

Table 2.3: The effects of NMDA antagonist treatment on glutamate receptor binding in rodent brain

Please see print copy for Table 2.3

Although in recent years it is commonly the NMDA receptor that is implicated in schizophrenia, disorders of other glutamate receptors could produce the appearance of an abnormally functioning NMDA receptor. For example, at physiologic concentrations NMDA receptors are blocked by magnesium. AMPA receptor-mediated depolarization of the membrane removes magnesium from the NMDA receptor, and allows the NMDA receptor to bind glutamate (Konradi and Heckers 2003). Therefore, abnormalities in the AMPA receptor could present as an abnormality of the NMDA receptor. Therefore non-NMDA glutamate receptors should be considered just as important as the NMDA receptor in the study of schizophrenia. As such, a study examining the effects of PCP treatment on AMPA receptor binding showed widespread reductions in binding of this receptor in rat brain, suggesting that hyperactivity of the AMPA receptor may contribute to PCP's neurotoxicity (Ellison et al. 1999a). In addition, AMPA receptor antagonists have been shown to inhibit PCP-induced neurotoxicity (Sharp et al. 1995), further highlighting the importance of non-NMDA glutamate receptors in NMDA hypofunction.

2.4 GABA

GABA is the major inhibitory neurotransmitter in the brain. It is synthesized from glutamate with the enzyme glutamic acid decarboxylase (GAD). There are two receptors that mediate GABA neurotransmission in the central nervous system (CNS); $GABA_A$ and $GABA_B$ (Matsumoto 1989). $GABA_A$ receptors are ligand gated chloride ion channels (Steiger and Russek 2004), while $GABA_B$ receptors are G-protein coupled (Kerr and Ong 1995). In addition to the GABA binding site, the $GABA_A$ receptor may also contain binding sites for benzodiazepines, barbiturates and convulsants (Matsumoto 1989). The presence of at least 2 different recognition sites on the $GABA_A$ receptor is suggested by biochemical data, which shows binding at the $GABA_A$ receptor to be

biphasic. High affinity sites can be labeled with [^{3}H]muscimol and are thought to be associated with the GABA recognition site. The low affinity sites, on the other hand, can be labeled with [^{3}H]bicuculline + thiocyanate, and are thought to be associated with the benzodiazepine binding site (Matsumoto 1989).

The $GABA_A$ receptor is believed to have a subunit structure of $\alpha_2\beta_2\gamma$ that is centered around a Cl^- ionophore (Steiger and Russek 2004). The binding of GABA and the GABA agonist muscimol, to recognition sites on the $GABA_A$ receptor opens Cl^- channels in the cell membrane, and thus inhibits the post-synaptic neuron (Steiger and Russek 2004). $GABA_A$ receptors are found throughout the CNS but are especially high in the superficial layers of the cortex (layers I-IV), thalamic nuclei, and the cerebellum of rat brain (Palacios et al. 1981). Inhibitory inputs to GABAergic interneurons can originate from other GABAergic neurons in the cortex, or from external sources such as nucleus basalis in the basal forebrain (Benes and Berretta 2001).

2.4.1 Schizophrenia

GABA has been implicated in schizophrenia from as early as 1980 when, like glutamate, low cerebrospinal fluid levels of GABA were found in schizophrenia patients (Van Kammen et al. 1980). Since then, studies have continued to provide strong evidence of GABAergic hypofunction in the schizophrenia brain. Reductions in GABA in nucleus accumbens and thalamus have been reported in schizophrenia (Perry et al. 1979). Decreased mRNA expression of the 67kDa isoform of glutamate decarboxylase (GAD67), the enzyme responsible for synthesizing GABA, has been consistently reported in the prefrontal cortex in schizophrenia (Akbarian et al. 1995b; Guidotti et al. 2000), as has increased binding of the $GABA_A$ receptor in several brain regions (Hanada et al. 1987; Benes et al. 1992b; Benes et al. 1996). In addition, studies have shown reductions in mRNA expression of the GABA membrane transporter (GAT1;

Ohnuma et al. 1999), and reductions in density of GABA uptake sites in the prefrontal cortex, amygdala, and hippocampus in schizophrenia (Simpson et al. 1989; Reynolds et al. 1990).

It has been suggested that both the increase in postsynaptic $GABA_A$ receptors and the decrease in presynaptic GAT1 reported in schizophrenia are possibly compensatory responses to a primary deficit in GAD67 mRNA expression (Lewis et al. 2005). In support of this, studies have shown no change in GABA subunit mRNA expression in schizophrenia (Akbarian et al. 1995a), suggesting that levels of $GABA_A$ receptors, as seen in radioligand binding, may be controlled by mechanisms downstream from GABA gene transcription and mRNA turnover (Kalkman and Loetscher 2003). On the other hand, there is evidence to suggest that alterations in $GABA_A$ receptors may occur independently of changes in GAD67mRNA, eg loss of neurons (Benes et al. 1991; Reynolds et al. 2001).

The reported deficit in GABA does not appear to be specific to schizophrenia, but common to several psychiatric disorders. Decreased GAD67 mRNA is not only found in schizophrenia but also in bipolar disorder (Guidotti et al. 2000). Furthermore, reduced concentrations of GABA have been reported in nucleus accumbens and the thalamus of Huntington's Chorea patients (Perry et al. 1979).

Considering that there is strong evidence of GABAergic hypofunction in the schizophrenia brain, it is unexpected that drugs, such as benzodiazepines, which increase $GABA_A$ receptor function, do not have much therapeutic effect (Coyle 2004). Current schizophrenia treatments do not specifically target the GABAergic system, however there are likely to be downstream effects on this system as studies do show alterations in GABAergic markers in antipsychotic drug treated rodents (Table 2.4).

Table 2.4: The effects of antipsychotic drug treatment on the GABAergic system in rodent brain

Please see print copy for Table 2.4

Based on these studies it appears that antipsychotic drug treatment can not explain the low levels of GAD67 reported in psychotic disorders, since drug therapy, if anything causes an increase in enzyme level. However, depending on the dosage and duration of antipsychotic drug treatment, it appears that its effects on $GABA_A$ receptor binding are varied. Lower antipsychotic drug doses coupled with shorter treatment durations appear to reduce $GABA_A$ receptor binding while higher doses coupled with extended treatment durations tends to increase binding.

2.4.2 NMDA receptor hypofunction

Olney et al. (1999) have suggested that GABA plays a major role in the mechanism of NMDA hypofunction-induced neurotoxicity, via NMDA-induced disinhibition of GABA. They suggest that it is the elimination of GABAergic inhibition that causes NMDA antagonists to excessively release glutamate and acetylcholine, resulting in neurotoxicity (Fig. 1.2).

In rodent models of NMDA receptor hypofunction, GABA-receptor agonists e.g. muscimol, have been found to prevent the neuronal vacuoles produced by PCP, ketamine and MK-801 (Olney et al. 1991; Farber et al. 2003). This is reportedly mediated by $GABA_A$ but not $GABA_B$ receptors. Systemic application of GABAergic agents, such as muscimol, also reverses the excessive release of neurotransmitter (Kim et al. 1999), and the neurotoxic action of NMDA antagonists (Olney et al. 1991; Sharp et al. 1994). Furthermore, GABAergic agents block the psychotomimetic actions of ketamine in humans (Knox et al. 1970; Bovill et al. 1971). This data suggests that GABA may play an important role in both the behavioural and biological effects of NMDA receptor antagonists.

There appear to be several similarities between the effects of NMDA receptor antagonists on the GABAergic system in rodent brain and changes in the GABAergic system reported in schizophrenia brain. NMDA receptors have been shown to modulate GAD67 expression as shown through rodent models. For example, MK-801 treatment has been shown to reduce GAD67 expression in dorsal and ventral striatum, olfactory tubercle, septum, and frontal and parietal cortices in rodents (Qin et al. 1994; Paulson et al. 2003). Similarly, reduced GAD67 mRNA has consistently been reported in the prefrontal cortex in schizophrenia (Akbarian et al. 1995b; Guidotti et al. 2000). In addition, it has recently been reported that chronic treatment with MK-801 results in a down-regulation of the expression of GAT1 in the frontal cortex of rats (Paulson et al. 2003). Furthermore, a study on rat brain slices demonstrated that in the limbic cortex, NMDA receptors on GABAergic neurons were more sensitive to the antagonistic effects of MK-801 as compared to NMDA receptors on pyramidal neurons (Li et al. 2002). This data further supports a role of GABA in NMDA receptor hypofunction, and

supports NMDA receptor hypofunction as a possible underlying mechanism contributing to schizophrenia.

2.5 Acetylcholine

Acetylcholine is a neurotransmitter that functions primarily in learning, memory, attention and motor control (Volpicelli and Levey 2004). The basal forebrain, including the nucleus basalis of Meynert, is the primary source of cholinergic afferents. Cholinergic afferents from the basal forebrain project to all layers of the cerebral cortex, and synapse with both GABAergic interneurons and pyramidal cells, accounting for 70-80% of cortical cholinergic input (Mesulam 1995). Approximately 20-30% of the cholinergic innervation of the cerebral cortex may derive from intrinsic cholinergic neurons (Johnston et al. 1981). There are two types of acetylcholine receptors, the nicotinic receptors, which are ion channels, and the muscarinic receptors, which are G-protein coupled.

There are 5 muscarinic cholinergic receptors, which mediate either excitatory or inhibitory neurotransmission. M1, 3, and 5 are reported to preferentially activate phospholipase C, whereas M2, and 4 inhibit adenylyl cyclase activity. Muscarinic receptors of the M1 subtype are primarily reported to be localised postsynaptically and are the most abundant of the muscarinic receptors in the cortex (Levey 1996; Mash et al. 1998). The M1 receptor has been shown to be localised on both cortical pyramidal cells and on cortical GABAergic interneurons. In contrast, M2 receptors are predominantly located presynaptically at the axonal terminals of the cholinergic neurons, where they inhibit acetylcholine release (Mash et al. 1985; Hoss et al. 1990; Billard et al. 1995). M4 receptors are postsynaptically located in the cortex (Levey et al. 1991). M1, 2, and 4 receptors are also located in other brain regions including the striatum, hippocampus, and thalamus (Hyde and Crook 2001).

The nucleus basalis of Meynert, located in the basal forebrain is about 90% cholinergic and is a major source of cholinergic input to the cortex (Mesulam et al. 1983). In the primate nucleus basalis there are acetylcholine, dopamine, GABA, serotonin and noradrenaline synapses on cholinergic cell bodies suggesting that multiple neurotransmitter systems can influence cholinergic outflow (Smiley and Mesulam 1999; Smiley et al. 1999). For example, studies show that dopamine release in nucleus accumbens acts to reduce the activity of inhibitory GABAergic efferents to the basal forebrain and therefore increase acetylcholine release from the basal forebrain (Moore et al. 1999). In addition, it has been shown that applying the $GABA_A$ agonist muscimol to the nucleus basalis of rats causes a striking decrease in the release of acetylcholine in the cerebral cortex (Casamenti et al. 1986).

There appears to be a strong relationship between the muscarinic and GABAergic systems. In addition to the above evidence, cortical GABAergic neurons have been found to receive the richest cholinergic innervation of all cortical cell types with numerous GABAergic interneurons containing muscarinic receptors (van der Zee and Luiten 1999). Moreover, more than half of all cholinergic local circuit neurons have been found to be immunoreactive for GAD (van der Zee and Luiten 1999). Further support for a relationship between the cholinergic and GABAergic systems is evidenced by studies on rat cortical slices showing that stimulation of cortical M1 muscarinic receptors inhibits GABA release (Hashimoto et al. 1994). On the other hand however, one in vivo study suggested that M1 receptors may actually *increase* extracellular concentrations of GABA as well as glutamate in rat medial prefrontal cortex (Sanz et al. 1997). It has also been suggested that M2 receptors inhibit GABA release in the striatum (Marchi et al. 1990; Raiteri et al. 1990) and the thalamus (Rowell et al. 2003). Further research is needed to determine the role muscarinic receptors play on GABA

release. It is possible that muscarinic receptors play different roles in various brain regions.

Cortical cholinergic innervation and muscarinic receptor density show an age-related decline in both humans and rodents (Geula and Mesulam 1989; Norbury et al. 2005; Tayebati et al. 2006). M1 and M2 receptors specifically have been shown to be reduced in striatum, nucleus accumbens, diagonal band of Broca, thalamus, septum, and frontal and parietal cortices in old rats compared to young rats (Narang 1995). As one of the major functions of acetylcholine is in memory, this age-related decline may provide an anatomical substrate for age-related changes in memory function.

2.5.1 Schizophrenia

Evidence suggests that both muscarinic and nicotinic acetylcholine receptors may play an important role in the neural mechanisms underlying the pathophysiology of schizophrenia, as there is an association of muscarinic and nicotinic receptor activity with the modulation of certain brain functions (cognition, attention, memory, motor control) that are altered in schizophrenia (Paterson and Nordberg 2000; Hyde and Crook 2001). It has been hypothesized that increased muscarinic cholinergic activity may be related to the negative symptoms of schizophrenia, while decreased cholinergic activity may be associated with the positive symptoms of schizophrenia (Tandon et al. 1991). Several studies have investigated muscarinic receptor binding in post-mortem schizophrenia tissue. Results have shown reduced M1 binding in hippocampus (Crook et al. 2000b), prefrontal cortex (Crook et al. 2000a; Crook et al. 2001), caudate-putamen (Dean et al. 1996), superior temporal gyrus (Deng and Huang 2005) and the anterior cingulate cortex (Zavitsanou et al. 2004b). M2 binding has also been found to be decreased in the caudate-putamen of schizophrenia subjects (Crook et al. 1999). Furthermore, using imaging studies, one group found reduced muscarinic receptor

availability in schizophrenia patients in striatum, thalamus, frontal, temporal and occipital cortices (Raedler et al. 2000).

Muscarinic activity has recently been suggested to be therapeutic in the treatment of schizophrenia. Anticholinergics, such as benztropine, are commonly used to treat antipsychotic drug-induced Parkinson-like side-effects, while atypical antipsychotic drugs (e.g. clozapine, olanzapine) also have anti-muscarinic properties, especially at the M1 receptor (Bymaster et al. 2003). However, muscarinic antagonists can produce schizophrenia-like psychotic symptoms in humans (Perry and Perry 1995).

Studies in rats have shown that chronic treatment with clozapine increases the levels of M1/4 binding in rat frontal cortex. Furthermore, chronic treatment with haloperidol, which does not contain direct muscarinic properties, was also shown to increase M1/4 binding (Crook et al. 2001). More recent studies have found that chronic olanzapine treatment also increases M1/4 and M2/4 binding in rat cortex (Terry et al. 2006), possibly contributing to the therapeutic properties of these drugs.

In addition to the anti-muscarinic properties of atypical antipsychotic drugs, there is evidence to suggest that muscarinic agonists may be beneficial in the treatment of schizophrenia. Clozapine has been shown to be a partial agonist at M4 receptors (Zorn et al. 1994; Olianas et al. 1997; Zeng et al. 1997), possibly contributing to the greater effectiveness of this drug in treating the negative symptoms of schizophrenia. In addition, xanomeline, an M1/4 preferring muscarinic cholinergic receptor agonist, produces some antipsychotic effects in rodents similar to clozapine and haloperidol and therefore may be effective as a treatment for schizophrenia (Shannon et al. 2000).

2.5.2 NMDA receptor hypofunction

Acetylcholine has been implicated in the underlying mechanism of NMDA receptor hypofunction-induced neuronal damage as detailed in section 1.5 (Olney et al.

1991; Olney and Farber 1995). In support of this, anticholinergic drugs, including scopolamine, benztropine and trihexylpheniidyl have been shown to prevent PCP-induced damage (Olney et al. 1991), while increased acetylcholine release has been hypothesized to contribute to the psychosis and neural damage induced by PCP (Kim et al. 1999). Studies show that administration of NMDA receptor antagonists in rats results in increased release of acetylcholine in the cortex and hippocampus by up to 500% (Giovannini et al. 1994; Kim et al. 1999), which is thought to cause neuronal damage by over-stimulating the post-synaptic neurons (Kim et al. 1999). Furthermore, this increase was shown to be reduced by treating the rats with GABAergic agonists (pentobarbital and muscimol; Kim et al. 1999), which suggests that GABAergic inhibitory neurons and GABA receptors are critically involved in NMDA antagonist-induced acetylcholine release. In accordance with the NMDA hypofunction hypothesis, it has been hypothesized that there is increased acetylcholine release in the basal forebrain in schizophrenia (Crook et al. 2001; Deng and Huang 2005), however this is yet to be confirmed.

2.6 Serotonin

Serotonin plays an essential role in a wide range of functions including neuronal development, motor control, appetite, mood and aggression (Breier 1995). It originates in the raphe nucleus of the midbrain, where it projects to the cortex and several other brain regions (Wilson and Molliver 1991). Fourteen receptor subtypes mediate the actions of serotonin, three of which ($5HT_{2A-C}$) belong to the $5HT_2$ receptor family, which are G-protein coupled. 5HT2 receptors are positively coupled to phospholipase C and mobilise intracellular calcium (Barnes and Sharp 1999).

Autoradiographic and immunohistochemical studies have shown that serotonin $5HT_{2A}$ receptors are widely distributed in the brain but are more numerous in the

cerebral cortex where they are primarily postsynaptically located (Pazos et al. 1985; Wilson and Molliver 1991). Autoradiographic studies of the cortex have shown that $5HT_{2A}$ receptors are more densely located in layers II-V (Joyce et al. 1993; Zavitsanou and Huang 2002). Immunohistochemical studies in the rat and primate have shown that $5HT_{2A}$ receptors are localized to pyramidal glutamatergic neurons and on GABAergic interneurons, however in the cortex they are reported to be predominantly located on pyramidal neurons (Willins et al. 1997; Jakab and Goldman-Rakic 1998; Xu and Pandey 2000). 5-HT_{2A} receptors located on GABA interneurons reportedly stimulate GABA release in the rat prefrontal cortex, and therefore, have an important role in regulating the inhibition of neuronal activity (Abi-Saab et al. 1999). 5-HT_{2A} receptors located on pyramidal neurons on the other hand, can *diminish* the effect of GABA currents in rat prefrontal cortex, thus reducing GABAergic inhibition (Feng et al. 2001). In addition, it has been shown that $5HT_{2A}$ receptors located on pyramidal neurons in the cortex can enhance excitatory output of pyramidal neurons (Aghajanian and Marek 1997). Supporting this, electrophysiological studies have shown that activated $5HT_{2A}$ receptors increase glutamate release (Aghajanian and Marek 2000). Therefore, it appears that serotonin and its receptors play an important role in the regulation of excitatory and inhibitory output.

2.6.1 Schizophrenia

Serotonin plays a role in promoting appropriate synaptic connections and in the maintenance and remodeling of synapses. It therefore plays an important role in neurodevelopment and may be important in the pathophysiology of schizophrenia (Lieberman et al. 1998). Furthermore, it is well known that serotonin plays a role in mood (especially depression; Jones and Blackburn 2002), which is altered in schizophrenia, further supporting a role of serotonin in this disease.

The strongest evidence implicating serotonin in schizophrenia is that the atypical antipsychotic clozapine has high affinity for serotonin $5HT_2$ receptors. Furthermore, LSD, a serotonin agonist, induces hallucinations and negative schizophrenia-like symptoms in humans and rodents by activation of $5HT_2$ receptors (Breier 1995).

Autoradiographic studies have reported a diversity of changes in 5HT2 receptor binding in schizophrenia, which appear to be region-specific (Table 2.5). Furthermore, there is strong evidence that suicide is associated with reduced levels of serotonin and/or its metabolites (Breier 1995), and increased $5HT_2$ receptor binding (Arango et al. 1990), which is relevant since 10% of schizophrenia patients commit suicide (NISAD 2004).

Serotonin interacts with many other neurotransmitter systems including the dopaminergic (Lieberman et al. 1998) and glutamatergic (Aghajanian and Marek 1999) systems, both of which are heavily implicated in schizophrenia.

Table 2.5: Summary of 5-HT_2 receptor binding in schizophrenia

Please see print copy for Table 2.5

Clozapine, the prototypical atypical antipsychotic, is considered one of the best treatments for schizophrenia to date. Although clozapine has affinity for a variety of receptors, it has high affinity for $5HT_{2A}$ receptors (and also less affinity for $5HT_{2C}$) (Brunello et al. 1995). Clozapine is considered more effective than the typical antipsychotic drugs, such as haloperidol, which mostly act on dopamine D2 receptors. Therefore, serotonin antagonism appears to be an important aspect in treating schizophrenia, and as such may be important in the underlying pathophysiology of this disease. Although serotonin may play a major role in clozapine's effectiveness, effects on cholinergic, adrenergic and dopaminergic systems may also be responsible for the greater efficacy of clozapine than haloperidol. However, other atypical antipsychotic

drugs such as olanzapine, risperidone, quietapine, and ziprasidone all also have high affinity for the $5HT_{2A}$ receptor (Meltzer et al. 2003).

Atypical antipsychotic drugs have serotonin antagonist properties while typical antipsychotic drugs do not. Therefore, it would be expected that these drugs would have differential effects on the serotonin system. Generally speaking, clozapine treatment down-regulates $5HT_{2A}$ receptors in rodent brain, while haloperidol treatment has no effect (Table 2.6). Furthermore, positron emission tomography (PET) studies have shown a decrease in cortical $5HT_{2A}$ receptor density in clozapine treated patients compared to drug-free patients (Trichard et al. 1998b).

Table 2.6: The effects of antipsychotic drug treatment on the serotonin system in rodent brain

Please see print copy for Table 2.6

2.6.2 NMDA receptor hypofunction

Studies have demonstrated a close relationship between the glutamatergic (especially NMDA) and serotonergic systems, which may be an important element in

the pathophysiology of schizophrenia. Activation of $5HT_{2A}$ receptors has been shown to increase the release of glutamate onto layer V pyramidal cells through a presynaptic mechanism (Aghajanian and Marek 1999). In addition, studies have shown that d-cycloserine, a positive modulator of NMDA receptors inhibits serotonergic function (Dall'Olio et al. 2000), while microdialysis studies have shown that NMDA receptor antagonists increase extracellular brain levels of serotonin (Martin et al. 1998). Likewise, knocking out NMDA receptor function in mice increases serotonin activity (Miyamoto et al. 2001). Finally, chronic PCP treatment has been shown to significantly decrease 5HT2 binding in rat brain synaptic membrane (Nabeshima et al. 1985b), while acute treatment has been shown to inhibit serotonin uptake (Hori et al. 1996).

It has been hypothesized that activation of $5HT_{2A}$ receptors located on GABAergic neurons restores inhibition to the NMDA receptor hypofunction network as studies have shown that $5HT_{2A}$ agonists, including LSD, prevent NMDA antagonist-induced neuronal injury (Farber et al. 1998). In contrast however, it has also been reported that $5HT_{2A}$ *antagonists* including atypical antipsychotic drugs prevent NMDA antagonist-induced psychosis and neurotoxicity (Sharp et al. 1994; Gleason and Shannon 1997). Therefore, it is clear that there is a relationship between serotonin and the NMDA glutamatergic system, albeit an unknown one, which may be important in the mechanism behind NMDA receptor hypofunction.

2.7 Cannabinoid

The endogenous cannabinoid system consists of the neurotransmitters anandamide and 2-arachidonylglycerol, and the cannabinoid receptors. To date, two cannabinoid receptors have been identified: CB1 and CB2. Both receptors are G-protein coupled (Childers and Breivogel 1998). While the CB2 receptor is located peripherally in tissues of the immune system, including the spleen, tonsils and thymus (Munro et al.

1993), the CB1 receptor is found throughout the CNS (Howlett et al. 2004). The highest densities of the CB1 receptor are reported in the basal ganglia, cerebellum and hippocampal formation. In human cortex, the highest densities are reported in association regions of the frontal lobe, and the limbic and temporal lobes (regions that are involved in higher cognitive functions; Glass et al. 1997). It is interesting that the brain has more CB1 receptors than any other G-coupled protein (Howlett et al. 2004), which may indicate the importance of this relatively unknown system in brain function. Activation of the CB1 receptor is thought to mediate the behavioural and physiological effects of both endogenous and exogenous (i.e. cannabis) cannabinoids in the brain (Ameri 1999). Several rodent studies have shown that cannabis intake causes decreased CB1 binding throughout the rat brain (Rodriguez de Fonseca et al. 1994; Romero et al. 1997; Romero et al. 1998).

The endogenous cannabinoid system, including CB1 receptors, is proposed to have a role in modulating neurotransmission by inhibiting the release of a variety of neurotransmitters, including glutamate and GABA (Schlicker and Kathmann 2001). Immunohistochemistry in the rat and primate has shown that CB1 receptors can be pre- or postsynaptically located on GABAergcic and glutamatergic neurons (Tsou et al. 1998; Katona et al. 1999; Ong and Mackie 1999; Katona et al. 2000) and are therefore likely to be colocalised with other receptors. This presynaptic localization is consistent with the proposed role of cannabinoids in modulating neurotransmitter release (Schlicker and Kathmann 2001; Iversen 2003). The mechanisms involved in this cannabinoid-mediated neurotransmitter inhibition are not known but it has been suggested that CB1 receptors are coupled to adenylyl cyclase and decrease Ca^{2+} conductance into the presynaptic neuron, whilst also increasing outward K^{+} conductance (Schlicker and Kathmann 2001; Iversen 2003).

2.7.1 Schizophrenia

Cannabis causes a range of effects many reminiscent of schizophrenia, including cognitive impairments and short-term memory impairments (Hollister 1986). The major psychoactive component of cannabis is Δ^9-tetrahydrocannabinol (Δ^9-THC) (Ameri 1999). Detectable levels in the blood or urine indicate that cannabis has been used within at least 5-7 days (Law et al. 1984).

A temporary form of psychosis can occur in some cannabis users, with many similarities to schizophrenia psychosis. This has led to the proposal of a cannabinoid hypothesis of schizophrenia which suggests that the symptoms of schizophrenia might be caused by an abnormal over-activity of the endogenous cannabinoid system in the brain (Emrich et al. 1997). Cannabis use is more common among schizophrenia patients than the general community (Kovasznay et al. 1997), while prolonged use of cannabis has been shown to trigger relapses of positive symptoms in schizophrenia patients (Linszen et al. 1994). One study however reported that cannabis use actually improved schizophrenia patients' symptoms (Peralta and Cuesta 1992). Whether cannabis use is a causative or secondary factor to schizophrenia is not known, however a recent study has reported that adolescent cannabis use increases the risk of schizophrenia sixfold (Arseneault et al. 2002).

Supporting an abnormal endogenous cannabinoid system in schizophrenia, increased cerebrospinal fluid levels of anandamide have been shown in schizophrenia patients (Leweke et al. 1999). Furthermore, increased binding of [^{3}H]CP-55940 to cannabinoid CB1 receptors in the dorsolateral prefrontal cortex of schizophrenia patients as compared to controls has been reported, which was independent of recent cannabis ingestion (Dean et al. 2001). Similarly, a study by our group found a 64% increase in [^{3}H]SR141716A binding to CB1 receptors in the anterior cingulate cortex in

schizophrenia subjects compared to controls (Zavitsanou et al. 2004a). Therefore, evidence suggests that there may be a relationship between the endogenous cannabinoid system and schizophrenia.

Little has been published regarding the possible modulation of the cannabinoid system by antipsychotic drugs. A recent study in rats however, showed that clozapine treatment reduced CB1 receptor density in rat nucleus accumbens, while having no effect in frontal cortex. Similarly, haloperidol treatment was shown to have no effect on CB1 receptor density in the frontal cortex (Sundram et al. 2005). Another study however reported that haloperidol treatment increased CB1 binding in the rat striatum and substantia nigra (Andersson et al. 2005).

2.7.2 NMDA receptor hypofunction

A role for cannabinoids in the NMDA receptor hypofunction hypothesis of schizophrenia has not yet been purported. However, cannabinoids have been shown to inhibit glutamate release in the basal ganglia and hippocampus (Schlicker et al. 1997; Szabo et al. 2000), and to reduce glutamate toxicity induced by NMDA antagonists in rat cortex (Hampson et al. 1998), showing that there is an interaction between these two systems. Furthermore, treatment with the NMDA receptor antagonist MK-801 has resulted in reduced CB1 mRNA in rodent brain (Mailleux and Vanderhaeghen 1994). It may be that the CB1 receptor plays an important role in *protection* against NMDA antagonist-induced neurotoxicity, via a reduction of NMDA-antagonist induced glutamate release. On the other hand however, CB1 receptors have been shown to inhibit GABA release (Katona et al. 1999), which could act to increase pyramidal excitation and contribute to neurotoxicity. More research is needed to determine the role of the cannabinoid system in NMDA receptor hypofunction.

2.8 Summary

After reviewing the literature it is evident that the PCC may play an important role in the pathology of schizophrenia. Recent human data suggests abnormalities in function of the PCC in schizophrenia. Furthermore, animal studies show that the PCC is the most susceptible brain region to NMDA receptor antagonist induced damage, implicating this brain region in the NMDA receptor hypofunction hypothesis of schizophrenia. While this hypothesis purports a primary abnormality in the glutamatergic system, it also suggests imbalances in several other neurotransmitter systems, including GABA, acetylcholine, serotonin, and cannabinoid. The GABAergic system provides the majority of neural inhibition to the brain and is thought to maintain a tonic inhibition over excitatory pathways to keep them from over acting. The cholinergic system interacts with the GABAergic system and it has been hypothesized that abnormalities in this system could possibly contribute to the symptomatology of schizophrenia. The serotonergic system has been implicated in schizophrenia due to its component in anti-schizophrenia medication. Finally, the cannabinoid system has a controversial role in the pathology of schizophrenia that still needs to be explored.

2.9 Aims of the Study

Part A: Investigate the fundamental neurochemical changes occurring in the PCC in schizophrenia by examining the key neurotransmitter systems.

1. Examine ionotropic glutamate receptor density including NMDA, AMPA and kainate in the PCC in schizophrenia.
2. Examine $GABA_A$ and muscarinic M1/2/4 receptor density in the PCC in schizophrenia.
3. Examine serotonin 5HT2 and cannabinoid CB1 receptor density in the PCC in schizophrenia.

Part B: Examine PCP-induced and antipsychotic drug-induced neurochemical changes in an animal model, in particular to examine key receptors that were altered in part A.

4. Examine locomotor activity induced by the chronic PCP and antipsychotic mouse models, in order to verify these models.
5. Examine the short and long-term effects on NMDA and muscarinic receptor binding following chronic PCP treatment
6. Determine if antipsychotic drug treatment has the ability to reverse the short-term changes or prevent the long-term changes in NMDA and muscarinic receptor density induced by chronic PCP treatment.

2.10 Significance of the study

The outcomes of this study will contribute to our understanding of the central neurochemical changes that occur in schizophrenia. This study will provide important information regarding neurotransmitter receptor imbalance in the PCC in schizophrenia and in an NMDA receptor hypofunction animal model. These new discoveries may

provide important information, not only regarding the pathology of schizophrenia, but also for designing new pharmacological treatments for this disease.

2.11 Hypotheses

1. There will be selective changes in neurotransmitter receptor density in the PCC in schizophrenia. Specifically, I hypothesize that there will be:
    a. Altered NMDA receptor density in schizophrenia.
    b. Altered $GABA_A$ and muscarinic receptor density.
    c. A relationship between muscarinic and GABA receptor densities.
    d. Altered serotonin and cannabinoid receptor density.
2. There will be selective changes in neurotransmitter receptor binding in the brains of PCP treated mice. There will be differential effects when examined in the short or long-term following PCP treatment.
3. There will be selective changes in neurotransmitter receptor binding in the brains of the antipsychotic drug treated mice. Clozapine will be more successful than haloperidol in preventing/reversing the PCP-induced receptor changes.

EXPERIMENTAL PART A:

EXAMINATION OF THE NEUROTRANSMITTER RECEPTOR BINDING PROFILES IN THE POSTERIOR CINGULATE CORTEX IN SCHIZOPHRENIA

Chapter 3: Differential alterations in ionotropic glutamate receptor binding in the posterior cingulate cortex in schizophrenia.

3.1 Introduction

The PCC is a posterior midline structure just above and posterior to the corpus callosum. Studies in rats and mice demonstrate structural damage, reversible neuronal alteration, neural disinhibition, and heat shock protein expression in the PCC during systemic administration of NMDA receptor antagonists (Sharp et al. 1994), indicating the importance of the PCC in NMDA hypofunction. Recent imaging studies have shown a relationship between the PCC, NMDA hypofunction, and schizophrenia symptomatology (Northoff 2004), while other studies have directly implicated the NMDA receptor in the pathology of schizophrenia (Gao et al. 2000; Nudmamud and Reynolds 2001; Zavitsanou et al. 2002). Although it is typically the NMDA receptor that is implicated in schizophrenia, abnormalities of any of the glutamate receptors could result in a condition that produces the appearance of an abnormally functioning NMDA receptor. Therefore, it is important to examine NMDA as well as non-NMDA receptors.

In view of the above evidence, the present study examined the hypothesis that ionotropic glutamate receptors are altered in the PCC in schizophrenia. Using quantitative autoradiography, the present study examined the binding of [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate to NMDA, AMPA and kainate glutamate receptors in the PCC of schizophrenia subjects compared to matched controls.

3.2 Materials and methods

3.2.1 Post-mortem brain tissue

Human brain tissue from the PCC (left hemisphere) was obtained from the New South Wales Tissue Resource Centre at the University of Sydney. Ethical approval for this study was granted by the University of Wollongong Human Research Ethics Committee (Approval No. HE99/22). Male Subjects without a known history of psychiatric illness and male subjects with a diagnosis of schizophrenia, matched for age and post-mortem interval (PMI), were used in this study (Tables 3.1 and 3.2). Subjects were excluded where there was extended PMI (>48 h), a comorbid diagnosis of substance abuse or dependency (that would meet diagnostic criteria according to DSM IV), a significant head injury, or any abnormality upon neuropathological examination. The diagnosis of schizophrenia was confirmed according to DSM-IV. After extensive review of all available medical records (e.g. private psychiatrist and general practitioner reports), a standardized clinical summary was constructed. The clinical summary was organised so that it can be audited for inter-rater reliability. The Diagnostic Instrument for Brain Studies (Harper et al. 2003) was then applied to the clinical summary and used to confirm the schizophrenia diagnosis according to DSM-IV. The control cases were confirmed through extensive contact with area health hospitals, the subject's family physician and next-of-kin. Medical records were reviewed to exclude any history of major psychiatric disorders or psychopatholology. Informed consent was obtained from the next-of-kin for the release of tissue and medical records for all cases.

Brains were cut in the coronal plane in approximately 1cm thick slices and the PCC was identified according to a standard human brain atlas (refer to plate 29, page

128, Mai et al. 1997). The PCC was dissected and immediately frozen at -80^{o}C until the assays.

Table 3.1 Demographic data, characteristics and medication status of schizophrenia subjects and controls

Cases	Age (yrs)	PMI (hrs)	pH	Manner/Cause of Death	Age at Disease Onset	Antipsychotic Medication at Death[#]	FRD
Schizophrenia							
1	67	5	6.4	Ischaemic heart disease	26	Thioridazine, benztropine, risperidone	1300
2	27	33	6.3	Suicide by hanging	19	Haloperidol, benztropine	342
3	44	27	6.6	Suicide by hanging	27	Haloperidol, benztropine	500
4	27	10	6.2	Clozapine toxicity	18	Clozapine	650
5	52	8	6.1	Ischaemic heart disease	21	Risperidone	525
6	51	21	6.0	Ischaemic heart disease	27	Thioridazine	400
7	27	39	6.2	Myocarditis	23	Clozapine	250
8	57	33	6.4	Cardiac arrhythmia	30	Olanzapine, zoloft, thioridazine	275
9	30	24	6.6	Suicide by CO poison	27	Clozapine	325
10	32	26	6.2	Suicide by hanging	19	Risperidone, Temazepam	780
Mean±SEM	41.1±4.7	22.6±3.7	6.3±0.06				
Control							
1	61	24	6.5	Ischaemic heart disease	N/A	Nil	
2	37	11	5.3	Pulmonary embolism	N/A	Nil	
3	60	13	6.3	Myocardial infarction	N/A	Nil	
4	19	21	6.6	Motorcycle accident[a]	N/A	Nil	
5	58	12	6.5	Ischaemic heart Disease	N/A	Nil	
6	55	20	N/A	Cardiac arrest	N/A	Nil	
7	73	10	6.2	Respiratory arrest	N/A	Nil	
8	18	33	6.1	Cardiomyopathy	N/A	Nil	
9	37	24	6.4	Electrocution	N/A	Nil	
10	43	13	6.4	Cardiac arrest	N/A	Nil	
11	48	24	6.7	Ischaemic heart disease	N/A	Nil	
Mean±SEM	46.3±5.3	18.6±2.2	6.3±0.1				

#Medication at time of death is based on toxicology reports. a: Massive chest injuries. Abbreviations: PMI: post-mortem interval, FRD: final recorded antipsychotic drug dose (chlorpromazine equivalents per day); All subjects are male. NB: Not all cases were used for each experiment. Tissue allocation was based on availability at the time of each experiment.

Table 3.2 Schizophrenia and control subjects used for [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate binding

	[^{3}H]MK-801	[^{3}H]AMPA	[^{3}H]Kainate
Schizophrenia	1-9	1-9	1-9
Control	1-9	1, 3-10	1-8, 10

Cases are described in detail in Table 3.1. Due to tissue availability, not all cases detailed in Table 3.1 were used in the present study.

3.2.2 Autoradiography

Coronal sections of 14μm thickness were cut at -18^{o}C using a cryostat, mounted onto gelatinised slides and stored at –20°C until they were used. All control and schizophrenia tissue sections were processed simultaneously to minimize experimental variance. Three sections per case were incubated to determine total binding and an additional two sections were processed to determine non-specific binding. Experiments were performed blind to all clinical details. The [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate receptor binding protocols were performed as previously detailed (Zavitsanou et al. 2002).

[^{3}H]MK-801: Sections were incubated at room temperature for 2.5 hrs in 30mM N-(2-Hydroxyethyl) piperazine-N`-2-ethane sulfonic acid (HEPES) buffer, pH 7.5, containing 100μM glycine, 100μM glutamate, 1mM ethylenediamine teraacetic acid (EDTA) and 20nM [^{3}H]MK-801 (specific activity 17.1Ci/mmol, PerkinElmer, USA). Non-specific binding was determined by incubating adjacent sections with [^{3}H]MK-801 in the presence of 20μM MK-801. Following the incubation, sections were washed twice for 20 min each at 0^{o}C in 30mM HEPES containing 1mM EDTA (pH 7.5), and then dried.

[^{3}H]AMPA: Sections were pre-incubated for 30min at 4^{o}C in 50mM TrisHCl buffer (pH 7.2), containing 2.5mM $CaCl_2$. Sections were then incubated at 4^{o}C for 60min in 50mM TrisHCl buffer (pH 7.2) containing 2.5mM $CaCl_2$, 100mM KSCN, and

65nM [^{3}H]AMPA (specific activity 45.5Ci/mmol, PerkinElmer). Adjacent sections were incubated with [^{3}H]AMPA in the presence of 1mM glutamate to determine non-specific binding. Following the incubation, sections were rinsed for 60 seconds at 4^{o}C in 50mM TrisHCl buffer (pH 7.2) containing 2.5mM $CaCl_2$, and then dried.

[^{3}H]Kainate: All sections were preincubated at 4^{o}C in 310mM Tris citrate buffer (pH 7.32) for 30min. Sections were then incubated in the same buffer containing 35nM [^{3}H]kainate (specific activity 58Ci/mmol, PerkinElmer) for 2 hrs at 4^{o}C. Non-specific binding was determined by incubating adjacent sections with [^{3}H]kainate in the presence of 1mM glutamate. Following the incubation, sections were rinsed twice for 1 min each in 4^{o}C buffer, then dipped once in ice-cold water and air-dried.

3.2.3 Quantitative analysis of autoradiograms

Quantification of binding sites was performed on a high resolution Beta Imager (BioSpace, France) as previously detailed (Cloez-Tayarani et al. 1997). The Beta Imager allows the detection of radioactivity directly from the tissue sections and is therefore superior to film-based autoradiography. Briefly, sections were placed inside the detection chamber of the Beta Imager. The levels of bound radioactivity in the brain sections were directly determined by counting the number of β-particles emerging from the tissue sections, which was followed by measuring the activities in the regions of interest using the Beta Vision Plus program (BioSpace, France). Specific binding was calculated by subtracting non-specific from total binding. The radioligand binding signal was expressed in counts per minute per square millimeter (cpm/mm^2) and with the use of standards, was converted to fmols/mg tissue equivalents. Division into superficial and deep cortical layers was based on visible laminar differences in radioligand binding. The percentage of total cortical layers that represented superficial

and deep layers was applied to adjacent Nissl stained sections for each case to determine equivalent cortical layers.

3.2.4 Statistical analysis

All the data was normally distributed, therefore parametric tests were used. Student's t-test was used to compare the levels of radioligand binding between different cortical compartments (e.g. superficial and deep cortical layers) in both schizophrenia and control groups. Student's t-test was used to compare the mean age, PMI, pH, freezer storage time, and brain weight between the two diagnostic groups. Separate ANCOVA analyses, controlling for age, PMI, and suicide were performed within each cortical compartment to compare the levels of radioligand binding in control and schizophrenia cases. Pearson's correlations were used to test for any effects of continuous descriptive variables including age, PMI, age of illness onset, pH, duration of illness, freezer storage time, brain weight, and final recorded antipsychotic drug dose, on receptor binding. Suicide, a non-continuous descriptive variable, was used as a grouping variable with t-tests to evaluate its effects on [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]kainate binding in the schizophrenia subjects. Pearson's correlations were used to test for any correlations between the three ligands. All tests were performed using the SPSS statistical package (SPSS Inc., Chicago, Illinois).

3.3 Results

3.3.1 Laminar distribution of [^{3}H]MK-801, [^{3}H]AMPA and [^{3}H]kainate binding in the PCC

Specific binding of [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate was observed in all cortical layers in the PCC. Non specific binding was less than 30% for [^{3}H]MK-801, and less than 5% for [^{3}H]AMPA and [^{3}H]kainate binding. [^{3}H]AMPA appeared to

display the highest density of binding sites followed by [^{3}H]MK-801. [^{3}H]Kainate displayed the lowest binding density. Binding sites labeled by the three ligands had differential distribution among the layers of the PCC. The principal laminar patterns of each binding site were similar in schizophrenia and control groups. [^{3}H]MK-801 and [^{3}H]AMPA binding showed a higher density in the superficial layers of the PCC, while [^{3}H]kainate binding showed a higher density in the deeper layers of the PCC (Fig. 3.1). Upon examination of Nissl stained sections, the laminar division of [^{3}H]MK-801 binding was determined to correspond to cortical layers I-III and IV-VI, while AMPA layers corresponded to I-II and III-VI. The visible lamination of [^{3}H]kainate binding was divided into layers I-IV and V-VI. There were no significant correlations between the three ligands. There were significant correlations in binding density between superficial and deep layers for NMDA and kainate, but not for AMPA (NMDA: $r = 0.918$, $p<0.01$; AMPA: $r = 0.198$, $p = 0.432$; Kainate: $r = 0.985$, $p<0.001$; Pearson correlation, 2-tailed).

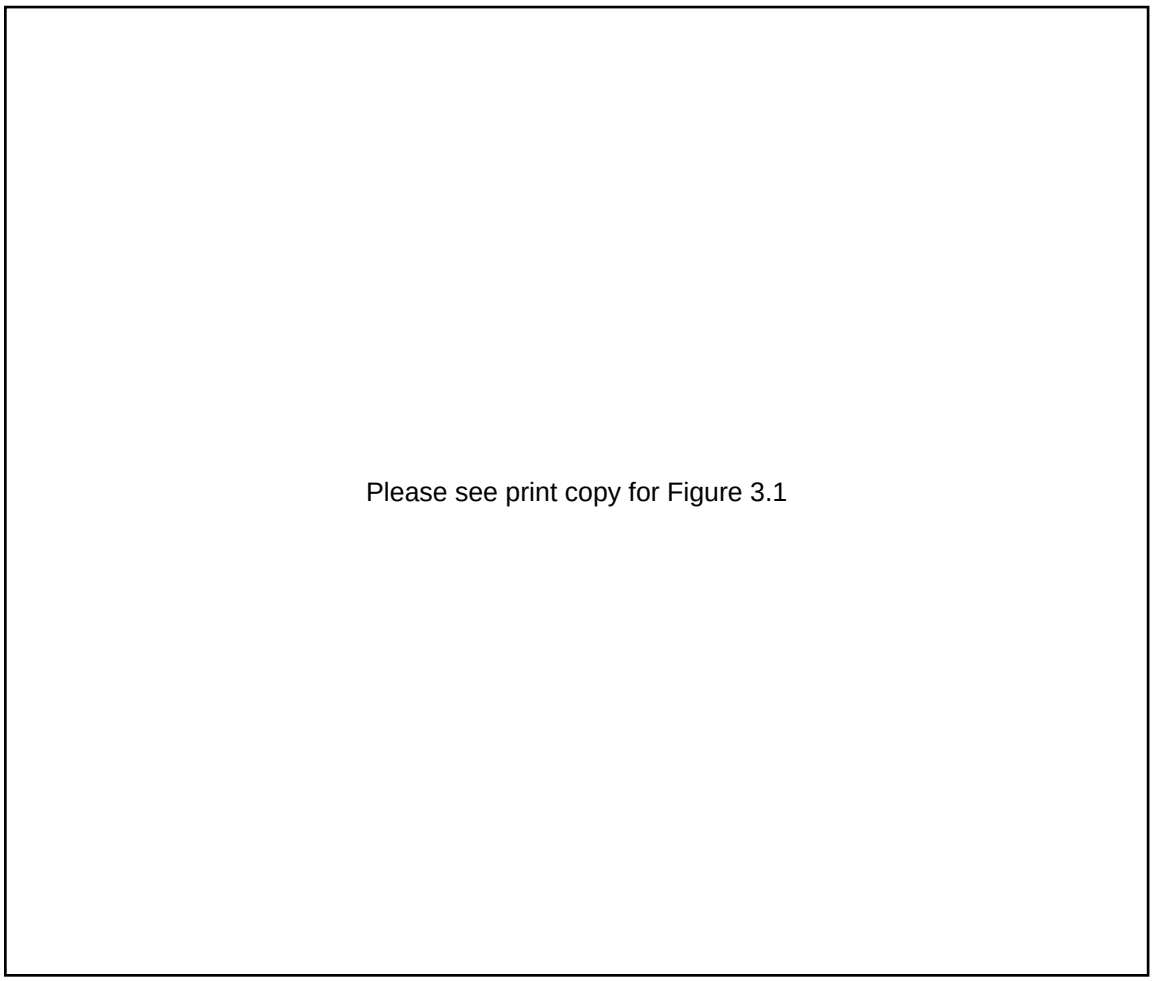

Figure 3.1 Digital autoradiograms obtained with the Beta-Imager showing [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate binding in the posterior cingulate cortex of one schizophrenia and one control case. The broken-line box on the right indicates where the section was taken. Abbreviations: cc, corpus callosum; PCC, posterior cingulate cortex; PoG, postcentral gyrus; STG, superior temporal gyrus. The line drawing was adapted from Mai et al. (1997).

3.3.2 Schizophrenia related effects on [^{3}H]MK-801, [^{3}H]AMPA, and [^{3}H]kainate binding in the PCC

Quantification indicated that [^{3}H]MK-801 binding was greater in the PCC in schizophrenia subjects than in normal controls. More specifically, there was a 41% increase of [^{3}H]MK-801 binding in cortical layers I-III of the schizophrenia group compared to the control group (200.3 ± 10.6 versus 141.3 ± 11.8 fmols/mg tissue, $F = 20.599$, $df = 1,16$, $p < 0.001$). In cortical layers IV-VI, a 30% increase in [^{3}H]MK-801

binding was observed in the schizophrenia group compared to the control group (150.8 ± 9.4 versus 115.8 ± 10.1 fmols/mg tissue, F = 11.806, df = 1,16, p = 0.004; Fig. 3.2A).

Quantification of [^{3}H]AMPA binding indicated no change in layers I-II (249.4 ± 23.9 versus 231.4 ± 25.4 fmols/mg tissue, F = 0.036, df = 1,16, p = 0.851) or layers III-VI (167.3 ± 15.2 versus 161.5 ± 18.6 fmols/mg tissue, F = 0.061, df = 1,16, p = 0.809) of the PCC between schizophrenia and control subjects (Fig. 3.2B).

Quantification of [^{3}H]kainate binding in layers I-IV and layers V-VI showed no significant differences between schizophrenia and control groups (layers I-IV: 13.2 ± 1.5 versus 12.6 ± 2.0 fmols/mg tissue, F = 0.073, df = 1,16, p = 0.791; layers V-VI: 17.4 ± 2.2 versus 17.7 ± 2.9 fmols/mg tissue, F = 0.673, df = 1,16, p = 0.426; Figure 3.2C).

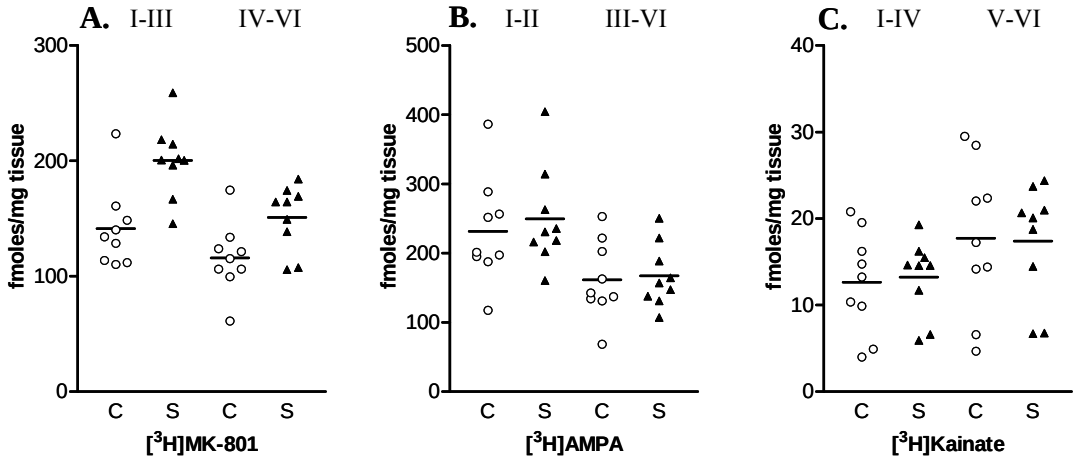

Figure 3.2 Scatterplots of [^{3}H]MK-801 (A), [^{3}H]AMPA (B), and [^{3}H]kainate (C) receptor binding in the posterior cingulate cortex of control (O) and schizophrenia (▲) cases. C: control; S: schizophrenia. Roman numerals indicate cortical layers.

3.3.3 Possible effects of continuous and non-continuous confounding variables

The mean age, PMI, pH, freezer storage time and brain weight did not differ between the schizophrenia and control groups. There were however, significant negative correlations between age and [^{3}H]AMPA binding in cortical layers I-II, (r = -0.572, p = 0.013) and between age and [^{3}H]kainate binding in cortical layers I-IV (r = -0.679 p = 0.002) and V-VI (r = -0.693, p = 0.001), when correlations were performed across the whole sample. There was a significant correlation between age of schizophrenia onset and [^{3}H]AMPA binding in cortical layers I-II (r = -0.709, p = 0.032). In addition, [^{3}H]MK-801 binding in both layer subdivisions (I-III & IV-V) was lower in schizophrenia cases that had committed suicide in comparison to cases that did not (layers I-III: 170.9 ± 16.1 fmols/mg tissue, n = 3 versus 215.0 ± 9.4 fmols/mg tissue, n = 6, t = 2.536, df = 7, p = 0.039; layers IV-V: 125.9 ± 19.3 fmols/mg tissue, n = 3 versus 163.3 ± 6.8 fmols/mg tissue, n = 6, t = 2.325, df = 7, p = 0.053). No other significant correlations were observed.

3.4 Discussion

This study examined the ionotropic glutamate receptor binding of NMDA, AMPA, and kainate in the PCC of schizophrenia subjects compared to matched controls. This study revealed an increase in [^{3}H]MK-801 binding to NMDA receptors in the schizophrenia group compared to controls, accompanied by no change in AMPA or kainate receptor binding. The principal laminar patterns that were observed in this study were similar in both control and schizophrenia groups for all three ligands. Furthermore, the laminar patterns that were reported here for each ligand are in agreement with earlier autoradiographic studies indicating high densities of NMDA and AMPA receptors in

superficial cortical layers and high densities of kainate receptors in deep cortical layers (Zavitsanou et al. 2002).

3.4.1 Ionotropic glutamate receptor binding in the PCC in schizophrenia

The present results have shown a significant increase in NMDA receptor binding accompanied by no change in AMPA or kainate receptor binding. This is the first study to report these changes in the PCC in schizophrenia. Previously, our laboratory found increased NMDA receptor binding in the anterior cingulate cortex (Zavitsanou et al. 2002), while several other reports have found upregulations of different binding sites of the NMDA receptor complex in several brain regions in schizophrenia (Kornhuber et al. 1989; Simpson et al. 1991; Aparicio-Legarza et al. 1998; Nudmamud and Reynolds 2001). However, unchanged [^{3}H]MK-801 binding has been reported in frontal and entorhinal cortices of schizophrenia subjects compared with controls (Kornhuber et al. 1989), suggesting that changes in NMDA receptors in schizophrenia are region-specific.

Tendolkar et al. (2004) recently reported dysfunctions in the PCC of schizophrenia subjects upon performing semantic memory tasks, while Northoff et al. (2004) showed decreased activation of the PCC during an episodic memory retrieval task in subjects treated with ketamine compared to controls. These findings in relation to the present study are interesting since NMDA receptors are critical for memory functions (Konradi and Heckers 2003). Therefore, the finding of altered NMDA receptors in the present study reinforces that the PCC might be an anatomical locus of glutamatergic deficiency in schizophrenia.

One explanation of the present findings is decreased glutamatergic transmission in the PCC in schizophrenia resulting in a compensatory upregulation of post-synaptic NMDA receptors. In agreement with this idea, reduced glutamate concentration in

cerebrospinal fluid of schizophrenia patients has been reported (Kim et al. 1980), however this has not yet been replicated directly in brain samples.

The present data on AMPA and kainate binding do not support this hypothesis of decreased glutamate transmission, as no change was found in AMPA or kainate receptor binding in the PCC in schizophrenia subjects compared to controls. AMPA and NMDA receptors are predominantly postsynaptic and are co-localised (Meador-Woodruff and Healy 2000), so a reduction in glutamate transmission would be expected to upregulate both NMDA *and* AMPA receptors. Furthermore, kainate receptors are predominantly presynaptic and act to regulate glutamate release. A reduction in glutamatergic transmission in the PCC in schizophrenia would therefore be expected to increase presynaptic kainate receptor binding to allow for more glutamate release. Furthermore, *increased* glutamate concentration has recently been reported in the prefrontal cortex and hippocampus of schizophrenia patients using magnetic resonance spectroscopy (van Elst et al. 2005).

Another possible explanation of the present findings is a phenomenon known as NMDA receptor hypofunction, as first proposed in rodents by Olney and Farber (1995). Cultured cortical neurons exposed to NMDA receptor antagonists have shown an upregulation of NMDA receptors (Williams et al. 1992). In addition, chronic PCP treatment has been shown to increase NMDA receptor subunit 1 expression in rat forebrain (Wang et al. 1999), and increase synaptic responses mediated by NMDA receptors without any changes in AMPA and kainate receptors (Yu et al. 2002). Furthermore, PCP has been shown to exacerbate symptoms in schizophrenia patients (Itil et al. 1967). Therefore, the increase in NMDA receptor binding that has been found in schizophrenia in the present study could be due to NMDA receptor hypofunction.

3.4.2 Possible effects of medication and suicide on the receptor binding in the PCC

As in most studies on schizophrenia that rely on post-mortem tissue, there is the potential confounding variable of antipsychotic drug exposure. All schizophrenia subjects used in this study received antipsychotic medication prior to death. The increase in NMDA binding observed in this study does not appear to be secondary to antipsychotic exposure since there was no correlation between NMDA binding in the PCC from schizophrenia subjects and final recorded antipsychotic drug dose. Furthermore, most antipsychotic medication does not target the glutamate system directly. Studies suggest that dopamine receptor blockers increase glutamate release (Yamamoto and Cooperman 1994; Olney and Farber 1995), which would be expected to down-regulate, not upregulate, NMDA (and non-NMDA) glutamate receptors. In agreement with this suggestion, animal data tends to show that antipsychotic drug treatment either decreases or has no effect on [^{3}H]MK801 binding to NMDA receptors in the cortex (Tarazi et al. 1996; Giardino et al. 1997).

The present study showed that death by suicide had a significant effect on NMDA receptor binding. Schizophrenia subjects that committed suicide had lower NMDA receptor binding levels than schizophrenia subjects that died from other causes. Interestingly, the suicidal subjects had NMDA binding levels similar to that observed in control subjects. This is the first time this has been reported in the PCC, but needs to be confirmed with a larger sample.

3.5 Conclusion

In conclusion, the present study clearly demonstrated for the first time increased [^{3}H]MK-801 binding to NMDA receptors accompanied by no change in AMPA or

kainate receptor binding in the PCC in schizophrenia. The NMDA receptor-specific changes that were found are therefore unlikely to be caused by a simple decrease in glutamatergic transmission. It is hypothesized that NMDA receptor hypofunction could be the basis for the increased NMDA receptor binding in the PCC in schizophrenia. It is unlikely however, that only a single system is altered in this region in schizophrenia due to the complex nature of this disease.

Chapter 4: Alterations of muscarinic and GABA receptor binding in the posterior cingulate cortex in schizophrenia

4.1 Introduction

The PCC, part of the corticolimbic system, has recently been shown to have an imbalance of glutamatergic neurotransmission in schizophrenia (Newell et al. 2005). It is unlikely however that this is the only abnormal system in this region, due to the complex nature that is schizophrenia. Animal and human studies have suggested that the GABAergic and cholinergic systems may also be altered in schizophrenia. Animal studies have shown that NMDA antagonist-induced damage in the PCC, can be attenuated by anticholinergic drugs such as scopolamine and benztropine, as well as barbiturates, which enhance activity at the GABA receptor channel complex (Olney et al. 1991). Furthermore, evidence from human autoradiographic studies suggests that muscarinic acetylcholine receptors and GABA receptors may play an important role in the neural mechanisms underlying the pathophysiology of schizophrenia (Benes et al. 1992b; Benes et al. 1996; Zavitsanou et al. 2004b; Deng and Huang 2005).

Muscarinic cholinergic receptors are associated with cognition, attention, memory and motor control, functions that are altered in schizophrenia (Hyde and Crook 2001). In addition, certain atypical antipsychotic drugs (e.g. clozapine and olanzapine) have strong anti-muscarinic properties (Hyde and Crook 2001). GABA is the major inhibitory transmitter in the brain. Studies show that GABAergic terminals make synaptic contact with cholinergic nucleus basalis neurons and that cholinergic terminals

make synaptic contact with GABAergic nucleus basalis and cortical neurons, suggesting a relationship between these two systems (Zaborszky et al. 1986).

Based on the above evidence, it is therefore important to examine muscarinic and GABA receptor systems in the PCC in schizophrenia. Using quantitative autoradiography, the present study for the first time investigated if the densities of muscarinic and GABAergic receptor systems are altered in the PCC in schizophrenia. [^{3}H]Pirenzepine and [^{3}H]AF-DX 384 were used to examine M1/M4 and M2/M4 muscarinic receptors respectively, while [^{3}H]muscimol was used to examine $GABA_A$ receptor binding.

4.2 Materials and methods

4.2.1 Post-mortem brain tissue

Post-mortem brain tissue (Table 4.1) was obtained from the same source as previously detailed in chapter 3. There were several cases that were common to the three different ligands used, allowing for correlations to be performed.

Table 4.1 Schizophrenia and control subjects used for [^{3}H]pirenzepine, [^{3}H]AF-DX 384, and [^{3}H]muscimol binding

	[^{3}H]Pirenzepine	**[^{3}H]AF-DX 384**	**[^{3}H]Muscimol**
Schizophrenia	1-3, 5-10	2-3, 5-10	1, 3-8, 10
Control	1-7, 9-10	1-4, 6-7, 9-10	1, 4-10

Cases are described in detail in table 3.1. Due to tissue availability, not all cases detailed in Table 3.1 were used in the present study.

4.2.2 Autoradiography

Coronal sections as previously detailed were used (refer to chapter 3). [^{3}H]Pirenzepine (Zavitsanou et al. 2004b), [^{3}H]AF-DX 384 (Piggott et al. 2002) and [^{3}H]muscimol (Zilles et al. 1991) binding were performed as previously described.

[^{3}H]Pirenzepine: Briefly, all sections were pre-incubated for 15 min at room temperature in 22mM HEPES (pH 7.5). Sections were then incubated for 90 min at room temperature in the same buffer with the addition of 10nM [^{3}H]pirenzepine (specific activity 86Ci/mmol, PerkinElmer, USA). Non-specific binding was determined by incubating adjacent sections with [^{3}H]pirenzepine in the presence of 10μM atropine. Following the incubation, sections were rinsed 3 times for 4 min each in 4°C buffer, and dipped once in ice-cold distilled water.

[^{3}H]AF-DX 384: All sections were pre-incubated for 15 min at room temperature in 10mM KH_2PO_4, 10mM Na_2HPO_4 (pH 7.4). Sections were then incubated for 1 hour in the same buffer containing 4nM [^{3}H]AF-DX 384 (specific activity 120Ci/mmol, PerkinElmer). Non-specific binding was determined by incubating adjacent sections in [^{3}H]AF-DX 384 plus 10μM atropine. Following incubation, sections were washed twice for 2 min each in room temperature buffer, and dipped once in distilled water.

[^{3}H]Muscimol: Briefly, all sections underwent three 5 min pre-incubations at 4°C in 50mM Tris citrate buffer (pH 7.0). Sections were then incubated for 45 min at 4°C in the same buffer containing 3nM [^{3}H]muscimol (specific activity 29.5Ci/mmol, PerkinElmer). Non-specific binding was determined by incubating adjacent sections in [^{3}H]muscimol plus 100μm GABA. Following incubation, sections were rinsed four times for 2 seconds each in 4°C buffer.

4.2.3 Quantitative and statistical analyses of autoradiograms

Quantification of binding sites was performed on a high resolution Beta Imager as detailed in chapter 3. Statistical analyses were also performed as detailed in chapter 3.

4.3 Results

4.3.1 Laminar distribution of [^{3}H]pirenzepine, [^{3}H]AF-DX 384 and [^{3}H]muscimol binding in the PCC

Specific binding of [^{3}H]pirenzepine, [^{3}H]AF-DX 384 and [^{3}H]muscimol was observed in all cortical layers of the PCC and was >95% of total binding for all three ligands. [^{3}H]Pirenzepine displayed the highest density of binding sites followed by [^{3}H]AF-DX 384, while [^{3}H]muscimol displayed the lowest density of binding sites (Fig. 4.1).

Binding sites labeled by these ligands appeared to have differential distribution patterns among the layers of the PCC. The principal laminar patterns of each ligand were similar in schizophrenia and control groups. [^{3}H]AF-DX 384 showed a homogeneous distribution across the layers of the PCC. In contrast, [^{3}H]pirenzepine and [^{3}H]muscimol both showed a laminar distribution pattern. [^{3}H]Pirenzepine binding was higher in the superficial layers of the PCC grey matter compared to the deeper layers. Inspection of Nissl stained sections indicated that this division corresponded to layers I-II and III-VI respectively. There was a significant positive correlation in binding density between the superficial and deep cortical layers ($r = 0.894$, $p<0.001$, Pearson Correlation, 2-tailed). In addition, a higher density of [^{3}H]muscimol binding was observed in the superficial layers of the PCC grey matter (layers I-IV) in comparison to the deeper layers (layers V-VI). There was a significant positive correlation in binding density between the superficial and deep cortical layers ($r = 0.958$, $p<0.001$, Pearson Correlation, 2-tailed).

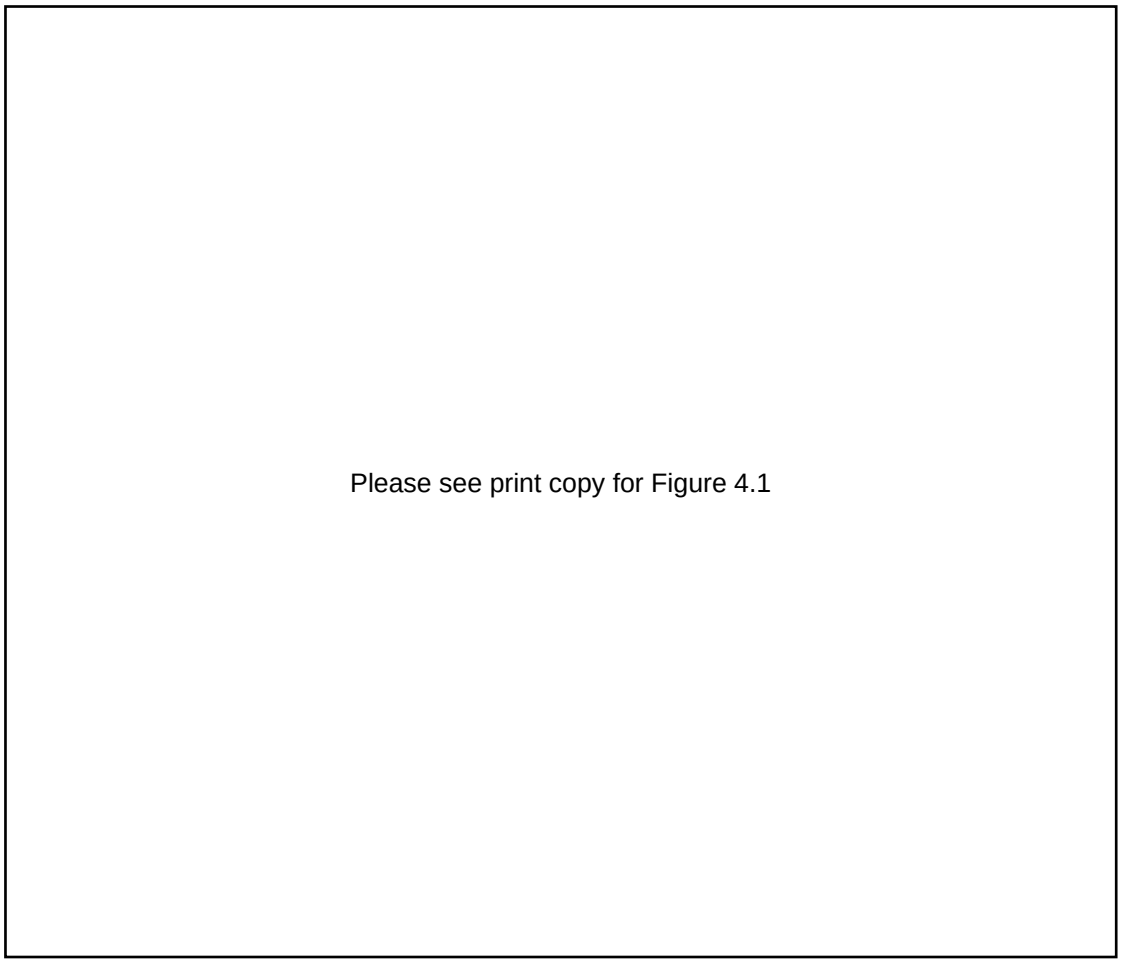

Figure 4.1 Digital autoradiograms obtained with the Beta-Imager showing [^{3}H]pirenzepine, [^{3}H]AF-DX 384, and [^{3}H]muscimol binding in the posterior cingulate cortex of one control and one schizophrenia case. The broken-line box on the right indicates where the section was taken. Abbreviations: cc, corpus callosum; PCC, posterior cingulate cortex; PoG; postcentral gyrus; STG, superior temporal gyrus. Line drawing was adapted from Mai et al. (1997).

4.3.2 Schizophrenia related effects on [^{3}H]pirenzepine, [^{3}H]AF-DX 384 and [^{3}H]muscimol binding in the PCC

Overall, [^{3}H]pirenzepine binding was significantly lower in the PCC in schizophrenia cases compared with controls (Fig. 4.1). In cortical layers I-II, a 13% decrease of [^{3}H]pirenzepine binding was found in the schizophrenia group compared with the control group (67.78 ± 3.02 versus 59.21 ± 4.52 fmols/mg tissue, $F = 17.568$, df = 1,16, $p = 0.001$). Similarly, in layers III-VI, a significant 22% decrease in

[^{3}H]pirenzepine binding was found in the schizophrenia group compared to the control group (55.88 ± 2.91 versus 43.46 ± 3.97 fmols/mg tissue, F = 29.972, df = 1,16, p < 0.001). Suicide had a significant effect on these results. [^{3}H]Pirenzepine binding in both layers I-II and III-VI was higher in schizophrenia cases that had committed suicide in comparison to schizophrenia cases that did not (layers I-II: 72.96 ± 2.59 fmols/mg tissue, n = 3 versus 52.35 ± 4.39 fmols/mg tissue, n = 6, t = -3.099, p = 0.017; layers III-VI: 54.96 ± 1.73 fmols/mg tissue, n = 3 versus 37.71 ± 4.16 fmols/mg tissue, n = 6, t = -2.784, p = 0.027). Therefore, excluding the suicide cases, there was a 24% decrease of [^{3}H]pirenzepine binding in the schizophrenia group compared with the control group (p = 0.002, Fig. 4.2A). Similarly, in layers III-VI, there was a significant 35% decrease in [^{3}H]pirenzepine binding in the schizophrenia (non-suicide) group compared to the control group (p<0.001; Fig. 4.2A). There was no difference in binding between the schizophrenia subjects that committed suicide and controls **(**layers I-II: 72.96 ± 2.59 fmols/mg tissue, n = 3 versus 67.78 ± 3.02 fmols/mg tissue, n = 9, t = -0.930, p = 0.374; layers III-VI: 54.96 ± 1.73 fmols/mg tissue, n = 3 versus 55.88 ± 2.91 fmols/mg tissue, n = 9, t = 0.172, p = 0.867).

Quantification of [^{3}H]AF-DX 384 binding across all layers of the PCC revealed no statistically significant differences between the schizophrenia and control groups (27.5 ± 2.4 versus 28.5 ± 4.0 fmols/mg tissue, F = 0.092, df = 1,14, p = 0.766).

Quantification of [^{3}H]muscimol binding in cortical layers I-IV of the PCC indicated a dramatic increase of 112% in the schizophrenia group in comparison with the control group (34.3 ± 4.0 versus 16.2 ± 2.0 fmols/mg tissue, F = 18.860, df = 1,14, p = 0.001; Fig. 4.2B). In layers V-VI, [^{3}H]muscimol binding was increased by 100% in the schizophrenia group compared with the control group (16.2 ± 2.0 versus 8.1 ± 1.2 fmols/mg tissue, F = 7.731, df = 1,14, p = 0.017; Fig. 4.2B).

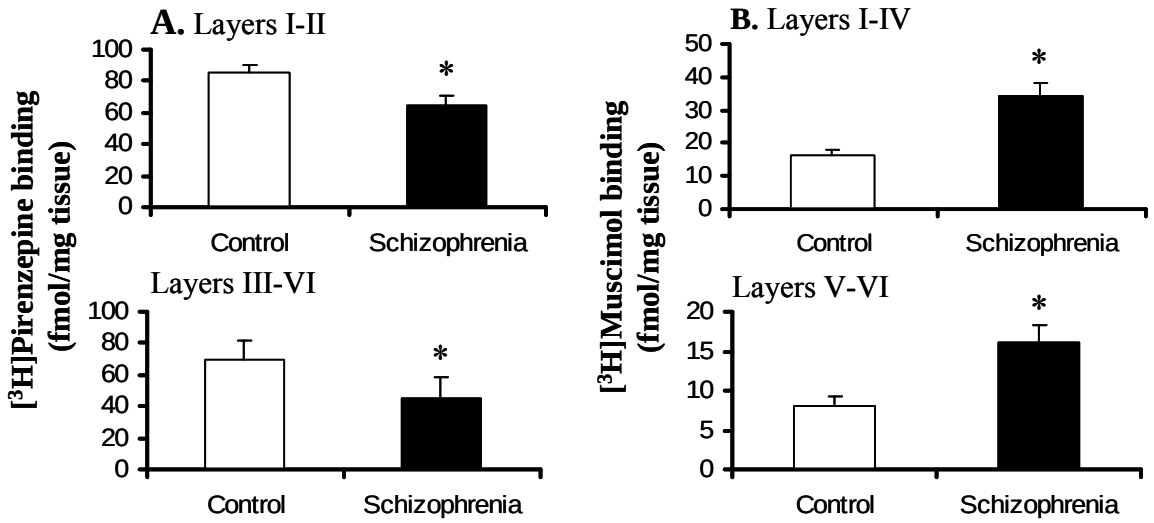

Figure 4.2 (A) Histogram of [^{3}H]pirenzepine binding in layers I-II and III-VI of the posterior cingulate cortex of schizophrenia (non-suicide) and control groups. **(B)** Histogram of [^{3}H]muscimol binding in layers I-IV and V-VI of the posterior cingulate cortex of schizophrenia and control groups. *: $p < 0.002$.

Interestingly, there was a significant negative correlation between [^{3}H]muscimol binding (layers I-IV) and [^{3}H]pirenzepine binding (layers III-VI) when measured across all cases (r = -0.562, $p = 0.036$; Fig. 4.3).

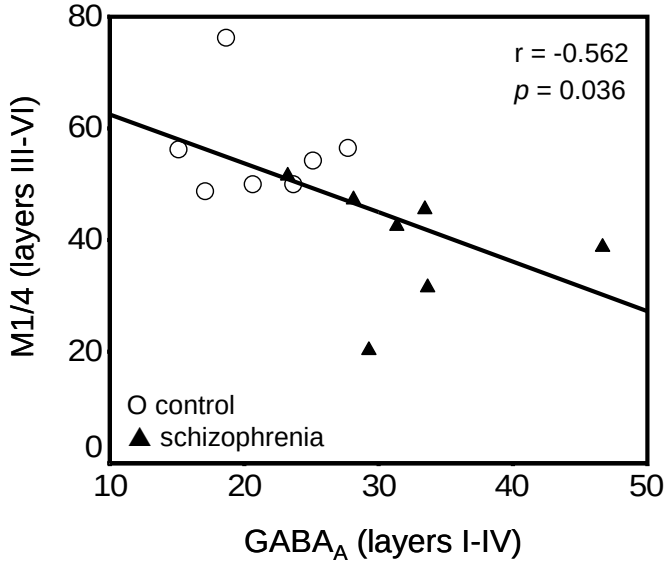

Figure 4.3 Significant correlations were found between $GABA_A$ receptor binding (layers I-IV) and M1/4 receptor binding (layers III-VI) in the posterior cingulate cortex. Values are in fmoles/mg tissue.

4.3.3 Possible effects of continuous and non-continuous confounding variables

The mean age, PMI, pH, freezer storage time and brain weight did not differ between the two groups. Overall, there were significant negative correlations between age of disease onset and [^{3}H]AF-DX 384 binding (r = -0.730, p = 0.040), and between tissue storage time and [^{3}H]AF-DX 384 binding (r = -0.631, p = 0.028) when measured across the whole sample. There were also significant negative correlations between age and [^{3}H]pirenzepine binding in cortical layers I-II (r = -0.559, p = 0.016) and III-VI (r = -0.473, p = 0.048) across the whole sample. Students t-test showed no effect of benztropine treatment on the binding of any of the ligands examined. There were no other significant correlations observed.

4.4 Discussion

The present study investigated the binding of the acetylcholine muscarinic receptor antagonists [^{3}H]pirenzepine and [^{3}H]AF-DX 384, and the $GABA_A$ receptor agonist [^{3}H]muscimol in the PCC of subjects with schizophrenia. These radioligands bind to M1/4, M2/4, and $GABA_A$ receptors respectively. A significant down-regulation of M1/4 muscarinic receptors and an up-regulation of $GABA_A$ receptors was observed in the PCC in schizophrenia. No changes in M2/4 receptor density were observed between the schizophrenia and control groups. The concentrations of ligands that were used in this study were primarily based on studies published previously by our laboratory (Zavitsanou et al. 2004b) and others (Piggott et al. 2002; Zink et al. 2004). The present study used 10nM [^{3}H]pirenzepine which labels 65% M1 receptors and 18.5% M4 receptors (Flynn and Mash 1993), while 4.8nM [^{3}H]AF-DX 384 labels all M2 receptors and most M4 receptors (Piggott et al. 2002). Similar concentrations of

[^{3}H]muscimol as used in this study have consistently been used previously to label $GABA_A$ receptors (Zilles et al. 1991; Zink et al. 2004).

4.4.1 M1/4, M2/4 and $GABA_A$ receptor binding in the PCC in schizophrenia

This study revealed a significant decrease in M1/4 receptor density in the PCC in schizophrenia. While the cause is not known, it has previously been suggested that the down regulation of M1/4 receptors seen in post-mortem schizophrenia brain, could be due to an increase in acetylcholine release from the cholinergic basal forebrain (Crook et al. 2001), where 80-90% of cortical cholinergic projections originate. Several studies have reported an increased dopamine concentration in the nucleus accumbens in schizophrenia (Bird et al. 1977; Bird et al. 1979; Mackay et al. 1982). Studies show that dopamine release from terminals in nucleus accumbens acts to reduce the activity of inhibitory GABAergic efferents to the basal forebrain and therefore increase acetylcholine release from the basal forebrain (Moore et al. 1999; Sarter et al. 1999). Furthermore, using the NMDA receptor hypofunction model of schizophrenia, it has been shown that administration of NMDA receptor antagonists produces an increase in acetylcholine release in rat PCC (Kim et al. 1999), suggesting that NMDA receptor hypofunction could be a possible mechanism of decreased M1/4 binding in the PCC in schizophrenia.

In contrast to the present finding of altered M1/4 muscarinic receptors in schizophrenia, this study found no significant difference in [^{3}H]AF-DX 384 binding in the PCC between schizophrenia and controls. Another study however, found *decreased* [^{3}H]AF-DX 384 binding in the striatum of schizophrenia subjects compared to controls (Crook et al. 1999). It has been suggested that in the cortex [^{3}H]AF-DX 384 binds predominantly to the M2 receptor, while in the striatum it binds primarily to the M4

receptor (Piggott et al. 2002). This may explain the difference between the present results and those reported in the striatum. However, it may also be that changes in [^{3}H]AF-DX 384 binding in schizophrenia are specific to the striatum. Furthermore, there is evidence that in addition to M2 postsynaptic receptors, there are M2 pre-synaptic receptors located on the terminal projections, which act to inhibit acetylcholine release (Vilaro et al. 1992). Therefore, altered amounts of neurotransmitters may not change the overall M2 receptor binding densities. One of the limitations of using receptor autoradiography is that pre- and post-synaptic receptor sites cannot be differentiated. More defined receptor binding methods are needed to clarify this. The present study did find however, a significant negative correlation between M2 binding and age of schizophrenia onset, suggesting that the older the age of onset, the lower the M2 binding. This correlation has also been reported in the anterior cingulate cortex (Zavitsanou 2005).

The examination of [^{3}H]muscimol binding in this study showed a dramatic increase in binding to $GABA_A$ receptors in the PCC in schizophrenia. This result is in agreement with what has been reported in the prefrontal and anterior cingulate cortices (Benes et al. 1992b; Benes et al. 1996) and in the caudate (Hanada et al. 1987). Furthermore, the laminar pattern observed with [^{3}H]muscimol is in accord with what has been reported previously in the PCC (Vogt et al. 1990). It has been suggested that the upregulation of $GABA_A$ neurons in the prefrontal and anterior cingulate cortices is due to a loss of GABAergic neurons in these regions in schizophrenia (Benes et al. 1991; Benes et al. 1992b; Benes et al. 1996). Although there is no evidence to suggest that this is occurring in the PCC, it is a possibility, and further research should be conducted to clarify this.

Alternatively, the increased $GABA_A$ binding observed in the present study could be a result of decreased GABAergic release or production in the PCC in schizophrenia. As reduced GAD67 expression has been found in the cortex in schizophrenia (Akbarian et al. 1995a), it is possible that this is causing reduced production of GABA. It is also possible that the increased $GABA_A$ receptor binding in the PCC is due to NMDA receptor hypofunction in this region. Blocked NMDA receptors (by MK-801) have been shown to reduce GABA transmission in rat PCC (Li et al. 2002), while also downregulating the expression of GAD in rat frontal cortex (Paulson et al. 2003). These studies indicate low levels of GABA, which in turn would be expected to cause upregulated $GABA_A$ receptor binding.

In contrast to the present findings of increased $GABA_A$ receptor binding in the PCC in schizophrenia, imaging studies have shown no change in binding to the $GABA_A$-benzodiazepine site (using [^{123}I]Iomazenil) in limbic cortical regions in schizophrenia (Busatto et al. 1997; Ball et al. 1998). However, as this is a separate binding site on the $GABA_A$ receptor, it is possible that there is an uncoupling in the regulation of the $GABA_A$ and benzodiazepine receptors in schizophrenia (Benes et al. 1997). Specific studies in the PCC are needed to confirm this.

The current study found a significant negative correlation between M1/4 (layers III-VI) and $GABA_A$ (Layers I-IV) receptor binding in the PCC, indicating interactions between these two systems. Due to the difference in layer subdivisions between these two receptors, it is uncertain if this interaction is in fact in the same cortical layers. While there is currently no additional data available in relation to these two systems directly interacting in the PCC, studies have shown that stimulating M1 receptors increases GABA release in the rat medial prefrontal cortex (Sanz et al. 1997). Therefore, it is possible that a decreased M1 receptor activity, as was found in the PCC

in schizophrenia, would lead to reduced GABA release, and in turn result in a compensatory upregulation of $GABA_A$ receptor binding.

4.4.2 Possible effects of medication and suicide on the receptor binding in the PCC

A potential confounding factor in all studies in schizophrenia is the possible effects of antipsychotic medication. Although we cannot completely rule out an effect of medication, it seems unlikely that the changes in [^{3}H]pirenzepine binding we have seen in this study are consequences of medication as the Pearson's analysis showed no relationship between [^{3}H]pirenzepine binding and final recorded antipsychotic drug dose in schizophrenia subjects. Furthermore, imaging studies have shown that both medication-free schizophrenia patients, and clozapine and olanzapine treated patients, have reduced muscarinic receptor binding in the cortex as measured with the non-specific ligand [^{123}I]iodoquinuclidinyl benzilate (QNB; Raedler et al. 2000; Raedler et al. 2003a; Raedler et al. 2003b). [^{123}I]IQNB binds to all 5 muscarinic receptors, therefore we cannot be sure about its effects specifically on M1 or M2 binding. [^{3}H]Pirenzepine however has recently been used to measure M1/4 binding in typical and atypical antipsychotic drug treated rats, showing an increase or no change in binding to this specific ligand (Crook et al. 2001). This therefore suggests that antipsychotic drug treatment may not be responsible for the observed downregulation of M1/4 receptor binding in the present study.

It should be noted that three subjects used in the present study were receiving benztropine, an anticholinergic drug, at the time of death. A study on human tissue has suggested that benztropine lowers [^{3}H]pirenzepine binding in the prefrontal cortex (Crook et al. 2001). However in the same report, rats treated with benztropine showed no change in [^{3}H]pirenzepine binding. The results from the present study suggest that

benztropine treatment has no effect on [^{3}H]pirenzepine, [^{3}H]AF-DX 384, or [^{3}H]muscimol binding in the PCC, as there were no differences in binding between these subjects and schizophrenia subjects not receiving benztropine. However, due to the small number of cases receiving this treatment at death, this result should be viewed as preliminary.

While no relationship was found between [^{3}H]muscimol binding in the PCC and final recorded antipsychotic drug dose, there are inconsistent findings in the literature as to the effects of antipsychotic medication on cortical $GABA_A$ binding. Animal studies have shown that clozapine and olanzapine act to decrease $GABA_A$ binding in rat cortex (Farnbach-Pralong et al. 1998); however an increase was also reported in the cortex of clozapine treated rats (Zink et al. 2004). More research is therefore needed to clarify the effects of antipsychotic drug dosage, treatment duration, and gender differences on cortical $GABA_A$ binding.

In the present study, schizophrenia subjects who had committed suicide had a higher density of M1/4 receptors in the PCC than schizophrenia subjects that died from other causes. Interestingly, the cases that did commit suicide had M1/4 binding densities similar to the control group. This effect of suicide on M1/4 binding in schizophrenia has been reported previously in the anterior cingulate cortex (Zavitsanou et al. 2004b). However, a similar study in the striatum found no effect of suicide on M1/4 binding (Dean et al. 1996), suggesting that there may be regional differences in the effect of suicide on this receptor system. In the current study inverse correlations were found between PMI and M1/4 binding in the schizophrenia group and between age and M1/4 binding in the control group. These correlations however are not responsible for the observed decrease in M1/4 binding in schizophrenia because the groups were age and

PMI matched. Furthermore, the correlation between age and M1/4 binding supports previous data showing an age-related decline in M1 receptor density (Narang 1995).

4.5 Conclusion

In conclusion, the decrease in M1/4 receptor density and the increase in $GABA_A$ receptor density in schizophrenia suggest an involvement of these two systems in the pathology of the PCC in schizophrenia. It is suggested that these changes are the result of increased cortical acetylcholine and decreased cortical GABA. The observed changes may be due to NMDA receptor hypofunction in this region. However, further research is needed to determine the mechanism of these changes. The examination of other neurotransmitter systems and their interactions with GABA and muscarinic receptors as well as NMDA receptors may help to determine the mechanisms of these changes.

Chapter 5: Alterations in serotonin and cannabinoid receptor binding in the posterior cingulate cortex in schizophrenia.

5.1 Introduction

It has been shown in chapters 4 and 5, that there are alterations in glutamatergic, gabaergic and cholinergic receptor binding in the PCC in schizophrenia (Newell et al. 2005; Newell et al. 2007c). These systems are known to interact with other systems which are implicated in schizophrenia, including the serotonergic and cannabinoid systems. Serotonin has been implicated in the pathology of schizophrenia since the discovery that clozapine, one of the most successful antipsychotic drugs for treating schizophrenia, was a $5HT_2$ receptor antagonist (Brunello et al. 1995; Jones and Blackburn 2002). In addition, clozapine and more specific $5HT_{2A}$ receptor antagonists inhibit the psychotic effects caused by NMDA receptor antagonists (Gleason and Shannon 1997). This therefore suggests an important relationship between the serotonergic and glutamatergic systems, which may be an important element in the pathophysiology of NMDA hypofunction and schizophrenia.

The endogenous cannabinoid system has recently been implicated in schizophrenia due to the association between cannabis use and schizophrenia. The endogenous cannabinoid system, including CB1 receptors, is proposed to have a role in modulating neurotransmission via affecting the release of other neurotransmitters (eg GABA). In accordance with this role, CB1 receptors have been shown to be localized presynaptically (Katona et al. 1999; Katona et al. 2000).

The present study examined the binding of [^{3}H]spiperone to $5HT_{2A}$ receptors, and [^{3}H]SR141716A and [^{3}H]CP-55940 to CB1 receptors in the PCC of schizophrenia subjects and matched controls.

5.2 Materials and Methods

5.2.1 Post-mortem human brain tissue

Human brain tissue was received as previously described in chapter 3. However, due to tissue availability, several different subjects were used compared to those used in chapters 3 and 4 (Table 5.1).

Table 5.1 Schizophrenia and control subjects used for [^{3}H]spiperone, [^{3}H]SR141716A, and [^{3}H]CP-55940 binding

	[^{3}H]Spiperone	**[^{3}H]SR141716A**	**[^{3}H]CP-55940**
Schizophrenia	1, 3-4, 8-9	1, 3-4, 8-9	1-2, 5-10
Control	1-3, 8-10	1-3, 8-10	1-2, 5-7, 9-11

Cases are described in detail in table 3.1. Due to tissue availability, not all cases detailed in Table 3.1 were used in the present study.

5.2.2 Autoradiography

[^{3}H]Spiperone (Zavitsanou and Huang 2002), [^{3}H]SR141716A (Zavitsanou et al. 2004a), and [^{3}H]CP-55940 (Herkenham et al. 1990) binding were performed as detailed previously.

[^{3}H]Spiperone: Sections were pre-incubated in buffer (170mM Tris, containing 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, and ascorbic acid; pH7.4) for 15min at room temperature. Sections were then incubated for 45min at room temperature in the same buffer containing 1.5nM [^{3}H]spiperone (90Ci/mmol, Amersham, UK), in the presence or absence of 10μM mianserin ($5HT_2$ antagonist). Sections were rinsed in ice-cold distilled water (quick dip), followed by 2x5min washes

in ice-cold buffer, with a final dipping in cold distilled water, and then dried with warm air.

[^{3}H]SR141716A: Sections were incubated for 60min at room temperature in 50mM Tris buffer (pH7.4) containing 1.5nM [^{3}H]SR141716A (52Ci/mmol, Amersham) in the presence of 0.01% bovine serum albumin (BSA). Non-specific labelling was determined by incubating additional sections with [^{3}H]SR141716A in the presence of 100μM HU210 (Tocris, MO, USA), a potent cannabinoid receptor agonist. After incubation, the sections underwent 2x30min washes in ice-cold buffer and were then dried with warm air.

[^{3}H]CP-55940: Sections were pre-incubated for 30min at room temperature in 50mM TrisHCl (pH 7.4) containing 5% BSA. Sections were then incubated in the same buffer with the addition of 10nM [^{3}H]CP-55940 (168Ci/mmol, PerkinElmer, USA) for 2 hours at room temperature. Non-specific binding was determined by incubating adjacent sections in 10nM [^{3}H]CP-55940 in the presence of 10μM CP-55940. Following incubation, the sections were washed three times. First in 50mM TrisHCl (pH 7.4) containing 1% BSA, for 1 hour at 4^{o}C. The second wash was in 50mM TrisHCl (pH 7.4) for 3 hours at 4^{o}C, and the third wash was in the same buffer for 5 mins at 4^{o}C. Finally, the sections were dipped briefly in milliQ water and air-dried.

5.2.4 Quantitative Analysis of Autoradiograms

Quantification was as described in chapter 3.

5.2.5 Statistical Analysis

All data was normally distributed. A t-test was conducted to test for any differences in age, PMI, pH, freezer storage time, or brain weight between the schizophrenia and control groups. T-tests were used to test for significant differences in

binding between control and schizophrenia for [^{3}H]spiperone, [^{3}H]SR141716A and [^{3}H]CP-55940. Pearson's correlations were used to test for any effects of continuous descriptive variables including age, PMI, age of illness onset, pH, duration of illness, freezer storage time, brain weight, and final recorded antipsychotic drug dose, on receptor binding. Pearson's correlations were also used to test for correlations between [^{3}H]spiperone, [^{3}H]SR141716A and [^{3}H]CP-55940 binding.

5.3 Results

5.3.1 Laminar distribution of [^{3}H]spiperone, [^{3}H]SR141716A and [^{3}H]CP-55940 binding in the PCC.

Specific binding of all three ligands was observed in the PCC. Non-specific binding was less than 30% for [^{3}H]spiperone and [^{3}H]SR141716A, and less than 5% for [^{3}H]CP-55940. [^{3}H]CP-55940 showed the greatest density of binding, followed by [^{3}H]SR141716A, with [^{3}H]spiperone showing the lowest density of binding in the PCC. Binding sites labeled by the three ligands appeared to have differential distribution among the layers of the PCC. The principal laminar patterns of each binding site were similar in schizophrenia and control groups. [^{3}H]Spiperone showed high binding in the middle cortical layers, and lower binding in the superficial and deep cortical layers. From examination of Nissl stained sections, the middle cortical layers were found to correspond to layers II-V, with the superficial and deep layers corresponding to I and VI respectively. [^{3}H]SR141716A binding showed a homogenous distribution in the PCC, while [^{3}H]CP-55940 showed a greater binding density in the superficial cortical layers (55.24 ± 3.14 fmols/mg tissue) compared with the deeper layers (50.29 ± 2.59 fmols/mg tissue, t = 2.324, df = 1,14, p = 0.035, paired 2-tailed t test). This laminar pattern was determined to correspond to layers I-II and layers III-VI (Fig. 5.1). There was a

significant positive correlation in binding density between the superficial and deep cortical layers (r = 0.740, p = 0.001, Pearson Correlation, 2-tailed).

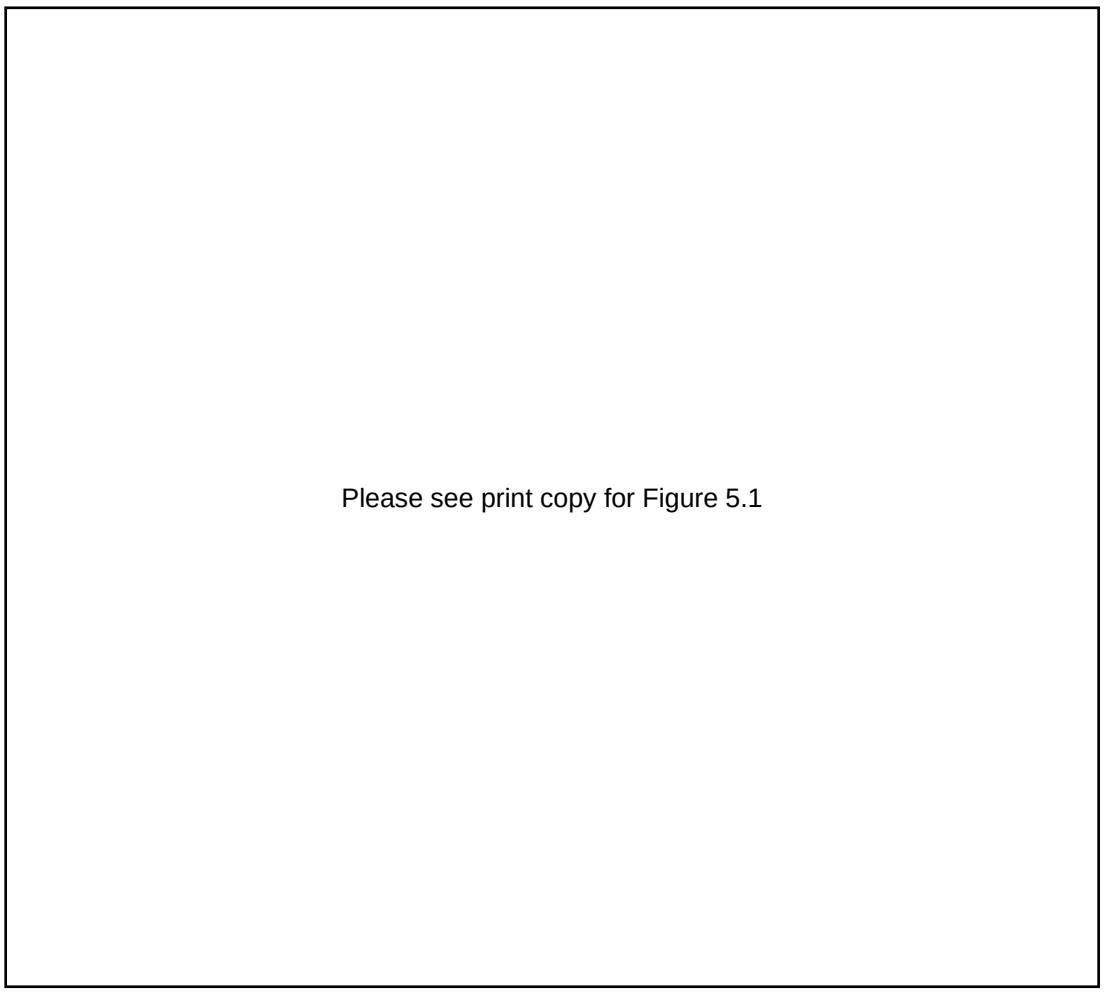

Figure 5.1 Digital autoradiograms obtained with the Beta-Imager showing [^{3}H]spiperone, [^{3}H]SR141716A and [^{3}H]CP-55940 binding in the posterior cingulate cortex of one control and one schizophrenia case. The broken-line box on the right indicates where the section was taken. Abbreviations: cc, corpus callosum; PCC, posterior cingulate cortex; PoG, postcentral gyrus; STG, superior temporal gyrus. Line drawing is adapted from Mai et al. (1997).

5.3.2 Schizophrenia related effects on [^{3}H]spiperone, [^{3}H]SR141716A and [^{3}H]CP-55940 binding in the PCC

Quantification of [^{3}H]spiperone binding in cortical layers II-V revealed a significant 34% decrease in [^{3}H]spiperone specific binding to $5HT_2$ receptors in the schizophrenia group as compared to the control group (13.52 ± 1.87 versus 20.48 ± 1.06 fmols/mg tissue; t = 0.578 , df = 1,9, p = 0.008; Fig. 5.2).

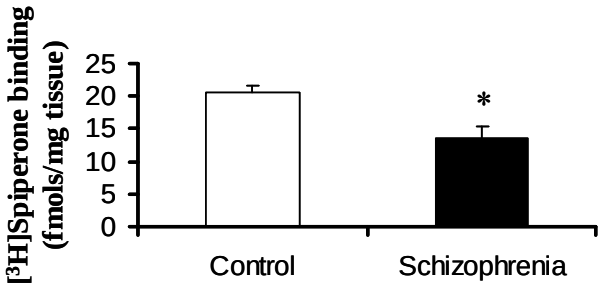

Figure 5.2 Histogram of [^{3}H]spiperone binding in layers II-V of the posterior cingulate cortex of schizophrenia and control groups. *: p = 0.008.

Quantification of [^{3}H]SR141716A binding indicated a significant 31% increase in [^{3}H]SR141716A specific binding to CB1 receptors in the schizophrenia group as compared to the control group (25.67 ± 2.05 versus 19.55 ± 1.47 fmols/mg tissue; t = 0.617, df = 1,9, p = 0.034; Fig. 5.3A).

Quantification of [^{3}H]CP-55940 binding indicated a significant 25% increase in binding in the superficial layers (I-II) of the schizophrenia group compared to the controls (61.31 ± 2.60 versus 49.17 ± 4.99 fmols/mg tissue; t = -2.156, df = 1,14, p = 0.049; Fig. 5.3B). In the deeper cortical layers (III-VI) there was no significant difference in [^{3}H]CP-55940 binding in the PCC in the schizophrenia group compared to controls (54.42 ± 1.94 versus 46.16 ± 4.49 fmols/mg tissue; t = 2.849 , df = 1,14, p = 0.114).

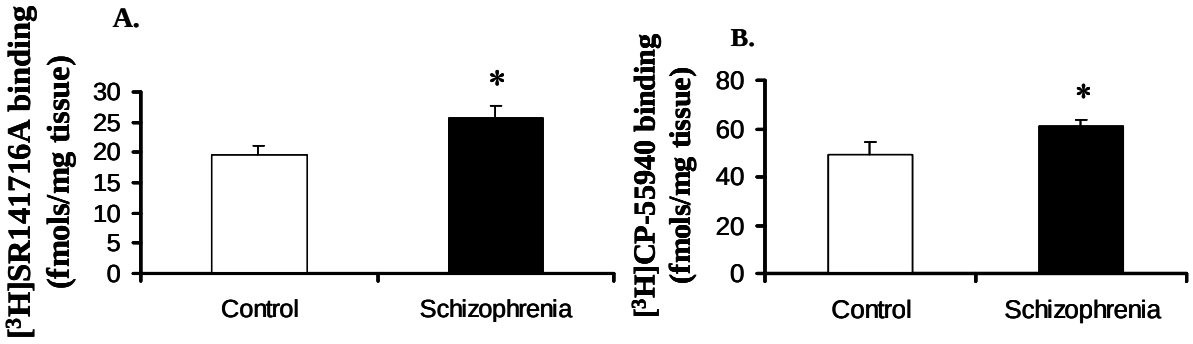

Figure 5.3 (A) Histogram of [^{3}H]SR141716A binding across all layers of the posterior cingulate cortex of schizophrenia and control groups. **(B)** Histogram of [^{3}H]CP-55940 binding in layers I-II of the posterior cingulate cortex of schizophrenia and control groups. *: $p < 0.05$.

Pearson's correlations showed there was a trend for a negative correlation between [^{3}H]SR141716A and [^{3}H]spiperone binding, although this did not quite reach significance (r = -0.577, $p = 0.06$). There was no correlation between [^{3}H]SR141716A and [^{3}H]CP-55940 binding in the PCC.

5.3.3 Possible effects of confounding variables

There were no differences in age, PMI, pH, freezer storage time, or brain weight between the control and schizophrenia groups for each ligand. Furthermore, in the schizophrenia cases, there were no significant correlations between [^{3}H]spiperone (r = 0.043, $p = 0.945$), [^{3}H]SR141716A (r = -0.188, $p = 0.762$), or [^{3}H]CP-55940 (superficial layers: r = 0.260, $p = 0.534$; deep layers: r = -0.390, $p = 0.339$) binding density and final recorded antipsychotic drug dose. There were however significant correlations between [^{3}H]spiperone binding and duration of brain storage (r = -0.832, $p = 0.010$) and between [^{3}H]CP-55940 binding in the deep layers and brain weight (r =

0.637, $p = 0.026$) when measured across all samples. No other significant correlations were observed.

5.4 Discussion

This study investigated the binding of the serotonin/dopamine receptor antagonist [^{3}H]spiperone, the cannabinoid receptor antagonist [^{3}H]SR141716A and the cannabinoid receptor agonist [^{3}H]CP-55940 in the PCC of subjects with schizophrenia compared to controls. There was a significant 34% decrease in [^{3}H]spiperone binding in layers II-V of the PCC in schizophrenia, accompanied by a significant 31% increase in [^{3}H]SR141716A binding across all layers, and a significant 25% increase in [^{3}H]CP-55940 binding in layers I-II of the PCC in schizophrenia compared to control subjects.

[^{3}H]Spiperone is a mixed dopamine/serotonin antagonist and has been shown to possess high affinity for both $5HT_2$ receptors and dopamine D2 receptors (Leysen et al. 1978; Seeman and Van Tol 1994). However, under the conditions used in the present study, [^{3}H]spiperone would bind predominantly to $5HT_2$ receptors. Recently, our laboratory showed that mianserin (a $5HT_2$ antagonist) displaces approximately 70% of [^{3}H]spiperone in human anterior cingulate cortex, supporting that most of [^{3}H]spiperone binding in the anterior cingulate cortex, and possibly other cortical regions, is to $5HT_2$ binding sites (Zavitsanou and Huang 2002). In addition, there are reported to be few dopamine D2 receptors in the PCC (De Keyser et al. 1988). Furthermore, studies have shown that spiperone is at least 2 orders of magnitude more selective for $5HT_{2A}$ versus $5HT_{2B/2C}$ receptors (Barnes and Sharp 1999). Therefore it is probable that the reduction in [^{3}H]spiperone binding in the PCC in schizophrenia reflects a reduction in $5HT_{2A}$ receptor density.

[^{3}H]SR141716A is a CB1 receptor antagonist that is reportedly very selective for CB1 receptors (Rinaldi-Carmona et al. 1994; Rinaldi-Carmona et al. 1996). [^{3}H]CP-

55940 however, is a non-specific cannabinoid receptor agonist, with equal affinity for CB1 and CB2 receptors (Felder et al. 1992; Pertwee 1999). Under the conditions used in this study, it would be expected that most binding of [^{3}H]CP-55940 is to the CB1 receptor. There are reported to be no, or minimal CB2 receptors in the CNS. Accordingly, in CB1 receptor knockout mice, no detectable [^{3}H]CP-55940 binding sites were observed in the brain (Zimmer et al. 1999). Therefore the CB1 receptor accounts for most, if not all, [^{3}H]CP-55940 and [^{3}H]SR141716A binding in the brain.

5.4.1 $5HT_{2A}$ receptor binding in the PCC in schizophrenia

The examination of [^{3}H]spiperone binding in this study showed a 34% decrease in binding to $5HT_{2A}$ receptors in the PCC in schizophrenia. This result, although preliminary, is in agreement with the 16% decrease reported in the anterior cingulate cortex in schizophrenia (Zavitsanou and Huang 2002), as well as the decreased binding consistently reported in the frontal cortex (Arora and Meltzer 1991; Burnet et al. 1996b; Dean and Hayes 1996; Dean et al. 1998). The laminar pattern observed in the present study has been reported previously in the PCC (Joyce et al. 1993).

The present finding of decreased $5HT_{2A}$ receptor binding in the PCC in schizophrenia could be a result of increased serotonin in schizophrenia. Several studies have reported increased serotonin in the striatum in schizophrenia (Crow et al. 1979; Korpi et al. 1986). Furthermore, the chronic PCP model of schizophrenia has shown increased serotonin levels in the mouse cortex (Nabeshima et al. 1985a). This would be expected to downregulate serotonin receptors. In support of this, it has been found that rats treated for 14 days with PCP had decreased [^{3}H]spiperone binding to 5HT2 receptors in the rat brain synaptic membranes (Nabeshima et al. 1985b). These findings support the hypothesis that NMDA blockade/dysfunction could be a contributing factor to the decreased $5HT_{2A}$ receptor binding that was found in the PCC in schizophrenia.

In chapter 3, increased NMDA receptor binding in the PCC in schizophrenia was reported (Newell et al. 2005), in which 10 of the subjects used were the same as in the present study. Interestingly, there was a significant negative correlation between $5HT_{2A}$ binding and NMDA binding in both upper ($r = -0.913$, $p < 0.001$), and lower ($r = -0.814$, $p = 0.004$) cortical layers. This suggests that as $5HT_{2A}$ binding decreases, NMDA binding increases. Electrophysiological studies have shown that activated $5HT_{2A}$ receptors increase glutamate release (Aghajanian and Marek 1999), which therefore would be expected to reduce NMDA receptors. Therefore, in the opposite manner, a reduction in $5HT_{2A}$ receptors would be expected to result in less glutamate release and therefore higher NMDA receptor binding. This relationship between NMDA and serotonin has been demonstrated previously using the NMDA receptor hypofunction animal model (Gleason and Shannon 1997).

Preliminary studies from our laboratory suggest decreased inhibitory GABA neurons in the PCC in schizophrenia (Newell et al. 2002). It is possible that the decreased $5HT_{2A}$ binding reported in the present study is a compensatory mechanism for excessive excitatory activity of the pyramidal cells due to decreased inhibitory GABA neurons, or due to an associated loss of $5HT_{2A}$ receptors located on GABAergic interneurons. However, this preliminary finding of decreased GABA neurons in the PCC is yet to be replicated or confirmed.

It is well accepted that schizophrenia patients that commit suicide have higher $5HT_{2A}$ receptor densities than patients that die of natural causes (Arango et al. 1990; Laruelle et al. 1993). Therefore, cause of death is an important factor to take into account when analysing serotonin receptor binding results. However, due to low sample size the effect of suicide on $5HT_{2A}$ receptor binding in the PCC could not be examined in the present study.

As with all studies that use post-mortem human tissue, it is important to discuss the possible effects of long-term antipsychotic drug treatment on the results. The present study found no correlation between final recorded antipsychotic drug dose and 5HT2 receptor binding, suggesting that antipsychotic drug treatment may not have caused the downregulated 5HT2 receptor density in the PCC in schizophrenia. In support of this, one study found that chronic (32 days) treatment with haloperidol (3mg/kg) and clozapine (30mg/kg) in male rats resulted in no change in $5HT_{2A}$ receptor mRNA cortical expression (Buckland et al. 1997). However, in a similar study, Burnet et al (1996a) found that while haloperidol treatment had no effect on 5HT2 binding, clozapine decreased 5HT2 binding in cingulate and frontal, but not piriform cortex. Several additional studies support Burnet's finding that clozapine down-regulates $5HT_{2A}$ receptors, while haloperidol treatment has no effect (Reynolds et al. 1983a; Wilmot and Szczepanik 1989; O'Dell et al. 1990). Therefore, the lack of a correlation between antipsychotic drug dose and [^{3}H]spiperone binding should be regarded with caution. It is interesting to note that in the present study, the one subject receiving haloperidol treatment had an exceptionally larger $5HT_{2A}$ binding (however, this case also committed suicide, which could have caused this increase). This should be examined in more detail.

Several PET studies on drug-free or drug-naive schizophrenia patients have shown no change in $5HT_{2A}$ receptor density compared to control subjects (Sedvall et al. 1995a; Sedvall et al. 1995b; Trichard et al. 1998a; Okubo et al. 2000), and a decrease in $5HT_{2A}$ receptor density in antipsychotic drug treated patients compared to drug-free patients (Trichard et al. 1998b). Therefore, it appears that atypical antipsychotic drug treatment can down-regulate $5HT_2$ receptors and could possibly contribute to the observed decrease in the schizophrenia subjects in the present study. However, whether

the decrease in $5HT_{2A}$ receptors seen in post-mortem schizophrenia tissue is a result of atypical antipsychotic drug treatment alone, or both a disease and drug effect, is unknown.

In contrast to the findings from the present study, one group found that $5HT_2$ binding, as labelled with [^{3}H]LSD, was actually increased in the PCC in schizophrenia (Joyce et al. 1993). Drug treatment would not be expected to influence binding in that study as several subjects were on no medication, while the remaining subjects were on typical antipsychotics which have been shown not to alter $5HT_{2A}$ binding (Reynolds et al. 1983a; Wilmot and Szczepanik 1989; O'Dell et al. 1990; Burnet et al. 1996a). However, several cases had committed suicide which could have contributed to the results of this earlier study.

5.4.2 CB1 receptor binding in the PCC in schizophrenia

This is the first study to report changes in cannabinoid receptor binding in the PCC in schizophrenia. Although the [^{3}H]SR141716A data should be viewed as preliminary due to the small sample size, the use of [^{3}H]CP-55940 with a larger number of cases confirms the finding of increased CB1 receptor binding in the PCC in schizophrenia. The PCC plays an important role in working memory function (Vogt et al. 1992). The cannabinoid system has also been implicated in memory functions (Reibaud et al. 1999). Therefore, it is possible that in schizophrenia, the deficits in working memory could be partially attributable to altered CB1 receptors in the PCC. Previous studies have reported varying results depending on the specific brain regions examined. No change in CB1 binding has been reported in the hippocampus and caudate-putamen in schizophrenia (Dean et al. 2001). Conversely, an increase of CB1 binding has been reported in the dorsolateral prefrontal (Dean et al. 2001) and anterior cingulate cortices (Zavitsanou et al. 2004a) in schizophrenia.

Due to the availability of two CB1 receptor ligands, the present study was able to evaluate the binding distribution of these two ligands. The homogenous distribution of [^{3}H]SR141716A binding to CB1 receptors that was observed in the PCC in the present study has been reported previously in the anterior cingulate cortex (Zavitsanou et al. 2004a). [^{3}H]CP-55940 binding in the PCC on the other hand showed a laminar distribution, with a greater binding density in the superficial cortical layers compared to the deeper layers. Previous studies have shown that the detection of this lamination in the PCC is quite subtle (Glass et al. 1997). Immunohistochemical studies examining the distribution of CB1 receptors in rat brain have shown that intensely stained cells are present in the superficial layers of the PCC (Tsou et al. 1998), which supports the current finding of denser CB1 binding in the superficial layers of the PCC. In other cortical regions however, CB1 binding shows bilaminar peaks, characterized by high binding in cortical layers I and VI and low binding in the middle layers (Glass et al. 1997; Biegon and Kerman 2001). It is interesting that two ligands that bind to the same receptor show different laminar patterns in the PCC. It is possible that each ligand binds to different sites on the CB1 receptor, or that they each bind to a different population of CB1 receptors (Shire et al. 1995).

Immunohistochemistry in the rat and primate has shown that CB1 receptors can be pre- or postsynaptically located on GABAergic and glutamatergic neurons (Tsou et al. 1998; Ong and Mackie 1999) and are therefore likely to be colocalised with other receptors. This presynaptic localization is consistent with the proposed role of cannabinoids in modulating neurotransmitter release (Schlicker and Kathmann 2001; Iversen 2003). In chapter 4, increased $GABA_A$ receptor binding in the PCC in schizophrenia was reported (Newell et al. 2007c),in which 13 subjects were the same as that used for [^{3}H]CP-55940 binding (6 control, 7 schizophrenia), and 8 subjects were

that same as that used for [^{3}H]SR141716A binding (4 control, 4 schizophrenia). It was interesting that a trend for a positive correlation between [^{3}H]CP-55940 and $GABA_A$ binding in the superficial cortical layers was found (r = 0.488, p = 0.091). Consistent with this finding, there was a significant correlation between [^{3}H]SR141716A and $GABA_A$ binding in upper (r = 0.803, p = 0.016) and lower cortical layers (r = 0.774, p = 0.024). This correlation suggests that as CB1 receptors increase, $GABA_A$ receptors increase. Although the exact relationship between these two systems in the PCC has not been reported, CB1 receptors have been shown to inhibit the release of GABA in human and rat hippocampus (Katona et al. 1999; Katona et al. 2000). Therefore, it is possible that CB1 receptors inhibit GABA release in the PCC also, possibly contributing to the upregulation of $GABA_A$ receptors in the PCC in schizophrenia. Alternatively, it is also possible that there is an increase in cell number or terminals in the PCC in schizophrenia, which could explain why both receptors are increased. CB1 receptors have also been reported to modulate glutamate release. However, using cases from a previous study in chapter 3 (Newell et al. 2005), no correlation was found between CB1 receptor binding (both ligands) and NMDA, AMPA, or kainate receptor binding.

It is unknown if the observed changes in CB1 receptor binding in schizophrenia could be attributable to antipsychotic drug treatment. Little has been published regarding this possible relationship. Preliminary studies however, suggest that antipsychotic drugs do not increase CB1 receptor density in the cortex (Sundram et al. 2005). Furthermore, in the present study no correlation was found between [^{3}H]CP-55940 or [^{3}H]SR141716A binding in the PCC and final recorded antipsychotic drug dose, suggesting that the increase in CB1 binding found in schizophrenia may not be due to antipsychotic drug treatment. In addition to antipsychotic drug treatment, it is possible that the abuse of cannabis could influence the status of CB1 receptors.

However, in the present study, none of the cases examined had recently used cannabis (based on zero THC detected), so it is unlikely that recent cannabis use is the cause of the changes in CB1 binding found in this study.

It is interesting that in schizophrenia, CB1 receptor binding is increased. The intake of cannabinoids in rat studies actually decreases CB1 binding (Oviedo et al. 1993; Rodriguez de Fonseca et al. 1994; Romero et al. 1997). This suggests that smoking marijuana may possibly be therapeutic in schizophrenia as previously suggested (Peralta and Cuesta 1992). However, it is known from clinical studies that marijuana can induce psychosis and worsen symptoms in schizophrenia patients (D'Souza et al. 2005). The data on this subject is still controversial, and more research is required in this area.

The trend for a negative correlation between [^{3}H]spiperone and [^{3}H]SR141716A binding densities suggests that there may be interactions between the cannabinoid and serotonin systems. Recent studies have shown that CB1 receptor agonists reduce serotonin synthesis in rat brain (Moranta et al. 2004; Moranta et al. 2006), while antagonists increase serotonin synthesis (Darmani et al. 2003), further suggesting a relationship between these two systems. This correlation could not be confirmed with [^{3}H]CP-55940 and [^{3}H]spiperone as there were only 4 common cases.

5.5 Conclusion

These results suggest an increase in CB1 and a decrease in $5HT_{2A}$ receptor densities in the PCC in schizophrenia. It is possible that antipsychotic drug treatment has contributed to the observed reduction in $5HT_{2A}$ receptor binding. The changes in CB1 receptor binding further support a role of the endogenous cannabinoid system in schizophrenia. Further investigation into the cannabinoid system in schizophrenia and in

NMDA receptor hypofunction animal models would help to determine its role in schizophrenia. The examination of a larger number of both schizophrenia and control cases abusing cannabis would help to determine the role it plays on CB1 binding.

EXPERIMENTAL PART B:

CONSEQUENCES OF NMDA RECEPTOR HYPOFUNCTION ON NEUROTRANSMITTER BALANCE IN A MOUSE MODEL

Chapter 6: Verifying the effects of phencyclidine, clozapine and haloperidol on locomotor activity in mice

6.1 Introduction

There have been several pharmacological models of schizophrenia developed over the years. One of the most recent models is that developed with the use of PCP and other similar NMDA receptor antagonists. PCP non-competitively blocks the NMDA subtype of glutamate receptor and chronic PCP treatment is considered to provide one of the best pharmacological models of schizophrenia to date, as it produces some core symptoms almost indistinguishable from the disease itself (Javitt and Zukin 1991; Jentsch and Roth 1999). Jentsch and Roth (1999) and others (Morris et al. 2005) have suggested that chronic PCP treatment, as opposed to a single acute treatment, is a more precise model of schizophrenia, as it more accurately represents schizophrenia symptomatology in humans and animals. Furthermore, they suggest that the biological changes induced by the chronic model are more reminiscent of changes that have been reported in schizophrenia.

In non-psychotic humans, PCP abuse induces a state of schizophrenia-like psychosis, showing both positive and negative schizophrenia-like symptoms, while in schizophrenia patients, PCP abuse further exacerbates symptoms. Furthermore in animals, including rodents, PCP treatment has been shown to produce a range of symptoms that are analogous to the positive and negative symptoms of schizophrenia (For review see Javitt and Zukin 1991; Jentsch and Roth 1999). One of the most common PCP-induced behaviours in rodents is increased locomotor activity, which is considered to correlate with positive schizophrenia symptomatology (Lipska and Weinberger 2000). Furthermore, this PCP-induced hyperlocomotion is thought to

provide a useful screening device for antipsychotic drugs (Hoffman 1992), and therefore may be considered an important behavioural change in an animal model of schizophrenia. More recently, a delayed hypolocomotor effect has also been reported in rats treated chronically with PCP, an effect which has been hypothesized to model negative schizophrenia-like symptoms (Hori et al. 2000; Audet et al. 2007).

Clozapine, the original atypical antipsychotic drug, is one of the most effective agents to treat the psychosis of schizophrenia patients, including patients that are otherwise unresponsive to antipsychotic drugs (Meltzer 2004). Haloperidol on the other hand, a dopamine D2 antagonist, is considered a typical antipsychotic which primarily treats the positive symptoms of schizophrenia. Studies have shown that clozapine and haloperidol administration to rodents both cause reductions in locomotor activity (Simon et al. 2000). Furthermore, each of these drugs has the ability to prevent PCP-induced hyperlocomotion (Gleason and Shannon 1997).

The aim of the present study was to examine the effects of chronic PCP, clozapine and haloperidol treatment on locomotor activity, in order to verify the effects of these drugs in the animal models to be applied in chapters 7, 8 and 9.

6.2 Materials and Methods

6.2.1 Animals

Twenty-four female C57Bl/6 mice, aged 9 weeks, were obtained from the Animal Resource Center, WA, Australia, and housed in groups of three. Food and water were available ad libitum. Mice were allowed 2 weeks to acclimatize to their new environment before any studies were conducted. The mice were housed in a reversed 12-hour light-dark cycle, in which lights were off from 09:00-21:00 and the mice were

treated and tested between 10:00 and 17:00. All experiments described in this study were approved by the University of Wollongong Animal Ethics Committee (AE02/21).

6.2.2 Drug Treatment

Phencyclidine Hydrochloride (PCP; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in normal saline to a concentration of 10mg/kg. Clozapine tablets (25mg; Sigma) were dissolved in saline to a concentration of 1.5mg/kg. Minimal hydrochloric acid was added to dissolve the clozapine tablets, and the pH was then neutralized with sodium hydroxide (La et al. 2006). Haloperidol (Sigma) was obtained in a concentration of 2mg/ml and was diluted with saline to an injection concentration of 1mg/kg. All injections were at a volume of 30*ul.* Drugs were injected subcutaneously into the outer thigh, and left and right thighs were alternated each day to reduce animal discomfort. Mice were injected once a day between 10:00am and 10:30am for 14 days. Mice were randomly assigned to their treatment groups (PCP, clozapine, haloperidol, or saline controls), with 6 mice allocated to each group.

6.2.3 Locomotor testing

All mice were introduced to the locomotor box 1 week prior to the experiment by placing them in the locomotor box for one hour. The locomotor box consisted of a large mouse cage (25cm wide x 40cm long x 15cm high) attached to a light source and split beam detector. The light source sends a beam across the cage. Each time the animal passes the beam, the split beam detector sends a signal to the counter, which records the event (Fig. 6.1).

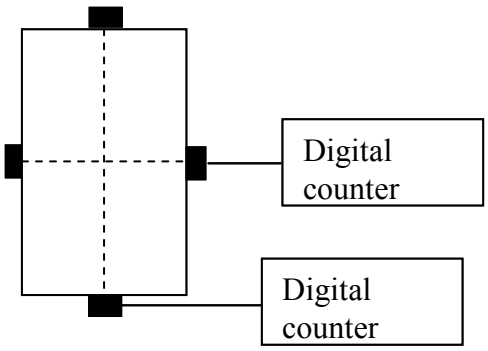

Figure 6.1 Diagrammatic representation of the locomotor box

Mice were habituated to the locomotor box for 20mins before measurements were taken. On days 2, 8, and 14 of treatment, locomotor activity was then measured for 1 hour prior to the daily injection (pre-injection phase – equivalent to 24 hours after the previous injection). The mice were then injected with their allocated drugs. On days 1, 2, 8, and 14, locomotor activity was also measured for 1 hour *after* the drug injection (post-injection phase).

6.2.4 Statistical Analysis

Two-way repeated ANOVA was used to determine any effects of treatment or time on locomotor activity. One-way ANOVA was employed to determine specific treatment effects on locomotor activity at each time point. Tukey's tests were used for post-hoc comparisons. Paired t-tests were used to test for differences in locomotor activity between the different treatment days.

6.3 Results

6.3.1 The effects of chronic PCP, clozapine, and haloperidol treatment on locomotor activity measured in the post-injection phase

The analysis of locomotor activity in the post-injection phase (60mins after the injection) of 14-day treatments showed a significant effect of treatment ($F(1,14) = 55.864$, $p<0.001$), but no significant effect of time ($F(3, 42) = 0.642$, $p = 0.526$), and no significant time x treatment interactions ($F(9, 42) = 0.160$, $p = 0.983$). Post hoc analysis showed that locomotor activity in the PCP treated mice was significantly greater than in the saline treated mice ($p<0.001$). In contrast, locomotor activity in the haloperidol treated mice was significantly reduced compared to the saline treated mice ($p = 0.002$). There was no significant difference between clozapine treatment and saline treatment in terms of locomotor activity ($p = 0.314$). However, it was observed that there was consistently lower activity in the clozapine-treated group versus the saline group (Fig. 6.3)

One-way ANOVA followed by Tukey's post hoc analysis showed that PCP treatment caused a significant increase in locomotor activity compared to saline treated mice, at all days measured ($p \leq 0.001$; Fig. 6.2). As the repeated ANOVA showed no effect of time, further t-test analyses between the treatment days were not conducted.

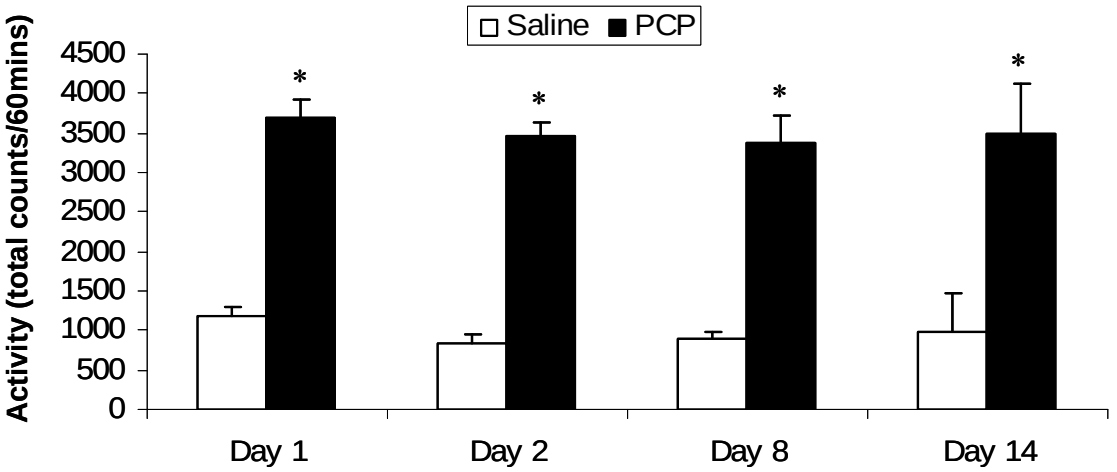

Figure 6.2 Locomotor activity of saline and PCP treated mice, as measured in the post-injection phase on days 1, 2, 8, and 14. ∗: $p \leq 0.01$, compared to saline treated mice.

One-way ANOVA followed by Tukey's post hoc analysis showed that locomotor activity in the haloperidol treated mice was significantly and dramatically (up to 96%) less than in the saline treated mice on days 1 ($p<0.001$), 2 ($p = 0.004$), and 8 ($p = 0.018$) but surprisingly this was not significant at day 14 ($p = 0.213$). As the repeated ANOVA showed no effect of time, further t-test analyses between the treatment days were not conducted.

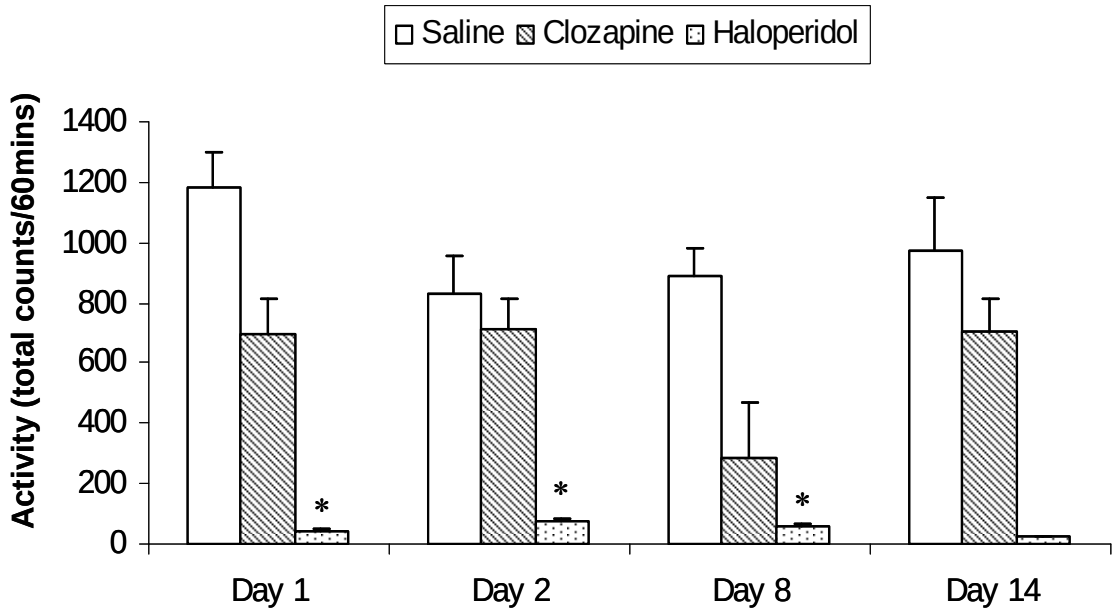

Figure 6.3 Locomotor activity of saline, clozapine, and haloperidol treated mice, measured in the post-injection phase on days 1, 2, 8, and 14. ∗: $p< 0.002$ compared to saline treated mice.

6.3.2 The effects of chronic PCP, clozapine, and haloperidol treatment on locomotor activity measured in the pre-injection phase

The analysis of locomotor activity in the pre-injection phase (measured 24 hours after the 1st, 7th, and 14th injections) of 14-day treatments revealed significant effects of time ($F(2, 36) = 11.670$, $p<0.001$) and treatment ($F(3, 18) = 3.314$, $p = 0.044$), and a borderline significant interaction between time and treatment ($F(6, 36) = 2.231$, $p = 0.062$). Post-hoc tests demonstrated that locomotor activity of PCP treated mice was significantly decreased compared to saline treated mice ($p = 0.037$; Fig. 6.4). However, the locomotor activity of clozapine ($p = 0.795$) and haloperidol ($p = 0.951$) treated mice was not significantly different compared to saline treated mice, when measured in the pre-injection phase (Fig. 6.5).

One-way ANOVA showed that on day 2 of treatment, there was a 20% reduction in locomotor activity in the PCP treated mice compared to saline treated mice, however this did not reach significance ($F = 3.541$, $p = 0.089$). On day 8, there was a significant 44% reduction in locomotor activity in the PCP treated mice versus saline treated mice ($F = 5.537$, $p = 0.040$), and on day 14, there was a 46% reduction in locomotor activity in the PCP treated mice versus the saline group ($F = 7.392$, $p = 0.022$).

There was a significant effect of time which is evident in figure 6.4. Paired t-test analysis showed that in the PCP treated mice, locomotor activity on day 8 was significantly less than activity on day 2 ($t = 2.458$, $p = 0.057$). In addition, activity on day 14 was less than activity on day 8, however this did not reach significance ($t = 2.196$, $p = 0.079$). Furthermore, locomotor activity on day 14 was significantly less than activity on day 2 ($t = 3.727$, $p = 0.014$).

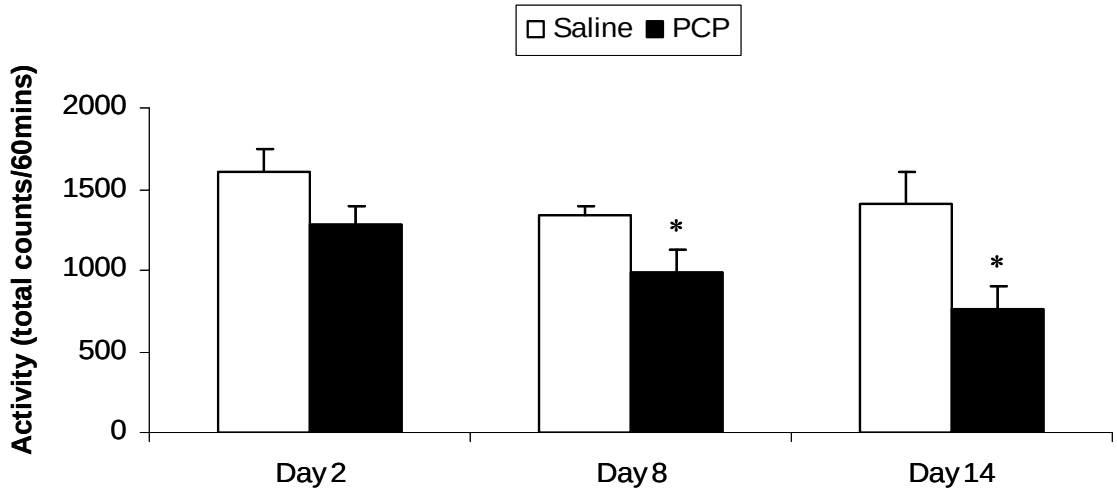

Figure 6.4 Locomotor activity of saline and PCP treated mice, measured in the pre-injection phase on days 2, 8, and 14. *: $p<0.05$ compared to saline treated mice.

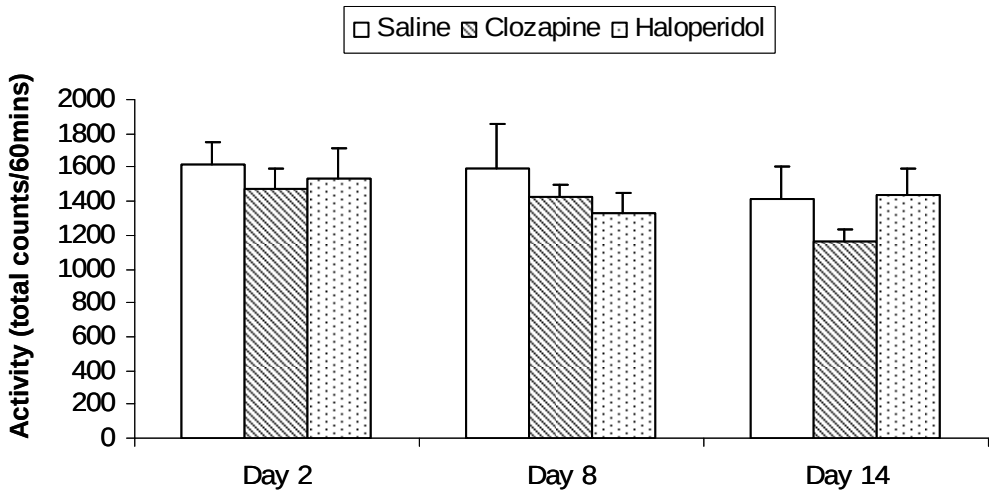

Figure 6.5: Locomotor activity of saline, clozapine, and haloperidol treated mice, measured in the pre-injection phase on days 2, 8, and 14, showing no statistical differences compared to the saline treated mice.

Although there were no differences in locomotor activity between clozapine and saline treated mice, there was a significant time effect of the clozapine treatment. Locomotor activity in the clozapine treated mice was significantly lower on day 14

compared to day 2 ($t = 4.253$, $p = 0.013$) and day 8 ($t = 8.285$, $p = 0.001$). There was no significant difference however between day 2 and day 8 ($t = 0.647$, $p = 0.553$).

There was no significant difference in locomotor activity in the haloperidol treated mice between days 2, 8 and 14.

6.4 Discussion

The main findings from this study were: 1) PCP administration produced severe hyperlocomotor activity immediately following administration, and this hyperlocomotion did not diminish with each subsequent injection; 2) PCP administration produced hypolocomotion when measured 24 hours after the injection, and this progressively became more severe over 14 days of treatment; 3) haloperidol treatment dramatically reduced locomotor activity in the post-injection phase of treatment, and this effect did not diminish over the treatment period; and 4) clozapine administration decreased locomotor activity in the post-injection phase of treatment, however not significantly so compared to saline treated mice.

The PCP model developed for this study was of a chronic nature. Nabeshima and his research group have demonstrated that the model used in the present study (i.e. PCP 10mg/kg/day, 14 days) mimics schizophrenia symptomatology in both mice and rats (Nabeshima et al. 1985a; Nabeshima et al. 1987; Noda et al. 1995; Noda et al. 2000). They have shown that this treatment regime produces psychotic-like symptoms as evidenced by increased locomotor activity and stereotyped behaviours (Nabeshima et al. 1987; Noda et al. 1995), in addition to producing negative schizophrenia-like symptomatology as demonstrated by increased immobility time in a forced swim test (Noda et al. 1995; Noda et al. 2000). Furthermore, Nabeshima and colleagues have shown that this treatment protocol produces some biological changes reminiscent of schizophrenia, such as dysfunctional cortical dopamine (Noda et al. 2000). The dosages

of clozapine and haloperidol used in this study were chosen based on information from previous studies, showing that these doses did have antipsychotic effects in PCP-treated rodents. The doses used were shown to have the ability to attenuate PCP-induced hyperlocomotion (Gleason and Shannon 1997).

An important unique aspect of this animal model is the treatment time. The majority of studies using PCP and antipsychotic drugs in animal models treat rodents, which are nocturnal, during the animals sleep time. In the present study, mice were housed in reversed photoperiod so they could be treated and their activity tested during their active time. Despite this, the results were consistent with previous studies treating animals during their sleep time.

Female mice were chosen for this project as it has been demonstrated that females are more sensitive to the neurotoxic (Fix et al. 1995) and behavioural (Frantz and Van Hartesveldt 1999) effects of NMDA receptor antagonists. While the use of male mice would have provided an interesting comparison of the gender effects of NMDA antagonism, this was beyond the scope of this study.

6.4.2 Locomotor activity during the post-injection phase

The PCP-induced hyperlocomotion observed in this study has been reported previously (Nabeshima et al. 1984; Gleason and Shannon 1997; Hori et al. 2000) and confirms that a state of psychosis has been induced. PCP-induced hyperlocomotion has previously been discussed as representing the positive symptoms of schizophrenia (Castellani and Adams 1981; Gleason and Shannon 1997; Lipska and Weinberger 2000), and is one of the common features of schizophrenia animal models. Gleason and Shannon (1997) and others (Ninan and Kulkarni 1998) have shown that the PCP-induced increase in locomotor activity is at least partly due to the actions of $5HT_{2A}$ receptors, while other studies have shown that dopamine is primarily involved in

producing PCP-induced hyperlocomotion (Nabeshima et al. 1983; Greenberg and Segal 1985).

Chronic treatment with haloperidol showed very dramatic decreases in locomotor activity to almost zero activity. This is a previously reported effect of haloperidol on rodents (Hoffman 1992) and because of the severity of this reduction, it suggests that it is not a specific antipsychotic mechanism. Furthermore, this is consistent with its reported effects in patients, in which akinesia occurs, and is a factor in non-compliance of medication (Hymowitz et al. 1986; Clinton et al. 1987). Therefore, when testing novel antipsychotics, locomotor activity should be examined to try and eliminate this major side effect of akinesia.

Chronic clozapine treatment showed a hypolocomotion effect when measured in the post-injection phase, however this was not significantly different compared to the saline treatment group. It may be that a larger number of subjects were required to show significance in this group, as the changes were not of the same magnitude as the haloperidol or PCP treated mice. It was expected that clozapine would cause a reduction in locomotor activity (although not to the same extent as haloperidol), as it has been reported previously in rodent studies (Gleason and Shannon 1997). Furthermore, it is well known that clozapine has acute sedative effects in patients (Marinkovic et al. 1994).

6.4.3 Locomotor activity during the pre-injection phase

The chronic PCP-induced hypolocomotion observed in the present study is a new phenomenon that has not previously been reported in mice. Hori et al (2000) and more recently Audet et al (2007) observed this PCP-induced hypolocomotion in rats treated with a similar dose and duration of PCP as in the present study. They discussed that this may represent the negative symptoms of schizophrenia. Furthermore, an earlier

study showed that withdrawal of PCP resulted in reductions in exploratory activity in rats (Spain and Klingman 1985), supporting the hypothesis that the hypolocomotion may represent negative symptoms. Thus, PCP-induced hypolocomotion may represent a form of depression/decreased motivation, and shows that there is a delayed effect of chronic PCP treatment. While it cannot be ruled out that this hypolocomotor effect is due to fatigue, studies by Audet et al. (2007) suggest that this is unlikely to be the case as when placed on a rotarod, PCP treated rats were able to run at a constant speed and were as good as controls in maintaining balance and avoiding falls.

The PCP-induced hypolocomotion observed in the present study is a phenomenon induced by chronic treatment as it was not significant at day 2 and became more significant over time. This therefore could add to the body of information suggesting that the chronic PCP model better represents schizophrenia symptomatology.

PCP-induced hypolocomotion is an interesting phenomenon and should be examined further. It is possible that it could be used to test the effectiveness of antipsychotic drugs in treating negative schizophrenia symptoms. $5HT_{2A}$ receptor antagonists (eg clozapine) and dopamine D2 antagonists (eg haloperidol) have been shown to reduce locomotor activity and also prevent PCP-induced hyperlocomotion in rodents (Gleason and Shannon 1997; Krebs-Thomson et al. 1998). It could therefore be hypothesized that $5HT_{2A}$ and D2 receptors may also be involved in PCP-induced hypolocomotion.

6.5 Conclusion

The use of this chronic PCP model has shown that it does emulate some schizophrenia-like symptomatology as evidenced by the positive schizophrenia-like hyperlocomotion and the negative schizophrenia-like hypolocomotion. Furthermore, this study has shown that the typical antipsychotic haloperidol, and the atypical

antipsychotic clozapine, at the doses used in the present study both have hypolocomotor effects as previously established. This study has therefore verified that these drugs, at the dosages used in this study are in fact effective at altering mouse behaviour. It would be interesting to examine changes in key neurotransmitters that result from the above PCP and antipsychotic drug treatments.

Chapter 7: Differential short and long term changes in NMDA receptor binding in mouse brain following chronic phencyclidine treatment

7.1 Introduction

Pharmacological and biochemical data has suggested a deficiency in NMDA-mediated glutamatergic transmission in schizophrenia. Post-mortem studies suggest altered NMDA receptor binding in several brain regions including the anterior and posterior cingulate cortices (Zavitsanou et al. 2002; Newell et al. 2005), superior temporal gyrus (Nudmamud and Reynolds 2001) and caudate putamen (Aparicio-Legarza et al. 1998). The most convincing evidence of the NMDA hypofunction hypothesis of schizophrenia is based on the fact that PCP, a psychotomimetic drug that blocks the NMDA subtype of glutamate receptor, produces a syndrome that appears similar to schizophrenia (Javitt and Zukin 1991). In addition to the effects in humans, a history of experimental investigations suggest that PCP administration may also model some of the behavioural symptoms of schizophrenia in non-human subjects (Jentsch et al. 1999). PCP arguably provides one of the best pharmacological models of schizophrenia, where NMDA hypofunction has been hypothesized to be the underlying mechanism of this behavioural syndrome and possibly of schizophrenia (Olney and Farber 1995).

To date, several studies have examined the behavioural effects of acute and chronic PCP treatment in humans and animals. PCP produces not only an acute psychotic reaction in normal humans but also "flashback" recurrences of that same psychosis (Allen and Young 1978). Furthermore, long lasting cognitive impairments in

episodic memory and attention have been reported in recreational users of the PCP analogue ketamine (Morgan et al. 2004). In addition to these long-term effects in humans, long-term cognitive and behavioural effects of PCP have been described in primates and rodents. A study by Jentsch et al. (1997b) showed that monkeys treated chronically with PCP developed cognitive deficits that were still present at least 4 weeks after PCP treatment, an effect that was mimicked in mice (Hashimoto et al. 2005). Nabeshima and his research group (and the study in chapter 6 of this thesis) have demonstrated that PCP treatment in mice, using the dosing regimen, 10mg/kg/day for 14 days, mimics positive and negative schizophrenia symptomatology (Nabeshima et al. 1985a; Noda et al. 1995; Noda et al. 2000). Using this same dosing regimen, Noda et al. (1995) reported that mice not only displayed hyperlocomotion, but also developed negative schizophrenia-like symptoms that were still present 3 weeks after chronic PCP treatment. Despite these obvious long-term behavioural effects of chronic PCP treatment, neurochemical substrate alterations in the long-term after chronic PCP treatment have not been investigated.

The aims of the present study were to: 1) identify any changes in NMDA receptor binding in mouse brain following chronic PCP treatment; 2) determine if there were differential short-and long-term effects on NMDA receptor binding following chronic PCP treatment and; 3) determine if there are region-specific changes in NMDA receptor binding following chronic PCP treatment.

7.2 Materials and Methods

7.2.1 Animals and Drug Treatment

Thirty female C57Bl/6 mice, aged 9 weeks, were obtained from the Animal Resource Center, WA, Australia, and housed in groups of three. Food and water were

available ad libitum. Mice were allowed 2 weeks to acclimatize to their new environment before any studies were conducted. The mice were housed in a reversed 12-hour light-dark cycle, in which lights were off from 09:00-21:00. Phencyclidine Hydrochloride (PCP; Sigma) was dissolved in normal saline to a concentration of 10mg/kg. All injections were at a volume of 30*ul.* Drugs were injected subcutaneously into the outer thigh, and left and right thighs were alternated each day to reduce animal discomfort. Mice were injected once a day between 10:00am and 10:30am. All experiments described in this study were approved by the University of Wollongong Animal Ethics Committee (AE02/21). The mice were randomly designated to either short or long-term treatment groups with 6 mice per group.

Short-term

1. PCP 14 days, sacrificed 1 hour after the final injection ($PCP_{14days\text{-}1hr}$)
2. PCP 14 days, sacrificed 24 hours after the final injection ($PCP_{14days\text{-}24hr}$)
3. Saline 14 days

Long-term

4. PCP 14 days followed by saline 14 days (PCP_{14days}+$Saline_{14days}$)
5. Saline 28 days

7.2.4 Receptor autoradiography

All mice were sacrificed with an overdose of sodium pentobarbitone anesthesia (120mg/kg i.p.). In order to minimize circadian variation in receptor density, all mice were sacrificed between 11:00am and 11:30am. Brains were immediately removed after death and frozen in liquid nitrogen. Coronal brain sections (14μm) were cut at -17^{o}C on a cryotome (Clinicut cryostat, Bright Instruments, England) and mounted onto microscope slides. [^{3}H]MK-801 autoradiography was performed as previously detailed in chapter 3 (Ekonomou and Angelatou 1999; Newell et al. 2005).

7.2.5 Quantification and statistical analysis

Detailed quantification procedures have been described previously (Huang et al. 2004). Briefly, all treated sections were placed on Kodak BioMax MR film. After exposure for 4 months, films were developed using Kodak GBX developer and fixed with Kodak GBX fixer. All films were analyzed by using a computer-assisted image analysis system, Multi-Analyst, connected to a GS-690 Imaging Densitometer (Bio-Rad, USA). Brain regions for quantification were identified based on a standard mouse brain atlas (Franklin and Paxinos 1997). Seventeen brain regions were selected for quantification including: Limbic – posterior cingulate cortex, anterior cingulate cortex, hippocampus, nucleus accumbens, and amygdala; Caudate-Putamen (CPu) – rostral and caudal subdivisions; Cortex – auditory, motor, sensory, and visual; Thalamus – lateroventral and medial thalamus (Fig. 7.1). Quantification of receptor binding in each brain region was performed by measuring the average density of each region in three adjacent brain sections. Values were then compared against an autoradiographic standard (Amersham, UK). Specific binding was calculated by subtracting non-specific from total binding. Receptor density in each brain region was analyzed using a one-way ANOVA. Tukey's post-hoc analysis was employed to determine differences between the short-term treatment groups. All data was analyzed using the SPSS statistical package.

7.3 Results

Specific [^{3}H]MK-801 binding was widely distributed throughout the mouse brains, although there were significant regional variations in density (Fig. 7.1). Non-specific binding was measured to be less than 30%. In all groups, the highest density of specific binding was distinctly observed in the hippocampus. Moderately high levels of

binding were observed in the auditory, visual, anterior cingulate, sensory and motor cortices. The thalamus and CPu subdivisions displayed moderate-low levels of [^{3}H]MK-801 binding, while the amygdala generally showed the lowest density of binding.

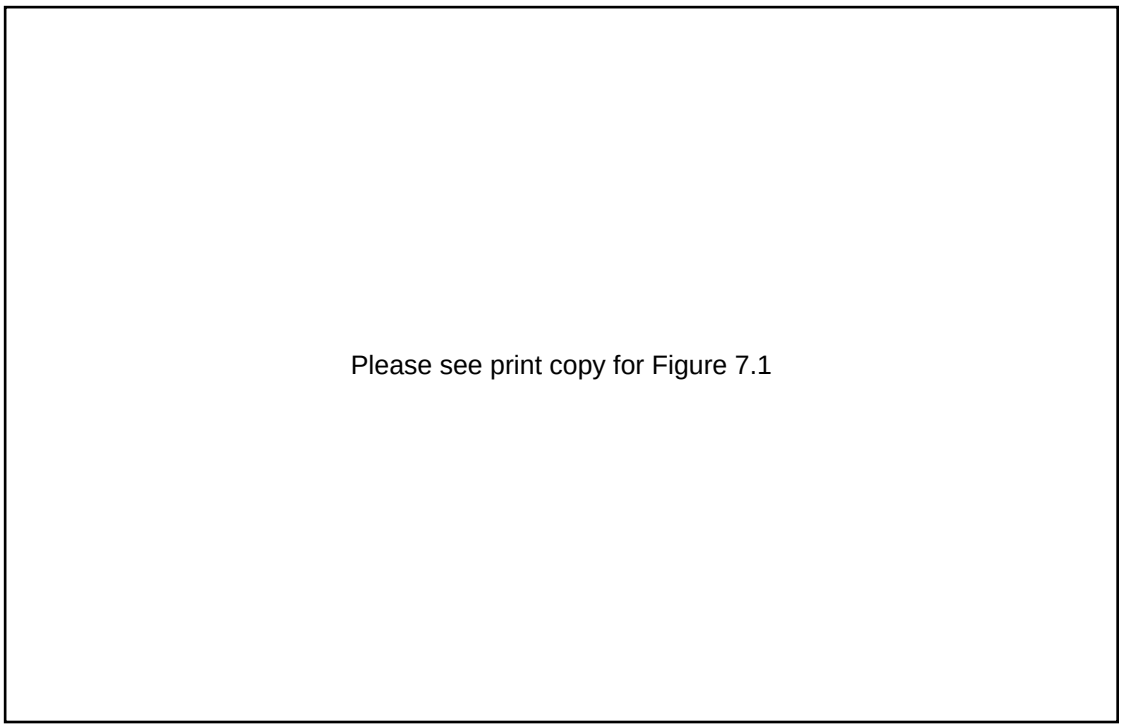

Figure 7.1 Representative autoradiographs of coronal brain sections which illustrate total [^{3}H]MK-801 binding (A', B', C'), and non-specific [^{3}H]MK-801 binding (A'', B'', C''). Mouse brain atlas image (A, B, C) adapted from Franklin and Paxinos (1997). Acb: nucleus accumbens, ACC: anterior cingulate cortex, ACtx: auditory cortex, Amyg: amygdala, D: caudal dorsal caudate-putamen, DL: rostral dorsolateral caudate-putamen, DM: rostral dorsomedial caudate-putamen, Hi: hippocampus, LVthal: lateroventral thalamus, M1/2: primary/secondary motor cortex, Mthal: medial thalamus, PCC: posterior cingulate cortex, S1: primary sensory cortex, V: caudal ventral caudate-putamen, VCtx: visual cortex, VL: rostral ventrolateral caudate-putamen, VM: rostral ventromedial caudate-putamen.

7.3.2 The short-term effects on [^{3}H]MK-801 binding following chronic PCP treatment

A significant effect of treatment was found in the hippocampus ($F = 14.377$, $p<0.001$), anterior cingulate cortex ($F = 4.479$, $p = 0.035$) and the caudal dorsal CPu ($F = 3.623$, $p = 0.054$; one-way ANOVA). PCP treatment for 14 days resulted in a significant 18% ($PCP_{14days\text{-}1hr}$) and 24% ($PCP_{14days\text{-}24hr}$) increase in [^{3}H]MK-801 binding

in the hippocampus compared to saline treated mice (Fig. 7.2). There was also a significant 17% increase in the anterior cingulate cortex and a significant 16% decrease in binding in the caudal dorsal CPu in the $PCP_{14days\text{-}1hr}$ group, when compared to saline treated mice. There were no significant changes in any other brain region examined in either of the PCP treated groups compared to the saline controls. Furthermore, there were no differences in [^{3}H]MK-801 binding between $PCP_{14days\text{-}1hr}$ and $PCP_{14days\text{-}24hr}$ treated mice in any brain region examined.

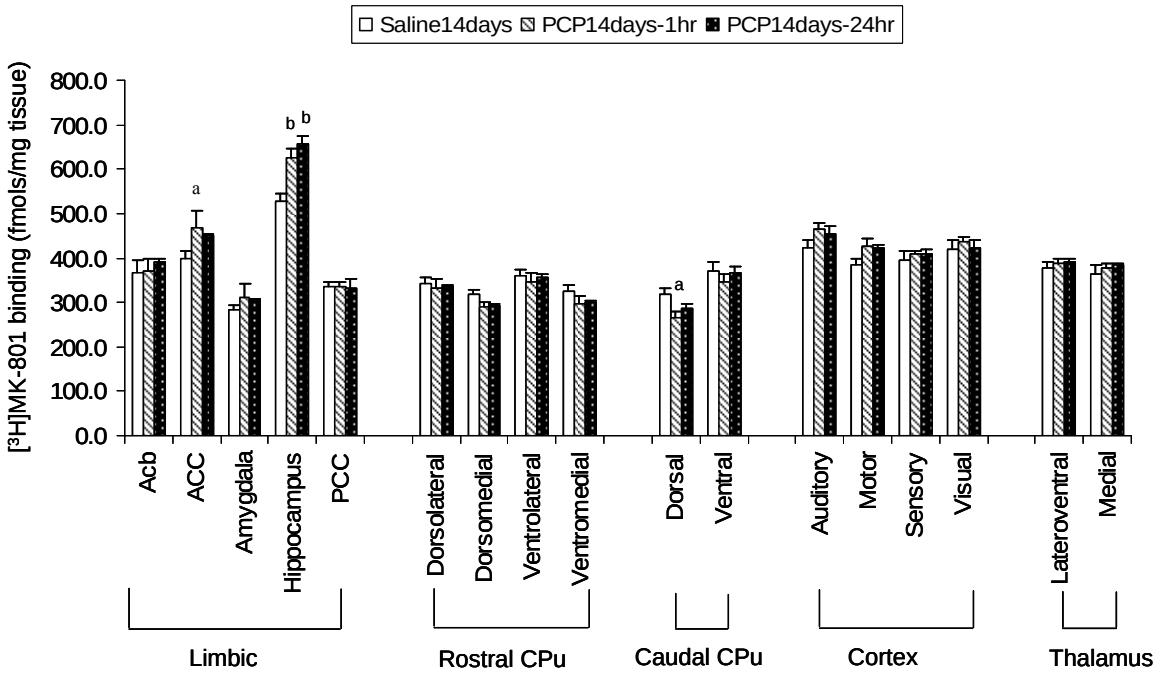

Figure 7.2. The short-term effects on [^{3}H]MK-801 binding in mouse brain following chronic PCP treatment. PCP14days-1hr: mice treated for 14 days with PCP and sacrificed 1 hour after the final injection; PCP14days-24hr: mice treated for 14 days with PCP and sacrificed 24 hours after the final injection. Acb: nucleus accumbens, ACC: anterior cingulate cortex, CPu: caudate putamen, PCC: posterior cingulate cortex ^{a}:$p \leq 0.05$, ^{b}:$p \leq 0.006$ compared to saline treated mice.

7.3.3 The long-term effects on [^{3}H]MK-801 binding following chronic PCP treatment

PCP treatment for 14 days followed by saline for 14 days (PCP_{14days}+$Saline_{14days}$) resulted in decreased [^{3}H]MK-801 binding in 15 out of 17 brain regions examined, when compared to saline treated mice (Fig. 7.3). The largest decrease was observed in the hippocampus (35%). This was followed by the sensory, visual, auditory and anterior cingulate cortices (29, 26, 24 and 23% respectively). The medial and lateroventral thalamus, caudal ventral CPu, posterior cingulate and motor cortices and nucleus accumbens showed reductions of 20, 23, 21, 19, 18, and 17% respectively. The lowest magnitude of change was observed in the rostral part of the CPu. The caudal dorsal CPu, although not significant, did show a trend towards decreased binding, while the amygdala, the region with the lowest binding density, showed no statistically significant change in the PCP treated mice versus the saline controls.

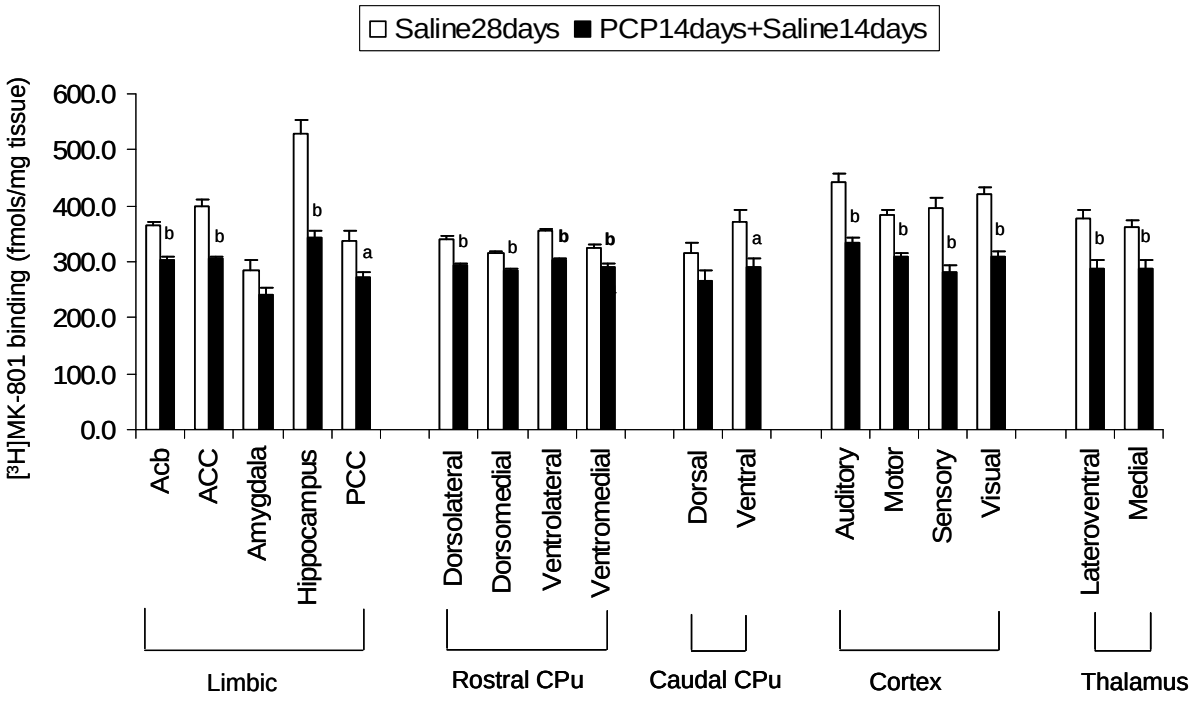

Figure 7.3. The long-term effects on [^{3}H]MK-801 binding in mouse brain following chronic PCP treatment. Acb: nucleus accumbens, ACC: anterior cingulate cortex, CPu: caudate putamen, PCC: posterior cingulate cortex, [a]:$p<0.05$, [b]: $p<0.008$ compared to saline treated mice.

7.4 Discussion

The present study has shown that: 1) NMDA receptor binding was significantly increased consistently in the hippocampus in mice treated with PCP for 14 days and sacrificed 1 or 24 hours after the last PCP treatment (short-term PCP treatment groups), compared to saline-treated controls; 2) NMDA receptor binding showed widespread reductions in mice treated with PCP for 14 days and sacrificed 14 days after the last PCP treatment (long-term PCP treatment group).

7.4.1 Distribution of NMDA receptor binding in control animals

The regional distribution of NMDA receptor binding sites in the mouse brain corresponded well to that previously reported in rat brain (Sakurai et al. 1991). The

highest levels of [^{3}H]MK-801 receptor binding were found in the hippocampus followed by cortical regions. The thalamus, CPu and amygdala showed moderate-low densities of NMDA receptor binding.

7.4.2 Short-term effects on NMDA receptor binding following chronic PCP treatment

Increased [^{3}H]MK-801 binding to NMDA receptors was found in the hippocampus of mice sacrificed 1 and 24 hours after the last PCP treatment, suggesting a strong involvement of this brain region in the short-term effects of PCP. This increase in the hippocampus, the area with the highest density of NMDA receptors of all brain regions examined, was the only change that was significant to both short-term groups. Interestingly, the hippocampus is well known to be involved in cognitive functions, such as learning and memory, that have been shown to be affected by PCP (Frederick et al. 1995; Newcomer and Krystal 2001). PCP is an NMDA receptor antagonist, and is thought to produce its psychotomimetic effects through this antagonistic action. It is therefore possible that the increased NMDA receptor binding observed in the present study may represent a regulatory mechanism that compensates for reduced NMDA receptor function following NMDA receptor blockade. In agreement with our data, Gao and Tamminga (1994; 1995) have reported an increase in NMDA sensitive [^{3}H]glutamate binding in the hippocampus 24 hours after a single administration of PCP or MK-801. Similarly, Hori et al. (2000), showed that after 14 days of PCP treatment, [^{125}I]MK-801 binding to NMDA receptors was increased in the hippocampus, however in contrast they also found increased binding in the thalamus, substantia nigra and infralimbic cortex. In support of increased NMDA activity in the short-term after PCP treatment, recent electrophysiological studies have shown hyperactive NMDA receptors

up to 5 days following subchronic PCP treatment (Arvanov and Wang 1999; Yu et al. 2002).

Upregulated NMDA receptor binding in the hippocampus in both of the short-term treatment groups was accompanied by significantly *reduced* NMDA receptor binding in the caudal dorsal CPu in the $PCP_{14days\text{-}1hr}$ group only. While the function of this subregion of the CPu is not yet known, it is known that the CPu is a brain region that plays an important role in movement. Striatal blockade of NMDA receptors is known to produce increases in locomotor activity (Hauber 1998). As major increases in locomotor activity were evident in the present study 1 hour after PCP treatment (refer to chapter 6), it is possible that this alteration of NMDA receptors in the caudal dorsal CPu could contribute to the abnormal motor activity.

The half-life of PCP has been reported to be approximately 4 hours in male rats (at a dose of ~13mg/kg; Massey and Wessinger 1990) and 1.5 hours in female rats (at a dose of ~20mg/kg; Nabeshima et al. 1984). Therefore in the short-term treatment groups, we cannot exclude the possibility that exogenous PCP has not been completely removed from the mouse brain cell membranes, especially in the animals sacrificed 1 hour after the last PCP treatment. However, we found no difference in NMDA receptor density in the 14-day PCP treated mice whether they were sacrificed 1 or 24 hours after the final PCP injection. Therefore, the observed changes are unlikely to be affected by the presence of exogenous PCP.

In the present study we examined NMDA receptor density following PCP treatment by incubating brain sections with a single concentration of [^{3}H]MK-801. Therefore this study provides information about changes in receptor density only, although changes in the binding affinity of the ligand for the receptor may have occurred. Previous studies using membrane preparations have reported changes in the

density of binding sites targeted by [^{3}H]TCP following PCP treatment (Massey and Wessinger 1990; Saransaari et al. 1993) as well as in the affinity of [^{3}H]TCP for its receptors (Massey and Wessinger 1990). However these studies provide no information about the anatomical localization of these changes.

7.4.3 Long-term effects on NMDA receptor binding following chronic PCP treatment

A widespread reduction in [^{3}H]MK-801 binding to NMDA receptors was observed in the mouse brain 14 days following chronic PCP treatment, suggesting that NMDA hypofunction occurs in this long-term phase of PCP treatment. This effect was more evident in the hippocampus where a 35% reduction of binding was found. Ellison et al. (1999b) showed that 5 days of high-dose PCP (~20 mg/kg/day) treatment resulted in reductions in [^{3}H]TCP binding to NMDA receptors in rat hippocampus measured 21 days following PCP treatment. However, this was accompanied by increased NMDA binding in the striatum, and no significant changes in the remaining brain regions examined. Taken together, although there is some dissimilarity, these data do suggest a strong involvement of the hippocampus in the long-term effects following PCP treatment.

Chronic PCP treatment has been shown to cause long-term cognitive/behavioural effects in primates and rodents. A study by Jentsch et al. (1997b) showed that monkeys treated chronically with PCP developed cognitive deficits that were still present at least 4 weeks after PCP treatment, while Noda et al. (1995), using the same dosing regimen as in the present study, showed that increased immobility time in swimming tests was still present 3 weeks after chronic PCP treatment. Furthermore, using 10mg/kg for 10 days, Hashimoto et al. (2005) reported that PCP induced cognitive deficits in mice that lasted for more than 6 weeks after PCP treatment.

However, neurochemical data explaining these long-term cognitive effects is not available. Finally, Kalinichev et al. (2005) treated rats for 7 days with PCP, followed by 7 days of withdrawal, and then challenged the rats with PCP (3.2mg/kg) on the 8th day. They found that the PCP challenge caused a major increase in locomotor activity, while rats that had not been pretreated with PCP showed no or only a small change, suggesting a long-term effect of the PCP pre-treatment. The present study reports that chronic PCP treatment induces reductions in NMDA receptor binding that persist long after completion of drug treatment. It is therefore a possibility that reduced NMDA receptor function might underlie the long lasting cognitive and behavioral impairments reported after prolonged blockage of NMDA receptors in humans or animals.

The long-term effects following chronic PCP treatment observed in the present study may be due to several factors including increased glutamate release (Moghaddam et al. 1997; Adams and Moghaddam 1998; Moghaddam and Adams 1998) and/or cell death/damage (Wozniak et al. 1998). Acute PCP administration has been reported to increase glutamate release in several brain regions including, nucleus accumbens, anterior cingulate cortex, posterior cingulate cortex, hippocampus and striatum (Liu and Moghaddam 1995; Moghaddam and Adams 1998; Noguchi et al. 1998). Interestingly, NMDA receptor downregulation was observed in all the above regions in the present study. It is possible therefore that decreased NMDA receptor binding occurred in the present study to compensate for continuous glutamate release. Furthermore, previous studies suggest that increased glutamate release can cause neuronal cell injury or cell death (Nishizawa 2001), which could contribute to the downregulated NMDA receptors. Consistent with this notion, chronic treatment with PCP in rats has been shown to cause cell death in multiple brain regions (Ellison and Switzer 1993; Ellison 1994).

The dosage of PCP used in the present study has been shown previously to induce both positive and negative schizophrenia-like symptoms in mice (Noda et al. 1995; Noda et al. 2000). Specifically, it was shown that doses lower than 10mg/kg did not produce these negative schizophrenia-like symptoms and hence were not used in the present study. However, at this dosage, PCP does not only target the NMDA receptors but also several other receptor systems including the dopamine, norepinephrine and serotonin transporters and the sigma receptor (Javitt and Zukin 1991). It may actually be the incorporation of these other neuronal systems that gives PCP its characteristic behavioural effects, and it is possible that the involvement of these systems has contributed to the observed changes in NMDA receptors. The involvement of additional receptor systems is currently under investigation.

Despite the fact the present findings do not reflect the changes in NMDA receptor binding density that have been reported in schizophrenia, it does not preclude NMDA hypofunction as being an underlying mechanism in schizophrenia. Furthermore, the present finding of widespread changes in NMDA receptor binding density in the long-term following chronic PCP treatment is consistent with the notion that schizophrenia is a disease that affects most if not all frontal cortical systems, limbic systems, basal ganglia and thalamus (Moghaddam 2003).

7.5 Conclusion

The present study has shown that chronic PCP treatment has differential short and long-term effects on NMDA receptor binding in mouse brain. The short-term effects following chronic PCP treatment were upregulated NMDA receptor binding in the hippocampus, accompanied by virtually no other changes, suggesting a regulatory mechanism in this region to compensate for strong NMDA blockade. In the long-term, it was found that chronic PCP treatment caused widespread reductions in NMDA

receptor binding, with the most pronounced reduction occurring in the hippocampus, indicating that the NMDA receptors in this region are more sensitive to chronic treatment than other brain regions. This downregulation may be due to several factors including increased glutamate release and/or cell death.

Chapter 8: Opposing short and long-term effects on M1/4 muscarinic receptor binding in mouse brain following chronic phencyclidine treatment

8.1 Introduction

The muscarinic cholinergic system has been hypothesized to contribute to the schizophrenia-like effects induced by PCP (Olney and Farber 1995). Supporting this cholinergic hypothesis, animal studies have shown that PCP-induced psychosis and neurotoxicity can be prevented by muscarinic cholinergic antagonist treatment (Olney et al. 1991). In addition, it has been shown that NMDA antagonist administration increases acetylcholine release in rat brain (Kim et al. 1999). Olney and Farber (1995) have suggested that the excessive release of acetylcholine on muscarinic receptors contributes to PCP-induced neural damage and psychosis. Some attempts to investigate the role of muscarinic receptors in PCP animal models have measured binding of the non-specific ligand [^{3}H]quinuclidinyl benzilate (QNB) in PCP treated rats (Ward and Trevor 1981; Ellison et al. 1999a).

The aims of the present study were to: 1) identify any changes in M1/4 receptor binding in mouse brain following chronic PCP treatment; 2) determine if there are differential short-and long-term effects on M1/4 receptor binding following chronic PCP treatment and; 3) determine if there are region-specific changes in M1/4 receptor binding following chronic PCP treatment.

8.2 Materials and Methods

8.2.1 Animals and Drug treatment

The same mice and drug treatments that were described in chapter 7 were used in the present study.

8.2.2 Receptor autoradiography, quantification and statistical analysis

Adjacent brain sections were used to those described in chapter 7. [^{3}H]Pirenzepine autoradiography was performed as previously detailed in chapter 4 (du Bois et al. 2005; Newell et al. 2007c)

Quantification was performed as previously described in chapter 7 except [^{3}H]pirenzepine sections were exposed to film for 7 weeks. Statistical analysis was as detailed in chapter 7.

8.3 Results

Specific [^{3}H]pirenzepine binding was observed in all brain regions examined, although there were regional variations in density (Fig. 8.1). Non-specific binding was observed to be less than 5%. The highest density of [^{3}H]pirenzepine binding was observed in the CPu, hippocampus, and nucleus accumbens. Moderate levels of binding were observed in the cortical regions, including the auditory, sensory, visual, motor and anterior cingulate cortices. Low levels of [^{3}H]pirenzepine binding were observed in the amygdala and posterior cingulate cortex, with the lowest density of binding distinctly occurring in the thalamus.

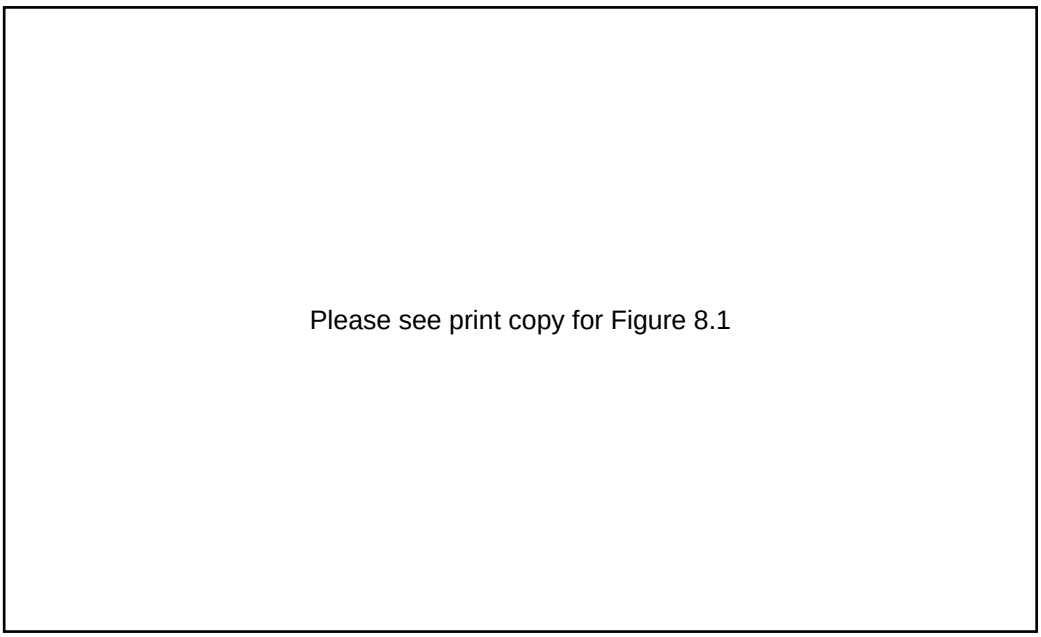

Figure 8.1 Representative autoradiographs of coronal brain sections which illustrate total [^{3}H]pirenzepine binding (A', B', C'), and non-specific [^{3}H]pirenzepine binding (A'', B'', C''). Mouse brain atlas image (A, B, C) adapted from Franklin and Paxinos (1997). Acb: nucleus accumbens, ACC: anterior cingulate cortex, ACtx: auditory cortex, Amyg: amygdala, D: caudal dorsal caudate-putamen, DL: rostral dorsolateral caudate-putamen, DM: rostral dorsomedial caudate-putamen, Hi: hippocampus, LVthal: lateroventral thalamus, M1/2: primary/secondary motor cortex, Mthal: medial thalamus, PCC: posterior cingulate cortex, S1: primary sensory cortex, V: caudal ventral caudate-putamen, VCtx: visual cortex, VL: rostral ventrolateral caudate-putamen, VM: rostral ventromedial caudate-putamen.

8.3.1 The short-term effects on [^{3}H]pirenzepine binding following chronic PCP treatment

The analysis revealed a significant effect of treatment in all brain regions examined (p<0.001; one-way ANOVA). Specifically, PCP treatment for 14 days (1hr and 24hr groups) increased [^{3}H]pirenzepine binding in the limbic system, CPu, cortex and thalamus compared to saline treated mice (Fig. 8.2). In the CPu, increases of 205-351% were observed (p<0.001). The nucleus accumbens showed an increase of >315% (p<0.001), while the anterior cingulate cortex showed an increase of >268% (p<0.001) and the hippocampus displayed an increase of 237% (p<0.001). The smallest increases in [^{3}H]pirenzepine binding were in the amygdala (~123%; p<0.001), posterior cingulate

cortex (~145%; $p<0.001$) and medial (70-82%; $p<0.001$) and lateroventral thalamus (56-71% ; $p<0.001$). There were no differences in [^{3}H]pirenzepine binding between $PCP_{14days\text{-}1hr}$ and $PCP_{14days\text{-}24hr}$ treated mice in any brain region examined.

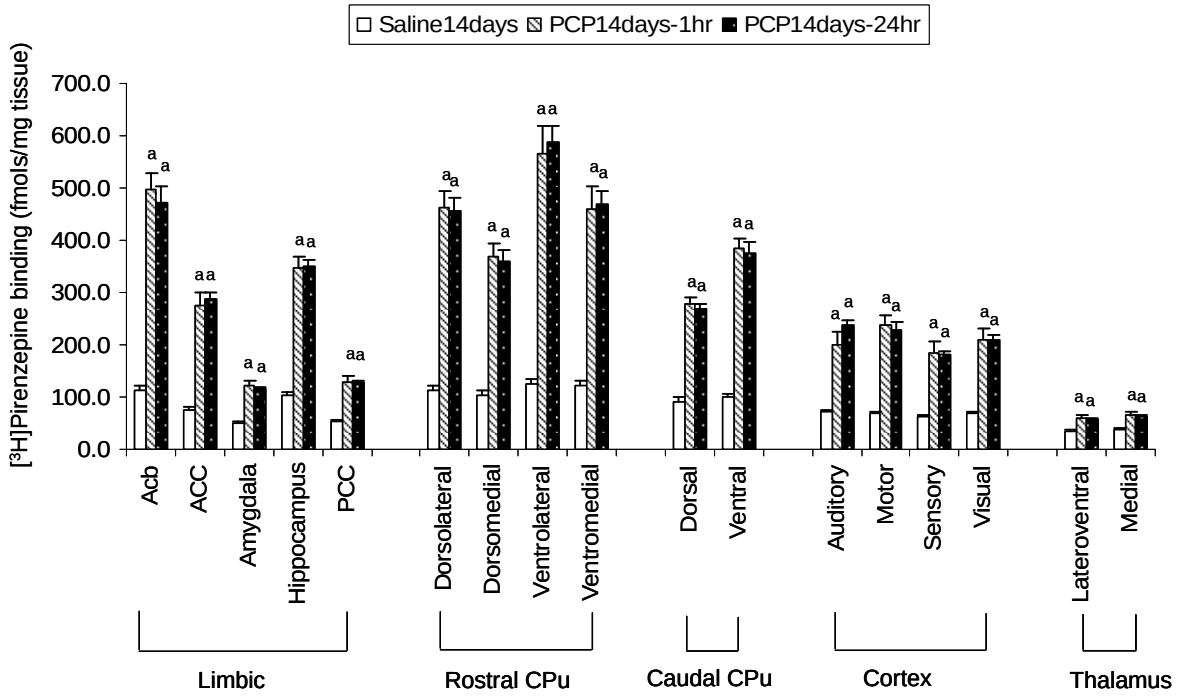

Figure 8.2: The short-term effects on [^{3}H]pirenzepine binding in mouse brain following 14 days of PCP treatment. PCP14days-1hr: mice treated for 14 days with PCP and sacrificed 1 hour after the final injection; PCP14days-24hr: mice treated for 14 days with PCP and sacrificed 24 hours after the final injection. Acb: nucleus accumbens, ACC: anterior cingulate cortex, CPu: caudate putamen, LV: lateroventral, PCC: posterior cingulate cortex, ^{a}: $p<0.001$ compared to saline treated mice.

8.3.3 The long-term effects on [^{3}H]pirenzepine binding following chronic PCP treatment

PCP treatment for 14 days followed by saline for 14 days (PCP_{14days}+$Saline_{14days}$) decreased [^{3}H]pirenzepine binding in the limbic system, CPu and cortex (ranging from 31-72%), but not in the thalamus in which no change was

found (Fig. 8.3). The largest decreases were observed in the rostral CPu (64-72%; $p<0.001$). The caudal CPu showed reductions of 55-62% ($p<0.001$), while the cortex showed reductions in the range of 46-54% ($p<0.001$). The limbic region showed a wide range of reductions, with high reductions occurring in the nucleus accumbens (66%; $p<0.001$), hippocampus (61%; $p<0.001$), and anterior cingulate cortex (56%; $p<0.02$), and relatively low reductions occurring in the posterior cingulate cortex (46%; $p<0.001$) and amygdala (31%; $p<0.001$).

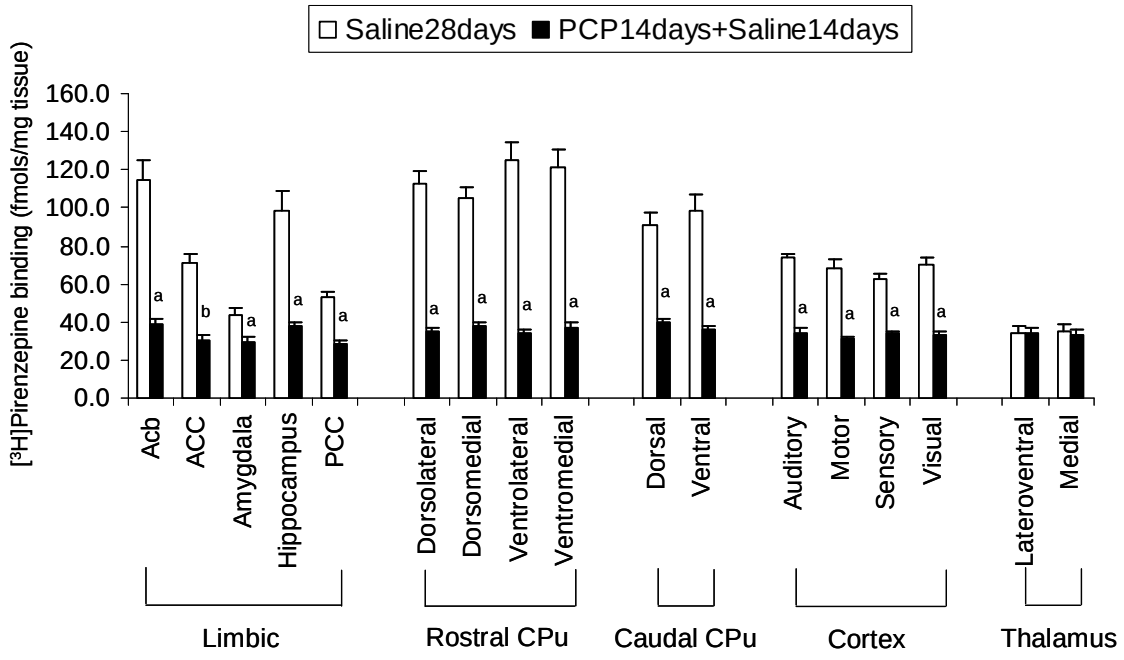

Figure 8.3: The long-term effects on [^{3}H]pirenzepine binding in mouse brain following 14 days of PCP treatment. Acb: nucleus accumbens, ACC: anterior cingulate cortex, CPu: caudate-putamen, PCC: posterior cingulate cortex, [a]: p<0.001, [b]:p<0.02

8.4 Discussion

The present study examined the density of muscarinic M1/4 receptors in mouse brain in both the short and long-term following chronic PCP treatment. The key findings from the present study were: 1) M1/4 receptor binding was increased in all limbic, CPu, cortex and thalamic regions in mice treated with PCP for 14 days and

sacrificed 1 or 24 hours after the last PCP treatment (short-term PCP treatment groups), compared to saline-treated controls; 2) M1/4 receptor binding was decreased in limbic, CPu and cortical regions, and unchanged in the thalamus, in mice treated with PCP for 14 days and sacrificed 14 days after the last PCP treatment (long-term PCP treatment group), compared to saline controls.

8.4.1 Distribution of M1/4 receptor binding in control animals

The regional distribution of [^{3}H]pirenzepine binding sites in the mouse brain corresponded well to that previously reported in rat brain (Narang 1995; du Bois et al. 2005). The highest density of binding was observed in the CPu, hippocampus and nucleus accumbens. Moderate binding was observed in the cortex, with low binding in the posterior cingulate cortex and amygdala. The lowest density of binding was observed in the thalamus.

8.4.2 M1/4 receptor binding in the short-term following chronic PCP treatment

Dramatically increased M1/4 binding was found in all brain regions examined in mice sacrificed 1 and 24 hours after the last PCP treatment. Although PCP does have direct muscarinic antagonist properties (Vincent et al. 1978), this is reportedly a much lesser component of its action compared to that at NMDA receptors (Morris et al. 2005), and has been suggested to occur at doses higher than 10mg/kg (Javitt and Zukin 1991). Nonetheless, we can not rule out that this upregulation is a compensatory result of muscarinic receptor blockade. Upregulation of muscarinic receptors, by up to 100%, has been found in several brain regions after chronic administration of muscarinic antagonists (Takeyasu et al. 1979; Ben-Barak and Dudai 1980; Wall et al. 1992).

It is probable that the present increase in M1/4 receptor binding is at least partly a downstream effect of NMDA receptor blockade. Further studies assessing the effects of the repeated administration of MK-801, a drug that is a more selective non-competitive NMDA receptor antagonists, are required to test this hypothesis.

In contrast to the present findings, a study by Ellison et al. (1999a) showed that 1 day after completing 5 days of PCP treatment (~20 mg/kg/day), there were no changes in muscarinic binding in rat brain except for a reduction in the septal area. It should be noted however, that this earlier study used [^{3}H]QNB, compared to our use of [^{3}H]pirenzepine to label muscarinic receptors. Pirenzepine is more specific to M1/4 (Flynn and Mash 1993) while QNB labels most if not all 5 muscarinic receptors (Bolden et al. 1992).

8.4.3 M1/4 receptor binding in the long-term following chronic PCP treatment

This study has shown that there is an opposite effect of chronic PCP treatment on M1/4 receptor binding in the long-term compared to the short-term following treatment. It has been shown in rats that NMDA antagonist treatment increases acetylcholine release in the rat cortex (Kim et al. 1999) and striatum (Hanania et al. 1999). In the short-term this may have overstimulated the muscarinic receptors and possibly contributed to the short-term upregulation of M1/4 receptors. However, prolonged activation of M1 receptors has been shown to induce downregulation of the receptors (Mullaney et al. 1993), possibly explaining the observed downregulation in the present study. Furthermore, studies have shown that chronic PCP treatment can cause cell death in several brain regions (Ellison and Switzer 1993; Ellison 1994). Therefore, we can not rule out that cell death has occurred with this prolonged dosing

regimen, contributing to the reduced M1/4 receptor binding in the PCP_{14days}+$Saline_{14days}$ treated mice.

In accordance with our results in the long-term group (PCP_{14days}+$Saline_{14days}$), one study found that treatment of rats for 10 days with PCP (10mg/kg/twice a day) caused a decrease in the number of [^{3}H]QNB binding sites in striatum, hippocampus, and cortex membrane preparations, 3 days following treatment (Ward and Trevor 1981). However, this study gives limited information as to specific brain regions affected by the treatment. Furthermore, the magnitude of reductions was much lower compared to our study, which may be a reflection of the longer time delay in our study (i.e. 14 days vs 3 days). An additional study using the non-specific [^{3}H]QNB ligand reported that 21 days after high-dose PCP treatment (~20 mg/kg/day, 5 days), there were reductions in [^{3}H]QNB binding in the anterior cingulate cortex, piriform cortex, nucleus accumbens, striatum, and septal area. However, they reported no change in the ventral thalamus, posterior cingulate cortex, amygdala, and hippocampus (Ellison et al. 1999a). The use of [^{3}H]QNB which labels all five muscarinic receptors may have masked any change in these regions in this earlier study.

The present study reported no change in M1/4 binding in the thalamus in the long-term following chronic PCP treatment. In addition, this study revealed a low concentration of M1/4 receptors in the thalamus, which is consistent with a study showing that the thalamus had one of the lowest concentrations of muscarinic receptors in rat brain (Cortes and Palacios 1986). This could indicate that the thalamus may not play an important role in M1/4-mediated functions/transmission, or that the PCP-induced alterations may not be large enough to be detected. Studies on schizophrenia post-mortem human tissue have also reported no change in M1/4 binding in the thalamus (Dean et al. 2004), which similarly may be due to a low density of M1/4

receptors in this region. In addition, reductions in M1/4 binding in the posterior cingulate cortex (Newell et al. 2007c), anterior cingulate cortex (Zavitsanou et al. 2004b), superior temporal gyrus (Deng and Huang 2005), CPu (Dean et al. 1996) and hippocampus (Crook et al. 2000b) have been reported in schizophrenia post-mortem brain, similar to that found in the present study.

8.5 Conclusion

The present study has shown for the first time opposing short and long-term effects on M1/4 muscarinic receptor binding in mouse brain following chronic PCP treatment. The short-term effect following chronic PCP treatment was upregulated M1/4 receptor binding in all limbic, CPu, cortex and thalamic regions examined. In contrast, in the long-term, dramatic reductions in M1/4 receptor binding were found in all the above regions except the thalamus which showed no change. Interestingly, these long-term findings correspond closely to the [^{3}H]pirenzepine binding changes reported in schizophrenia brain.

Chapter 9: Can the phencyclidine-induced neurotransmitter alterations be reversed or prevented by antipsychotic drug treatment?

9.1 Introduction

Chronic PCP treatment in humans and animals produces a behavioural syndrome, with many symptoms analogous to those present in schizophrenia (Jentsch and Roth 1999). Changes in neurotransmission are thought to underlie this PCP-induced behavioural syndrome. It has been shown in chapters 7 and 8 that there are differential short and long-term effects on NMDA and muscarinic receptor density following chronic PCP treatment. Studies have shown that certain drugs including some antipsychotics can prevent PCP-induced behaviours and neurotransmission alterations when administered prior to, or in conjunction with PCP (Gleason and Shannon 1997; Arvanov and Wang 1999; Andreasen et al. 2006). No study has however first induced a state of psychosis with PCP and then examined if subsequent antipsychotic drug treatment have the ability to reverse/prevent the PCP-induced neurotransmitter receptor changes.

The present study aimed to test if: 1) 14-day clozapine or haloperidol treatment was able to alter NMDA and M1/4 receptor binding in normal mouse brain; 2) acute (1-day) or chronic (14-day) clozapine or haloperidol treatment could reverse the short-term changes observed in NMDA and M1/4 receptor binding following chronic PCP treatment (refer to chapters 7 & 8) and; 3) 14-day clozapine or haloperidol treatment

could prevent the long-term changes observed in NMDA and M1/4 receptor binding 14 days following chronic PCP treatment (refer to chapters 7 & 8).

9.2 Materials and Methods

9.2.1 Animals and Drug Treatment

Sixty female C57Bl/6 mice, aged 9 weeks, were obtained from the Animal Resource Center, WA, Australia, and housed in groups of three. Food and water were available ad libitum. Mice were allowed 2 weeks to acclimatize to their new environment before any studies were conducted. The mice were housed in a reversed 12-hour light-dark cycle, in which lights were off from 09:00-21:00. All experiments described in this study were approved by the University of Wollongong Animal Ethics Committee (AE02/21).

Phencyclidine Hydrochloride (PCP; Sigma) was dissolved in saline to a concentration of 10mg/kg (Noda et al. 1995). Clozapine tablets (25mg; Sigma) were dissolved in saline to a concentration of 1.5mg/kg. Minimal hydrochloric acid was added to dissolve the clozapine tablets, and the pH was then neutralized with sodium hydroxide (La et al. 2006). Haloperidol (Sigma) was obtained in a concentration of 2mg/ml and was diluted with saline to an injection concentration of 1mg/kg. All injections were at a volume of 30*ul.* Drugs were injected subcutaneously into the outer thigh, and left and right thighs were alternated each day to reduce animal discomfort. Mice were injected once a day between 10:00am and 10:30am. Mice were randomly assigned to their treatment groups, with 6 mice allocated to each group.

1. Chronic antipsychotic drug treatment

Clozapine 14 days ($Clozapine_{14days}$)

Haloperidol 14 days ($Haloperidol_{14days}$)

Saline 14 days ($Saline_{14days}$)

2. Chronic PCP treatment followed by short-term antipsychotic drug treatment

PCP 14 days followed by clozapine 1 day (PCP_{14day}+$Clozapine_{1day}$)

PCP 14 days followed by haloperidol 1 day (PCP_{14days}+$Haloperidol_{1day}$)

PCP 14 days (PCP_{14days})

3. Chronic PCP treatment followed by chronic antipsychotic drug treatment

PCP 14 days followed by clozapine 14 days (PCP_{14days}+$Clozapine_{14days}$)

PCP 14 days followed by haloperidol 14 days (PCP_{14days}+$Haloperidol_{14days}$)

PCP 14 days followed by saline 14 days (PCP_{14days}+$Saline_{14days}$)

Saline 28 days ($Saline_{28days}$)

The doses of clozapine and haloperidol chosen for the present study were based on their reported ability to reverse or prevent PCP-induced behavioural changes including hyperlocomotion (Gleason and Shannon 1997) and deficits of pre-pulse inhibition (Andreasen et al. 2006). As behaviour is mediated by neurotransmission, it is expected that the ability of these drugs to reverse or prevent PCP-induced behavioural changes represents a change in neurotransmission that we may be able to identify in the mouse brain.

9.2.2 Receptor autoradiography, quantification and statistical analysis

All mice were sacrificed with an overdose of sodium pentobarbitone anesthesia (120mg/kg i.p.). In order to minimize circadian variation in receptor density, all mice were sacrificed between 11:00am and 11:30am. Brains were immediately removed after death and frozen in liquid nitrogen. Coronal brain sections were cut at -17^{o}C on a cryotome (Clinicut cryostat, Bright Instruments, England) and mounted onto microscope slides. [^{3}H]MK-801 and [^{3}H]pirenzepine autoradiography was performed as

previously detailed in chapters 3 and 4 (Newell et al. 2005; Newell et al. 2007c). Quantification was also performed as detailed previously (refer to chapters 7 and 8). For each of the 3 treatment categories, one way ANOVA followed by Tukey's post-hoc analysis was performed to test for significant differences between groups. All data was analyzed using the SPSS statistical package.

9.3 Results

9.3.1 The effects of 14-day clozapine and haloperidol treatment on [^{3}H]MK-801 binding

There was a significant effect of treatment in all regions except caudal dorsal CPu (p<0.05; one-way ANOVA; Table 9.1).

Fourteen day clozapine treatment caused a widespread increase in [^{3}H]MK-801 binding, affecting 14 out of 17 brain regions examined, when compared to saline treated mice. The largest change occurred in the hippocampus (45%), followed by the motor cortex (40%), anterior cingulate cortex (39%), auditory cortex (35%) and nucleus accumbens (32%). Moderate changes occurred in the sensory cortex (26%), lateroventral thalamus (23%), amygdala (21%), and the visual cortex (21%). The smallest significant changes occurred in the posterior cingulate cortex (17%) and the CPu subregions (17-19%).

Haloperidol treatment for 14 days appeared to have a slightly greater effect on [^{3}H]MK-801 binding than clozapine treatment. It caused significant increases in [^{3}H]MK-801 binding in 16 out of 17 brain regions examined, when compared to saline treated mice, with the caudal dorsal CPu the only region which showed no significant change. The largest change in [^{3}H]MK-801 binding was once again seen in the hippocampus (55%), followed by the anterior cingulate cortex (48%), motor cortex

(42%), nucleus accumbens (38%), auditory cortex (37%), sensory cortex (34%), and visual cortex (30%). Moderate increases were found in caudal ventral CPu (29%), amygdala (27%), and posterior cingulate cortex (26%). The lowest significant changes were in the thalamus (23%) and the rostral CPu (22-26%).

There were no significant differences in [^{3}H]MK-801 binding between Clozapine$_{14days}$ and Haloperidol$_{14days}$ treated mice in any of the brain regions examined. However, it was observed that haloperidol treated mice had consistently higher [^{3}H]MK-801 binding than clozapine treated mice.

Table 9.1 [^{3}H]MK-801 binding densities in brains of mice treated with saline, clozapine, or haloperidol for 14 days

Brain area	Mean ± S.E.M. (fmols/mg tissue)			One-way ANOVA		*P*-value, Tukey's post hoc		
	$Saline_{14days}$	$Clozapine_{14days}$	$Halperidol_{14days}$	F[3,20]	*P*-value	$Saline_{14days}$ vs. $Clozapine_{14days}$	$Saline_{14days}$ vs. $Haloperidol_{14days}$	$Clozapine_{14days}$ vs. $Haloperidol_{14days}$
Limbic System								
Acb	366.4 ± 29.2	486.5 ± 14.2	507.7 ± 23.1	9.802	**0.005**	**0.028**	**0.005**	0.832
ACC	398.6 ± 15.8	556.6 ± 22.9	592.9 ± 30.8	19.045	**0.000**	**0.001**	**0.000**	0.566
Amygdala	283.8 ± 11.0	346.0 ± 16.2	361.3 ± 11.9	5.781	**0.017**	**0.048**	**0.015**	0.705
Hippocampus	528.8 ± 16.2	770.5 ± 26.7	823.8 ± 23.0	49.397	**0.000**	**0.000**	**0.000**	0.242
PCC	335.3 ± 11.5	395.1 ± 9.2	424.5 ± 14.0	15.060	**0.000**	**0.010**	**0.000**	0.242
Rostral part of the caudate putamen								
Dorsolateral	341.6 ± 13.2	408.9 ± 8.3	431.8 ± 15.9	12.311	**0.001**	**0.017**	**0.001**	0.534
Dorsomedial	316.3 ± 13.5	373.2 ± 4.9	389.3 ± 14.6	9.185	**0.003**	**0.032**	**0.003**	0.702
Ventrolateral	358.6 ± 14.4	425.7 ± 9.1	445.7 ± 17.7	9.569	**0.003**	**0.031**	**0.003**	0.670
Ventromedial	324.1 ± 14.2	379.3 ± 5.5	395.4 ± 15.7	7.741	**0.006**	**0.051**	**0.006**	0.731
Caudal part of the caudate putamen								
Dorsal	317.8 ± 15.4	329.6 ± 19.9	364.8 ± 11.9	2.570	0.118	—	—	—
Ventral	371.1 ± 20.1	441.6 ± 29.1	479.3 ± 12.8	7.818	**0.007**	0.084	**0.006**	0.465
Cortex								
Auditory cortex	423.8 ± 15.3	574.0 ± 16.1	581.8 ± 35.7	12.868	**0.001**	**0.003**	**0.001**	0.975
Motor cortex	382.7 ± 14.2	536.3 ± 13.3	543.5 ± 23.1	22.509	**0.000**	**0.000**	**0.000**	0.963
Sensory cortex	394.8 ± 20.4	500.5 ± 20.2	529.2 ± 14.9	14.367	**0.000**	**0.003**	**0.000**	0.537
Visual cortex	419.6 ± 18.9	511.4 ± 18.4	546.6 ± 28.1	7.644	**0.008**	**0.044**	**0.007**	0.518
Thalamus								
LV thalamus	376.6 ± 15.5	463.4 ± 18.6	466.9 ± 18.6	8.415	**0.004**	**0.009**	**0.007**	0.989
Medial thalamus	363.9 ± 21.1	417.5 ± 16.7	447.7 ± 12.8	6.315	**0.011**	0.116	**0.009**	0.470

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex

9.3.2 The effects of 14-day clozapine and haloperidol treatment on [^{3}H]pirenzepine binding

There was a significant treatment effect in all brain regions examined (p<0.001; one-way ANOVA; Table 9.2).

Clozapine treatment for 14 days increased [^{3}H]pirenzepine binding in limbic, CPu, cortex and thalamic brain regions. The largest change occurred in rostral ventrolateral CPu (403%), while the smallest changes were in medial and lateroventral thalamus (100% and 87% respectively). The limbic system showed increases in the range of 180-390%. The rostral CPu showed increases of 301-403%, while binding in the caudal CPu was increased by 216-291%. The cortex showed increases of 261-338%.

Haloperidol treatment for 14 days also increased [^{3}H]pirenzepine binding in limbic, CPu, cortex, and thalamic regions. The largest change occurred in rostral ventrolateral CPu (337%), while the smallest changes were in medial and lateroventral thalamus (50% and 45% respectively). The limbic system showed increases of 99-302%. The rostral CPu was increased by 232-337%, while the caudal CPu showed increases of 173-250%. Finally, the cortex showed increased [^{3}H]pirenzepine binding in the range of 192-269%.

Clozapine$_{14days}$ treatment caused a greater increase in [^{3}H]pirenzepine binding than Haloperidol$_{14days}$ treatment, reaching significance in the amygdala (29%), hippocampus (25%), posterior cingulate cortex (23%), sensory cortex (18%) and medial (25%) and lateroventral (22%) thalamus.

Table 9.2 [^{3}H]Pirenzepine binding densities in brains of mice treated with saline, clozapine, or haloperidol for 14 days

Brain area	Mean ± S.E.M. (fmols/mg tissue)			One-way ANOVA		*P*-value, Tukey's post hoc		
	$Saline_{14days}$	$Clozapine_{14days}$	$Haloperidol_{14days}$	F[3,20]	*P*-value	$Saline_{14days}$ vs. $Clozapine_{14days}$	$Saline_{14days}$ vs. $Haloperidol_{14days}$	$Clozapine_{14days}$ vs. $Haloperidol_{14days}$
Limbic System								
Acb	113.8 ± 7.3	503.3 ± 40.9	453.2 ± 36.4	44.067	**0.000**	**0.000**	**0.000**	0.523
ACC	75.0 ± 5.7	368.0 ± 26.4	302.3 ± 17.6	68.100	**0.000**	**0.000**	**0.000**	0.061
Amygdala	51.4 ± 2.2	144.0 ± 14.6	102.2 ± 4.9	32.329	**0.000**	**0.000**	**0.001**	**0.008**
Hippocampus	103.3 ± 4.6	467.6 ± 47.4	350.6 ± 20.1	38.771	**0.000**	**0.000**	**0.000**	**0.036**
PCC	52.3 ± 3.1	162.9 ± 13.4	125.2 ± 1.5	58.690	**0.000**	**0.000**	**0.000**	**0.011**
Rostral part of the caudate putamen								
Dorsolateral	112.1 ± 8.5	520.0 ± 48.0	435.0 ± 28.6	43.532	**0.000**	**0.000**	**0.000**	0.190
Dorsomedial	104.6 ± 8.1	422.0 ± 32.1	348.0 ± 21.1	53.510	**0.000**	**0.000**	**0.000**	0.086
Ventrolateral	125.4 ± 8.4	632.1 ± 53.9	549.4 ± 34.0	53.672	**0.000**	**0.000**	**0.000**	0.286
Ventromedial	122.2 ± 9.2	491.1 ± 41.3	443.4 ± 25.8	49.143	**0.000**	**0.000**	**0.000**	0.484
Caudal part of the caudate putamen								
Dorsal	91.6 ± 6.9	290.1 ± 24.5	250.6 ± 10.7	52.217	**0.000**	**0.000**	**0.000**	0.181
Ventral	100.8 ± 5.9	394.2 ± 25.5	353.9 ± 17.1	89.283	**0.000**	**0.000**	**0.000**	0.257
Cortex								
Auditory cortex	71.9 ± 2.1	268.8 ± 29.1	210.7 ± 7.4	40.758	**0.000**	**0.000**	**0.000**	0.070
Motor cortex	68.2 ± 4.8	298.9 ± 23.0	251.8 ± 12.0	64.201	**0.000**	**0.000**	**0.000**	0.106
Sensory cortex	62.9 ± 1.6	227.5 ± 17.7	186.1 ± 7.8	58.248	**0.000**	**0.000**	**0.000**	**0.049**
Visual cortex	69.8 ± 2.4	255.0 ± 30.6	204.0 ± 5.7	31.043	**0.000**	**0.000**	**0.000**	0.132
Thalamus								
LV thalamus	35.6 ± 2.5	66.6 ± 5.5	51.8 ± 2.0	18.019	**0.000**	**0.000**	**0.018**	**0.030**
Medial thalamus	36.3 ± 2.9	72.9 ± 6.0	54.6 ± 1.9	20.569	**0.000**	**0.000**	**0.015**	**0.015**

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex

9.3.3 [^{3}H]MK-801 binding in the brains of mice treated for 14 days with PCP followed by 1 day of antipsychotic drug treatment

There was a significant effect of treatment in the anterior cingulate cortex, hippocampus, caudal dorsal CPu, and auditory, motor and sensory cortices ($p<0.002$; one-way ANOVA; Table 9.3).

PCP treatment for 14 days followed by a single clozapine injection (PCP_{14days}+$Clozapine_{1day}$) caused a significant 15% increase in [^{3}H]MK-801 binding in the caudal dorsal CPu compared to the PCP_{14days} treated mice. There were no significant differences in [^{3}H]MK-801 binding in any other brain region between PCP_{14days}+$Clozapine_{1day}$ treated mice and PCP_{14days} treated mice. Compared to saline treated mice however, PCP_{14days}+$Clozapine_{1day}$ treatment resulted in significantly increased [^{3}H]MK-801 binding in the hippocampus, anterior cingulate cortex, and auditory and sensory cortices, and a borderline significant increase in the motor cortex.

PCP treatment for 14 days followed by a single haloperidol injection (PCP_{14days}+$Haloperidol_{1day}$), resulted in a significant increase in [^{3}H]MK-801 binding in the hippocampus (20%) and sensory cortex (17%), and a borderline significant increase in the motor cortex (19%) and caudal dorsal CPu (14%) when compared to the PCP_{14days} treated group. Compared to saline treated mice, PCP_{14days}+$Haloperidol_{1day}$ treatment resulted in increased [^{3}H]MK-801 binding in the hippocampus, anterior cingulate cortex, and auditory, sensory and motor cortices.

There were no significant differences in [^{3}H]MK-801 binding between PCP_{14days}+$Clozapine_{1day}$ and PCP_{14days}+$Haloperidol_{1day}$ treated mice in any brain region examined.

Table 9.3 [^{3}H]MK-801 receptor densities in brains of mice treated with saline, PCP, clozapine, and/or haloperidol for 14 days

Brain area	Mean ± S.E.M. (fmols/mg tissue)				One-way ANOVA		*P*-value, Tukey's post hoc				
	$Saline_{14days}$	PCP_{14days}	PCP_{14days}+ $Clozapine_{1day}$	PCP_{14days}+ $Haloperidol_{1day}$	F[3,20]	*P*-value	A vs. B	A vs. C	B vs. C	B vs. D	C vs. D
Limbic System											
Acb	366.4 ± 29.2	372.0 ± 25.2	401.0 ± 12.7	437.6 ± 9.5	2.277	0.124	—	—	—	—	—
ACC	398.6 ± 15.8	468.3 ± 37.5	493.3 ± 16.5	542.2 ± 23.2	9.080	0.001	0.024	0.001	0.372	0.893	0.177
Amygdala	283.8 ± 11.0	309.5 ± 31.6	308.3 ± 10.6	328.9 ± 13.9	1.203	0.351	—	—	—	—	—
Hippocampus	528.8 ± 16.2	624.0 ± 21.3	648.6 ± 31.2	749.3 ± 42.7	9.774	0.000	0.031	0.000	0.102	0.931	0.041
PCC	335.3 ± 11.5	334.9 ± 10.3	362.6 ± 12.0	353.9 ± 18.0	0.860	0.482	—	—	—	—	—
Rostral part of the caudate putamen											
Dorsolateral	341.6 ± 13.2	331.9 ± 20.9	345.1 ± 11.3	362.9 ± 13.1	0.778	0.521	—	—	—	—	—
Dorsomedial	316.3 ± 13.5	290.0 ± 11.4	312.2 ± 10.2	331.3 ± 11.6	0.197	0.154	—	—	—	—	—
Ventrolateral	358.6 ± 14.4	346.6 ± 21.4	365.9 ± 10.3	383.5 ± 12.7	1.080	0.381	—	—	—	—	—
Ventromedial	324.1 ± 14.2	298.4 ± 16.2	320.7 ± 10.4	339.1 ± 12.0	1.535	0.238	—	—	—	—	—
Caudal part of the caudate putamen											
Dorsal	317.8 ± 15.4	266.9 ± 11.7	309.5 ± 3.7	305.4 ± 5.9	4.492	0.015	0.934	0.815	0.991	0.044	0.076
Ventral	371.1 ± 20.1	344.4 ± 17.5	385.4 ± 5.6	407.9 ± 17.5	2.591	0.083	—	—	—	—	—
Cortex											
Auditory cortex	423.8 ± 15.3	464.7 ± 12.1	519.0 ± 13.8	503.4 ± 20.6	7.632	0.002	0.002	0.010	0.903	0.121	0.359
Motor cortex	382.7 ± 14.2	424.8 ± 20.5	454.4 ± 17.7	506.4 ± 25.1	6.617	0.004	0.060	0.003	0.275	0.670	0.052
Sensory cortex	394.8 ± 20.4	408.2 ± 7.3	462.2 ± 12.2	480.7 ± 21.0	6.677	0.003	0.029	0.007	0.853	0.099	0.025
Visual cortex	419.6 ± 18.9	436.3 ± 12.1	463.4 ± 16.7	468.2 ± 28.1	1.035	0.415	—	—	—	—	—
Thalamus											
LV thalamus	376.6 ± 15.5	387.5 ± 10.1	421.8 ± 11.5	413.3 ± 13.7	2.811	0.067	—	—	—	—	—
Medial thalamus	363.9 ± 21.1	378.5 ± 10.9	389.5 ± 8.6	397.8 ± 17.1	0.916	0.452	—	—	—	—	—

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex.

A: $Saline_{14days}$

B: PCP_{14days}+$Clozapine_{1day}$

C: PCP_{14days}+$Haloperidol_{1day}$

D: PCP_{14days}

NB: A vs. D statistical results is presented in chapter 7 (Fig 7.2)

9.3.4 [^{3}H]Pirenzepine binding in the brains of mice treated for 14 days with PCP followed by 1 day of antipsychotic drug treatment

There was a significant effect of treatment in all brain regions examined (p<0.001; one-way ANOVA; Table 9.4).

PCP_{14days}+$Clozapine_{1day}$ treated mice showed reduced [^{3}H]pirenzepine binding in nucleus accumbens, amygdala, hippocampus, caudal CPu, and thalamus compared to PCP_{14days} treated mice. The binding in these regions was reduced between 20 and 26%. Compared to saline treated mice, PCP_{14days}+$Clozapine_{1day}$ treatment resulted in increased [^{3}H]pirenzepine binding in all brain regions examined, however this did not reach significance in visual cortex or lateroventral thalamus.

PCP_{14days}+$Haloperidol_{1day}$ treated mice showed significantly reduced [^{3}H]pirenzepine binding in nucleus accumbens, amygdala, and thalamus compared to the PCP_{14days} treated mice. The binding in these regions was reduced by 21-24%. When compared to saline treated mice however, PCP_{14days}+$Haloperidol_{1day}$ treatment caused increased [^{3}H]pirenzepine binding in all brain regions examined, with significance not being reached in lateroventral thalamus.

There were no significant differences in [^{3}H]pirenzepine binding in any brain region between PCP_{14days}+$Clozapine_{1day}$ and PCP_{14days}+$Haloperidol_{1day}$ treated mice.

Table 9.4 [^{3}H]Pirenzepine receptor densities in brains of mice treated with saline, PCP, clozapine, and/or haloperidol

Brain area	Mean ± S.E.M. (fmols/mg tissue)				One-way ANOVA		*P*-value, Tukey's post hoc				
	$Saline_{14days}$	PCP_{14days}	$PCP_{14days-1hr}$+ $Clozapine_{1day}$	PCP_{14days}+ $Haloperidol_{1day}$	F[3,20]	*P*-value	A vs. B	A vs. C	B vs. C	B vs. D	C vs. D
Limbic System											
Acb	113.8 ± 7.3	498.4 ± 28.7	379.0 ± 17.0	391.3 ± 32.6	58.836	0.000	**0.000**	**0.000**	0.979	**0.011**	**0.023**
ACC	75.0 ± 5.7	276.4 ± 23.0	251.0 ± 15.8	260.7 ± 20.4	34.781	0.000	**0.000**	**0.000**	0.977	0.723	0.914
Amygdala	51.4 ± 2.2	121.2 ± 9.8	88.7 ± 5.3	94.8 ± 4.4	28.400	0.000	**0.000**	**0.000**	0.864	**0.004**	**0.033**
Hippocampus	103.3 ± 4.6	348.2 ± 21.4	278.5 ± 14.1	347.8 ± 25.9	43.790	0.000	**0.000**	**0.000**	0.061	**0.045**	1.000
PCC	52.3 ± 3.1	128.1 ± 13.0	99.1 ± 9.4	108.5 ± 6.0	16.281	0.000	**0.002**	**0.000**	0.827	0.102	0.406
Rostral part of the caudate putamen											
Dorsolateral	112.1 ± 8.5	461.2 ± 34.0	380.5 ± 20.6	401.3 ± 40.2	35.121	0.000	**0.000**	**0.000**	0.952	0.211	0.448
Dorsomedial	104.6 ± 8.1	368.5 ± 23.9	309.4 ± 22.9	327.6 ± 32.6	29.781	0.000	**0.000**	**0.000**	0.943	0.301	0.601
Ventrolateral	125.4 ± 8.4	566.8 ± 53.4	477.5 ± 13.9	499.3 ± 52.2	32.646	0.000	**0.000**	**0.000**	0.975	0.354	0.585
Ventromedial	122.2 ± 9.2	459.3 ± 44.7	389.8 ± 16.8	414.3 ± 42.6	26.572	0.000	**0.000**	**0.000**	0.945	0.425	0.745
Caudal part of the caudate putamen											
Dorsal	91.6 ± 6.9	279.5 ± 11.1	222.3 ± 5.1	244.7 ± 22.9	46.348	0.000	**0.000**	**0.000**	0.598	**0.015**	0.238
Ventral	100.8 ± 5.9	384.5 ± 19.4	291.8 ± 8.6	327.4 ± 32.5	47.789	0.000	**0.000**	**0.000**	0.540	**0.007**	0.166
Cortex											
Auditory cortex	71.9 ± 2.1	201.6 ± 24.2	182.3 ± 12.2	169.7 ± 44.6	5.563	0.009	**0.023**	**0.048**	0.984	0.963	0.861
Motor cortex	68.2 ± 4.8	237.2 ± 20.4	211.2 ± 13.2	217.2 ± 17.2	35.180	0.000	**0.000**	**0.000**	0.989	0.599	0.768
Sensory cortex	62.9 ± 1.6	185.6 ± 19.7	153.7 ± 8.9	172.3 ± 12.6	28.158	0.000	**0.000**	**0.000**	0.629	0.245	0.846
Visual cortex	69.8 ± 2.4	209.8 ± 22.7	143.7 ± 30.1	200.1 ± 18.7	8.896	0.001	0.076	**0.004**	0.310	0.192	0.992
Thalamus											
LV thalamus	35.6 ± 2.5	60.9 ± 3.5	46.1 ± 3.3	47.0 ± 3.0	10.336	0.000	0.083	0.071	0.997	**0.022**	**0.041**
Medial thalamus	36.3 ± 2.9	66.4 ± 4.4	48.5 ± 3.3	49.8 ± 2.7	12.807	0.000	0.054	**0.040**	0.991	**0.009**	**0.021**

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex.

A: $Saline_{14days}$

B: PCP_{14days}+$Clozapine_{1day}$

C: PCP_{14days}+$Haloperidol_{1day}$

D: PCP_{14days}

NB: A vs. D statistical results is presented in chapter 8 (Fig 8.2)

9.3.5 [^{3}H]MK-801 binding in the brains of mice treated for 14 days with PCP followed by 14 days of antipsychotic drug treatment

There was a significant effect of treatment in all brain regions examined except the amygdala and caudal dorsal CPu (p<0.005; one-way ANOVA; Table 9.5). There were however no significant differences in [^{3}H]MK-801 binding between PCP_{14days}+$Saline_{14days}$, PCP_{14days}+$Clozapine_{14days}$ or PCP_{14days}+$Haloperidol_{14days}$ treated mice in any region of the limbic system, CPu, cortex or thalamus. When compared to saline treated mice, PCP_{14days}+$Clozapine_{14days}$ treatment resulted in decreased [^{3}H]MK-801 binding in 13 brain regions, with the largest reduction occurring in the hippocampus (35%). PCP_{14days}+$Haloperidol_{14days}$ treatment, when compared to saline treatment, resulted in decreased [^{3}H]MK-801 binding in 15 out of 17 brain regions, with the largest reduction again occurring in the hippocampus (30%).

Table 9.5 [^{3}H]MK-801 receptor densities in brains of mice treated for 28 days with saline, PCP, clozapine, and/or haloperidol

Brain area	Mean ± S.E.M. (fmols/mg tissue)				One-way ANOVA		*P*-value, Tukey's post hoc					
	$Saline_{28days}$	PCP_{14days}+ $Saline_{14days}$	PCP_{14days}+ $Clozapine_{14days}$	PCP_{14days}+ $Haloperidol_{14days}$	F[3,20]	*P*-value	A vs. B	A vs. C	A vs. D	B vs. C	B vs. D	C vs. D
Limbic System												
Acb	366.1 ± 4.5	305.7 ± 5.1	295.5 ± 12.3	291.7 ± 9.6	19.033	0.000	0.000	0.000	0.000	0.914	0.740	0.988
ACC	400.4 ± 9.5	303.1 ± 2.5	299.1 ± 19.8	300.9 ± 17.2	13.203	0.000	0.002	0.000	0.001	0.990	0.996	1.000
Amygdala	284.6 ± 20.0	242.0 ± 13.1	257.6 ± 9.2	253.9 ± 5.6	1.737	0.197	—	—	—	—	—	—
Hippocampus	530.0 ± 22.5	341.9 ± 14.1	367.4 ± 17.6	367.8 ± 9.6	28.672	0.000	0.000	0.000	0.000	0.746	0.666	1.000
PCC	336.3 ± 18.4	270.7 ± 10.0	254.5 ± 4.6	268.0 ± 7.5	9.380	0.001	0.012	0.001	0.005	0.828	0.999	0.873
Rostral part of the caudate putamen												
Dorsolateral	339.7 ± 5.9	292.5 ± 4.6	290.4 ± 12.1	278.4 ± 12.5	7.655	0.002	0.013	0.013	0.001	0.999	0.698	0.813
Dorsomedial	314.5 ± 4.9	284.9 ± 3.2	272.4 ± 11.5	260.9 ± 10.0	7.859	0.001	0.081	0.012	0.001	0.699	0.163	0.751
Ventrolateral	356.2 ± 3.3	301.8 ± 5.4	299.1 ± 14.3	285.6 ± 13.7	8.585	0.001	0.008	0.008	0.001	0.998	0.663	0.796
Ventromedial	324.9 ± 5.0	291.8 ± 4.0	281.8 ± 10.4	271.4 ± 10.4	7.919	0.001	0.044	0.010	0.001	0.820	0.274	0.797
Caudal part of the caudate putamen												
Dorsal	316.9 ± 17.9	267.5 ± 16.9	276.6 ± 4.6	263.7 ± 11.9	2.928	0.066	—	—	—	—	—	—
Ventral	370.5 ± 21.0	290.2 ± 15.8	309.3 ± 4.1	287.0 ± 13.3	6.185	0.005	0.012	0.088	0.009	0.868	0.999	0.806
Cortex												
Auditory cortex	441.3 ± 15.5	334.3 ± 8.5	345.7 ± 10.1	329.6 ± 4.2	28.363	0.000	0.000	0.000	0.000	0.875	0.988	0.692
Motor cortex	382.1 ± 11.0	310.4 ± 6.4	298.5 ± 16.7	293.3 ± 10.5	15.363	0.000	0.001	0.000	0.000	0.883	0.652	0.989
Sensory cortex	395.1 ± 18.6	280.2 ± 13.6	322.9 ± 10.7	323.5 ± 6.5	13.179	0.000	0.000	0.013	0.006	0.204	0.127	1.000
Visual cortex	420.3 ± 13.8	308.1 ± 9.8	347.6 ± 10.0	313.9 ± 9.5	20.707	0.000	0.000	0.007	0.000	0.293	0.989	0.303
Thalamus												
LV thalamus	376.3 ± 16.1	288.4 ± 14.7	319.2 ± 7.4	307.9 ± 7.8	9.188	0.001	0.000	0.028	0.008	0.366	0.719	0.934
Medial thalamus	361.1 ± 14.3	288.1 ± 15.0	308.6 ± 8.9	296.7 ± 9.4	6.854	0.003	0.003	0.069	0.013	0.723	0.964	0.935

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex.

A: $Saline_{28days}$

B: PCP_{14days}+$Saline_{14days}$

C: PCP_{14days}+$Clozapine_{14days}$

D: PCP_{14days}+$Haloperidol_{14days}$

9.3.6 [^{3}H]Pirenzepine binding in the brains of mice treated for 14 days with PCP followed by 14 days of antipsychotic drug treatment

Analysis revealed a significant effect of treatment in all brain regions except the thalamus (p<0.001; one-way ANOVA; Table 9.6). However, in all limbic, CPu, cortex and thalamic brain regions examined, there were no significant differences in [^{3}H]pirenzepine binding between PCP_{14days}+$Saline_{14days}$, PCP_{14days}+$Clozapine_{14days}$, and PCP_{14days}+$Haloperidol_{14days}$ treated mice. When compared to saline treated mice, PCP_{14days}+$Clozapine_{14days}$ and PCP_{14days}+$Haloperidol_{14days}$ treatments both decreased [^{3}H]pirenzepine binding in all brain regions examined except the thalamus, which showed no change.

Table 9.6 [^{3}H]Pirenzepine receptor densities in brains of mice treated for 28 days with saline, PCP, clozapine, and/or haloperidol

Brain area	Mean ± S.E.M. (fmols/mg tissue)				One-way ANOVA		*P*-value, Tukey's post hoc					
	$Saline_{28days}$	PCP_{14days}+ $Saline_{14days}$	PCP_{14days}+ $Clozapine_{14days}$	PCP_{14days}+ $Haloperidol_{14days}$	F[3,20]	*P*-value	A vs. B	A vs. C	A vs. D	B vs. C	B vs. D	C vs. D
Limbic System												
Acb	114.6 ± 10.8	38.8 ± 2.6	37.0 ± 3.9	33.7 ± 1.7	53.118	0.000	0.000	0.000	0.000	0.993	0.871	0.964
ACC	70.8 ± 5.4	30.8 ± 2.4	31.9 ± 2.9	27.0 ± 0.6	38.589	0.000	0.000	0.000	0.000	0.995	0.854	0.728
Amygdala	43.4 ± 4.3	29.8 ± 1.9	26.1 ± 0.4	27.2 ± 0.8	9.660	0.001	0.006	0.001	0.002	0.759	0.901	0.991
Hippocampus	98.8 ± 10.2	38.0 ± 1.3	35.4 ± 1.7	33.8 ± 1.4	31.858	0.000	0.000	0.000	0.000	0.988	0.952	0.997
PCC	52.9 ± 3.1	28.5 ± 2.0	25.2 ± 0.9	26.9 ± 1.6	39.117	0.000	0.000	0.000	0.000	0.714	0.957	0.942
Rostral part of the caudate putamen												
Dorsolateral	113.0 ± 6.4	35.3 ± 1.8	36.2 ± 3.7	33.3 ± 1.0	117.392	0.000	0.000	0.000	0.000	0.998	0.967	0.927
Dorsomedial	104.8 ± 6.5	37.5 ± 1.9	39.5 ± 4.1	36.3 ± 1.0	79.669	0.000	0.000	0.000	0.000	0.976	0.994	0.918
Ventrolateral	124.9 ± 9.1	34.2 ± 1.7	35.9 ± 4.5	31.0 ± 1.4	91.999	0.000	0.000	0.000	0.000	0.991	0.948	0.857
Ventromedial	121.6 ± 8.9	37.1 ± 2.2	38.4 ± 4.0	35.1 ± 1.3	80.976	0.000	0.000	0.000	0.000	0.997	0.986	0.945
Caudal part of the caudate putamen												
Dorsal	90.5 ± 6.9	40.0 ± 1.7	38.0 ± 2.1	37.6 ± 1.8	46.120	0.000	0.000	0.000	0.000	0.977	0.972	1.000
Ventral	98.1 ± 8.4	36.4 ± 1.4	33.8 ± 2.0	31.2 ± 2.0	51.213	0.000	0.000	0.000	0.000	0.972	0.848	0.979
Cortex												
Auditory cortex	73.6 ± 2.6	34.4 ± 2.5	29.2 ± 1.3	29.1 ± 1.4	111.741	0.000	0.000	0.000	0.000	0.553	0.553	1.000
Motor cortex	68.5 ± 4.0	31.0 ± 1.5	29.0 ± 2.5	27.0 ± 0.5	68.529	0.000	0.000	0.000	0.000	0.901	0.547	0.919
Sensory cortex	62.6 ± 2.9	33.8 ± 1.7	30.4 ± 0.4	30.0 ± 1.0	70.109	0.000	0.000	0.000	0.000	0.608	0.533	0.999
Visual cortex	69.8 ± 4.2	32.8 ± 1.8	27.4 ± 0.8	27.7 ± 1.3	57.157	0.000	0.000	0.000	0.000	0.592	0.598	1.000
Thalamus												
LV thalamus	34.4 ± 3.8	34.0 ± 2.8	28.0 ± 1.0	30.0 ± 1.2	1.327	0.297	0.999	0.363	0.654	0.424	0.722	0.961
Medial thalamus	34.9 ± 3.7	33.3 ± 2.6	27.5 ± 0.7	28.9 ± 0.8	1.917	0.163	0.967	0.205	0.368	0.394	0.616	0.982

Abbreviations: Acb: nucleus accumbens, ACC: anterior cingulate cortex, LV: lateroventral, PCC: posterior cingulate cortex.

A: $Saline_{28days}$

B: PCP_{14days}+$Saline_{14days}$

C: PCP_{14days}+$Clozapine_{14days}$

D: PCP_{14days}+$Haloperidol_{14days}$

9.4 Discussion

The main findings from the present study were: (1) 14-day clozapine and haloperidol treatments both caused widespread increases in NMDA receptor binding in mouse brain; (2) 14-day clozapine and haloperidol treatments both increased M1/4 receptor binding in all limbic, CPu, cortex and thalamic brain regions examined, with clozapine causing significantly greater increases in thalamolimbic regions compared to haloperidol treatment; (3) However, both 1 day and 14-day clozapine or haloperidol treatments were unable to correct the NMDA or M1/4 receptor changes induced by 14 days of PCP treatment and; (4) Furthermore, 14-day clozapine or haloperidol treatment was also unable to prevent the changes in NMDA or M1/4 receptor density observed in the 14 days following PCP treatment.

9.4.1 The effects of chronic antipsychotic drug treatment on NMDA receptor binding in mouse brain

Clozapine treatment for 14 days resulted in increased NMDA receptor binding in the limbic system, rostral CPu, cortex and laterovental thalamus, with no change occurring in the medial thalamus and caudal CPu. Previous studies report inconsistent effects of clozapine treatment on NMDA receptors. A study using a much larger dose of clozapine (30mg/kg/day) found that treatment in male rats for 21 days resulted in a 20% increase in [^{3}H]MK-801 binding in the dentate gyrus, but a 20-30% decrease in the anterior cingulate cortex (Giardino et al. 1997). They found no change in striatum, nucleus accumbens or hippocampus. Another high dose study treated male rats with clozapine (30mg/kg/day) for 3 months and found increased [^{3}H]CGP 39653 binding to NMDA receptors in the insular and parietal cortices. However, they found no change in [^{3}H]MK-801 binding in these same areas (Ossowska et al. 1999). Electrophysiology

studies in male rats have shown that clozapine at high concentrations (>100nM) suppresses NMDA activity; while at low concentrations (1-10nM) it enhances NMDA activity in several brain regions (Banerjee et al. 1995). This could possibly cause the different effects reported in the above studies compared to the present study, in which a low dose of clozapine was used. A third study however, using low doses of clozapine (0.1 and 1.0mg/kg/day) reported that treatment of male rats for 3 months did not change [^{3}H]TCP binding in cortex, striatum or hippocampus (Scarr et al. 2002). Species or methodological differences including differences in gender, treatment dosage and duration, and ligand used might account for the differences observed between these studies.

Clozapine has affinity for a wide variety of receptor systems, including serotonin, muscarinic cholinergic, dopamine, histamine and adrenergic (Moore 1999), however it is not exactly known how it regulates the NMDA receptor. Evidence suggests that it could be mechanisms downstream from dopamine D2 antagonism that causes the clozapine-induced NMDA upregulation (Chen and Yang 2002; Wittmann et al. 2005). However, at the low dose used in the present study, clozapine is unlikely to have strong effects on the D2 receptor. It therefore may be that clozapine has caused this upregulation via several indirect mechanisms. For example, clozapine is known to have serotonin and muscarinic antagonist properties (Moore 1999), both of which could influence the glutamatergic system (Aghajanian and Marek 1999; Aghajanian and Marek 2000; Grishin et al. 2005). Studies have also suggested that clozapine may have a direct influence on the glutamatergic system (Lidsky et al. 1993; Banerjee et al. 1995). However, more research is required to undertstand the mechanism(s) of clozapine induced changes in NMDA receptor binding density.

Haloperidol treatment for 14 days resulted in increased NMDA receptor binding in all brain regions examined, except caudal dorsal CPu. This supports the results of a previous study, which reported that rats treated with haloperidol (0.24mg/kg/day) for 14 days showed an upregulation of [^{3}H]TCP binding in whole brain membranes (Byrd et al. 1987), however their study provided no information as to specific brain regions affected. Several other studies have also reported NMDA receptor upregulations in haloperidol-treated rodents (Ulas et al. 1993; Riva et al. 1997; Ossowska et al. 1999).

Haloperidol is primarily a dopamine D2 receptor antagonist. It is therefore expected that this increase is the result of a mechanism downstream from D2 antagonism. Several studies have shown that haloperidol increases glutamate release in rat (See and Chapman 1994; Yamamoto and Cooperman 1994; Olney and Farber 1995; See and Lynch 1995). Therefore, it would be expected that haloperidol would *down-regulate* NMDA receptors. However, by blocking presynaptic D2 receptors, haloperidol increases extracellular dopamine (Pucak and Grace 1994; Liegeois et al. 2002), which has been shown to result in activation of postsynaptic D1 receptors, which enhance NMDA receptor currents (Harvey and Lacey 1997; Lee et al. 2002; Pickel et al. 2006). Consistent with this notion, haloperidol treatment in rats has been shown to enhance NMDA receptor responses (Banerjee et al. 1995; Arvanov et al. 1997) possibly contributing to the haloperidol-induced NMDA receptor upregulation observed in the present study. Furthermore, haloperidol has been suggested to be a partial agonist of the strychnine-insensitive glycine binding site on the NMDA receptor (Fletcher and MacDonald 1993) which could contribute to the NMDA upregulation observed in the present study.

No significant differences in NMDA receptor binding were observed between clozapine$_{14day}$ and haloperidol$_{14day}$ treated mice. When compared to saline$_{14day}$ treated

mice however, haloperidol treatment consistently caused a greater increase in NMDA receptor binding, and significantly affected two more brain regions than clozapine treatment. A common property of these two antipsychotic drugs is that haloperidol is a potent and clozapine is a weak D2 antagonist. This may suggest that the NMDA upregulation induced by these drugs is could be due to their D2 antagonist properties. Haloperidol's slightly more widespread increase in NMDA receptor density may therefore be due to its stronger dopaminergic component. Furthmore, this D2 antagonim of the drugs may contribute to the widespread nature of the NMDA receptor changes observed. Dopamine D2 receptors are distributed in many brain regions, including striatum, cortex and limbic areas, and they have been reported to regulate the glutamatergic system (Hatzipetros and Yamamoto 2006). Therefore, it would be expected that there would be widespread changes in NMDA activity as a consequence of the widespread nature of dopamine receptors in the brain.

Clozapine is well accepted to be more effective in treating schizophrenia and in preventing NMDA-induced neurotoxicity than haloperidol (Farber et al. 1993; Olney and Farber 1994). Therefore it would be expected that clozapine would have a different binding profile to haloperidol in mice. It may be that clozapine's greater effectiveness is not actually due to its effects on NMDA receptors, but rather its effectiveness on other systems such as the serotonergic or muscarinic system. While NMDA receptor hypofunction is hypothesized to contribute to the underlying cause of schizophrenia, it is the downstream effects on other systems such as dopamine, muscarinic and serotonin which is believed to cause the symptoms.

In chapter 7, it was reported that 14 day PCP treatment resulted in increased NMDA receptor binding primarily in the hippocampus when measured 1 or 24 hours after the final PCP treatment (Newell et al. 2007b). Clozapine and haloperidol have

shown in the present study to have more widespread effects in the short term compared to the short term effects of chronic PCP. In the long-term however, chronic PCP treatment caused a widespread downregulaiton of NMDA receptors, suggesting that possibly this upregulation observed following clozapine or haloperidol treatment could be therapeutic.

9.4.2 The effects of chronic antipsychotic drug treatment on M1/4 receptor binding in mouse brain

Clozapine treatment for 14 days increased M1/4 binding in all limbic, CPu, cortex and thalamic brain regions examined. It therefore had a more widespread effect on M1/4 binding than it did on NMDA receptor binding. Clozapine is known to have M1 antagonist properties (Bymaster et al. 2003). Therefore the observed upregulation in [^{3}H]pirenzepine binding may represent a compensatory response to M1 antagonism by clozapine. There are limited studies examining the effects of antipsychotic drugs on muscarinic receptor binding. However, one recent study found that male rats treated with clozapine for 3 months had increased [^{3}H]pirenzepine binding in the frontal cortex (Crook et al. 2001), consistent with the findings in the present study.

Haloperidol treatment for 14 days, like clozapine treatment, increased M1/4 binding in the limbic system, CPu, cortex and thalamus. This finding is consistent with a study that found haloperidol (1mg/kg) treatment for 3 months increased [^{3}H]pirenzepine binding in male rats (Crook et al. 2001). In contrast however, a recent study reported no effect of haloperidol (2mg/kg) treatment on [^{3}H]pirenzepine binding in male rats following 3 or 6 months of treatment (Terry Jr et al. 2006).

Haloperidol is primarily a dopmaine D2 antagonist. Therefore the haloperidol-induced increase in M1/4 receptor density is likely to occur indirectly via this antagonism. It has been shown that dopamine receptor stimulation in the nucleus

accumbens reduces GABAergic efferents to the basal forebrain resulting in increased excitability of the cholinergic neurons and increased acetylcholine release (Moore et al. 1999; Sarter et al. 1999). Therefore, it would be expected that dopamine receptor antagonism would have the opposite effect; to reduce acetylcholine release from the basal forebrain which would be expected to result in a compensatory upregulation of acetylcholine muscarinic receptors, as found in the present study. Supporting this, studies have shown that PCP-induced acetylcholine release was reversed by the injection of haloperidol into nucleus accumbens (Moore et al. 1999).

Studies on schizophrenia post-mortem tissue have reported decreased [^{3}H]pirenzepine binding in several brain regions (Crook et al. 2000b; Crook et al. 2001; Zavitsanou et al. 2004b; Newell et al. 2007c). Furthermore, in chapter 8 it was shown that there is reduced [^{3}H]pirenzepine binding in mouse brain in the long-term following chronic PCP treatment (Newell et al. 2007a). It is therefore logical that clozapine and haloperidol, which to some extent treat schizophrenia symptoms and prevent PCP-induced psychosis, would act to upregulate M1/4 receptor binding.

A prominent finding in this study was that in every brain region examined, clozapine$_{14day}$ treatment increased M1/4 binding to a greater extent than haloperidol$_{14day}$ treatment. This was expected because clozapine has direct muscarinic properties while haloperidol is thought not have these properties. This increase however only reached significance in thalamolimbic regions, suggesting that clozapine has a greater effect on thalamolimbic regions than haloperidol. Atypical antipsychotic drugs have been shown to target limbic regions more so than typical antipsychotic drugs (Worrel et al. 2000), which may contribute to their superior efficacy in treating schizophrenia. Furthermore, clozapine's muscarinic properties are thought to contribute to the improvement in cognitive symptoms in schizophrenia patients. Haloperidol does not have muscarinic

antagonist properties and has a limited effect on negative and cognitive schizophrenia symptoms (Lewis 2002). It may therefore be clozapine's stronger effect on the muscarinic system in thalamolimbic regions that contributes to its unique effect on cognitive schizophrenia symptoms.

9.4.3 The effects of acute antipsychotic drug treatment on NMDA receptor binding in PCP-treated mouse brain

PCP treatment for 14 days followed by a single treatment of clozapine (PCP_{14days}+$Clozapine_{1day}$), caused virtually no change in NMDA receptor density when compared to PCP_{14days} treated mice. This suggests that the single clozapine injection has had no effect on NMDA receptor binding in the PCP-treated mouse brains. In schizophrenia, clozapine effectively treats the symptoms. However this treatment effect is not immediate (Fabrazzo et al. 2002), which could explain why the single clozapine injection in the present study showed no change in NMDA receptor density compared to PCP only treated mice. It may be that one day of treatment is not long enough for the neurons to respond and adapt to the treatment, or a higher dose may be required. Furthermore, a more specific target on the glutamatergic system may be required.

There were significant increases in NMDA receptor binding density observed between PCP_{14days}+$Clozapine_{1day}$ and $Saline_{14days}$ treated mice in several brain regions including the hippocampus and anterior cingulate cortex. However, as there were no differences in NMDA binding in these regions between PCP_{14days} and PCP_{14days}+$Clozapine_{1day}$ treated mice, it suggests that these changes are not an effect of clozapine but rather the PCP treatment.

There were no differences in NMDA receptor density between PCP_{14day}+$Clozapine_{1day}$ and PCP_{14day}+$Haloperidol_{1day}$ treated mice although there was a consistently greater increase in the PCP_{14day}+$Haloperidol_{1day}$ treated mice. Compared to

PCP_{14days} treated mice, PCP_{14day}+$Haloperidol_{1day}$ treated mice showed a significant increase in NMDA receptor binding in the hippocampus and visual cortex with borderline significant increases in caudal dorsal CPu and sensory cortex. This suggests that this single injection may be beginning to have a treatment effect on the NMDA receptor system. Based on the results from the 14 day antipsychotic drug treatments, in which haloperidol had a more widespread effect on NMDA receptor binding than clozapine treatment, it was expected that haloperidol would also have a stronger effect on the PCP-treated mice. This suggests that a stronger dopamine antagonism has a greater effect on the NMDA receptors in the "diseased" state copmpared to weak D2 antagonism.

9.4.4 The effects of acute antipsychotic drug treatment on M1/4 receptor binding in PCP-treated mouse brain

PCP_{14day}+$Clozapine_{1day}$ treated mice showed reduced M1/4 binding in nucleus accumbens, amygdala, hippocampus, caudal dorsal, and caudal ventral CPu, and medial and lateroventral thalamus compared to the PCP_{14days} treated mice. Similarly, PCP_{14day}+$Haloperidol_{1day}$ treated mice showed reduced M1/4 binding in nucleus accumbens, hippocampus and thalamus. This is interesting since the 14-day clozapine and haloperidol treatments actually worked to *increase* M1/4 binding in normal mouse brain. Discontinuing the PCP treatment may be beginning to reduce binding towards what was observed in the PCP_{14day}+$Saline_{14day}$ group. However, because there was no difference in M1/4 binding between $PCP_{14days\text{-}1hr}$ treated mice and $PCP_{14days\text{-}24hr}$ treated mice (see chapter 8), this would suggest that the 1 day of PCP withdrawal is not causing this reduction. Therefore, it is possible that clozapine and haloperidol treatment of the "diseased" state has a different effect to clozapine and haloperidol treatment of the normal state. Alternatively, this reduction may actually be the effect of acute clozapine

or haloperidol treatment, whereas the increase observed in the $Clozapine_{14days}$ and $Haloperidol_{14days}$ treatment groups may be due to chronic treatment.

9.4.5 The effects of chronic antipsychotic drug treatment on NMDA and M1/4 receptor binding in PCP-treated mouse brain

No significant differences in NMDA or M1/4 receptor binding were observed between PCP_{14day}+$Clozapine_{14day}$, PCP_{14day}+$Saline_{14day}$ and PCP_{14day}+$Haloperidol_{14day}$ treated mice. This demonstrates that 14 day treatment with clozapine or haloperidol following 14 days treatment with PCP does not have the ability to return NMDA or M1/2 receptor binding to the levels observed in non-PCP treated mice. Furthermore, it demonstrates that these antipsychotic drug treatments do not have the ability to prevent the long-term changes in NMDA and M1/4 receptor binding that were observed 14 days following PCP treatment.

PCP_{14day}+$Haloperidol_{1day}$ treatment significantly increased NMDA receptor binding in the hippocampus compared to the 14 day PCP treatment group. It was therefore anticipated that 14 days of haloperidol treatment would also increase NMDA binding. It is possible that in the long-term the brain may have been damaged beyond repair as chronic PCP treatment has been shown to kill neurons (Ellison and Switzer 1993; Ellison 1994). On the other hand however, it may be possible that these two drugs do not target the key systems required to prevent the long-term PCP-induced neurotransmitter receptor changes. It may be that a more specific target on the glutamatergic and/or muscarinic systems may be required to help prevent the long-term changes. Alternatively, a longer treatment period may be required to normalize the NMDA receptor binding. In schizophrenia, treatment is a long-term course of action. The brain may require a longer treatment time to adapt and change its neuronal state in response to the clozapine or haloperidol treatment.

9.5 Conclusion

Clozapine and haloperidol 14-day treatments both caused widespread increases in NMDA receptor binding. These increases may be due to several factors, the most likely of which is downstream effects from dopamine antagonism. Clozapine and haloperidol treatments both increased M1/4 binding in the limbic system, CPu, cortex and thalamus, while clozapine had a larger effect than haloperidol. This could possibly be attributed to clozapine's direct muscarinic properties, while haloperidol may work indirectly via its D2 antagonism. Specifically, clozapine appeared to target the thalamolimbic regions significantly more so than haloperidol, suggesting that this may be parts of its superior efficacy. Despite their effectiveness in treating schizophrenia, clozapine and haloperidol treatments, for 1 or 14 days, were unable to return NMDA or M1/4 receptor binding to control (non-PCP) levels or prevent the long-term changes in NMDA and M1/4 receptor binding following PCP treatment.

Chapter 10: Conclusions and Recommendations

10.1 Overall Conclusion

The present study has shown for the first time that there are specific neurotransmitter receptor alterations in the PCC in schizophrenia. Furthermore, this study has shown that there are differential effects of PCP treatment on neurotransmitter receptors in the short and the long-term following treatment.

This study showed that there are specific changes in the glutamatergic system as evidenced by increased NMDA receptor density in the PCC in schizophrenia, but unchanged AMPA and kainate receptor density. This specific increase in NMDA receptor density is an effect that is unlikely to be due to changes in glutamate levels, but possibly an upregulation to compensate for NMDA hypofunction. In addition, the present study demonstrated specific alterations in the cholinergic system as shown by decreased M1/4 receptor density, but unchanged M2/4 receptor density in the PCC in schizophrenia. Major increases in $GABA_A$ receptor density were found in the PCC in schizophrenia. Whilst the exact mechanism causing these muscarinic and gabaergic receptor alterations is not yet known, a possible increased acetylcholine and down regulated GABA stimulation in the PCC of schizophrenia is suggested, as has been reported in NMDA hypofunction animal models. The observed changes may therefore stem from NMDA receptor hypofunction in this region. Further neurotransmitter alterations were observed in the PCC in schizophrenia, namely in the serotonergic and cannabinoid systems. This study showed a reduction in 5HT2 and an increase in CB1 receptor density in the PCC in schizophrenia and therefore provides support for a role of the endogenous cannabinoid system and serotonergic system in schizophrenia.

However, the reduction in 5HT2 receptor density may be a consequence of long-term antipsychotic drug exposure.

The present study showed that there are differential short and long-term effects on neurotransmitter receptor density following chronic PCP treatment in mice. These results suggest that the long-term more accurately represents a state of NMDA hypofunction than the short-term as shown by a widespread downregulation of the NMDA receptor. Although this long-term status of NMDA receptors does not reflect the NMDA receptor changes reported in post-mortem brain in schizophrenia, it does not preclude NMDA hypofunction as being an underlying mechanism for the NMDA receptor changes observed in the PCC in schizophrenia. It is possible that both NMDA receptor changes in schizophrenia and the mouse model are a result of NMDA hypofunction, but with different mechanisms. In the human tissue, it is hypothesized that there is a regulatory mechanism to cause an upregulation of receptors to compensate for the hypofunction, while in the mouse model, this may not be occurring. Furthermore, recent genetic evidence suggests that NMDA hypofunction may occur in the schizophrenia brain (Hahn et al. 2006). It is possible that the increased binding observed in the post-mortem schizophrenia tissue is due to antipsychotic drug effects as it was shown in the present study that clozapine and haloperidol treatment increased NMDA receptor binding. However, it was also found that clozapine treatment of the diseased (i.e PCP-treated) state had no effect on NMDA receptor density while haloperidol treatment only had a minor effect, suggesting that antipsychotic drug treatment may not cause an upregulation in schizophrenia.

The changes in M1/4 receptor density observed in the long-term PCP model closely resemble post-mortem changes reported not only in the PCC in schizophrenia, but also in several other brain regions. As the changes in the animal model stem from

NMDA hypofunction, this provides further support that the downregulated M1/4 receptors reported in the PCC in schizophrenia could also be due to NMDA hypofunction. The present study showed that clozapine and haloperidol increase M1/4 binding in normal brain suggesting that antipsychotic drug treatment may not contribute to the observed findings of downregulated M1/4 receptor density in the PCC in schizophrenia. Furthermore, it was shown that in the diseased (PCP-treated) state, chronic treatment with these two antipsychotic drugs had no effect on M1/4 binding.

The combination of human and animal studies has demonstrated that neurotransmitter imbalance in specific brain areas plays an important role in schizophrenia. This imbalance may underlie the pathology of schizophrenia and assist in the identification of therapeutic targets for treatment of schizophrenia.

A limitation of the studies reported in this thesis is the use of a single radioligand concentration in the autoradiography experiments. Therefore, these studies provide information about changes in receptor density only, while changes in binding affinity for the ligands to the receptors may have occurred. Autoradiographic studies performed with the aim of determining affinity require substantial amounts of tissue, much more than what was obtained in the present study. Therefore, it was unfeasible in the present series of studies to examine binding affinity. Instead, the focus of these experiments was a comparison of receptor density between experimental groups.

10.2 Recommendations for Further Research

Based on findings in the present thesis, recommendations for further research are listed below.

1) The examination of antipsychotic-free schizophrenia patients as well as a psychiatric comparison group will strengthen the importance of the post-mortem neurotransmitter receptor findings in the PCC in schizophrenia.

2) The analysis of additional neurotransmitter systems in the PCP model will help us to link the findings in this model with the findings in schizophrenia.
3) Further research is recommended to establish the underlying causes of the neurotransmitter receptor changes in the PCP model. For example, testing for cell death, determining neurotransmitter levels in both the short and long-term following treatment.
4) As schizophrenia is widely regarded as a neurodevelopmental disorder, examinations of the consequences of PCP treatment at important neurodevelopmental stages (eg perinatal period, adolescent period) on neurochemical balance, would give further insight into the NMDA hypofunction hypothesis of schizophrenia.
5) There is much research suggesting that there may be a genetic link to schizophrenia. Therefore, further research examining how candidate genes for schizophrneia (eg neuregulin-1) interact with the NMDA receptor could be conducted. In addition, examination of neurotransmitter alterations in animal models of altered genetic expression (eg neuregulin-1 knockdown) may give some insight into the possible mechanisms of neurotransmitter change found in the postmortem schizophrenia tissue.

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