Understanding the role of dopamine D4 receptor regulation of mesolimbic dopamine function in a rat model of schizophrenia

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Abstract

The project concentrated on characterising the effect of D4 receptor activation in the context of an animal model relevant to schizophrenia (phencyclidine pretreatment) and elucidating the mechanisms involved. Behavioural studies measured selective attention to motivational stimuli, through measurement of latent inhibition of conditioned learning (LI), and episodic memory measured by novel object recognition (NOR), behaviours which are dysfunctional in schizophrenia. Subchronic PCP pre-treatment for five days disrupted LI and induced behavioural deficits in NOR, replicating previous findings using seven days pre-treatment. A412997 reversed deficit in NOR but not in LI. However, the neural the mechanisms these processes are as yet unclear.

A better understanding of the physiology of cortical-limbic circuits is important in elucidating the neurophysiological mechanisms underlying dopamine-mediated processes which are vital for normal behaviour, and which may be abnormal in schizophrenia. The project examined the neuropharmacology underlying these behavioural processes, both in normal animals, and in animals pretreated with phencyclidine. Focusing on the role of D4 receptors. To achieve these aims fast-scan cyclic voltammetry was used to measure electrically stimulated dopamine release in nucleus accumbens, in rat brain slices *in vitro*. The selective dopamine receptor agonist A412997 caused a decrease in electrically stimulated dopamine release which was abolished in animals pretreated with PCP. This inhibitory effect of A412997 was blocked by D4 specific antagonist L-741,742.

Gene expression of dopamine D4 receptors, as well as in other dopamine receptors (D1, D2, D3, D5) in response to sub-chronically pre-treated with PCP was significantly changed in different regions of rat brain, as well as these pre-treatment as modelling relevant to schizophrenia produced changes on the basal level of dopamine and its metabolites in the same brain areas.

Taken together with behavioural data this demonstrated changes in D4-receptor mediated regulation of accumbal dopamine function after PCP pretreatment, suggesting a role for these receptors in schizophrenia.

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‘All praises and gratitude to the Almighty’

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aca	anterior commissure
aCSF	artificial cerebrospinal fluid
ADHD	Attention deficit hyper activity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
cDNA	Complementary DNA
COMT	Catechol-O-methyltransferase
Ct	Cycle threshold
DA	Dopamine
ddH2O	Double-distilled water
DOPAC	3, 4-dihydroxyphenylacetic acid (DOPAC)
DR	Dopamine receptor
FCX	Frontal cortex
FSCV	Fast scan cyclic voltammetry
GABA	Gama amino butyric acid
GluR	Glutamate receptor
HPLC	High pressure liquid chromatography
5-HT2	5-hydroxytryptamine
HVA	Homovanillic acid
Hz	Hertz
iGluR	inotropic glutamate receptor
KA	Kinate
LI	Latent inhibition
mGluR	Metabotropic glutamate receptor
MK-801	Dizocilpine
nA	Nano Ampere
NAcS	Nucleus accumbens shell
NMDAR	N-methyl-aspartate receptor
NOR	Novel object recognition
NR	Rat NMDA subunits
NRGI	Neurogulin1

PCP	Phencyclidine
PFC	Prefrontal cortex
q	Chromosome
qPCR	Quantitative real time PCR
SEM	Standard error of the mean
SNc	substantia nigra compacta
Tm	melting temperature
V	voltammetric
VTA	ventral tegmental area

Chapter One

1. Introduction

Schizophrenia is a major mental disorder that affects 1% of the world's population (Vinson and Conn, 2012). It is characterized by deep disturbances in mental function, behavior and emotion, disruption of perception and judgment human (Neill *et al*., 2014). Symptoms of schizophrenia are classified into three main groups namely positive, negative and cognitive (Lewis and Lieberman, 2000). The positive symptoms include hallucination, delusions, disturbed behavior, thought and speech. The cognitive deficits include deficits in working memory, attention and executive function (Fraga *et al*, 2011). Negative symptoms include social withdrawal, depression and a reduction in affective expression (Andreasen *et al*., 2012). Schizophrenia presents primarily in young adulthood with symptoms causing serious economic and social problems in communities (Goff *et al*., 2001). One of the major challenge of neuroscience is to understand the neurobiological basis of schizophrenia because the disease affect various characteristic of human that include perception, thinking and self-awareness (Berrettini , 2000). The development of animal models that mimic the human condition in terms of pathology and behavior may be the key to elucidating these fundamental mechanisms, leading to improved therapies and better prediction of efficacy of novel treatment in patients (Abdul -Monim *et al*., 2007).

Amongst patients whose symptoms do respond well to drug treatment, many show debilitating side effects which reduce drug compliance, and compromise their ability to function in society, and maintain employment. These findings emphasise the need for alternative strategies for the treatment of schizophrenia, in turn demanding a better understanding of the neurophysiological and neurochemical abnormalities which underlie schizophrenia.

The dopamine hypothesis of schizophrenia has underpinned schizophrenia research for several decades and has clearly proven its experimental value. However, despite evidence for the role of the dopaminergic dysfunction in schizophrenia, this theory, cannot explain all the observations, and theories relating to other transmitter system including the amino acids, glutamate and GABA, Nevertheless, the identification of neurotransmitter abnormalities in schizophrenia is important for understanding the aetiology of the disease as well as for the development of new therapeutic strategies (Carlsson et al., 2001).

1.1 Aetiology of schizophrenia

The neurodevelopment hypothesis of schizophrenia is characterized by a combination of the susceptibility to genetic factors and environmental risk factors that, cause various changes leading to schizophrenia, however there were no specific identification of aetiological influences of these factors (Sullivan *et al*., 2003).

1.1.1 Genetics factor associated with schizophrenia

Several studies focusing on family, twin and adoption indicate that genetic factors are a significant part of the aetiology of schizophrenia and demonstrated that it is a genetic disease (McGuffin *et al*., 1994). In 2001, Goff showed that the liability for developing schizophrenia occurs more in biological relatives than in adoptive relatives. Similarly other studies found that the disease was related to the existence in biological parents and not in the adoptive parents and increased risk of the disease appeared in the first degree relative with schizophrenia (Gershon *et al* 1988; Maier *et al* 1993). Also twin studies indicate higher risk of sharing the disease in monozygotic twins (50 %) than in dizygotic (15 %) twins suggesting important genetic influence to increase risk of schizophrenia (Farmer *et al*., 1987; Goff, 2001).

Goff *et al*., (2001) indicate that there are number of genes which each provide some vulnerability for the disease. Several studies have identified chromosomal regions connected with schizophrenic susceptibility (McGuffin *et al*., 1994; Berrettini, 2000) .In addition (Blouin *et al*., 1998) and (Brzustowicz *et al*., 2000) have provided a linkage to chromosome (1q21-q22 and 13q32) with heterogeneity of schizophrenia, which could provide a clue to identify the susceptible genes that influences in schizophrenia.

The neurodevelopment hypothesis suggests that interaction between multiple genes provokes a cascade of neuropathological events starting during gestation and progressing into adulthood, which could be established and directed by environmental impacts (Stachowiak *et al*., 2013). In addition they hypothesize that genes associated with schizophrenia produce a protein which can alter and affect the neurotransmitter systems and the development of multiple neural circuits. Change in protein level resulting from changes in gene expression may, for example affect dopamine receptor expression (Araki *et al*. 2007). However the correlation between gene code expression and protein expression or receptor function remains to be tested (Duan *et al*., 2003).

Variant genes with their protein product that are associated with the pathology of schizophrenia include genes coding for dopamine receptor D1-D4 (DRD1-4), Neurogulin1 (NRGI), dysbindin (DTNBPI), Catechol-O-methyltransferase (COMT), metabotropic glutamate receptor (Tandon *et al*., 2008)

The mode of the disorder transmission is unknown and many environmental factors play important roles; in addition there are several genes with small effect which interact with the main effect genes and related environmental factors to explain the aetiology of the disease (Schwartz *et al*., 2000). Moreover most of association studies focusing on the aetiology of schizophrenia show that this genetic risk expresses its effect directly on dopamine function, via dopamine receptor and dopamine transporters (Talkowski *et al*., 2007), suggesting that dopamine dysfunction in schizophrenia in addition to genetic studies related to dopamine genes and receptors are required to understand the pathogenesis of schizophrenia: however the association between genetic process and dopaminergic pathophysiology is poorly understood (Moran *et al*., 2014).

1.1.2 The interaction between gene and environment factors

It has been reported that 83% of variation in risk for schizophrenia is accounted for by genetic factors and 17% is accounted for by environmental factors (Cannon *et al*., 1997). Different environmental factors have been suggested as important in the aetiology of schizophrenia, including both biological and psychosocial risk factors. The psychological and biological risk factors which lead to schizophrenia include a history of substance abuse, early prenatal and postnatal life adversity and psychosocial illness (Moran *et al*., 2014). Moreover viral infections during the fetal development and infancy have been indicated as possible causes of schizophrenia due to poor maternal nutrition (Berrettini, 2000). These affect the individual during different periods of their life from perinatal and antenatal periods, early and late childhood, adolescence and early adulthood (Cannon *et al*., 1998). It is scientifically challenging to differentiate how genes and environment interact to lead to dopamine dysfunction in addition to find the predisposing genes (Howes and Kapur, 2009).

1.2 Biochemical theories (Dopamine, glutamate)

Schizophrenia research for several decades were supported by dopamine hypothesis However, despite evidence for the role of the dopaminergic dysfunction in schizophrenia, this theory, cannot explain all the observations, and theories relating to other transmitter system including the amino acids, glutamate and GABA have become increasingly recognised (Carlsson *et al*., 2001).

1.2.1 The dopamine hypothesis of schizophrenia

In 1966, Van Rossum had recommended a hypothesis that dopamine pathways may be overactive in schizophrenia, based on the fact that dopamine–mimetic drugs caused hallucinations and neuroleptics caused rigidity. In addition post-mortem studies and data from *in vivo* experiments using the dopamine D2 selective antagonists, both suggested a high density of D2 receptors in schizophrenic brains (Seeman , 1987).

Carlsson (1988) demonstrated that the dopamine hyperactivity in schizophrenia can be mimicked by the effect of dopamine-releasing agent like amphetamine: when amphetamine is chronically abused it can induce psychotic symptoms that mimic schizophrenia, especially the positive symptoms. Related to this, early antipsychotic drugs act on dopamine in the brain, and the therapeutic effect of these drugs was due to their ability to block dopamine receptor and decrease dopamine transmission in the brain. In support of this hypothesis, it was found that binding efficacy of these drugs to dopamine D2 receptors was positively correlated with their clinically effective dose (Carlsson and Lindqvist., 1963).

1.2.2 Dopamine release under the control of glutamatergic neurons

Dopamine neurons are controlled by corticostriatal-glutamatergic neurons directly or by GABAergic interneurons, through direct or indirect action. Increase in dopamine function is as a result of reduced glutamate function and this depends on whether the effects on the direct or the indirect predominate. Thus, a reduction in glutamate function, induced by N-methyl-D-aspartate (NMDAR) antagonists may cause elevation of dopamine release (Carlsson *et al*., 2001).

Extensive interest had focussed on the possible role of glutamate in schizophrenia (Kim et al 1980; Goff and Coyle, 2001). On the basis that phencyclidine (PCP) in addition to

other non-competitive NMDA antagonist, such as ketamine and MK801 induced a psychotic condition mimicking schizophrenia and exacerbated psychotic symptoms in schizophrenics, a key role for glutamate receptors particularly the NMDA receptor, has been suggested (Abdul-Monim *et al*., 2003; Jentsch and Roth, 1999). Enhanced dopaminergic activity could be mediated by hypoglutamatergia, leading to an impairment of feedback control induced by failure of indirect pathway. In normal condition there is a balance between the direct and indirect pathway, however when a releasing agent such as amphetamine enhanced the dopamine function this will lead to activation of the negative feedback regulation resulting in a strong overweight of the indirect glutamatergic /GABAergic pathway (Carlsson *et al*., 2001). Figure 1.1.

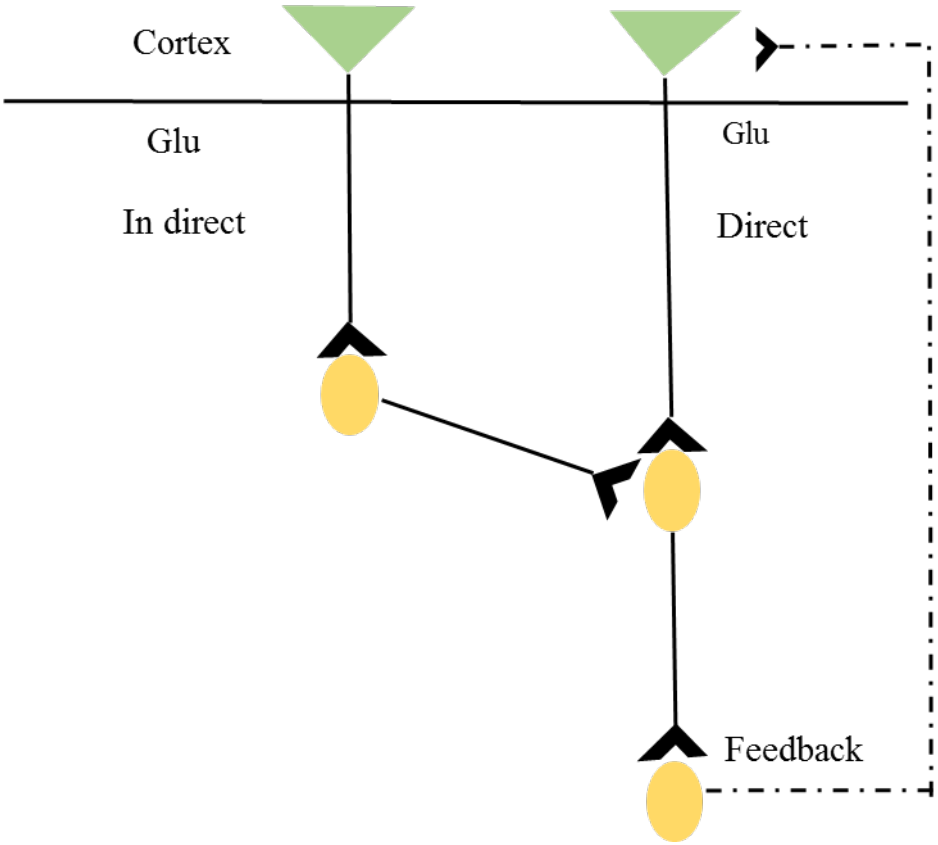


Fig 1.1: A hypothetical scheme shows the cortical regulation of the general effect of direct glutamatergic pathway (direct) and indirect glutamatergic /GABAergic pathway (indirect) on the action of amino acids in striatum and the thalamus. From (Carlsson et al., 2001).

1.2.3 Glutamate and the Glutamate hypothesis of schizophrenia

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) and is present in about 60% of neurons and 40% of synapses. Glutamate is synthesized in the brain from glutamine and used as excitatory neurotransmitter by cortical pyramidal neurons (Javitt, 2007). Synaptic transmission (pre and postsynaptic) modulated by glutamate actions by different types of ionotropic receptors (iGluR, NMDA, AMPA, and kainate), that are directly attached to cation channels, and their activation induces fast synaptic actions which may result in longer-term changes in excitability. Metabotropic (mGluR, group I, II, III) receptors are present as eight subtypes of G-protein coupled receptor Group I are excitatory, Group II and Group III inhibit adenylate cyclase (Blackshaw et al., 2011); Figure 1.2.

1.2.4 Glutamate receptors

The action of glutamate is through either, ligand gated ion channels (ionotropic receptors), comprising NMDA and AMPA / kainate receptors or G-protein coupled receptors (metabotropic receptors), (Javitt ., 2007).

1.2.4.1 Inotropic receptors
-NMDA Receptors

NMDA receptors contain a recognition site for glutamate in addition to two sites for the amino acids glycine and D-serine. NMDA receptors comprise several subunits, Rat NMDA subunits [NR1, NR2 (A,B, C,D), NR3A] and in human NR3B (Cull – Candy *et al*., 2001). NMDAR subunit can be used for therapeutic advantage. Several studies demonstrated the role of NMDA receptor in development learning and memory (O'Brien *et al*., 1998; Dingledine *et al*., 1999). Tsien et al. (1996) demonstrated that mice lacking NMDA receptors showed abnormal synaptic currents, potentiating the pyramidal cells of hippocampus and exhibiting impaired spatial memory (Tsien *et al.*, 1996).

NMDA receptors are blocked non-competitively by PCP and its analogues ketamine and dizocilpine (MK-801). PCP has the ability to induce symptoms relevant to schizophrenia-like psychotic symptoms (Abdul-Monim et al., 2003; Jentsch and Roth, 1999) providing a link between glutamate dysfunction and the pathophysiology of schizophrenia to glutamatergic dysfunction (Javitt, 2007).

-AMPA / kainate Receptors

AMPA / kainate receptors are a second type of ionotropic receptors for glutamate neurotransmission and are often classified as sub-types of the same receptors. However, AMPA receptors comprise combination of GluR1-4 subunits, although kainate receptors comprise GluR5-7 and KA1 and KA2 subunit, suggesting they may be separate competitive types (Javitt, 2007).

-Metabotropic receptors

The principle role of glutamatergic metabotropic receptors is regulating the release of presynaptic glutamate and postsynaptic sensitivity (Javitt, 2007), thus providing a regulating action on transmitter release. Eight distinct mGluRs have been identified, which are generally separated into three groups (see figure 1.2).

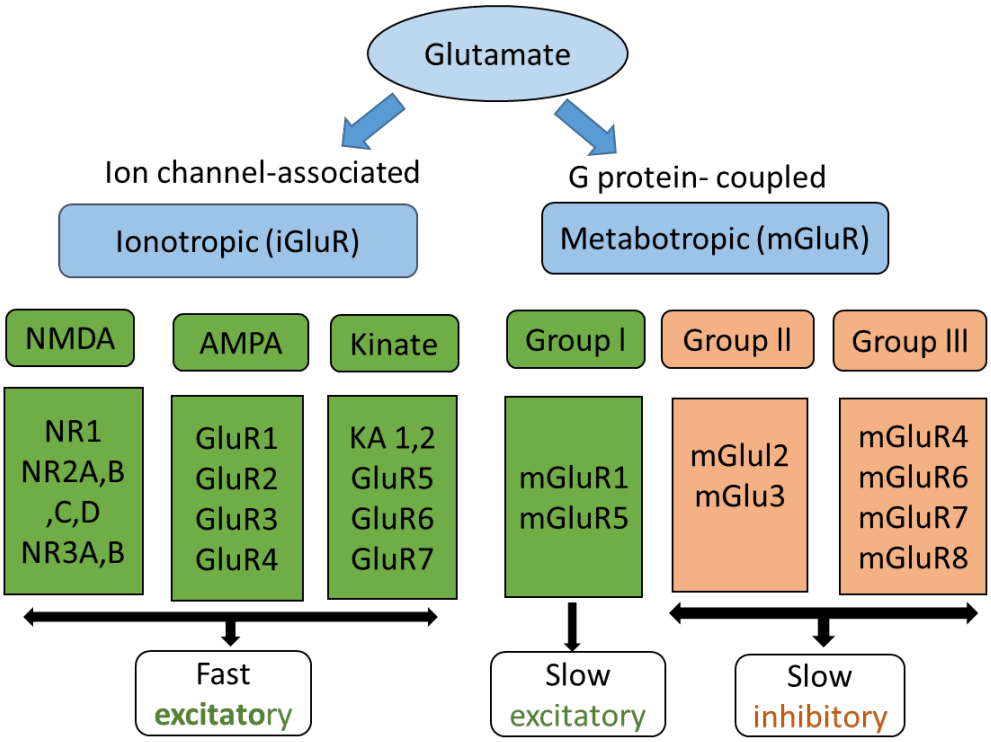


Figure1.2: Classification of Glutamate receptor. From Blackshaw (Blackshaw *et al*., 2011), Glu (glutamate receptor), Rat NMDA subunits [NR1, NR2 (A,B, C,D), NR3A], KA(Kinate receptor).

1.2.5-The Glutamate Hypothesis of Schizophrenia

The glutamate hypothesis of schizophrenia has been derived from some clinical observation after using PCP as anesthetic agent which enhances damage in glutamate areas of brain and changes in glutamate receptors.

Pharmacologically PCP is a non-competitive antagonist at NMDA-type glutamate receptors, and it has a psychotomimetic characteristic in humans (Javit and Zukin, 1991). Schizophrenia relevant symptoms are produced by PCP in healthy volunteers and abusers (Jentsch and Roth, 1999). In addition PCP enhances psychotic symptoms in schizophrenia patients (Lerich *et al*., 2004). Notably, unlike amphetamine which produces only positive symptoms (see above), PCP evokes a full spectrum of psychotic symptoms, including positive, negative and cognitive. Thus it appears likely that the main underlying cause of schizophrenia relates to glutamatergic dysfunction and that this interacts with dopamine systems to produce symptoms.

In animal experiments PCP and MK-801 evoke behavioral abnormalities (Jentsch and Roth., 1999), which relate to abnormalities seen in schizophrenia. This led to the proposal that schizophrenia results from glutamate deficiency (Lerich *et al*., 2014). Systemic administration of PCP or MK-801 in animals produced (1) increased dopamine cell firing rate in ventral tegmental area (VTA), (2) increased extracellular dopamine concentration in the nucleus accumbens (NAc: Freeman and Bunney,1984) and (3) increased dopamine metabolism (Lerich *et al*.,2014). Therefore changes in dopamine function appear to be intrinsically linked to the glutamatergic effects evoked by blockade of NMDA-receptors.

1.2.6-Dopamine receptors

Historically, dopamine receptors were divided to two functional types: D1 and D2 both of which belong to G-protein mediated metabotropic receptors (Carlsson *et al*., 1957). More recently, using molecular biology approaches, five dopamine receptor subtypes have been identified (Beaulieu and Gainetdinov, 2011). D1-like receptors comprise D1 and D5 receptor subtypes, which stimulate adenylyl cyclase, producing an excitatory effect. On the other hand, D2-like receptor comprises D2, D3 and D4 receptor subtypes and inhibit adenylyl cyclase and are inhibitory (figure 3). All dopamine receptors are found in brain and in the periphery,and the different sub-types show distinct regional distribution, with only some areas of overlap (Beaulieu and Gainetdinov, 2011; Neill, 2014).

-D1 receptor

D1 receptors are widespread in areas receiving dopaminergic innervation. They are mostly found in the striatum, the NAc, thalamus, hypothalamus and limbic system (Missale *et al*., 1998). Gene coding expression of D1 mRNA was indicated in highest levels in the, nucleus accumbens, caudate-putamen and olfactory tubercle (Fremeau *et al*., 1991).

-D5 receptor

The D5 receptors are much more sparsely distributed in the rat brain than D1 receptors, and are restricted in hippocampus, parafasicular nucleus of the thalamus and the lateral mammillary nucleus (Meador *et al*., 1992). Generally D5 receptor are structurally

similarity to D1 and both are characterized by activation of adenylate cyclase (Missale *et al*., 1998).

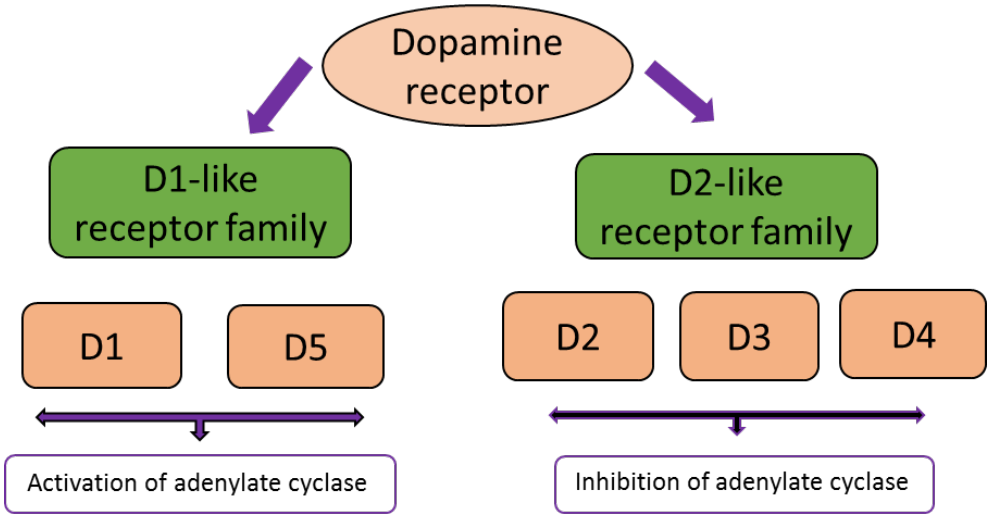


Fig1. 3: Types of dopamine receptors (D1-D5).

-D2 receptor

Dopamine D2 like receptors (D2, D3 and D4) are major target for action of typical and a typical antipsychotics which are used in the treatment of schizophrenia. They show differential regional and cellular distribution in the midbrain, cerebellum, basal ganglia, hippocampus, and cerebral cortex, thus indicating their distribution for area specific regulation of dopamine neurotransmission (Khan *et al*., 1998).

D2 receptor have a widespread distribution in striatum, NAc, limbic system and basal ganglia (Missale *et al*., 1998). It is found that the major of antipsychotic drugs are effective antagonist of D2 subtypes (Reynolds, 2005).

-D3 receptor

The D3 receptor has a particular distribution to limbic areas (Landwehrmeyer *et al*., 1993) and it is found at the low levels in the hippocampus and various cortical layers (Bouthenet *et al*., 1991). D3 receptors have generated some interest as a target for antipsychotic action (Reynolds , 2005).

-D4 receptor

The D4 receptor suggested to be localized in the area that relevant to the neuropathology of schizophrenia such as frontal cortex, limbic areas and medial temporal lope (Tol *et al*., 1992).

1.3 D4R and Schizophrenia

The dopamine receptor D4 (D4R) is a member of the dopamine D2 receptor family. It is distributed widely in different regions of human and rodent's brain including frontal cortex (FCx), hippocampus and entorhinal cortex, which are important regions for memory and cognition function (Mrzljak *et al*., 1996) and lower densities in NAc, dorsal striatum and the thalamus. Several studies have shown an important role of D4R in cognitive disturbances including in schizophrenia and in attention deficit disorder (Thomas *et al*., 2009; Furth et al., 2013). Furthermore, the elevation of D4R density in the brains of schizophrenia patients has led to the suggestions of a role of D4R stimulation in the pathophysiology of schizophrenia (Seeman *et al*., 1993).

Schizophrenia patients suffer from differentiated symptoms, positive, negative in addition to cognitive deficits that are only had partial responsive to current antipsychotic therapies, particularly, cognitive dysfunction (perseveration, working and reference memory deficits, decreased vigilance) which represents as a social integration and functional recovery problem (Newman-Tancredi *et al*. 2008). Some antipsychotics such as haloperidol are D2 and D3 receptors antagonist, and improve positive symptoms but fail to treat cognitive or negative symptoms (Harvey and Davidson, 2002; Meltzer *et al*., 1999). Clozapine an atypical antipsychotic antagonizes D2, D3 and D4 receptors (D4R), in addition to blockade serotonin receptor. It is thought that the combined D2 and serotonin receptor blockade results in reduced extrapyramidal symptoms and increased dopamine level in frontal cortex leading to improvements in negative and cognitive symptoms (Meltzer *et al*., 2003). The pharmacological characteristics of D4R are very similar to D2R and D3R receptors, however there are important distinguishing feature for D4R among which is the higher affinity for clozapine (Leyson, 2000; Van Tol *et al*., 1991) suggesting that clozapine atypical profile aspect may be attributed to actions at D4. However it is showed that positive symptoms of schizophrenia were not treated by selective D4 antagonist (Bristow *et al*., 1997; Corrigan et al., 2004). Therefore the role of D4R interaction in the actions of antipsychotics remains open to discussion.

Recently, it was proposed that there are no specific treatments for improvement or prevent the cognitive impairment associated with schizophrenia (CIAS). However various clinical trials such as recent meta - analysis indicated that atypical antipsychotic drugs (APDs), such as clozapine, risperidone, and lurasidone have the ability to achieve improvement in some domains of cognition more than typical APDs such as haloperidol in some patients with schizophrenia, Furthermore Preliminary evidence suggested that verbal learning and memory, executive function and verbal fluency improved by olanzapine but not working memory, visual learning and memory and attention, in addition it proposed that atypical antipsychotic drugs appear to act better than typical neuroleptics with regard to cognitive function (Meltzer and McGurk, 1999; Woodward et al., 2005). It was reported that there was an improvement for cognition in specific domains, especially episodic memory (Woodward et al., 2005; Desamericq et al., 2014; Meltzer, 2015). Consequently, it is important to develop novel and superior treatments for CIAS (Meltzer, 2015) and this has led to greater attention to pharmacological and genetic models of CIAS in animal studies (Young et al., 2009). NOR is one of the of the most widely employed behaviours used to study cognitive deficits induced by subchronic PCP treatment in rodents (Jentsch et al., 1998; Warburton and Brown, 2015; Grayson et al., 2015;), see chapter 3.

Increase of different dopamine receptors activity through dopamine release has been suggested in cortex and hippocampus by atypical APD as a favourable approach for treating CIAS (Ichikawa et al., 2002). The contribution of D4R in cognitive function and the efficacy of atypical APDs is not well understood (Miyauchi et al., 2017). It has been found that D4Rs are expressed in brain regions such as the PFC and hippocampus which are important for cognition and there D4R mRNA was highly expressed in monkey PFC layer V glutamatergic neurons in addition to parvalbumin-positive GABAergic interneurons (de Almeida and Mengod, 2010) which have been implicated in CIAS (Gonzalez -Burgos et al., 2015). It was therefore proposed that the excitability of parvalbumin-positive interneurons and PFC pyramidal neurons is attributed to D4R activation (Zhong and Yan, 2016) and because of its predominance in parvalbumin interneurons, de Almeida and Mengod (2010) hypothesized that D4R could be integral target in development of antipsychotic therapy.

In addition it has been found that D4R has the ability to regulate GABAergic receptor activity in in pyramidal neurons in the PFC (Graziane et al., 2009; Wang et al., 2002). It

was postulated that the efficacy of clozapine in treating schizophrenia was attributed to D4R antagonism (Van Tol et al., 1991). However many researchers have argued this hypothesis in clinical studies (Mansbach et al., 1998; Jentsch et al., 1999; Hertel et al., 2007; Rondou et al., 2010) because D4R antagonists such as L-745,870 and sonepiprazole were ineffective in clinical trials arguing against the use of D4R antagonists in treating schizophrenia (Kramer et al., 1997; Corrigan et al., 2004).

However, recently data from a temporal delay task in normal rats, indicated that the selective D4R agonist (A-412997) has the ability to improve NOR performance and increase extracellular DA in the medial PFC, indicating that D4R agonist may be a potential therapeutic target for CIAS (Woolley et al., 2008). In addition it was found that the D4R agonist, PD168077, reversed the NOR deficit induced by sub-chronic PCP in rats (Sood et al., 2011) an effect which was recently replicated by Miyauchi et al.,(2017).

Furthermore the DR4 antagonist, L-745,870, diminished the efficacy of lurasidone in the object retrieval task, whereas the ability of lurasidone in the same task was potentiated by DR4 agonist Ro10-5284 (Murai et al., 2014). These finding suggest that D4R activity may be important to some atypical psychotic drugs ability to improve CIAS and indicate that D4R stimulation contributes to NOR, as well more complex tasks relevant to schizophrenia (Miyauchi et al., 2017).

1.4 Dopamine pathways

One of three major projections of dopamine (figure 1.4), the mesolimbic pathway is thought to be very important in the pathophysiology of schizophrenia (Vallone *et al*., 2000). It is composed of dopaminergic neurons projecting from VTA to NAc and frontal cortex, and forms part of limbic cortical circuity (Neill *et al*., 2010).

It is found that projections of dopamine neurons to NAc modulate behaviours particularly which facilitate reward seeking (Redgrave *et al*., 1999). In addition, the projections of dopamine to prefrontal cortex are involved in cognitive processes such as motor planning (Braver et al., 1999). Furthermore the mesolimbic dopamine neurons play an important role in mediating cognitive and affective functions, and there is also an association between the mesolimbic pathway and the antipsychotic effects of dopamine receptor antagonist (Kerwin , 1996).

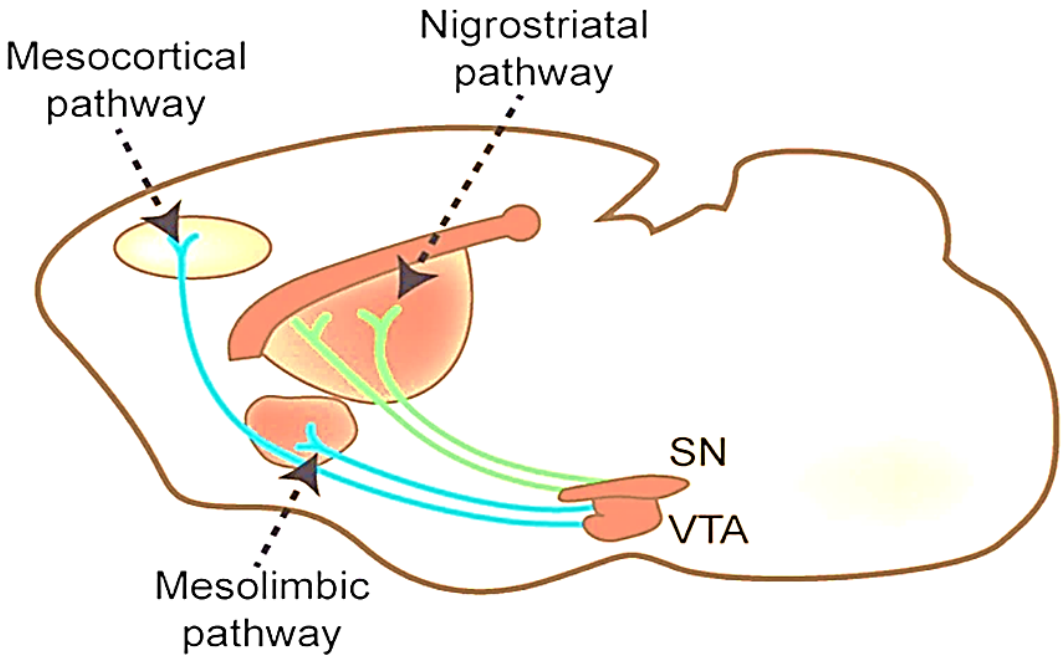


Figure 1.4: Three major projections of dopamine in brain. From Money and Stanwood, 2013. Projections constituting three majors pathways for dopamine, first, the mesocortical pathway originates from the ventral tegmental area (VTA) to different regions of the frontal cortex, this pathway appears concerned in some aspects of learning and memory (Feldman *et al*., 1997; Le Moal and Simon, 1991). Second, the mesolimbic pathway arises from the midbrain VTA and innervates the ventral striatum (NAc) (Feldman *et al*., 1997. This pathway is implicated in influencing motivated behaviour (Koob , 1992). Third, the nigrostriatal pathway originates from the substantia nigra compacta (SNc) which innervates the dorsal striatum (caudate-putamen), this pathway is concerned in the control of movement and its degeneration causes Parkinson's disease, (Vallone et al., 2000).

1.5 Dopamine dysfunction in schizophrenia

It has been found that disruption in dopamine cell activity affects many different functions in the brain including motor planning, reward seeking and cognition (Sesack and Carr, 2002). In addition excessive dopamine transmission is thought to cause delusions and hallucinations which are the positive symptoms of schizophrenia (Farde, 1997).

Carlsson (2001) hypothesized that midbrain dopamine neurons are regulated by PFC through a balance between direct accelerating influences and indirect braking effects. This hypothesis is supported by studies on animal models of schizophrenia showing that treatments that cause reduction in PFC outflow promote behaviours that are associated with dopamine transmission enhancement in NAc (Tzschentke, 2001).

Rodent pharmacological models relevant to schizophrenia have assessed the role of D4R in the regulation of the cognitive symptoms of schizophrenia. Interestingly it is reported that both D4 agonist and antagonist have beneficial impact of cognition and memory (Newman -Tancredi et al., 2008). It was shown that activation of D4R was implicated in memory consolidation by using D4 receptor agonist PD168,077 in rodent (Bernaerts and Tirelli, 2003). Sood et al. (2011) showed that D4R activation using the agonist PD168077 (3 and 10 mg/kg) can reverse cognitive dysfunction induced by subchronic PCP pre-treatment in novel object recognition (NOR). In contrast in cognition test in primates it was found that D4 receptor antagonists reversed the deficit in working-memory tasks induced by stress (Arnsten et al., 2000) or deficit induced by chronic PCP pre-treatment (Jentsch et al., 1999). Newman -Tancredi et al. (2008) suggested that modest level of D4 receptor activation may be a required for novel antipsychotic agents to improve cognitive symptoms of schizophrenia.

1.6 Models of schizophrenia

Animal models relevant to schizophrenia have undergone many developments through biological and psychiatric research (Jentsch and Roth, 1999). Although no animal model can reflect all aspects of neuropsychiatric disease such as schizophrenia, this can be divided into more simplified types that represent the main symptoms of schizophrenia (van den Buuse, 2010; Eyles et al., 2012), such as latent inhibition (LI) for positive symptoms, NOR for cognitive and sucrose preference tests for negative symptoms (see below).

One of the important selective attentional task is LI, which is disrupted in schizophrenic people and by psychomimetic drugs (Moore, 1959; Baruch *et al*., 1988; Barak and Weiner, 2011). It is based on the ability of the subject to ignore irrelevant stimuli: thus LI is the reduction of associability of a stimulus when it is pre-exposed without

reinforcement (Lubow and Moore, 1959) and it has been envisaged as a process of learning to ignore irrelevant stimuli (Weiner and Feldon, 1992). It is shown that the effect of preexposure is reduced in human studies (i.e. LI is disrupted) in positively symptomatic schizophrenia sufferers, and on this basis LI has been suggested as model for studying mechanisms of positive symptomatology in animal models (Hemsley et al., 1987; Weiner, 1990; Gray et al., 1995)

Negative symptoms can be assessed using sucrose preference tests for anhedonia, and social interaction tasks, while cognitive dysfunction can be assessed by using a variety of learning and memory tasks, including mazes and NOR (Van den Buuse *et al*., 2005). Working memory impairments are not delay dependent, thus provide useful animal models for drug development in schizophrenia (Lee and Park, 2005).

In schizophrenia, failure in learning basic life skills, social problem solving and the capacity for work or schooling are associated with difficulties in memory and learning (Green *et al*., 2000). The development in therapeutics to treat these difficulties can be improved by identifying good, high quality cognitive tasks that can be used for testing drugs in pre-clinical tests (Dudchenko *et al*., 2013).

1.7 Phencyclidine (PCP) models of schizophrenia

In the recent decades, the prospective role of the NMDA-type glutamate receptors in schizophrenia has been investigated by several studies and considered as a major research subject due to the propsychotic effect of PCP. PCP abusers show behavioural changes similar to the positive and negative symptoms relevant to schizophrenia (Allen and Young, 1978). PCP is a non-competitive NMDA receptor antagonist which is acts on the PCP binding-site. However at high doses it will also act on other receptor types, including dopamine, noradrenaline and serotonin (Thornberg and Saklad, 1996). These observation led to PCP induced psychosis being used as a pharmacological model for studying schizophrenia (Olney *et al*., 1999).

It was suggested that an animal model which contains a neurodevelopmental dysfunction, mesolimbic DA overactivity, hippocampal pathology, gender differences and vulnerability to stress could be hypothesised as factors in the pathophysiology of

schizophrenia (Feldon and Weiner, 1992). There are various animal models relative to schizophrenia, which attempt to replicate human schizophrenics' symptoms, although, it was suggested that it has been difficult to find a specific model that is able to completely replicate all the symptoms (Dawe *et al*., 2009).

Amphetamine increases dopamine release in animal models relative to schizophrenia and produces the positive symptoms of schizophrenia such as hyperactivity and behavioural disinhibition. In addition LI and prepulse Inhibition (PPI) modelling sensorimotor gating, are reduced in animals treated with amphetamine (Kilts , 2001) and both models showed deficits in patient with schizophrenia (Morgan *et al*., 2003). However in comparisons to PCP, animals treated with amphetamine show no deficits in social withdrawal representing negative symptoms of schizophrenia (Dawe *et al*., 2009). Also dopamine transporter knockout mice (DAT-KO) do show stereotypic behaviour, hyperactive behaviour, and cognitive impairments, but do not show any social withdrawal symptoms, thus they are not able to replicate the negative symptoms of schizophrenia (Lipska and Weinberger, 2000).

Another animal models relevant to schizophrenia, Stable tubule-only polypeptide (STOP) null mice, shows deficits in behaviours modelling positive (Fitzgerald *et al*., 1991) , and negative symptoms (Torres *et al*., 2005) and deficits PPI and LI modelling abnormal sensorimotor gating (Verma *et al*., 2008). They also show deficits in memory tasks (Dawe and Ratty, 2007), although, it has not determined to what extent these relate to cognitive deficits in working memory (Dawe *et al*., 2009).

In 2009, Dawe *et al* proposed that animal models based on pre-treatment with NMDA antagonist represents the animal models most relevant to schizophrenia as they exhibit the classic triad of schizophrenic symptoms, positive, negative and cognitive symptoms of the disease, and are similar to those which are observed in healthy individuals infused with ketamine.

In monkeys treated with PCP twice a day for 14 days there was a deficit in a PFC-dependent object retrieval task, which was reversed by acute clozapine (Jentsch et al., 1997). In addition in 2008, Mao *et al* indicated that over a 7 month period, PCP significantly reduced the frequency and duration of primate social behaviour which reflects the negative symptoms observed in in schizophrenia patients.

In assessing appropriate animal models of disease, they should be judged against three main areas of validity (Broekkamp, 1997): construct validity (the extent to which they replicate the theoretical neurobiological basis of the condition); face validity (to what extent the model produces changes which are similar to the symptoms of the condition); and predictive validity (the extent to which the model shows a similar pharmacological response, or lack of it, to the disease state).

Many different animal models relevant to schizophrenia have been suggested and tested, however the question remains as to which animal model is the most appropriate as a model of the human disease (Dawe *et al*., 2009). In the studies presented here sub-chronic PCP pre-treatment was chosen as it has been shown to exhibit a good degree of predictive, face and construct validity, and because it meets the need for animal models reflecting the various domains related to the symptom of schizophrenia, including positive, negative and cognitive symptoms (Allen and Young, 1978).

Thus, it has been suggested that one of the advantages of chronic PCP models is the ability to translate findings to primates over other animal models relevant to schizophrenia, in addition it is better than acute PCP models due to the absence of impaired motor function and motivation (Jentsch and Roth, 1999).

It was suggested that surgical lesions in the early postnatal period and monitors the behavioural consequences of the ventral hippocampus lesions is one of the two major research lines have been used to model the pathophysiology of schizophrenia according to the neurodevelopmental hypothesis of this disease (Lipska and Weinberger, 1993; Lipska *et al*., 1995), however this model have been reported to show little to no neuropathological abnormalities reported in the temporal lobe of schizophrenics (Jongen *et al*., 2004). The second research line it was suggested to interfere with the development of the central nervous system, this line of research involves prenatal stress, induced with high glucocorticoid levels (Shalev and Weiner, 2001; Koenig *et al*., 2002) these studies induces behavioural changes in animals exposed to prenatal stress or glucocorticoids related to paradigms associated with schizophrenia during the final week of gestation similar to amphetamine-induced locomotor behaviour and disrupted sensorimotor gating(Shalev, and Weiner, 2001; Koenig *et al*., 2001, Koenig *et al*., 2002). In addition another approach involves selective prenatal disruption of neuronal circuitry in rats, by using anti-mitotic drug methylazoxymethanol acetate (MAM) to treat the pregnant rats at

a specific embryonic time-point (Talamini *et al*., 1998). As a neurodevelopmentalanimal model of schizophrenia, MAM treatment could be used however, behavioural assessment of MAM as a model of schizophrenia is lacking.

Thus, modelling of the pathophysiological aspects of schizophrenia is one of the greatest difficulties in the neurobiological research of this disease, although to model schizophrenia in animals different hypotheses have been used based on the neurodevelopmental hypothesis of this disease (Lewis and Murray, 1987; Lillrank *et a*l., 1995; Stöhr *et al*., 1998)

1.8 Dopamine hypothesis in schizophrenia

The positive symptoms of schizophrenia can be induced by hyperdopaminergia, which is considered a key feature for studying symptoms (Meyer-Lindenberg *et al*., 2002). However, disruption of glutamatergic transmission following use of PCP tends to include a wide range of schizophrenia symptoms such as hyperactivity and cognitive disturbances, paranoia, hallucinations, and negative symptoms (Neill *et al*., 2010). This is supported by Carlson study in 2013, which reveal that the development of schizophrenic symptoms depend on abnormality in dopaminergic neurotransmission. Increased subcortical release of dopamine that enhances D2 receptor activation leads to increases the positive symptoms of schizophrenia which include delusions and hallucinations (Shen *et al*., 2012). O'Donnell and Grace, (1998) thought that enhanced positive symptoms could be as a result of cortical pathway disruption through the NAc (O'Donnell and Grace, 1998). Previous research has shown that the final common pathway of positive symptoms of schizophrenia is subcortical dopamine disruption (Pycock *et al*., 1980; Howes and Kapur, 2009), leading to the dopamine hypothesis of schizophrenia based on the enhancement of symptoms. Thus D2-like receptor antagonists provide the mechanism of action of typical antipsychotic drugs (Seeman and Lee, 1975; Creese *et al*., 1976). The mainstream of dopamine neurons exist in the midbrain comprising cell bodies of VTA and the substantia nigra that innervate NAc, dorsal striatum and cortex (Haber, 2014). In 2002, Meyer -Lindenberg et al proposed that hypodopaminergia in frontal cortex contributes to negative symptoms and cognitive deficits, and hyperdopaminergia in NAc contributes to positive symptoms. It was suggested that in schizophrenia striatal hyperdopaminergia is associated with

elevated dopamine function in mesolimbic (ventral striatum; NAc) dopaminergic pathway (Kegeles *et al*., 2010). In 2013, Meltzer *et al*. demonstrated that NMDAR such as PCP are psychotomimetic, and when administered to adult rodents cause schizophrenia-like behavioural deficits including changes in recognition and working memory, anhedonia and hyper-locomotor activity. Importantly these changes can be reverse by antipsychotic drugs, explaining the expected effect of dopaminergic, cholinergic and glutamatergic neurotransmitter systems, and supporting the validity of the NMDAR antagonist model.

Recently Hill, (2015) suggest that females are more susceptible to the cognitive disturbances caused by PCP administration which is supported the use of female rats in this study. Previous studies have reported that stages of the oestrus cycle do not affect rats' performance ability in many behavioural tasks, including NOR or reversal learning task, (Sutcliffe *et al*., 2007; McLean *et al*., 2009), and indeed they perform better than male rats in cognitive task such as NOR due to sex hormones (oestrogen and/or progesterone) that modify memory retention in rodents (Sutcliffe *et al*., 2007), suggesting that female rats are entirely appropriate for such scientific research (Tomlinson *et al*., 2015). However, it was proposed that sex differences observed in behavioural experiments in perception and recognition test reflect different response criteria and there was no effect for sex differences (Barr-Brown and White, 1971).

1.9 PCP effects on Dopaminergic Neurotransmission.

Previous research has shown that acute PCP administration leads to augmentation of dopamine release in the PFC and NAc (McCullough and Salamone, 1992). This increase in extracellular dopamine induced by PCP is attributed to overactive of dopaminergic neurons actions in VTA, which is attributed to increased activity in the cell bodies of mesolimbic neurones projecting to NAc and FCx, which are located in VTA, and due to the inhibition effect of dopamine reuptake by PCP in the PFC and NAc (Freeman & Bunney, 1984). However, as mentioned above chronic PCP administration leads to decrease the concentration of extracellular dopamine (Jentsch & Roth, 1999), whereas acute administration of PCP has the opposite effect. Thus alterations in dopamine neuro-transmission may contribute to abnormalities in working memory produced by PCP

(Preedy, 2016) as previous research has shown that dopamine neurotransmission in the PFC participates in working memory (Sawaguchi and Goldman-Rakic, 1991). Consequently PCP produces constant dysfunction effects in PFC which are similar to those seen in schizophrenia (Thomson *et al*., 2011). Toyooka *et al* (2002) indicated that PCP antagonized NMDAR, and produced excitatory amino acid neurotransmission inhibition which could attributed to PCP induced behavioural changes.

1.9.1 Acute and long term PCP exposure

One of the most important current discussions in studies of PCP effect on animal model relevant to schizophrenia, is to differentiate the biological and behavioural effects between acute and chronic PCP exposure. There have been several studies reporting that both regimens are able to induce serious behavioural symptoms which are similar to schizophrenia in humans, although there were significant differences between acute versus chronic PCP administration depending on the dose and route of administration (Jentsch and Roth, 1999). Table 1.1and 1.2.

Rodent	**Acute Exposure**	**Repeated Exposures**
Frontal cortex function	Impaired	Impaired
Temporal cortex function	Impaired	Preserved
Sensorimotor gating	Impaired	Unknown
Motor function	Impaired	Preserved
Motivation	Impaired	Preserved
Associative processes	Impaired	Preserved
Social behaviour	Reduced	Reduced
Locomotion	Increased	Augmented response to stress or amphetamine

Table 1.1. Effect of acute versus long term PCP exposure on rodent. From Jentsch and Roth, (1999).

Krystal *et al*. (1994) noted that generally acute administration of NMDAR antagonists such as ketamine elicits delusion and hallucination usually within the visual domain. These symptoms are similar to those produced by d-lysergic acid diethylamide (LSD) intoxication but not those seen in schizophrenia. In contrast, the study by Allen and Young (1978) indicated that PCP abusers showed more consistent symptoms that observed and developed in schizophrenic patients, in addition there are multiple behavioural features that are related to long-term PCP abuse such as social incompetence, emotional lability, poor interpersonal relationships, poor attention span and concentration. These features provide evidence of a strong relationship between long-term, but not acute, PCP abuse and psychopathology seen in chronic schizophrenic symptoms (Jentsch and Roth, 1999).

Moreover, a number of studies of regional cerebral blood flow showed that significant differences do exist in regard to the biological effects different administration regimes of PCP. Although acute administration of NMDA receptor antagonists such as ketamine significantly augments frontal cortical blood flow in normal humans or schizophrenic patients (Vollenweider *et al*., 1997; Breier *et al*., 1997), in contrast prior evidence observed reduction in frontal blood flow and glucose utilization in an individuals with chronic PCP abuse (Wu *et al*, 1991; Hertzman *et al*, 1990), which is similar to the investigation of cognitive deficit of schizophrenia that correlate with reduced frontal blood flow and glucose utilization (hypofrontality: Weinberger *et al*., 1986; Andreasen *et al*, 1992). Hence chronic, but not acute PCP exposure could produce a psychopathological states that are similar to chronic schizophrenia (Jentsch and Roth, 1999).

Furthermore previous research findings in animals showed that acute administration of NMDA receptor antagonist of PCP or ketamine augments forebrain dopaminergic transmission (Deutch *et al*, 1987; Verma and Moghaddam, 1996; Jentsch *et al*, 1997a), while there is a decline in frontal dopamine transmission due to chronic PCP administration (Jentsch *et al*, 1997b).

In 2003, Cochran et al, confirmed that long-term PCP exposure could play an important role in produce a model relevant to schizophrenia with induced change in rat brain. Recently similarly reported by Preedy (2016), after replicated PCP exposure of prefrontal cortex (PFC) may lead to suppression of PFC activity.

Table 1.2 summarises the effect of acute and long term PCP/ ketamine exposure in humans which reveals that both regimens can induce psychosis, hallucination, delusion, thought disorder and withdrawal which are similar to schizophrenic symptoms in humans, but that the effects of chronic exposure to PCP were generally more continuous (Allen and Young, 1978; Pearlson, 1981; Malhotra et al., 1996). However there are qualitative differences regarding the effect of these different dosing regimens. Long-term PCP abuse is more associated with psychotic symptoms similar to schizophrenia, importantly the chronic symptoms of schizophrenia (negative and cognitive symptoms) than symptoms induced by single dose PCP exposure (Jentsch and Roth, 1999).

Humans	**Acute Exposure**	**Repeated Exposures**
Psychosis	Intense (hours)	Intense (days to weeks)
Hallucinations	Visual illusions (hours)	Auditor and paranoid (days to weeks)
Delusions	Yes (hours)	Frequently religious (days to weeks)
Thought disorder	Yes (hours)	Yes (days to weeks)
Affect	Euphoric to catatonic (hours)	Anxious, labile, or paranoid (days to weeks)
Cognition	Impaired (transiently)	Impaired (persistently)
Frontal blood flow	Increased (transiently)	Decreased (persistently

Table1. 2. Differing Psychiatric and Biological Effects of Acute Versus Long-Term PCP in humans. From Jentsch and Roth(1999).

1.10 Behaviours assessing schizophrenia

One of the most useful tools in describing the treatment and pathogenesis of human disease is using animal models. In case of schizophrenia neuroscientists are faced with difficult challenges due to unknown the aetiology of the disease and the difficulty in

assessing many symptoms of the disorders in animal models including in rodents. However, these challenges have not discourage neuroscientists who are keen to benefit from even slight interfering between animal behaviours and schizophrenia (Powell and Miyakawa, 2006).

Animal model relevant to schizophrenia are considered as an important tool to reflect the disease symptoms observed in schizophrenic patients. In addition the interaction between the environmental factors (Thomas *et al*., 2001) and the susceptible genes (Sullivan *et al*., 2003) play an important role in presentation of some symptoms of schizophrenia in humans and rodents, and it is considered that assessing schizophrenia related behaviours in animal model will help in determining the pathophysiology of schizophrenia and understanding the gene/behaviour association (Powell and Miyakawa, 2006).

One of the most useful advantages of measuring schizophrenia related behaviours in animal model is help to understand the associations between the multiple factors that could lead to schizophrenia such as environmental, molecular and genetic associations, and consider the causal interaction between the genetic associative with schizophrenia and environmental alterations and behavioural abnormalities (Powell and Miyakawa, 2006). Furthermore, animal models of relevant to schizophrenia provide a useful method for identifying the pathogenesis of the disorder: in particularly using animal models is useful in understanding the aetiology or the causal factor for the disease which help in development of molecular and cellular alterations studies in rodent. For example it was demonstrated that the reduction in the activity of NMDA receptors in neuregulin 1 (NRG1) heterozygous mice, was as similar to the NMDA receptors dysfunction seen in particular brain area in patient with schizophrenia (Stefansson et al., 2002).

1.11 Behavioural methods used in the study:

1.11.1 Novel object recognition (NOR) and schizophrenia:

Episodic memory is the ability to recall an event in time and place "What? Where? When?" which is considered as one of the cognitive symptoms in schizophrenia (Le Cozannet et al., 2010). Among the main symptoms of schizophrenia, cognitive deficits associate with functional outcome and are not controlled with current antipsychotic therapies (Le Cozannet *et al*., 2010; Young *et al*., 2009). Working memory is one of the

seven domains of cognitive function that is disrupted in schizophrenia: others include attention/vigilance, visual learning and memory, processing speed, verbal learning and memory, reasoning and problem solving, and social cognition. There is a particular need for neuropsychological test to investigate these domains (Young et al., 2009): one of these tests is NOR, which is used in this study.

Several studies in schizophrenia and other neuropsychiatric disorders have focused on NOR in rodents as a model of the deficits in declarative memory (Meltzer *et al*., 2011 ; Neill et al., 2010; Lyon et al.,2012), which is identified as one of the seven primary cognitive domains that are abnormal in schizophrenia (Rajagopal *et al*., 2014). The NOR paradigm is represented by differential exploration of familiar and novel objects used to measure visual working memory deficits and can be assessed by measuring the time spent exploring each object (Neill *et al*., 2010 ; Grayson *et al*., 2015: see chapter 2 for more details).

It is reported that in some human disorders with the exception of schizophrenia few studies have been focused on the advantages of NOR test as a model for studying cognitive impairment. In addition there are multiple factors that favour NOR as a test for cognitive dysfunction, including its basic dependence on animals innate preference for novel stimuli (there is no water or food restriction so it is not stressful); it is ethologically relevant; it is robust and flexible; it provides data in short period of time; and it is not expensive to run (Korosoglou *et al*., 2012).

One of the three major symptoms of schizophrenia is cognitive symptoms (O'Grada and Dinan, 2007; Keefe and Harvey, 2012), and the cognitive symptoms play an important role in deciding the result and effect of schizophrenic patient ability to rehabilitate inside society (Harvey and McClure, 2006; O'Grada and Dinan, 2007 ; Christensen, 2007). Sub chronic PCP treatment, is used as a model relevant to schizophrenia during NOR task induce cognitive deficits. It was suggested that D4R may improve recognition memory, it was found that PD168077, a D4R agonist, (3 and 10.0 mg/kg) reversed recognition memory deficits induced subchronic PCP suggesting a participation of the D4R in aspects of memory processing (Sood *et al*., 2011).

In the current studies, NOR testing essentially provided a positive control for the second behaviour experiment, LI. The aim of using NOR was to check that the previously

reported disruption of NOR by PCP (Grayson et al., 1997; Sood et al., 2011; Neill et al., 2010) was attained in the current studies, and to determined that this deficit could be restored by a second D4R agonist (A412997), supporting the previous finings of Sood *et al.*, (2011) with PD168077.

1.11.2 Latent inhibition (LI) and schizophrenia

LI is consider as a moderately complex model that is theoretically associated with schizophrenic disorder hypothesis (Geyer and Moghadda, 2002). LI refers to the observation that repeated exposures to a sensory stimulus decreases the subsequent conditioning to that stimuli (Weiner *et al*., 1988). In patients with schizophrenia, deficiency in LI have been reported (Gray *et al*., 1995): however these deficits are only observed in patients during the acute phase of schizophrenia (Swerdlow *et al*., 1996). This type of behavioural model relevant to schizophrenia defined as "process of learning to ignore irrelevant stimuli seen in humans and animals" (O'Callaghan *et al*., 2014) has been used widely in animals to study aspects relevant to schizophrenia (Lubow, 2010).

In rodents, LI can be elucidated in both traditional and instrumental conditioning procedures (Weiner, 2003). For example in a modified cued fear conditioning model (Caldarone *et al*., 2000), the animals which are pre-exposed to a conditioning signal are not subsequently able to form an association between this signal and the unconditioned stimulus (foot-shock). In this model the animal basically disregards the tone/shock association as a result of their previous learning that the tone is irrelevant, and so can be ignored. Studies have shown that LI depends on dopamine function in NAc (reviewed in Gray *et al*., 1995; Moser *et al*., 2000).

1.11.3 Method used in this study:

1.11.3.1 Fast scan cyclic voltammetry (FSCV)

FSCV is an electrochemical techniques used for monitoring the release and uptake dynamics of endogenous monoamine levels both *in vivo* (Robinson *et al*., 2003; Lin , 2006) and *in vitro,* including in brain tissue and in artificial cerebrospinal fluid (Rice *et al*., 1994; Jones *et al*., 1995). Dopamine is a monoamine neurotransmitter which can be

measured by FSCV by electrochemical detection through voltage-dependent oxidation and reduction: because dopamine can be oxidized at low potentials leading to a current flow in the nA range, this can be measured at the carbon fiber microelectrode (CFM), (Syslová *et al*., 2012). Thus FSCV provides a means measuring dopamine in real time, at sub-second (e.g. 100 ms) time resolution. This study proposed that in NAc shell (NAcS), dysfunctions in glutamate signalling disrupts glutamate-dopamine regulation involving dopamine D4R controlled mechanisms. By using FSCV in rat brain slices *in vitro*, the aim of the study was to measure the effect of the dopamine D4R agonist, A412997 on dopamine release in NAcS and to discover whether PCP pre-treatment affected this modulation (see chapter two).

1.11.3.2 Quantitative real time PCR (qPCR)

qPCR is defined as "the reliable detection and measurement of products generated during each cycle of the polymerase chain reaction (PCR) process which are directly proportionate to the amount of template prior to the start of the PCR process" (Ginzinger, 2002). It is provides a relatively easy and cost-effective, high sensitivity assay, thus requiring only a small amount of RNA, which can provide a large amount of data relatively quickly wThis has made it a useful method in molecular diagnostics, virology and bioinformatics (Johnson *et al*., 2004; Wrobel *et al*., 2004). To amplify small amount of DNA, qPCR uses primers and includes a fluorescent signal (SYBR green: Brenda *et al*., 2011), with the levels of fluorescence monitored by computerized thermocycler. Specificity and efficiency parameters are important to the design of SYBR green-based primers, which is achieved by finding a pair of primers that are specific for the target genes, do not produce primer dimers or result in short amplicon (specificity). In addition, the primers should be able to produce data that are consistent and reproducible (efficiency) (Brenda *et al*., 2010).

Using qPCR, this study investigated the expression of genes coding for dopamine receptors in brain tissue taken from rats which had been pre-treated with PCP in a model relevant to schizophrenia, and compared this to tissue taken from saline pre-treated animals (see chapter four).

1.11.3.3 High pressure liquid chromatography HPLC

Seperation techniques which can be defined as the method of separating component chemical compounds in a mixed solution by using chromatographic column, then the compounds can be purified or identified and quantified (Meyer., 2013). The most frequent application of HPLC is analytical which is used for identifying and quantifying endogenous chemicals including from *in vivo* measurements, like brain microdialysates or *in vitro* like brain slices and post mortem tissue homogenates. In this study HPLC with electrochemical detection (HPLC- ED) was used for monitoring of dopamine and its metabolites in different brain tissue homogenates (NAc, FCx and VTA) from sub chronically pretreated with PCP to test the effect of this pre-treatment, on the basal level of dopamine and its metabolites (see chapter five).

1.12. Brain area involved

In patients with schizophrenia, it is noted that the core deficits are significantly related to dysfunction in frontal lobe (Goldman-Rakic, 1987). In humans and monkeys behavioural deficits such as social withdrawal, perseveration, impulsivity, flattened affect and cognitive dysfunction are associated with frontal lobe lesions (Goldman-Rakic, 1987; Petrides and Baddely, 1996). In addition both Barch (2004) and Goldman-Rakic (2004) noted that one of the cognitive dysfunctions observed in schizophrenic patient is working memory disorder which is hypothesised to be a consequence of from dopamine dysregulation in prefrontal cortex (PFC). However, there is substantial evidence that positive symptoms are related to abnormal dopamine function in NAc (e.g. Gray et al., 1995): for this reason dopamine-releasing drugs (e.g. amphetamine) provoke primarily positive symptoms, and typical antipsychotic drugs, which act primarily on D2 receptors, are mainly effective at controlling positive symptoms. Sub-chronic exposure of PCP can produce behavioural disorders mimicking all three types of symptom, consistent with a dysfunction of PFC and NAc which leads to the concept that long-term PCP administration as a useful model to induce schizophrenic symptoms (Jentsch and Robert, 1999). Within NAc, two compartments can be distinguished, the NAc core and NAc shell (NAcS): dopamine concentrations were greater in NAcS than NAc core (Deutch and Cameron, 1992). It hypothesised that the dopamine innervations of the NAcS is

associated with limbic function, while that of NAc core is related to sensorimotor function linked to the nigrostriatal system (Deutch and Cameron, 1992).

1.13 Aim and Research Question

The overall aim of this study is to understand the neurochemical changes in dopamine D4R function after PCP pre-treatment, which will give clues to changes occurring in schizophrenia, perhaps leading to novel pharmacological therapies for the condition.

1.13.1 Research Question

What are the neurochemical changes in mesolimbic pathway, particularly involving D4R, evoked by sub-chronic PCP pre-treatment?

1.13.2 Objective

1-Measure the effects of drugs interacting with D4R on LI and NOR.
2-Describe long term changes in NAcS dopamine following sub chronic PCP, and test whether D4R agonist modified dopamine release.
3- Identify changes in the expression of genes coding for the dopamine receptors in different regions of the brains (NAc, VTA and FCx) of PCP-pretreated rats
4-Measure basal level of dopamine and its metabolites following sub chronic PCP in NAc, VTA and FCx

Achieving these aims expands our knowledge about the neurochemical changes in brain of PCP pretreated animals as a model relevant to schizophrenia, though modulating D4R and suggest the possible use of D4R agonist for the treatment of schizophrenia, which could be useful in improving cognitive symptoms. Moreover, it would provide us with insights about the importance of changes in dopamine receptor expression and in the turnover rate of dopamine suggestive of changes occurring in the pathogenesis of schizophrenia

Chapter Two

Chapter 2- Effect of D4 agonist on stimulated dopamine release in nucleus accumbens brain slices *in vitro*

2.1 Introduction

Dopamine has an important role in the CNS due to its involvement in motivation, cognition, mood, reward and movement, where it induces an excitatory as well as inhibitory stimulations which leading to generate postsynaptic action potential (Syslová *et al*., 2012). Measuring dopamine release and transporter activity in brain is one common application of voltammetry methods (Yorgason *et al*., 2011). FSCV is a dynamic electrochemical detection method that provides a tool to investigate the effects of monoamine uptake releasers and inhibitors on dopamine function (John and Jones, 2007).

FSCV is used with carbon-fibre microelectrodes, which provides good chemical and spatial resolution. Microscope guidance provides precise placing for carbon-fibre microelectrodes into specific regions of rat brain slices to allow the measurement and quantification of different aspects of neurotransmitter release such as: peak concentration, rate of release and duration of release. FSCV has greatly helped us to understand the role of mesolimbic dopamine system mediating the processes of cognition, reward and motor activity. FSCV has attracted more attention than many other techniques that measure endogenous dopamine in the brain in real time due to subsecond temporal resolution (Robinson et al., 2003) and less tissue disruption because of the smaller size of carbon-fibre microelectrodes (Chen and Budygin, 2007). Furthermore FSCV provides the ability to measure dopamine concentration at higher frequencies up to 10 Hz and the detection of dopamine overflow related phasic neuronal activity, related to reward in the rat nucleus accumbens (Phillips *et al*., 2003; Day *et al*., 2007)

In the FSCV experiments described here, brain slices were used, where only activity in local circuits was measured, since function of 'long-loop' circuits involving input from other areas of brain was removed *(*Cho *et al*., 2007).

The aims of the experiments described in this chapter were (1) to characterise the effect of selective D4R agonist (A412997) on electrically stimulated dopamine release and (2) to assess whether these effects were changed by PCP pretreatment, modelling schizophrenia.

To achieve these aims FSCV was used to measure electrically stimulated dopamine release in NAcS, in rat brain slices *in vitro*. The first objective was to characterize the effect of A412997 on stimulated dopamine release, and the second objective was to ascertain whether this effect was changed in animals pretreated with PCP.

2.1.1 *In Vitro* Fast Scan Cyclic Voltammetry procedure

Fast scan cyclic voltammetry provides an analytical chemical measurement of dopamine in rat brain slices. The basic action of FSCV depends on the electroactive properties of neurotransmitters. The applied voltage scan causes current flow at the working electrode, which provides the background signal (non-Faradaic current). When compounds oxidise or reduce they produce a small additional (Faradaic) current which can be measure. Due to the stability of the non-Faradaic current over time, the Faradaic current, derived from the chemical of interest, can be separated from the full signal by background (non-Faradaic) subtraction. The change in Faradaic current is attributable to dopamine oxidation to dopamine o-quinone and reduction of dopamine-o-quinone back to dopamine, causing movement of electrons between dopamine which can be measured as current and is proportional to dopamine concentration, (Stamford *et al*., 1995),(figure 2.1)

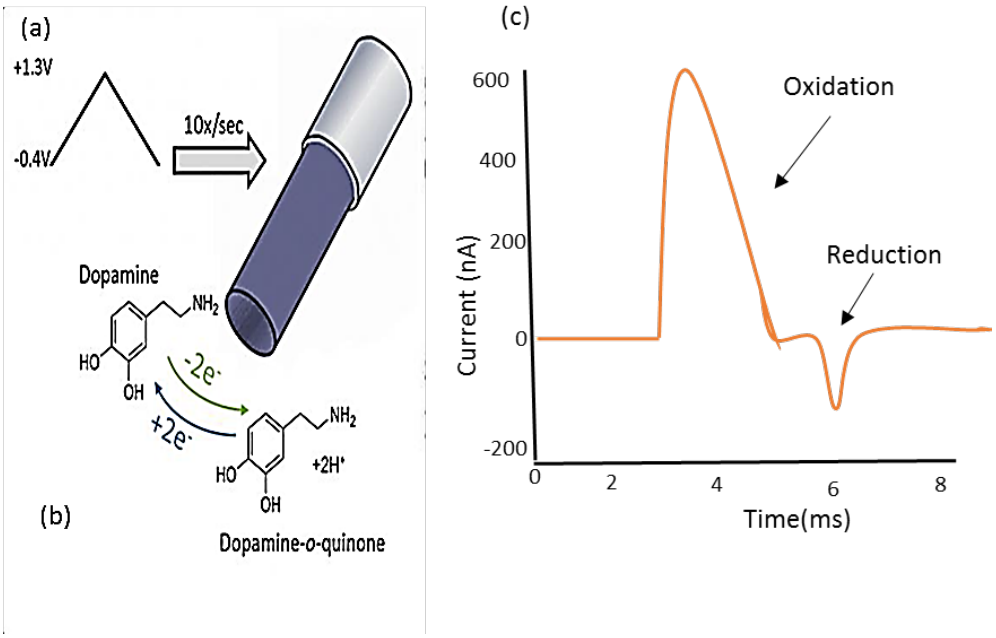


Figure 2.1: Dopamine detection by using FSCV. Image (a) and (b) from (Phillips and Wightman, 2003).

(a) Applied of a voltammetric scan from -0.4 to + 1.3V at 10 Hz (ten scan per second).

(b) Dopamine oxidation and reduction result in transfer of electrons that is detected as current at the recording electrode during this reaction.

(c) Background-subtracted signal due to oxidation – reduction, is seen as peaks at +600mV and –200mV respectively which identified the release of dopamine.

2.2 Materials and Methods

2.2.1 FSCV set up

FSCV equipment

The FSCV recordings were made in a custom built slice chamber (approx. 1 ml capacity: Biomedical Workshops, University of Leicester), superfused continuously with oxygenated artificial cerebrospinal fluid (aCSF), comprising 126 mM NaCl, 11 mM d - glucose, 2.4 mM $CaCl_2$, 2.5 mM KCl, 1.2 mM $MgCl_2$, 0.4 mM L-ascorbic acid, 1.2 mM NaH_2PO_4 and 25 mM $NaHCO_3$, at pH 7.4, heated to 33 ± 1°C using a thermostatically controlled peltier heater (Biomedical Workshops, University of Leicester). Superfusion flow rate was maintained at 1.4 ml/min using a Minipuls3 peristaltic pump (Gilson, UK). For FSCV recordings the carbon fibre working electrode was linearly scanned with a triangular waveform from -0.4 V to 1.3 V to -0.4 V at 400 V/s, versus an Ag/AgCl reference electrode placed remotely in the tissue chamber, at a rate of 10 Hz (10 scans per s). Waveform delivery and recording of resultant current was by a computer running Demon Voltammetry software (Wake Forest Innovations; NC, USA; Yorgenson *et al*., 2011) via a potentiostat and headstage circuit (Chem Clamp, Dagan Instruments USA).

2.2.1.1 FSCV Recording

The carbon fibre tip of the working electrode was inserted in the NAcS below the slice surface using a micromanipulator under microscopic guidance. Away from the working electrode, the reference electrode was placed at convenient location in the tissue chamber. Electrical dopamine release was evoked every 3 mins by a 4 ms, 10 pulse stimulation (monophasic, 300 µA) using concentric pole stimulating electrode (FHC; ME, USA) which was placed 100-200 µm from the working electrode in NAcS (Figure 2.2). By using Demon software, voltage waveforms (10 Hz) were applied to electrodes and the resulting changes to current were recorded and analyzed. A specific background signal generated by the recording electrode was subtracted to yield the Faradaic current caused by dopamine oxidation and reduction and by observation of the cyclic voltammograms dopamine was confirmed in each recording.

The location of the NAcS was determined by using rat brain atlas by Paxinos and Waston (1998) Figure 2.3. Baseline recording was commenced immediately with voltammetric scans applied at a frequency of 10Hz.

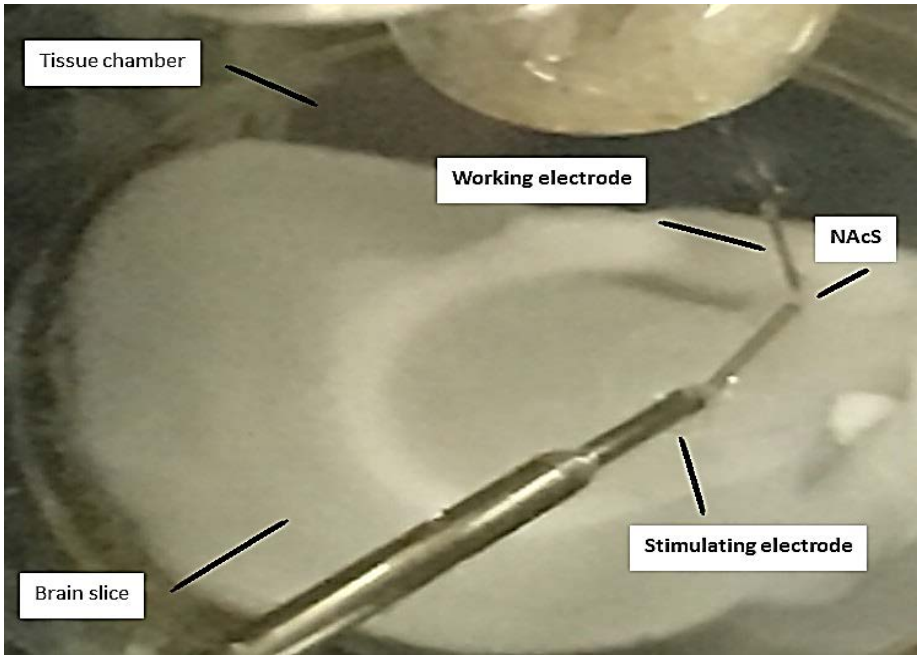


Figure 2.2. Rat brain slice within the FSCV tissue chamber, where the working electrode and the stimulating electrode in the area of NAcS.

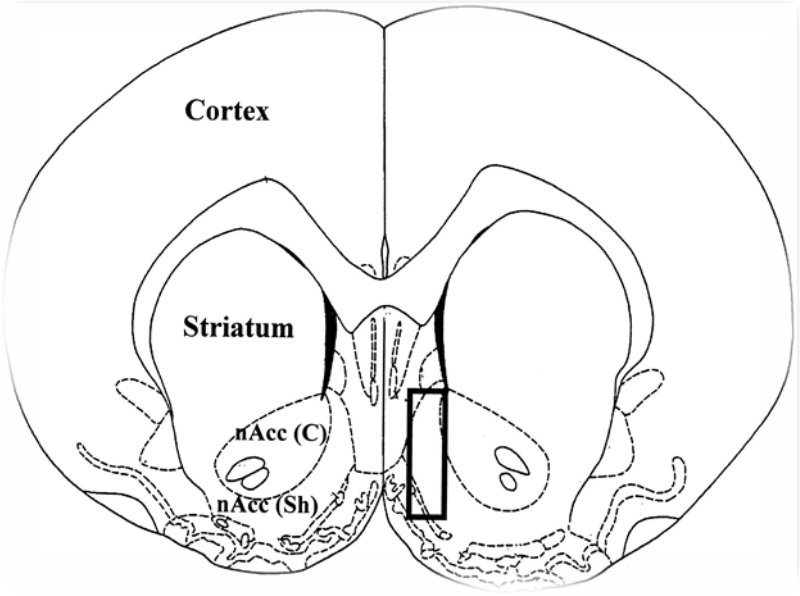


Figure 2.3. The location of the NAcS as shown by rat brain atlas by Paxinos and Waston (1998).

2.2.1.2 Working electrodes fabrication

Working electrodes were fabricated in the lab according to the description of Clarke et al. (2010), under a microscope. Carbon fibre (7 µm diameter; Good fellow, Cambridge Ltd.) was threaded into the fused silica capillary (OD 90 µm, ID 20 µm; CM Scientific, Cambridge, UK) under ethanol, and then it was left to dry overnight. A small amount of epoxy (Devcon, 5 min epoxy, ITW Polymers, Danvers, MA) was applied over the tip of the capillary and part of the fibre, and the fibre was pulled gently through the capillary to create an epoxy seal, leaving approximately 5 mm of fibre exposed. The electrode assembly was then left overnight to dry. A gold pin connector (MillMax, 0667; ID 0.6 mm; Farnell Electronics, Leeds, U.K.) was attached to the end of the silica tube that was not epoxied, using silver epoxy conductive adhesive (Circuitworks CW2400, Farnell Electronics, Leeds, U.K) (figure 2.4a), then left to dry and cure overnight. The carbon fibre tip cut to a length of 100 to 150 µm (Figure 2.4b) using iris scissors under the microscope.

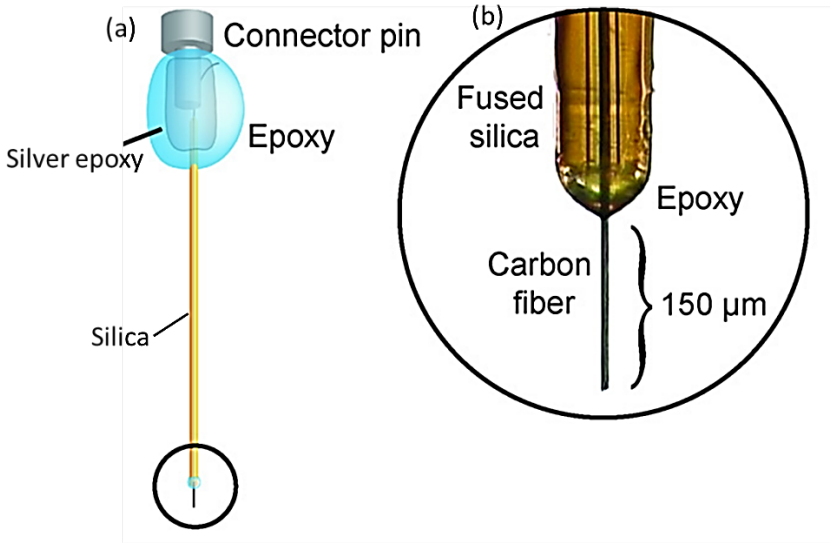


Figure 2.4: fabrication of working electrode. From Clark *et al*. 2010

(a) Picture of finished working electrode. Attachment of silver pin connector provides attachment to the FSCV system. The plug is fixed by silver epoxy on the silica tube; this connection also maintains electrical contact with the carbon fiber at the end, leading to allow transmission of signal at the carbon fiber to the FSCV system.
(b) Magnitude picture of working electrode tip. Extension of 150-200um carbon fiber tip from the end of the silica tube that was insulated with epoxy. To measure dopamine release the carbon fiber tip will be inserted into the nucleus accumbens shell.

2.2.1.3 Working Electrode Calibration

Electrochemically preconditioning of working the electrode was achieved by scanning at 60 Hz (-0.4 to + 1.3 vs Ag/ AgCl, 400V/s) for 10-15 minutes. Then the working electrodes were tested for a good voltammetric signal at 10 Hz. Determination of the sensitivity of each working electrode was by producing calibration curve. Every morning of experimental day prior to in-slice use, electrodes were preconditioned using the procedure described above. Measurement the electrode sensitivity on the day of experiment were taken from the working electrode's response to 5µM standard dopamine for 50 seconds The advantage of daily calibration also allowed the conversion of the dopamine signal (nA) to dopamine concentration (µM). Good sensitive electrode was used continuously for all experiment until sensitivity was lost.

2.2.1.4 Fabrication of Ag/AgCl reference electrodes

Silver wire (1.5 cm length) was inserted into the socket of a connector pin (Newark) and was epoxied by using the silver epoxy then it was left to cure overnight. The silver wire was then, dipped in 2 M KCl and electrolytically coated in AgCl, using a chloriding unit (NPI Electronics, Tamm, Germany: see appendix).

2.2.2 Brain slices *in vitro*

2.2.2.1 Animals

Juvenile female Wistar rats (80 – 120 g) from Central Research Facility, University of Leicester were used in all *in vitro* FSCV studies. Prior to use animals were housed under standard laboratory conditions with free access to food and water. All the experimental procedures were carried out under appropriate personal and project license authority under the Animals (Scientific Procedures) Act, UK 1986, and with local ethical review body approval.

2.2.2.2 Tissue preparation

Animals were humanely killed by cervical dislocation (with no anaesthesia), then decapitated and the skin overlying the skull was removed using dissection scissor. After that by using bone cutter, the cranium was removed carefully in order not to damage the brain, and then a flat spatula was inserted along the base of the skull to gently lift the brain. The brain was placed in ice-cold low-sodium slicing artificial cerebrospinal fluid (S.aCSF), comprising 250 mM sucrose, 11mM d-glucose, 25mM $NaHCO_3$, 0.1 mM $CaCl_2$, 2.5mM KCl, 1.2mM NaH_2PO_4, 4mM $MgCl_2$ and 0.4 mM L- ascorbic acid adjusted to PH 7.4, and transferred to the laboratory. The purpose of using this fluid is to suppress neuronal transmission during slicing. The brain was transferred onto petri dish and the cerebellum and olfactory bulbs were removed using razor blade, then by using cyanoacrylate glue (Loctite 'Precision Max' super glue), the caudal end of the cut brain was glued to a the mounting block of a vibrotome tissue slicer (1000 classic vibrating vibrotome (MO, USA)) and the glue allowed to dry for 2 minutes. The mounting block was fixed in the vibrotome specimen bath and the brain was sliced in ice-cold oxygenated S.aCSF. Coronal brain slices (400µM) including the NAc were cut, and transferred to oxygenated experimental aCSF at room temperature, composed of 126 mM NaCl, 11 mM d - glucose, 2.4 mM $CaCl_2$, 2.5 mM KCl, 1.2 mM $MgCl_2$, 0.4 mM L-ascorbic acid, 1.2 mM NaH_2PO_4 and 25 mM $NaHCO_3$, at pH 7.4. The slices having the NAc shell (NAcS) were chosen and taken by identifying the anterior commissure (aca) on gross examination. Each slice was cut along the midline to provide two unilateral slices at each level, using dissecting scissors. The slices were then allowed to recover from trauma of slicing for 60 mins in oxygenated aCSF at room temperature before FSCV recording then they were transferred to the FSCV tissue chamber superfused continuously with warmed aCSF (33°C) at flow rate 1.4 ml/ min and left their for at least (5 -10) mins in order to equilibrate. The slices were sourced from different numbers of animals for all the experiments as mentioned below: for experiment 1, (n = 25) slices from rats (n = 5), for experiments 2, (n = 40) slices from rats (n = 10), and for experiments 3, (n = 30) slices from rats (n = 10). In each case experimental conditions were distributed across slices from different animals, such that no more than two of any condition came from the same brain.

2.2.3 FSCV recording in brain slices *in vitro*

2.2.3.1 Experiment 1: Determination of the effective dose of A412997 on electrically stimulated dopamine release in non- pretreated rats

2.2.3.1.1 The aim of the experiment

Characterise the effective concentrations of selective dopamine receptor D4R agonist (A412997) on electrically stimulated dopamine release in non-pretreated rats (Juvenile Female Wistar rats).

2.2.3.1.2 FSCV Recording and protocol of experiment 1:

After equilibration of the slice in the recording chamber, a concentric bipolar tungsten stimulating electrodes (125 µm diameter: FHC Inc, USA) and a carbon fibre FSCV electrode were placed in NAc shell, and 10 electrical stimulation trains (10 pulses; 300 µA, 4ms, 50 Hz) were applied at 3 minute intervals. After 2 baseline stimulations (6 min), the A412997 was applied in the superfusate for four stimulations (12 min) while the control slices received no drug, and finally four post drug (washout) stimulations were applied (Figure 8). Five different A412997 concentrations were used (50 nM, 100 nM, 500 nM, 1 µM and 2 µM) to determine the effective dose of A412997 on dopamine release that evoked electrically in non pretreated animals (See figure 2.5).

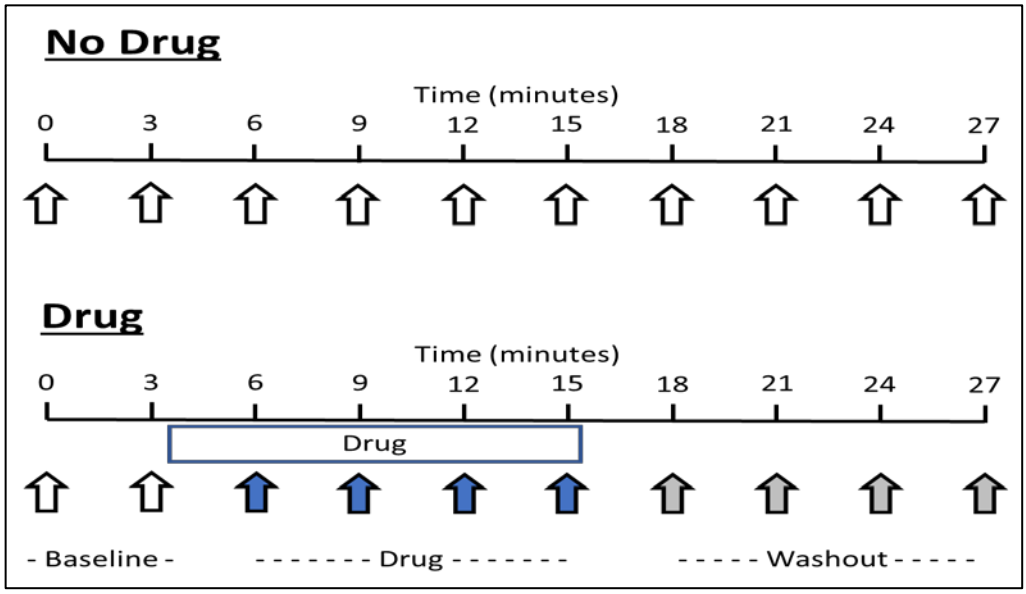


Figure 2.5. Summary of stimulation schedule for experiment 1. The first protocol (No drug) for the control condition and protocol 2 (Drug) was used for A412997. It involved 10 stimulation within 3 min interval. Protocol 1: In control experiments, slice were continuously perfused with aCSF. Protocol 2: in experimental condition using A412997, slice received aCSF for the first 3 minutes (Two stimulations), then slice perfused with (aCSF + A412997) for 12 minutes (four stimulations), finally the final 12 minute for washout period (four stimulations) that perfused with aCSF only.

2.2.3.2 Experiment 2: Confirmation that effect of A412997 was reserved by D4 antagonist (L-741,742) in non-pre-treated animals

2.2.3.2.1 FSCV recording and protocol

A concentric bipolar tungsten stimulating electrode, and a carbon fibre FSCV electrode were placed in NAc shell, and 14 electrical stimulation trains (10 pulses; 300 µA, 4 ms, 50 Hz) were applied at 3 minute intervals, as described above. After 2 baseline stimulation, the D4 antagonist (L-741,742) was applied in the superfusate for four stimulations in some slices, while other slices continued to be superfused with aCSF. Following this A412997 (2 µM: the effective concentration that attenuated dopamine release in experiment 1) was applied in the superfusate for four stimulation, either alone, or alongside L-741,742 and finally four post drug (washout) stimulations were applied (Fig. 2.7). Control slices were superfused throughout the 14 electrical stimulations with aCSF containing no drug. The peak stimulated dopamine release was measured.

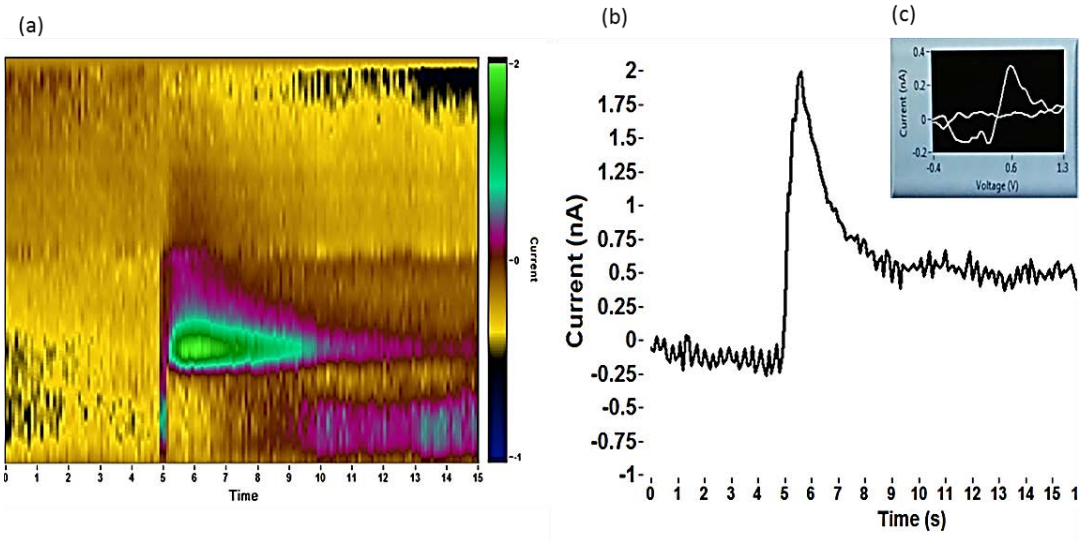


Figure 2.6. Example FCV data from a single slice before perfusion of D4 agonist A412997 (2 µM) for saline slice. (a) Colour plot and (b) current versus time plot of electrically stimulated (red bar) dopamine release during baseline period. (c) The characteristic cyclic voltammograms identifying the release of dopamine.

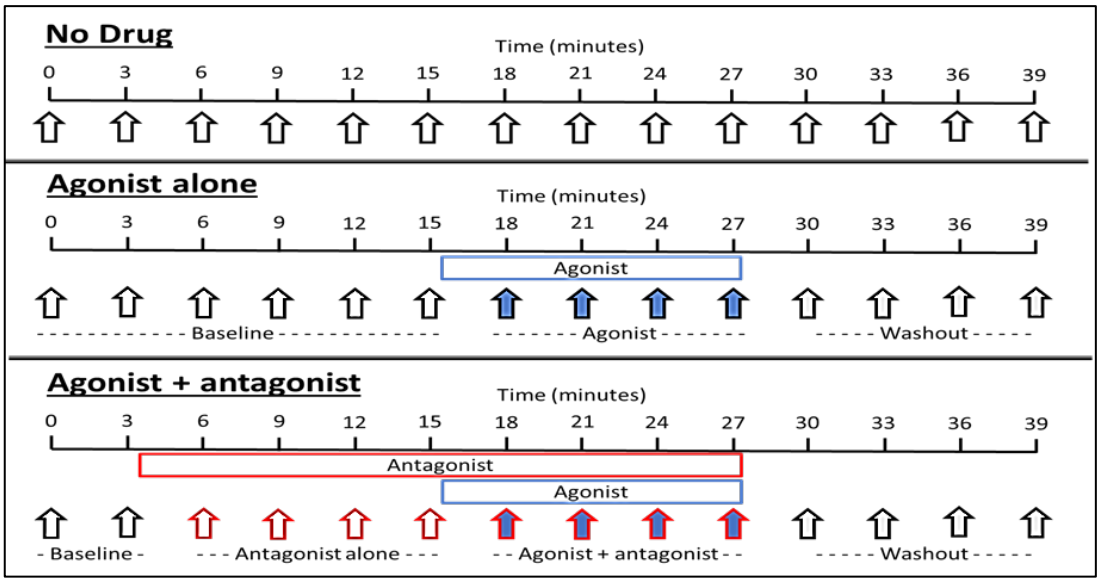


Figure 2.7. Summary of stimulation schedule for experiment 2. The first protocol (No drug) for the control condition and protocol 2 (Agonist) was used for A412997. Protocol 3 (Agonist + Antagonist) were used for A412997 + L-741,742. It involved 14 stimulation within 3 min interval. Protocol 1: In control experiments, slice were continuously perfused with aCSF. Protocol 2: in experimental condition using A412997, slice received aCSF for the first 15 minutes (Six stimulations), then slice perfused with (aCSF + A412997) for 12 minutes (four stimulations). Protocol 3: The first two stimulation (Base line) no drug, then perfused antagonist alone (L-741,742 + aCSF) for 12 minutes (four stimulation, (Red), then perfused agonist + antagonist (A412997 + L-741,742 + aCSF) for another 12 minutes (four stimulations) finally, 12 minute for washout period (four stimulations) that perfused with aCSF only.

2.2.3.3 Experiment 3: Effect of PCP pre-treatment A412997 mediated attenuation of nucleus accumbens dopamine release.

2.2.3.3.1 Aim of these experiment

Determine the effect of PCP pre-treatment on D4 mediated modulation of dopamine release.

2.2.3.3.2 Pre-treatment

For the pre-treatment cohort (Experiment 3 only,), PCP hydrochloride, (sigma-Aldrich, UK) was dissolved in sterile 0.9% saline. Rats (n = 10), (were divided to two groups, one group (n = 5) were pre-treated with PCP (2 mg/kg, i.p.) and the second group (n = 5) pre-treated with vehicle (0.9% saline) via intra peritoneal route (1 ml/kg) twice daily for 5 days. A 7-day washout period were given to rats in order to ensure that the animals were drug-free at the time of testing. Following washout, animals were killed, the brains removed and sliced as described above.

2.2.3.3.3 FSCV recording and protocol

Fast-scan cyclic voltammetry recordings were made by applying a potential waveform (-0.4 to +1.3 to -0.4 V; 400 V/s), and dopamine was measured in background subtracted current signal at an applied voltage of c600 mV. Ten electrical stimulation (10 pulses; 300 µA, 4 ms, 50 Hz) were applied at 3 minute intervals. After 2 baseline stimulations, A412997 (2 µM) was included in the superfusate for four stimulations (12 min) (control slices received no drug). A further four post drug (washout) stimulations were applied (Figure 2.5).

2.2.4 Chemicals and drugs

1- A412997 hydrochloride (N-(3-Methylphenyl)-4-(2-pyridinyl)-piperidine acetamide) a highly selective dopamine receptor D4R agonist (Tocris Bioscience, UK), was dissolved to a stock concentration of 10 mM in distilled water, and frozen at – 80°C. On the day of experiments the stock was diluted to a final volume 20 ml with aCSF. Five different concentrations were used from A412997 (50 nM, 100 nM, 500 nM, 1µM and 2µM).

2- L741,742 hydrochloride (5-(4-Chlorophenyl)-4-methyl-3-(1-(2-phenylethyl) piperidin-4-yl) isoxazole hydrochloride), a selective D4R antagonist (Tocris Bioscience, UK), was dissolved in DMSO, to make a 10 mM stock solution, which was frozen at – 80°C. The stock solution was dissolved in aCSF on each experiment day at two different concentrations, 10 µM and 50 µM.

2.2.5 Data analysis

Data analysis was performed using Demon Voltammetry Analysis Software. Based on maximum current generated during dopamine release (peak-dopamine signal, nA), the maximum extracellular dopamine concentration (peak-dopamine concentration in µM) was calculated. Data in this experiment were analysed using a two-way analysis of variance (ANOVA), (Stimulus number x Drug) for experiment 1 and 2, and using 3-way ANOVA (Stimulus number X Drug application X pre-treatment) for experiment 3, followed by Dunnett's post hoc test and Tukey's post hoc test. All statistical analysis were performed using IBM SPSS statistical 24.

2.3 Results

2.3.1 Experiment 1

The effects of different doses of A412997 on electrically stimulated dopamine release are shown on figure 2.8.One-way ANOVA showed a significant main effect of treatment ($F[15,114] = 7.631$; $p < .01$), which was entirely accounted for by a significantly higher baseline release in the slices treated with 1 µM A412997 ($p < .001$ compared to all other conditions: Tukey's multiple comparison test): no other differences were significant. It is not clear why these slices showed a bigger response than others, since, up to and including the baseline stimulation period, all slices had received exactly the same treatment. It is unlikely that this had a major impact on the interpretation of the experiment, since the normalised responses were similar in these slices: however, the data from 1 µM treatment with A412997 should be treated with some caution. See table 2.1.

Over 10 repeated electric stimulations at 3 min intervals, there was no significant change in the magnitude of the stimulus-evoked dopamine release in control slices not treated with any drug (no drug condition: n = 5: fig 2.8). The DR4 agonist, A412997, at concentrations of 50 nM, 100 nM, 500 nM, 1µM and 2 µM in the superfusate caused a concentration-dependent decrease in electrically stimulated dopamine release (Figure 2.8a). Two-way ANOVA showed a main effect of stimulus number ($F(9, 216) = 7.768$; $p < 0.0001$) and of drug ($F(5, 24) = 6.531$; $P < 0.001$) and a significant interaction between drug and stimulus number ($F(45, 216) = 1.751$; $p < 01$). In the control slices (no drug condition), post hoc analysis revealed no change over repeated stimulations (Figure 2.8b). The results also show that, although there was some indication of a small decrease during the lowest three different concentrations of A412997 (50 nM, 100 nM, 500 nM), this was not significant: however statistically significant decreases were seen with the two higher concentrations of A412997 (1 µM and 2 µM). There was also a highly significant linear trend in the effect of different concentrations of A412997 during the drug application ($R2 = 0.5539$, ($F(1, 24) = 31.35$; $p < 0.0001$) confirming the concentration-dependence of the effect, Fig (2.7b). It was found that, following the end of A412997 superfusion (washout period) the stimulated dopamine release exhibit limited, dose-dependent return toward baseline over the subsequent four stimulations: after 50 nM, 100 nM, 500 nM and 1µM, A412997 responses showed a partial return (75%) to baseline,

while after 2 μM A412997, there was little sign of reinstatement of the response. Thus the results reveal that the D4 agonist caused a clear concentration-dependent depression of stimulated dopamine release.

Exp. (1)	No drug	A412997				
		50 μM	100 μM	500 μM	1 μM	2 μM
Mean	0.0767	0.0776	0.0610	0.0942	0.2343**	0.0901
SEM	0.0291	0.0092	0.0063	0.0178	0.0655	0.0108
Exp. (2)	No drug	2μM A412997	2μM A41299 + 10μM L741, 742		2μM A412997 + 50μM L741, 742	
Mean	0.0256	0.0264	0.0617		0.0223	
SEM	0.0008	0.0016	0.0160		0.0022	
Exp. (3)	PCP	Saline	PCP + 2μM A412997		Saline+2μM A412997	
Mean	0.0407	0.0314	0.0611		0.0497	
SEM	0.0039	0.0037	0.0142		0.0123	

Table 2.1. Comparison of electrically-stimulated dopamine release during the baseline period (S1 and S2, before drug application). In experiment (1) the 1 μM A412997 group showed a significantly higher baseline stimulated release compared to control (no drug: ** $p < .001$ compared to all other conditions: Tukey's multiple comparison test), but there were no other significant differences for the other groups (A412997; 50 nM, 100 nM, 500 nM or 2 μM). In experiment (2), measuring the reversal of the effect of A412997 (2 μM), by D4 antagonist (L741, 742) in non-treated animal, baseline stimulated release was similar across all treatment groups. In experiment (3), measuring the effect of PCP (2 mg/kg, i.p., twice daily for 5 days) on the A412997 (2 μM) mediated attenuation of electrically stimulated dopamine release in pre-treated animals, PCP pre-treatment had no effect on the baseline stimulated release.

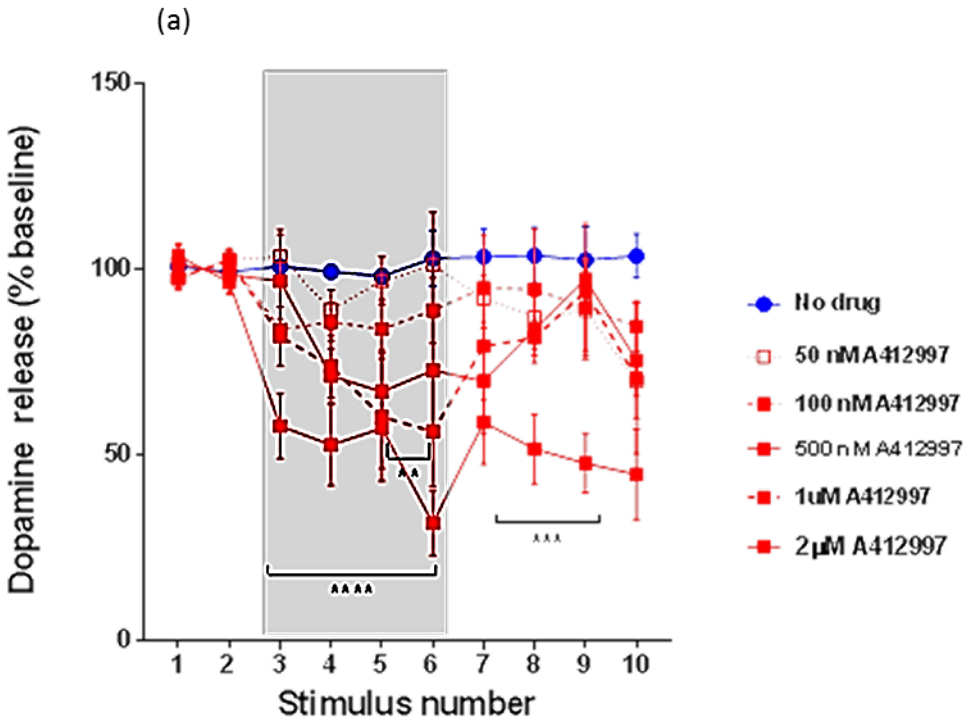


Figure 2.8a. Concentration - dependent effect of A412997 (50 nM, 100 nM, 500 nM, 1µM and 2 µM)), compared to control (no drug). Data show the time course of the effects over 10 stimulations at 3 mins intervals. Where drugs were given, they were included in the superfusate during stimulations S3-S6, indicated by gray panel. Data are mean ± SEM (n = 10 for each condition. ** *P < .01; P <* *** *.001;* **** *p < .0001;* statistically significant difference from baseline release in each condition: Dunnett's post hoc test, based on a significant interaction from ANOVA; n = 5.

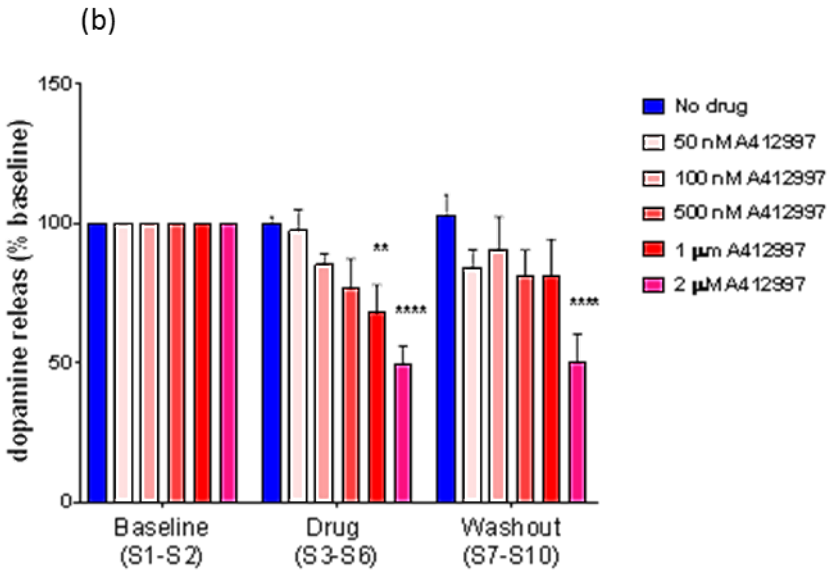


Figure 2.8b. Mean electrically stimulated dopamine release during baseline (S1-S2), drug (S3-S6), and washout period (S7-S10). Data are mean ± SEM. ** *P < .01; P <* *** *.001;* **** *p < .0001;* statistically significant difference from baseline release in each condition: Dunnett's post hoc test, based on a significant interaction from ANOVA; n = 5.

2.3.2 Experiment 2

The effect of the D4R antagonist, L-741,742, on attenuation of electrically stimulated dopamine release in NAcS are shown in figure (2.9a and b). Baseline stimulated release was similar across all treatment groups, see table 2.1. 54As indicated in the figure, L-741,742 at two concentration (10 µM and 50 µM) was applied alone (S3- S6) and concomitantly with A412997 (2 µM) in the superfusate (S7 – S10). This experiment replicated the previous depressant effect of A412997 (2 µM: Figure 2.8), which was entirely prevented by L-741,742 (10 µM), while at this concentration L-741,742 alone had no effect. Statistical analysis using ANOVA demonstrated a significant main effect of drug ($F(3, 36) = 10.049$; $p < 0.001$), but no significant main effect of stimulus number ($F(13, 468) = 1.070$; $p > 0.05$), however there was a significant interaction between drug and stimulus ($F(39,468) = 3.148$; $p < 0.001$). Post hoc analysis (Dunnett's post hoc test) showed a significant decrease in stimulated dopamine release during A412997 superfusion, which was abolished by L-741,742. At the high concentration (50 µM) the antagonist effects were quite variable, but overall appeared to enhance the stimulated release, both alone and in the presence of A412997, although, for the most part the differences were not significant. It is likely that at the high concentration, the drug is having a non-specific, perhaps toxic, effect.

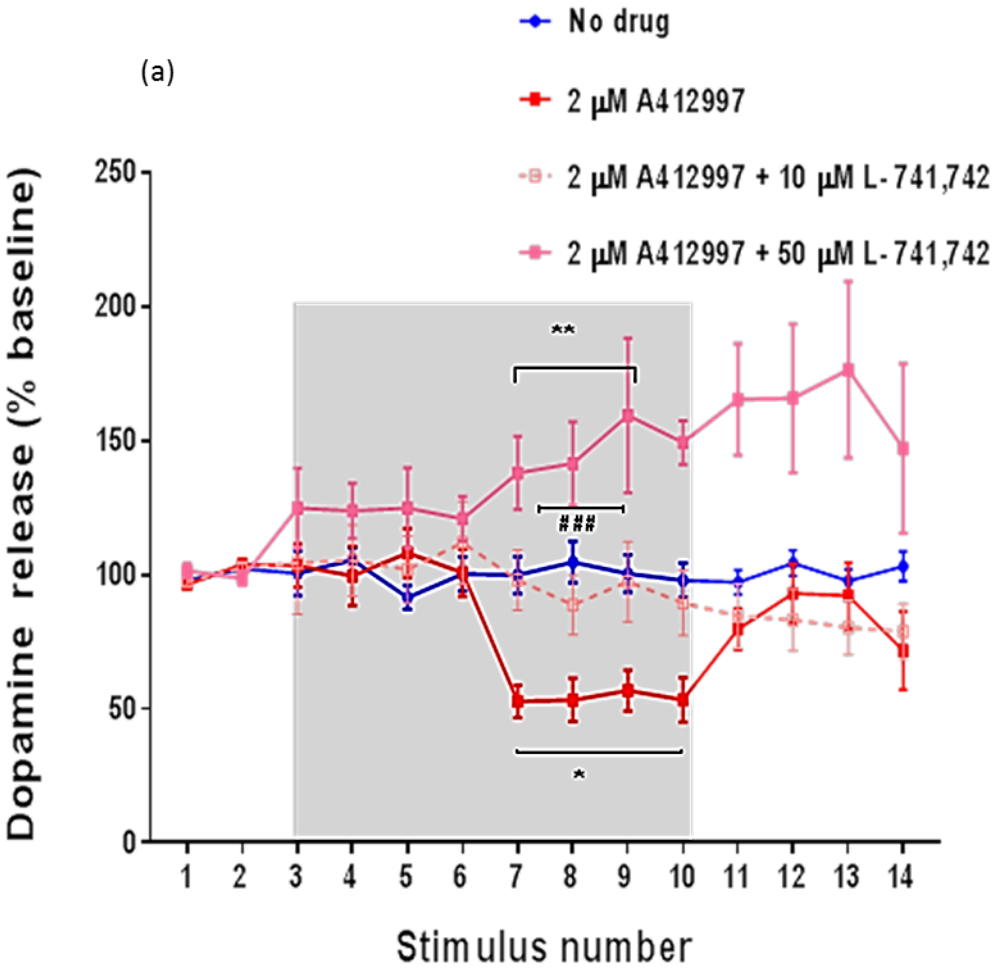


Figure 2.9a. Reversal of the effect of A412997 (2 µM), by D4 antagonist (L741, 742) in non-treated animal. Time course of the effects over 14 stimulations at 3 min intervals: L741, 742 alone was applied in the superfusate during stimulations S3−S6, and was applied with 2uM A412997 during stimulations S7−S10 indicated by the grey panel. Data are mean ± SEM. **p < 0.01; ***p < 0.001; Dunnett's post hoc test, based on a significant interaction from ANOVA; n = 10. ##p < 0.01; ###p < 0.001. Significant reversal of the A412997 effect by L741, 742 (Tukey's HSD).

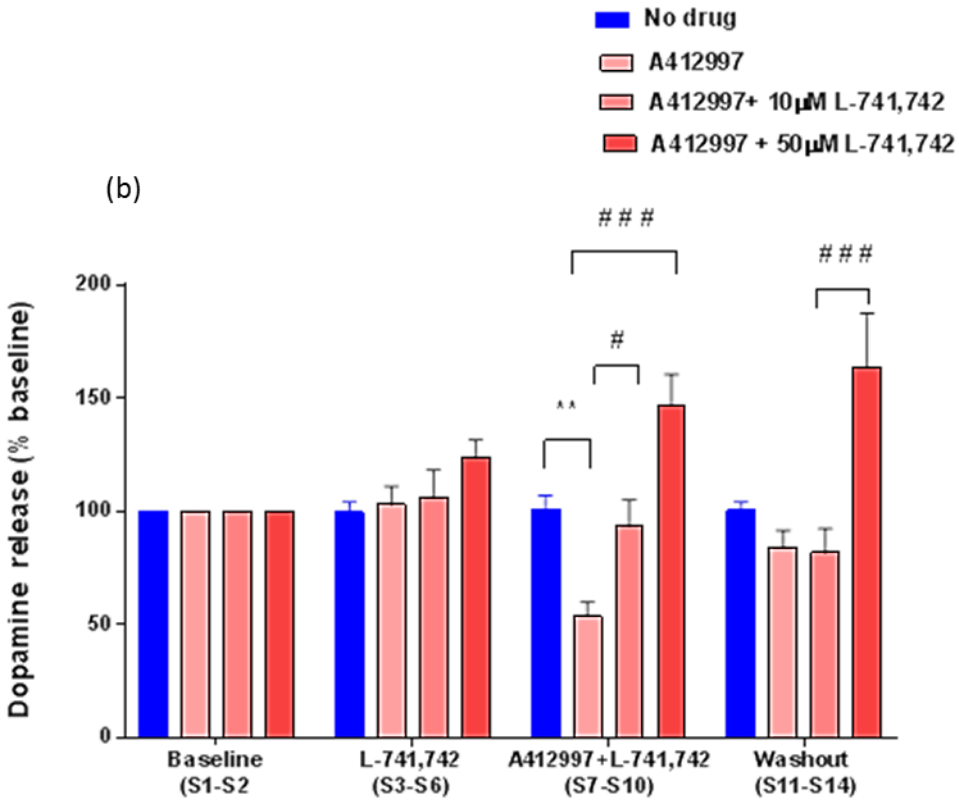


Figure 2.9b. Mean electrically stimulated dopamine release during baseline (S1−S2), A412997 (S3−S6), and A412997 + L741, 742 (S7 - S10) and post drug (S11−S14) periods. Data are mean ± SEM. **p < 0.01; ***p < 0.001; Dunnett's post hoc test, based on a significant interaction from ANOVA; n = 10. ##p < 0.01; ###p < 0.001. Significant reversal of the A412997 effect by L741, 742 (Tukey's HSD).

2.3.3 Experiment 3

To ascertain whether PCP pre-treatment affected attenuation of electrically stimulated dopamine release caused by A412997, tissue from saline (control) or PCP pretreated animals was used. PCP pre-treatment had no effect on the baseline stimulated release (saline pretreated, 0.043 ± 0.008 µM: PCP pretreated, 0.055 ± 0.010 µM: $t(28) = 0.911$, $p = .370$). See table 2.1 for all the data

ANOVA showed a main effect of PCP pre-treatment ($F(1, 22) = 5.948$; $p < .05$) and a main effect of A412997 ($F(1, 22) = 11.867$; $p < .01$) but no main effect of stimulus number ($F(9,198) = .872$; $p = .552$). There was a significant interaction PCP X A412997 ($F(1, 22)$ $p < .05$). Based on the significant interaction, post hoc analyses (Tukey's multiple comparisons test) were used to ascertain the differences. Secondary analysis compared the mean values during each separate treatment session (S1 - S2; S3 – S6; S7 – S10). Three way ANOVA showed main effect of Stage x A41 Interaction ($F(2, 44) = 5.778$; $p < .01$), while none of the other interactions involving stimulus were significant, although stage x PCP and Stage x PCP x A41 both approached significance, finally A412997 showed different effect on stimulated dopamine release in PCP and saline pretreated animals (Figure 2.10).

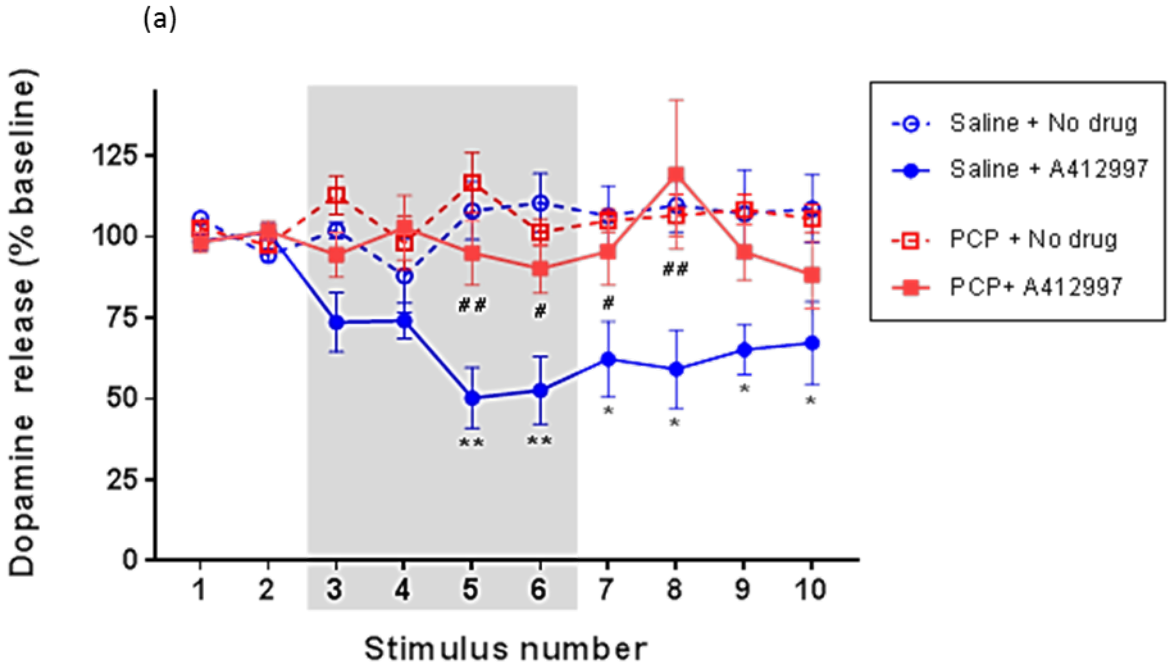


Figure 2.10a. Reversal of the effect of PCP (2 mg/kg, i.p., twice daily for 5 days), by D4R agonist, A412997 (2 µM) on electrically stimulated dopamine release in pre-treated animals. (a) Time course of the effects over 10 stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S3−S6, indicated by the grey panel. Data are mean ± SEM. * $p < 0.05$; ** $p < 0.01$; significant difference from saline no drug group; # $p < 0.05$; ## $p < 0.01$, significant difference between saline and PCP pretreated groups (Tukey's Post hoc test) n = 10.

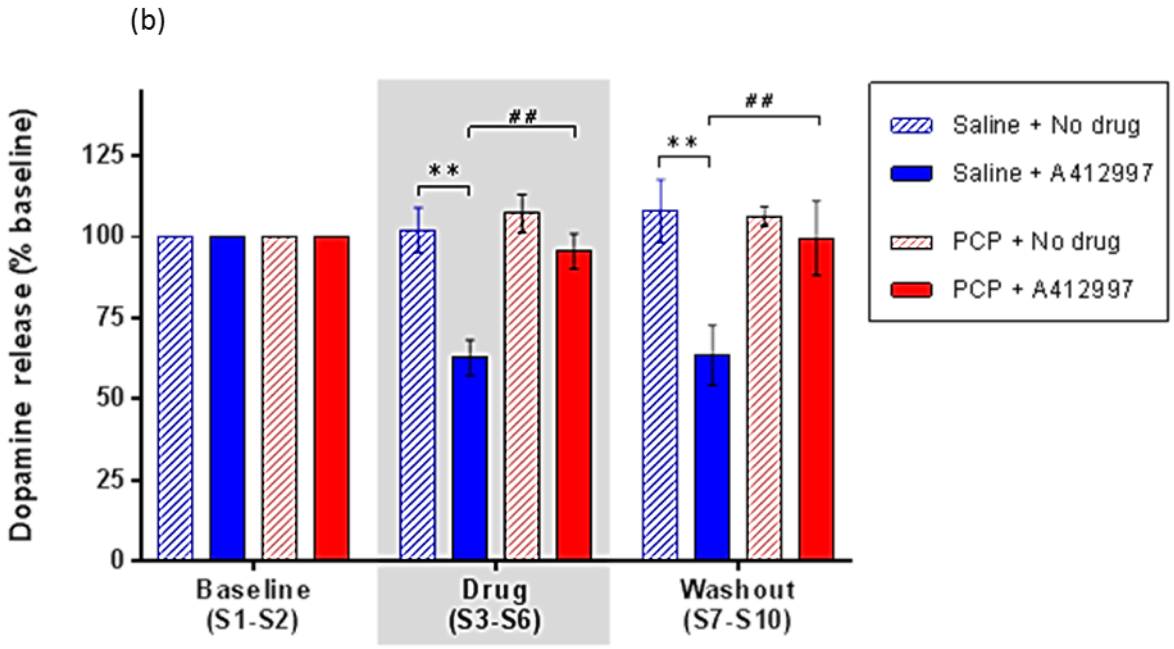


Figure 2.10b. Mean electrically stimulated dopamine release during baseline (S1−S2), drug (S3−S6), and post drug (washout periods), (S7−S10). Data are mean ± SEM. * $p < 0.05$; ** $p < 0.01$; significant difference from saline no drug group and from the appropriate PCP pretreated groups; # $p < 0.05$; ## $p < 0.01$, (Tukey's Post hoc test) n = 10.

2.4 Discussion

This experiment set out to establish whether or not the dopamine D4R agonist, A412997, had any effect on electrically-stimulated dopamine release and to determine the effective dose that has the ability to modulate the dopamine release in NAcS after application of electrical stimulation. The results provided a straightforward demonstration of a concentration-dependent attenuation of stimulated dopamine release, and that the attenuation caused by A412997 (2µM) was completely reserved by dopamine D4 antagonist L-741,742 in non-pretreated animals. However the effect of A412997 on dopamine release was completely eliminate in PCP pretreated animals, in a model of schizophrenia.

In experimental 1, D4R agonist (A412997) showed concentrations dependent effect for the doses tested (50 nM, 100 nM, 500 nM and 1 µM, 2 µM) with a significant effect for the two highest doses (1 µM and 2 µM). Experiment 2 showed that the inhibitory effect of A412997 on electrically-stimulated dopamine release was blocked by concomitant application of the D4 specific antagonist L-741,742, at doses which alone produced no effect, confirming that the effect of A412997 was mediated via D4Rs. This agrees with findings in electrophysiological studies on rat brain slices by Good *et al*., 2013, that dopamine initiates a depolarizing inward current and rise the spontaneous firing of lateral habenula neurons, which was imitated by A412997 and blocked by D4R antagonist L741, 742. The finding is also consistent with findings of past studies by Melis *et al*. (2005), which found that the effect of D4R partial agonist PD168077 was blocked by L-745,870 (1 µg) a selective D4R antagonist. In addition Rowley *et al*., 1996 mentioned to the role of D4R particularly in treatment of schizophrenia. This finding supports our study that DR4 antagonist (L-741,742) has no effect on electrically-stimulated dopamine release by itself but reversed the effect of the DR4 agonist, A412997. The effective concentration (2µM) of A412997 was chosen as it showed a consistent attenuation of stimulated dopamine release, which was reversed by antagonist (L-741,742). Some caution is required since the response did not recover during washout in all the slices. However, the fact that the response did recover in many slices (e.g. fig 2.9) and that the attenuation of stimulated release was reversed by the D4R antagonist suggests that the reductions measured are due to a true receptor mediated effect, and are unlikely to be due to toxicity.

Experiment 3, replicated the finding from experiment 1, that A412997 attenuated electrically stimulated dopamine release in control slices, in this case saline pretreated rather than non-pretreated. Importantly in PCP pretreated brains this effect was entirely abolished: that is A412997 had no effect on electrically stimulated dopamine release in slices taken from PCP pre-treated animals.

Kocsis *et al*. (2014), noted that dopamine receptor D4R has been involved in different psychiatric disorders such as schizophrenia, autism and attention deficit hyperactivity disorder (ADHD): however the mechanisms are poorly understood but they contributed these psychiatrics disease to neuronal defect particularly within cortical networks. Several studies have revealed that dopamine release is strongly modulated by D4R which has an important role in schizophrenia and has a strong connection to clinical and neuropathological demonstration of the disease (Rowley *et al*., 1996; Lauzon *et al*., 2010 ; Kocsis *et al*., 2014). For example, by using post-mortem tissue analyses for putamen of patients with schizophrenia, it was found that increase the level of D4R to 6-fold in comparison to the control (Seeman *et al*., 1993). This is further supported by post-mortem analyses by Murray *et al*. (1995) which reported a 2-fold elevation in D4R level in NAc of patients with schizophrenia, while with the same objective, Lahti *et al*. (1996) conducted numerical experiments on D4R and found the elevation of D4R binding is particularly in entorhinal cortex of schizophrenic patients.

However, several studies of post-mortem D4R binding found no difference in the level of striatal D4R in patients with schizophrenia relative to controls (Reynolds and Mason, 1995). Similarly Mulcrone and Kerwin (1996) concluded that there were no differences in D4R level by post-mortem analysis of frontal cortex in patients with schizophrenia.

One of the challenges in understanding the function of D4R, is that there is no successful antagonist at this receptor in clinical trials (Kramer *et al*., 1997). However, in the current study a full agonist at D4R, A412997 was used which has the selectivity to D4 receptor and is reported not to bind to other dopamine receptors or proteins (Moreland *et al*., 2005). In addition, the finding is consistent with findings of past study by Van Tol *et al*., 1991, which confirmed that high doses of A412997 were blocked by clozapine, and previous research contributed the high affinity of D4 antagonist, clozapine for D4R to a

hypothetical role of D4R in the pathophysiology of schizophrenia (Hrib, 2000; Wong and Van Tol, 2003).

One of the most important characteristic of A412997 is that it did not produced features of drug abuse at various dose tested in a behavioural study done by Tzschentke, (2007). D4R located in NAc core (Khan *et al*., 1998) and NAcS (Svingos *et al*., 2000) whereas at NAc core elevated dopamine level contributed to locomotor activity and at NAcS elevated level were responsible for rewarding feature of the drug (Sellings and Clarke, 2003). A412997 did not produce reward related behaviour, so can be used for the treatment of behaviour disorder related to schizophrenia without the drug abuse liability associated with current psychostimulants drugs.

One explanation could be that in spite of D4R presence in these location, its relative abundance in relation to D2 and D3 receptor, is much lower. Hence amphetamine activity in these area could be mediated through induction of dopamine activity to D2 and D3 receptors, in the conditioned place preference paradigm, in contrast to amphetamine, A412997 did not mediate reward related behaviour (Khan *et al.,* 1998; Woolley *et al*., 2008).

2.5 Conclusion

Repeated electrical stimulation facilitated the measurement of PCP effect on stimulated dopamine release in slice of NAcS allowing investigation of the influence of D4Rs on stimulated dopamine release, through measuring the effect of A412997.

Application of the dopamine D4R agonist, A412997, caused a decrease in electrically stimulated dopamine release in NAcS, which was entirely abolished in animals pretreated with PCP, in a model of schizophrenia. In addition the inhibitory effect of A412997 on electrically-stimulated dopamine release was blocked by concomitant application of the D4 specific antagonist L741, 742, at doses which alone produced no effect, which confirmed a specific D4R mediated mechanism. These changes are important in understanding of the role of dopamine D4 receptors in an animal model of schizophrenia.

Taken together with behavioural data [experimental chapter 3] this suggests abnormal D4-receptor mediated regulation of accumbal dopamine release after chronic PCP pretreatment, giving clues to dysfunctions occurring in schizophrenia, and providing a potential novel treatment strategy. The results presented here may facilitate improvements in the neurochemical basis for D4R modulation and suggest the potential use of D4R agonist for the treatment of schizophrenia. Further neurochemical studies both *in vivo* and *in vitro* are recommended to encourage future attention to NAcS which is an important area functionally and structurally for pharmacological treatment of schizophrenia.

Chapter Three

Chapter 3: Effects of sub-chronic PCP pre-treatment on latent inhibition and novel object recognition and investigation of reversal by A412997

3.1 Introduction

The experiments described in this chapter consist of two behavioural tests: (1) NOR to assess impaired episodic memory, modelling a cognitive symptom in schizophrenia (Le Cozannet *et al*., 2010), and (2) LI, a task that assesses selective attention, dysfunction of which represents more the positive symptoms of schizophrenia (Gray *et al*., 1995: See chapter 1). In this study the first experiment aimed to replicate work by Sood *et al*. (2011) who showed that activation of D4R can enhance cognitive dysfunction in NOR: the D4R agonist PD168077 (3 and 10 mg/kg) reversed the NOR deficit induced by seven days subchronic PCP pre-treatment. However, owing to uncertainly about the specificity of PD168077 (see below), in this study a different D4R agonist A412997 was used to test its reversal effect on NOR deficit induced by five days subchronic PCP pre-treatment animals. In addition, NOR experiments provided a positive control, to check that the previously reported disruption of NOR by PCP (Grayson et al., 1997; Sood *et al*., 2011; Neill *et al*., 2010) was achieved in these rats, and also aimed at ascertaining that this deficit could be restored by a pure D4R agonist (A412997), supporting the previous findings with the partial agonist, PD168077 (Sood *et al*., 2011).

The effect of sub-chronic PCP on LI was examined, since it provides a model of positive symptomatology. On the basis that PCP exacerbates symptoms of schizophrenia in all three behavioural domains of schizophrenia, it was anticipated that LI would be disrupted in PCP pre-treated rats. The second experiment extended this to test whether A412997 would also reverse disruption of LI induced by PCP pre-treatment.

Chronic PCP produces behavioural changes in people similar to deficits seen in all three domains of schizophrenia. However most symptoms of schizophrenia, such as thought disorder, delusions and hallucinations cannot be measured objectively in rodents, Nevertheless, there are a number of behaviour changes which have been observed in

animal models similar to those seen in schizophrenia patients such as behaviours connected with increased dopaminergic transmission (dopaminomimetic induced hyperlocomotion), cognitive deficits (impaired performance in a spatial memory test) and social withdrawal (reduced contact with unfamiliar partners), (Lipska and Weinberger, 2000, van den Buuse, 2010), thus providing useful pharmacological models for schizophrenia in which these symptoms were induced by PCP (Schijndel and Martens, 2010) (Bubeníková-Valešová *et al*., 2008). Therefore, the subchronic PCP model provides a valid method for studying the pathophysiology and aetiology of schizophrenia (Lipska and Weinberger, 2000). However, little is known about its action on animal models of positive symptoms. Hence the experiments described here aimed (1) **to assess whether subchronic PCP pre-treatment caused a disruption in LI, modelling positive symptomatology, and (2) to assess whether the dopamine D4 agonist A412997 could reverse PCP-induced deficits in both NOR and LI.**

A412997 is D4R full a agonist which has the ability to penetrates the blood brain barrier (Moreland *et al*., 2004), and it does not produce drug dependence (Sellings and Clarke, 2003). In the previous documented report by Sood *et al*. (2011), another D4R agonist, PD168077 was used: however, using receptor binding experiments *in vitro* Newman-Tancredi et al (2008) showed that PD168077 may act as a partial (rather than a full) agonist, therefore we cannot be certain that its behavioural effect is only through activating D4 receptors. Hence, A412997 was used in the present study to attempt to replicate the effect of PD168077, using the full D4R agonist, A412997.

Behavioural experiments

3.1.1 Effect of subchronic PCP pre-treatment on LI

To date, behavioural experiments in animal model of schizophrenia using sub-chronic PCP pretreatment have focused on behaviours modelling cognitive (e.g. NOR, Grayson *et al*., 2007; Sood *et al*., 2011) and negative (e.g. set shifting, McLean *et al*., 2008) symptoms, rather than positive symptoms (Cadinu *et al*., 2017).

In LI, the repeated pre-exposure (PE) of a stimulus impairs subsequent conditioning to that stimulus, and it is thought to model aspects of behaviour which are dysfunctional in positively symptomatic schizophrenia sufferers (Gray *et al*., 1995). It has been found that

LI disruption occurs in acute, positively symptomatic schizophrenic patients (Weiner 2003; Lubow 2005). Moreover, amphetamine, which increases dopamine release, and can induce and exacerbate positive symptoms of schizophrenia (Weiner, 2003), causes a disruption of LI in both people and in rats. This disruption represents deficits in selective attention which may underlie aspect of positive symptoms of schizophrenia (Weiner and Feldon, 1992; Weiner, 2003), and because of that LI has been suggested as model for studying mechanisms of positive symptoms in animals (Hemsley *et al*., 1987; Weiner , 1990; Gray *et al*., 1995; Moser et al., 2000).

Thus the dependence on accumbal dopamine function for LI has been well established (Weiner and Feldon, 1992; Gray *et al*., 1995). The behaviour study was concentrated on selective attention to motivational stimuli, through measurement of LI of conditioned learning which is a behaviour which is dependent on dopaminergic activity in the mesolimbic pathway and which is dysfunctional in schizophrenia. This study investigated the effect of sub-chronic PCP pre-treatment on LI in rats.

3.1.2 Aim of the Experiment 1

The aims were to investigate the effect of subchronic PCP and test the hypotheses that subchronic PCP pretreatment would disrupt LI. In this experiment NOR was also measured to check that, under the experimental conditions used, the effect of PCP pretreatment on NOR was replicated.

3.1.3 Experiment 2 Effect of A412997 on PCP-induced disruption of LI and NOR

The NOR paradigm is used to measure visual working memory deficits. In 2010, Neill *et al.* reported episodic memory in patient with schizophrenia can be modelled by visual learning in rats using NOR. Hence, changes in PCP induced cognitive impairments in animal model of schizophrenia illuminate the neurobiological changes in schizophrenic patients. NOR involves differential exploration of familiar and novel objects based on the animal's previously learned information about the objects. Object recognition can be assessed by measuring the time spent exploring each object (Neill *et al*., 2010). During NOR, animals which were sub-chronically treated with PCP, failed to discriminate between novel and familiar objects (Grayson *et al*., 2007; McLean et al., 2009; Idris et al 2010). PD168077 induced dose dependent reversal of PCP effect (Sood *et al*., 2011):

However, there is debate about the specificity of this drug (Newman -Tancredi et al., 2008; see above), so replicating the finding with a full agonist (A412997) was required.

3.1.4 Aim of the Experiment 2

Experiment 2 aimed to ascertain whether the pure D4 agonist (A412997) showed a similar restoration of NOR as previously shown with the D4 partial agonist PD168077 (Sood *et al*., 2011), and to ascertain whether the PCP-induced disruption of LI could be restored by A412997

3.2 Materials and Method

3.2.1 Animals

The experiments used female Lister-hooded rats (200 – 270g at the start of the experiment), housed four animals to a cage with food provided *ad libitum* under controlled temperature, humidity and lighting (12 h light/12 h dark, lights on at 07:00). Experiments were carried out under appropriate project and personal license authority in accordance with the UK Animals (Scientific procedures) Act, 1986, and with appropriate local Animal Welfare and Ethical Review Body (AWERB) approval.

3.2.2 PCP pre-treatment

PCP hydrochloride (Sigma-Aldrich, UK) was dissolved in 0.9 % saline and diluted to appropriate dose (2 mg/ml). Animals were sub chronically pretreated by the intra peritoneal route with PCP (2 mg/kg) or saline (1 ml/kg) twice daily for 5 days followed by a 7 days drug washout period, to ensure that behavior is not influenced by residual drug effect. (Grayson *et al*., 2007).

3.2.3 LI procedure

Behavioral training and testing were conducted in a bank of four identical behavioral experimental chambers (30 cm w X 30 d X 30 h, above grid floor: Med Associates, USA). Each was housed in a sound-attenuating outer box ventilated by a fan and illuminated by a single house light mounted on one wall, 25 cm above the floor. A removable drink spout was situated via a 2-cm hole in the left wall, the hole was centered 3cm above the grid

floor and 10.5 cm from the back of the chamber. Licks were monitored by using an electronic contact lickometer (Med Associates Inc., model ENV-251, USA).
Shock was delivered via the grid floor (0.4-cm-diameter bars at 1.5-cm intervals) from a shock generator linked to a shock scrambler (Med Associates, USA). By using a Med Associates, USA audiogenerator a tone stimulus was delivered from an overhead speaker. Animals' licking at the drinking spout was measured through a lickometer circuit (Med Associates. Details), interfaced to a desk top computer running MedPC control software. The LI procedure used conditioned suppression of licking for water, to assess the level of conditioning in a conditioned emotional response paradigm. Since the testing relies on the animals licking for water in the test chamber, they were maintained on restricted water during the testing, and trained to lick in the test chamber prior to the conditioning. The procedure consisted of four stages:

1-Handling and water restriction: - In the week before testing, animals were placed on a restricted water regime, where there was no access to water for 19 hours (4:00 p.m. to 11:00 a.m.), for four days per week: they had free access to water for the remaining three days. This regime of water restriction was found to be the minimum required to ensure that the animals were motivated to lick during the testing sessions (see below). During this time, animals were handled daily (20 min each), to acclimatize them before being tested.
2- Lick training: - The thirsty animals were trained to lick for water in the test chamber. This was done daily for 5 days, ensuring they reached criterion on three parameters: Lick delay (the time from the water becoming available to the first lick) < 60 sec; Time to complete licks 600 < 3 min; Total licks in 10 min > 1000. Those who did not achieve these criteria were trained as above for further days until they did. Following each lick training session, the animals were returned to full water availability for 4 hours in their home cage.
3- Once lick trained to criterion, the testing started.

The LI procedure itself was conducted over four days. On Day 1 (preexposure), the animals in the PE group were placed in the box with no water available and presented with 40 tones (5 sec; 15 dB above background) at 1 min intervals. Non-preexposed (NPE) animals were placed in the box for an equivalent length of time, but did not receive tone presentations (Table.3.1). On Day 2-Conditioning: All animals, both NPE and PE,

received two tone-foot shock pairings. Each animal was placed in the box, with no water available. After 5 min they received tone (5 sec) followed immediately by foot shock (0.35 mA; 1 sec 1 s train of 6 ms pulses, 25 Hz; 0.3 mA; delivered through the grid floor). This was repeated 5 min later, and 5 min after that the animals were removed from the box. On Day 3 –Testing: Each animal was placed in the box, with water available. The animals' licking was monitored continuously with a contact lickometer circuit (Med Associates), and the number of licks counted electronically. After 90 licks had been completed, the tone was presented until a further 10 licks were completed. The time for licks 80 to 90 (i.e. immediately before the tone), and licks 90 to 100 (i.e. the time during the tone) were measured as Time A and Time B respectively, and the suppression ratio (SR) was calculated: Time A/ (Time A + Time B). A suppression ratio of 0.0 indicated complete suppression of licking, while a suppression ratio on 0.5 indicated continuation of licking at a constant rate (i.e. no suppression). In each of these stages, whether water was available in the recording box or not, animals were returned to free water availability in their home cage for 4 hours after the completion of the session.

3- Extinction testing. After a three day rest period without testing, the testing procedure was repeated over four consecutive days, and time to onset of licking, Time A and Time B were recorded as above, and the SRs were calculated. After each extinction session, animals were returned to free water availability in their home cage for 4 hours. On completion of extinction testing animals were returned to full water availability in their home cage.

3.2.4 NOR procedure

For NOR testing, a black plexiglass testing arena (60 cm wide X 60 cm high, 60 cm long), was used. The procedure was as described by Grayson *et al*., (2007). Briefly, at least 9 days after the completion of the PCP (or saline) pre-treatment rats were habituated for 20 min in test arena on two consecutive days. The following day behavioural testing took place: each rat was placed into one corner of the arena for 3 mins habituation. It was then removed from the arena and placed in a holding cage for 1 minute inter-trial interval (ITI), while the arena was cleaned with 10% ethanol to prevent any lingering olfactory cues, then two identical objects (metal cylinders, 10 cm high by 8 cm diameter) were placed in opposite corners of the box, 10 cm from the edge. After that the acquisition trial was

started by introducing the rat into the arena in one of the corners not occupied by an object and left to explore the identical objects for 3 min (figure 3.1). Then the animal was placed again in the holding cage for 1 min ITI, while the arena was again wiped with 10% ethanol, before the retention trial. For this trial one object was similar to those used in acquisition trial (familiar object) and one novel object (glass jar, 10 cm high x 10 cm diameter) were placed in the corners of the arena (figure 1). The animal was returned to the testing arena and allowed to explore the familiar and novel objects for 3 min. On completion of the retention trial, the animal was returned to the home cage, and the objects and arena were cleaned with 70% ethanol, before testing the next animal. The positions of the novel and familiar objects was counterbalanced across animals.

All experiment were filmed and recorded on video using a camera (HD Pro Webcam C920), linked to a desktop PC. Object exploration was scored by two independent experimenters, who were blind to the experimental groups. For timing the exploration sessions, a PC based timer was used (Chron Me) [http://online-stopwatch.chronme.com/] which gave a time stamp for exploration and a total exploration time. Object exploration was defined by touching or sniffing the object with the forepaws while sniffing, licking or sniffing (Grayson *et al*., 2007).

For investigating the time of both objects during the acquisition and retention trial and discrimination index (DI: Grayson et al., 2007) was calculated by using the following formula:

$$DI = \frac{(\text{Time spent exploring novel object} - \text{Time spent exploring familiar object})}{(\text{Time spent exploring novel object} + \text{Time spent exploring familiar object})}$$

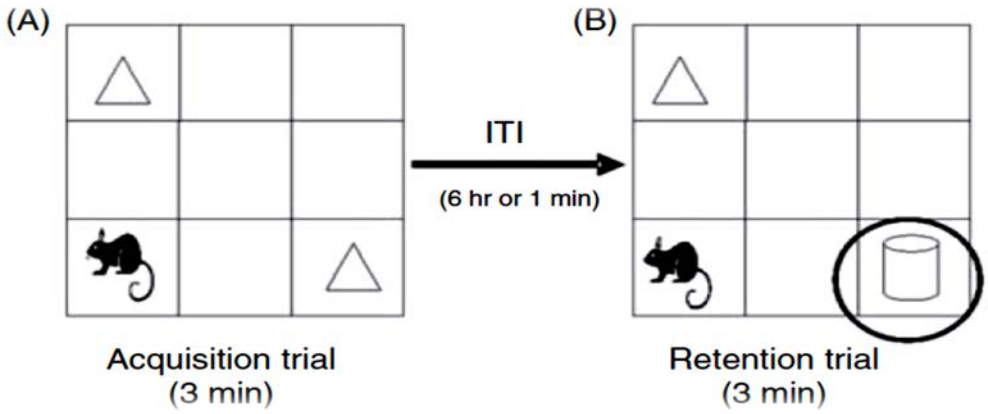


Figure 3.1: Novel object recognition task procedure. (A) For an acquisition trial two identical objects were placed in opposite corners and the animal introduce into the arena in one of the other corners. (B) For retention trial, the familiar object in one corner and the novel object was place in the same position, the animal introduced into the arena as for the acquisition stage. From Sood *et al*, 2011.

3.2.5 Experiment 1: Effect of subchronic PCP on LI and NOR

The experiments were performed using female Lister-hooded rats (200 – 270g at the start of the experiment), housed four animals to a cage with food provided ad libitum under controlled temperature, humidity and lighting (12 h light/12 h dark, lights on at 07:00).

3.2.5.1 Procedure

Female Lister-hooded rats (n = 24; 200 – 270g at the start of the experiment) were randomly assigned to four experimental groups, according to pre-treatment condition (saline or PCP) and exposure (NPE or PE): (table 3.1). They were pre-treated with PCP (2 mg/kg, i.p. n = 12) or saline vehicle (1 ml/kg, i.p. n = 12) twice per day for five days, then left for 10 days before testing, first with NOR then with LI. For NOR, briefly, rats were habituated for 20 min in test arena on two consecutive days. For testing, each rat received 3 mins habituation, followed by 1 min ITI, then 3 min acquisition with two identical objects in the arena, followed again by ITI. Finally they were tested with one familiar and one novel object in the arena, for 3 min. Experiment were filmed and recorded and object exploration was scored by two independent experimenters, who were blind to the experimental groups. DI were calculated from the exploration times. For detailed description of the procedure see section 3.2.4.

Group	Pre-treatment (daily for 5 days)	Pre-exposure (tone)
PCP/NPE	Phencyclidine	0
PCP/PE	Phencyclidine	40 min
SAL/NPE	Saline	0
SAL/PE	Saline	40 min

Table 3.1. The experimental groups PCP (PE) and Saline (PE) are both pre-exposed to non-aversive tone (40x5 sec tone presentations at 1 minute intervals) in Day 1 of experiment.

Seven days later they were tested with LI. Briefly this comprised (1) Water restriction, habituation and lick training for at least 7 days; (2) Preexposure (LI Day 1): 40 x 5 s tones; 15dB above background; (3) Conditioning (LI Day 2): 2 pairings of 5 s tone (15dB above background) with foot shock (0.3 mA, 1 sec) at 5 min intervals; (4) Test (LI Day 3): including exposure to non-aversive tone and mild foot shock (0.3 mA, 1 sec), with scheduled presentation of water; (5) Testing in extinction (LI Day 7 to 10): including exposure to non-aversive tone with scheduled presentation of water. For detailed description of the procedure see section 3.2.3.

3.2.5.2 Data Analysis

Time A and Time B were measured (see above) and the SR was calculated for each test session. Time A, Time B and SRs were submitted to ANOVAs for the between subject effects of drug and pre-exposure, while repeated testing days were added as a within-subject variable. SR data were analysed using a three-way analysis of variance (ANOVA), (Drug x Exposure x Day) followed by Fisher's LSD.

3.2.6 Experiment 2: Effect of A412997 on PCP-induced disruption of LI and NOR

Female Lister hooded rats (n = 24: 225 – 250g at the start of the experiment) were randomly assigned to six experimental groups according to chronic pre-treatments (saline or PCP), acute treatment immediately prior to conditioning (saline or A412997) and exposure (NPE or PE). They were pre-treated with PCP (2 mg/kg, i.p. n = 12) or saline vehicle (1 ml/kg, i.p. n = 12) twice per day for five days, then left for 10 days before LI testing. See table (3.2) for the treatment protocol.

Group	treatment (20 min before the test start)	Does
PCP/NPE	Saline	0.9 % in 1 ml/kg, i.p., n = 4
PCP/PE	Saline	0.9 % in 1 ml/kg, i.p., n = 4
SAL/ NPE	Saline	0.9 % in 1 ml/kg, i.p., n = 4
SAL/PE	Saline	0.9 % in 1 ml/kg, i.p., n = 4
PCP/PE	A412997	5 mg/kg in 1ml/kg, i.p., n = 4
SAL/PE	A412997	5 mg/kg in 1ml/kg, i.p., n = 4

Table (3.2) Summary of the treatment protocol for the day of test over the five test days for LI experiment, and for the retention trial for NOR experiment, n = 24. Note that due to time constraints, this is not a fully balanced design (see discussion for justification).

3.2.6.1 Procedure

Ten days after the end of pre-treatments, LI testing was carried out as described in section 3.2.3, to measure the effect of the D4 receptor agonist (A412997) on sub-chronic PCP reversal of LI. Briefly this comprised: (1) Water restriction, habituation and lick training for at least 7 days; (2) Preexposure (LI Day 1): 40 x 5 s tones; 15dB above background; NPE animals remained in the recording box for an equivalent time, but without tone presentation; (3) Conditioning (LI Day 2): 2 pairings of 5 s tone (15dB above background) with foot shock (0.3 mA, 1 sec) at 5 min intervals. Animals were injected by the intra peritoneal route with A412997 (5 mg/kg in 1 ml/kg 0.9% saline) or saline in a volume of (1 ml/kg) 20 min before the start of conditioning. Drug doses were calculated as the base equivalent weight; (4) Test (LI Day 3): including exposure to non-aversive tone with scheduled presentation of water; (5) Testing in extinction (LI Day 7 to 10): including exposure to non-aversive tone with scheduled presentation of water. For detailed description of the procedure see section 3.2.3.

After the completion of the LI experiment, and following at least 5 days in the home cage with food and water *ad libitum*, the same animals were tested with NOR, as previously

described. Briefly, rats were habituated for 20 min in test arena on two consecutive days. The following day behavioural testing took place. 20 min before testing, animals were injected with A412997 (5 mg/kg, i.p.) or saline (1 ml/kg, i.p.,) and returned to the home cage: the acute drug treatment for each animal was the same as in the LI experiment. For testing, each rat received 3 min habituation, followed by 1 min ITI, then 3 min acquisition with two identical objects in the arena, followed again by ITI. Finally they were tested with one familiar and one novel object in the arena, for 3 min. Experiment were filmed and recorded and object exploration was scored by two independent experimenters, who were blind to the experimental groups. DI were calculated from the exploration times. For detailed description of the procedure see section 3.2.4.

3.2.6.2 Data Analysis

For LI data, the SRs (see section 3.1.5) were submitted to two separate 3-way mixed-design ANOVA, each analysing specific aspects, since, due to the unbalanced design (see below for discussion), full factorial analysis was not possible. First, to assess whether PCP pre-treatment disrupted LI, providing a replication for experiment 1, a three-way mixed design ANOVA was performed with Day (test day 1 to 5), pre-treatment (saline or PCP) and exposure (NPE or PE) as the independent variables. This analysis used only data from animals treated with saline at test. The second analysis tested whether A412997 reversed the effect of PCP on preexposure using Kruskal –Wallis test (non-parametric) between groups analysis, pre-treatment (saline or PCP) and acute treatment (saline or A412997). This analysis used only preexposed animals as n = 16.

For NOR data, a three-way ANOVA was used: Chronic X Acute X Object, where chronic refers to pre-treatment (saline or PCP), acute refers to treatment with saline or A412997 and object refers to object position (left and right in acquisition trial or novel and familiar in retention trial), with drug treatment as the between factor and object exploration as the within factor. In each case, interactions were further interrogated using planned comparisons using paired or independent t-tests with Bonferroni correction.

DI data were analysed by using one-way way ANOVA followed by independent sample t-tests with Bonferroni corrections. One-sample t-tests were also used to test whether DI differed significantly from 0 (no discrimination) in each case. P value < 0.05 was

considered a significant. All statistical analysis were performed using IBM SPSS statistical 24.

3.3 Results

Experiment 1 - Effect of sub-chronic PCP on LI and NOR

3.3.1. LI

No significant difference in baseline licking was observed between PCP-pretreated and saline-pretreated animals, two-way ANOVA of Time A revealed no significant effect for preexposure [(6.425 ± 4.747); $F(1, 28) = 0.821$; $p > 0.05$] or pre-treatment [$F(1, 28) = 0.807$; $p > 0.05$], (M ±SEM) for saline (6.406 ±4.748) and PCP (2.112 ± 0.305) during baseline licking (figure 3.2a). As well as for time B, two-way ANOVA revealed no significant effect for preexposure [(120.01 ±60.07); $F(1, 28) = 0.474$; $p > 0.05$], or pre-treatment [$F(1, 28) = 0.390$; $p > 0.05$], (M ±SEM) for saline (117.981±55. 47) and PCP (78.33 ±28.98), nor any interaction between them [$F(1, 28) = 0.678$; $p > 0.05$] during test day one. (Figure 3.2.b). However, there was a lot of variability in the Time B data, making clear conclusions difficult to draw. At least some of this variability was due to changes in baseline licking: therefore SR was used for the main analysis, as this takes into account baseline lick rate (Time A) in assessing the level of suppression.

Saline pre-treated animals showed LI, as expected, with reduced learning (indicated by an increase in the SR) to the PE stimulus, compared to the NPE stimulus (SR [NPE] = 0.055 ± 0.019; n = 8: SR [PE] = 0.416 ± 0.077; n = 8: figure 3.3). However, animals pre-treated with PCP showed no LI, due to attenuation of the PE effect (SR [NPE] = 0.025 ± 0.005; n = 8: SR [PE] =0.148 ± 0.056; n = 8: figure 3.4). The data reveals a significant differences between the preexposed groups (Saline and PCP), [$t(28) = 3.914$; $p < 0.01$].

A three-way ANOVA (Day x Drug x Exposure) showed significant main effects of Day ($F(4,112) = 4.028$, $P < 0.01$) and of Exposure ($F(1, 28) = 44.285$, $P < 0.001$) but not of Drug ($F(1, 28) = 3.359$, $P = 0.078$), although this did approach significance. The interaction between drug x exposure was significant $F(1, 28) = 6.256$, $P = 0.019$, but the interaction between day x drug $F(4,112) = 2.232$, $P = 0.07$), and between day and exposure were not significant ($F(4,112) = 0.585$; $p = 0.67$).

The drug by exposure interaction was further interrogated using Fisher's LSD test of *a priori* planned comparisons of degree of LI shown in each of the two drug treatment groups (i.e. NPE vs PE in the saline pretreated and PCP pretreated animals) on each test day. These confirmed that saline pretreated animals exhibited LI across all five testing days, while PCP pretreated animals showed no significant LI on any test day. In addition, there were no significant differences in SR of NPE animals between the saline and PCP pretreated groups ($p > .05$: Fisher's LSD test).

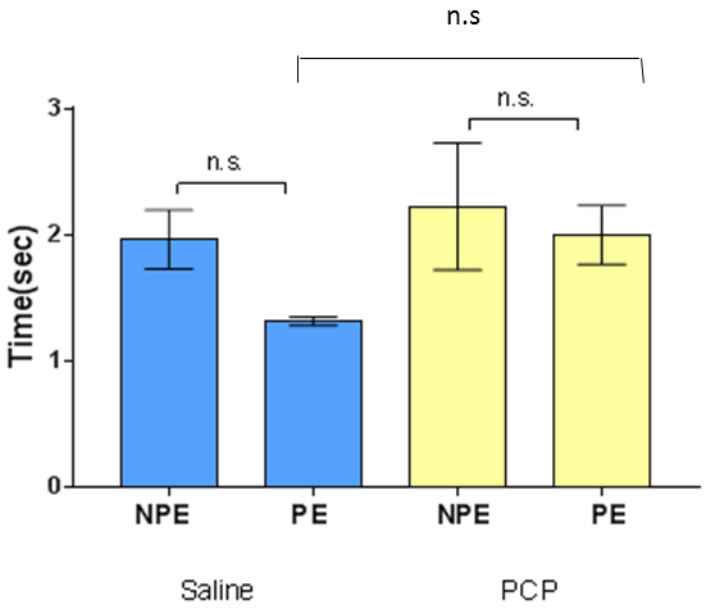


Figure 3.2a: Test day 1, Baseline licking: mean time taken for 10 licks before the onset of tone (Time A), during the first test session. Data are mean ± SEM, n = 8 per group. (n.s., not significant).

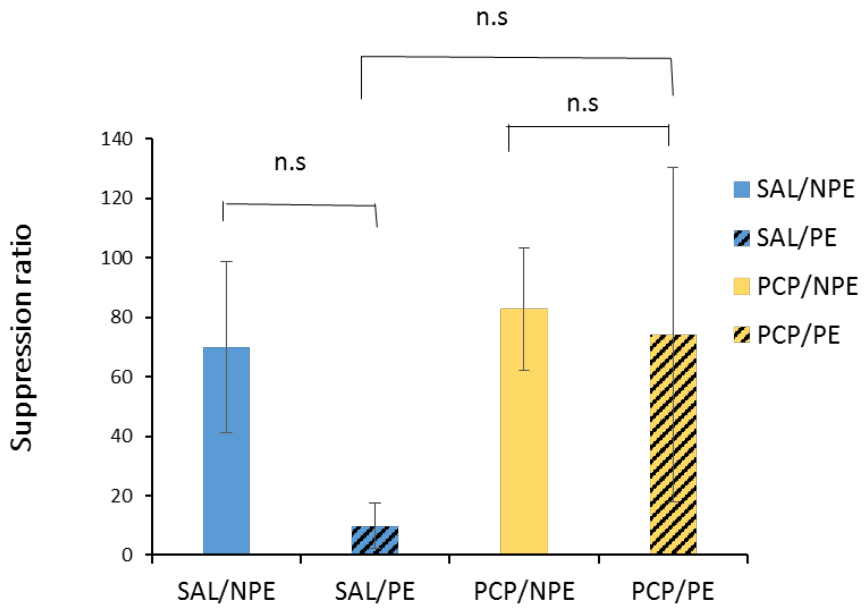


Figure 3.2b: Test day 1, mean time taken for 10 licks (Time B), during the first test session. Data are mean ± SEM, n = 8 per group. No significant effect for exposure or pre-treatment on LI during test day one for Time B.

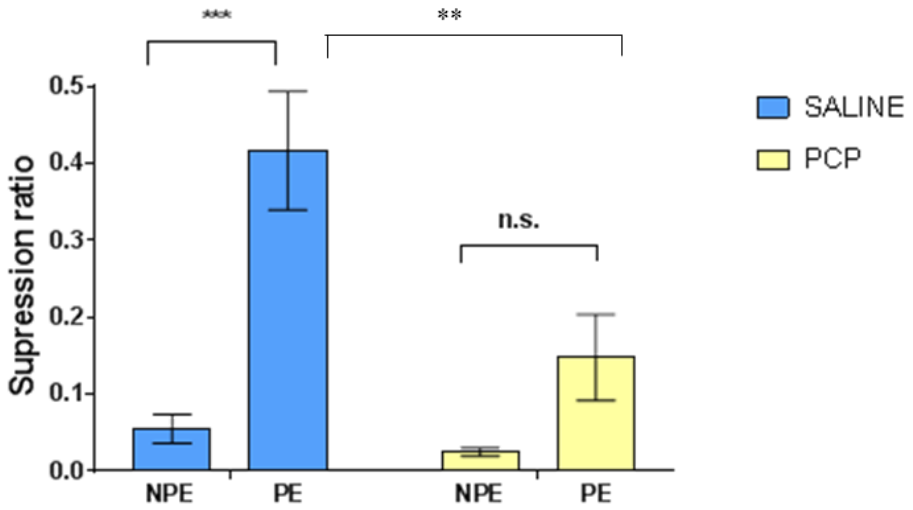


Figure 3.3. Effect of PCP on LI in testing day (Day 1).The SR in day 1 test (after tone-foot shock pairing) is shown. Weaker conditioned response (i.e. higher SR) in the pre-exposed groups (PE) compared to the non-pre-exposed groups (NPE) reflect the LI effect. Data are mean ± SEM SRs, where a value of zero indicates full suppression (good learning) and a value of 0.5 indicates no suppression (poor learning). Data are mean ± SEM, n = 8 per group. ** $P < 0.01$, *** $P < 0.001$; significant differences between groups; Fisher's LSD test (n.s., not significant).

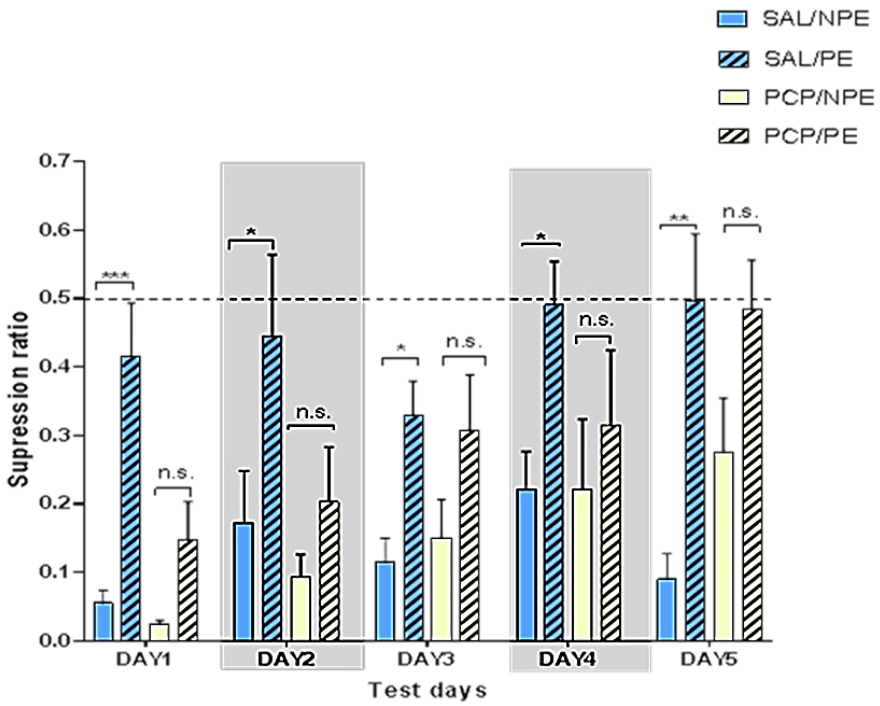


Figure 3.4. In the 5-days paradigm, PCP (2mg/kg in 1 ml/kg) pre-treatment showed no effect on LI due to attenuation of the PE effect. However, saline pre-treated animals showed LI with significantly reduced learning to the PE stimulus, (0.5 = no suppression (dotted line), 0.0 = maximal suppression over five days of testing in extinction (no shock). Data are mean ± SEM, n = 8 per group. *** P < .001; ** p < .01; * p < .05; significant differences between groups; Fisher's LSD test (n.s., not significant).

Experiment 2: Effect of D4R agonist, A412997, on PCP-promoted disruption of LI and NOR

3.2.1. Effect of PCP pre-treatment on LI

Preexposure to the tone caused reduced learning, indicated by a higher SR in saline pretreated animals, which was not seen in PCP pretreated animals, thus replicating the finding from experiment 1 (figure 3.5

Due to the small numbers of animals in this experiment (n = 16), the data were analysed non-parametrically. Kruskal-Wallis test revealed a statistically significant differences in preexposure for saline pretreated groups [X^2 (2, n = 16) = 8.05, p = 0.04]. A Mann-Whitney *U* test revealed significant differences in Saline PE (*Md* = 0.46, n = 4) and Saline NPE (*Md* = 0.035, n = 4), *U* = 0.00, z = -2.309, p = 0.02, r = 0.6. Non parametric test confirmed that LI was present in saline pretreated animals, but not in PCP pretreated animals. Notably, as in experiment 1, the effect of PCP pre-treatment was largely confined to the PE group with no effect of NPE animals.

3.2.2 Effect of A412997 on reduced effect of preexposure in PCP pretreated animals

Due to the small numbers of animals in this experiment, the data were analysed non-parametrically. Treatment with A412997 (5 mg / kg, i.p.), twenty minutes prior the LI experiment did not reverse the effect of PCP on preexposure, and therefore on LI. A Kruskal-Wallis test revealed no significant differences between the groups, and there is no effect for A412997 on the reduction of the preexposure effect induced by PCP pre-treatment [X^2 (2, n = 16) = 3.308, p = 0.347]. Thus PCP pre-treatment reduced the SR, indicating a reduction in the effect of preexposure, but this effect was not reversed by A412997. (Figure. 3.6).

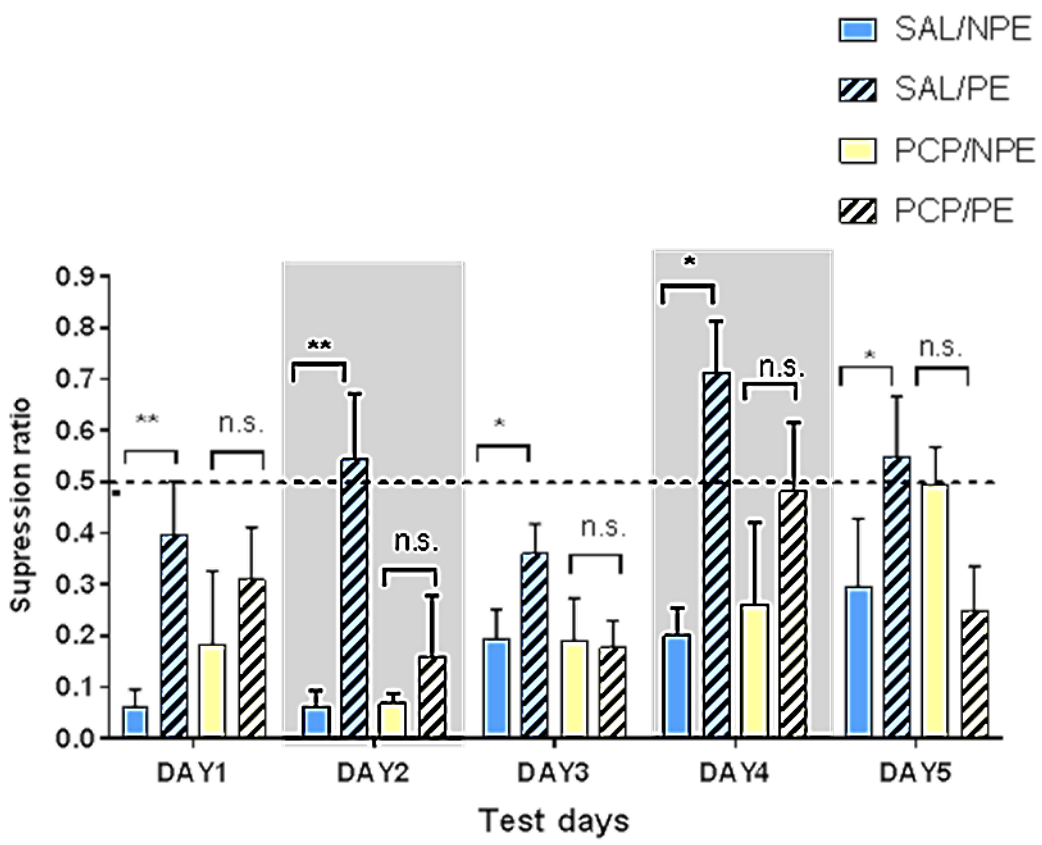


Figure 3.5. In the 5-days paradigm, PCP (2mg/kg in 1 ml/kg) pre-treatment showed no effect on LI due to attenuation of the PE effect. However, saline pre-treated animals showed LI with significantly reduced learning to the PE stimulus, (0.5 = no suppression, 0.0 = maximal suppression) over five days of testing in extinction (no shock). Kruskal-Wallis test revealed a statistically significant differences in preexposure for saline pretreated groups [X^2 (2, n = 16) = 8.05, p = 0.04]. A Mann- Whitney *U* test revealed significant differences in Saline PE (*Md* = 0.46, n = 4) and Saline NPE (*Md* = 0.035, n = 4), *U* = 0.00, z = -2.309, p = 0.02, r = 0.6. Non parametric test confirmed that LI was present in saline pretreated animals, but not in PCP pretreated animals. Data are mean ± SEM, n = 4 per group. ** P < .01; * p < .05; significant differences between groups; (n.s., not significant).

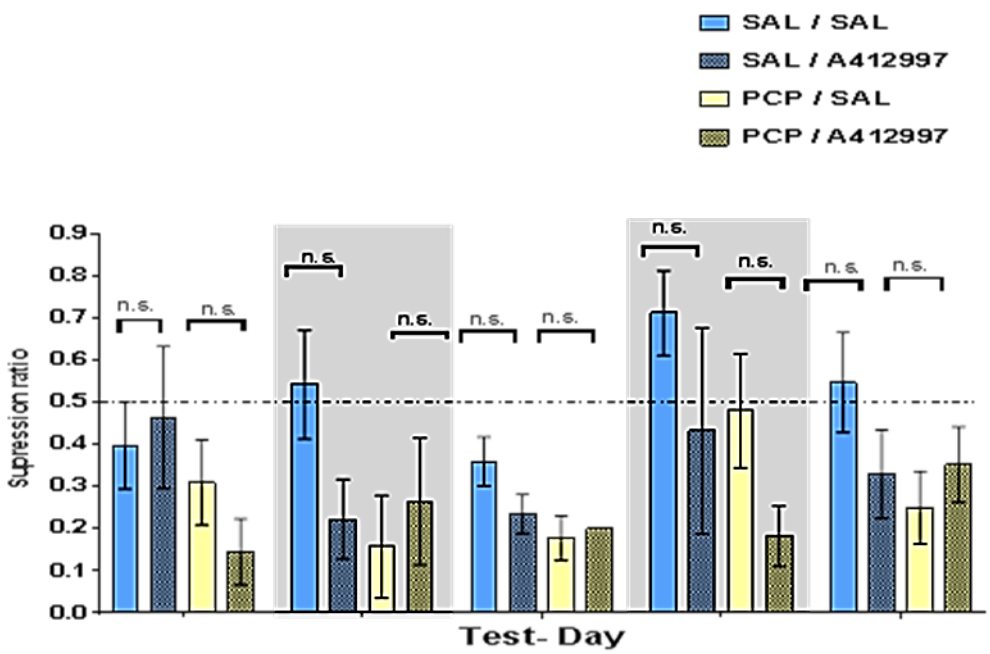


Figure 3.6. Effect of dopamine agonist A412997 (5 mg/kg., i.p.) 20 minutes before starting the experiment on preexposed animals (n = 4 for each treatment). A Kruskal-Wallis test revealed no significant differences between the groups, and there is no effect for A412997 on the reduction of the preexposure effect induced by PCP pre-treatment [X^2 (2, n = 16) = 3.308, p = 0.347]. Hence, there is no significant effect for A412997 on the reduction of the preexposure effect induced by PCP pre-treatment. Data are mean ± SEM. (n.s., not significant)

3.2.3 Effect of PCP pre-treatment and A412997 on NOR

After completion of LI testing, animals underwent NOR, testing the effect of the D4R agonist A412997 to reverse the sub chronic PCP induced deficit in NOR. Data from one animal (saline pretreated) could not be used due to the video being corrupted. During acquisition, analysis with a three way ANOVA revealed no significant side bias for exploration (main effect of side ($F(1, 19) = 1.729$; $p > 0.05$, no effect of pre-treatment on exploration time ($F(1, 19) = 0.07$; $p > 0.05$), no effect of D4R agonist A412997 ($F(1, 19) = 1.214$; $p > 0.05$), and no significant interaction between the three ($F(1, 19) = 3.183$; $p > 0.05$), (figure 3.7a).

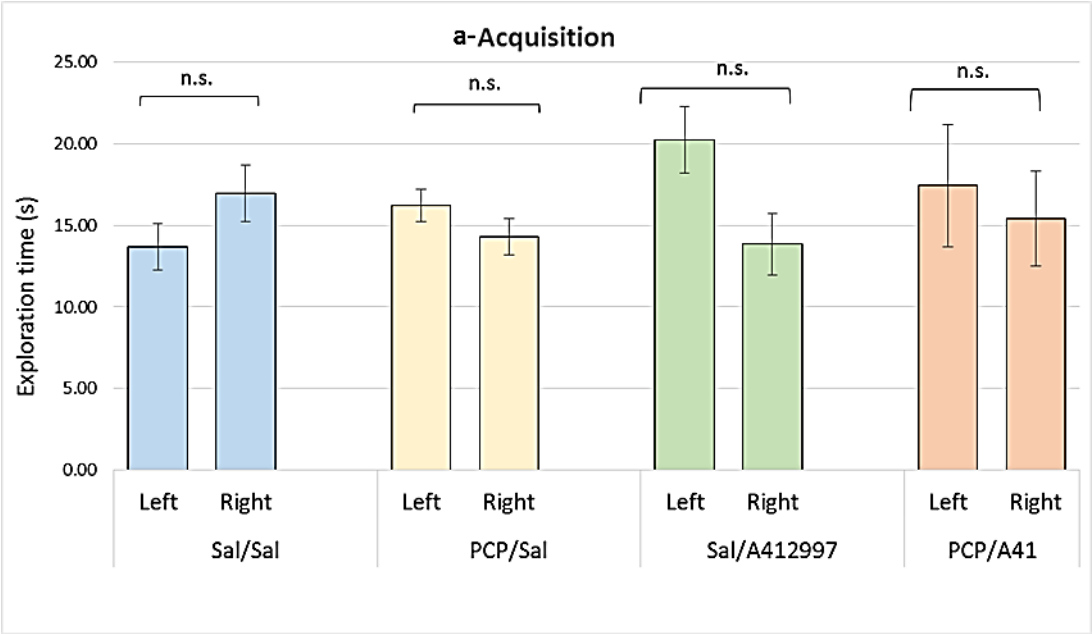
At test, there was a significant main effect of novelty (the exploration time for familiar and novel objects), ($F(1, 19) = 53.484$; $p < 0.001$), and a significant effect of A412997 ($F(1, 19) = 0.095$; $p < 0.01$), but no significant main effect of pre-treatment ($F(1, 19) = 0.069$; $p > 0.05$). There was significant interaction between pre-treatment and A412997 ($F(1, 19) = 0.182$; $p < 0.05$), and between novelty and A412997 ($F(1, 19) = 5.052$; $p < 0.05$), in addition between the three (novelty x pre-treatment x A412997), ($F(1, 19) = 11.424$; $p < 0.01$), but no significant interaction between novelty and pre-treatment ($F(1, 19) = 0.594$; $p > 0.05$).

Paired t-test on individual groups showed a significant time spent exploring the novel object versus the familiar object in the saline pretreated animals treated with saline ($t(6) = 5.316$, $p = 0.002$), but not in PCP pretreated animals treated with saline ($t(7) = 1.835$, $p = 0.109$), confirming a PCP induced disruption of NOR.

However, on the day of test, animals pretreated with PCP and treated with A41299 (5 mg/kg), showed a significant increase in the time exploring the novel object in comparison with the familiar object ($t(3) = 3.590$, $p = 0.037$), thus A412997 restored the ability to discriminate between the two object (figure 3.7b): that is, it reversed the effect of PCP, while having no effect in saline pretreated animals.

Examination of DIs confirmed this view as two way ANOVA showed a significant main effect of A412997 [$F(3, 19) = 6.239$; $p < 0.05$], but not for pre-treatment [$F(3, 19) = 0.137$; $p > 0.05$]. There was a significant interaction between the two [$F(3, 19) = 15.462$; $p < 0.01$]. In addition independent sample t-test showed a significant differences in DI between saline and PCP pretreated animals [$t(13) = 2.984$, $p = 0.01$)], showing a

disruption of NOR by PCP, and between the DI for sub-chronic PCP animals treated with A412997 ($t(10) = -5.282$, $p = 0.001$) compared with sub-chronic PCP animals acutely treated with saline, indicating a reversal of the effect of PCP by A412997. Moreover, saline pretreated animals, but not PCP pretreated animals, showed significant discrimination between the objects, since the DI was significantly above zero (saline, [($t(6) = 4.37$; $p = 0.005$): PCP, [($t(7)$; $p = 0.14$)], and significant discrimination in PCP pretreated animals treated with A412997 [($t(3) = 6.282$; $p = 0.008$]: Figure 3.8)



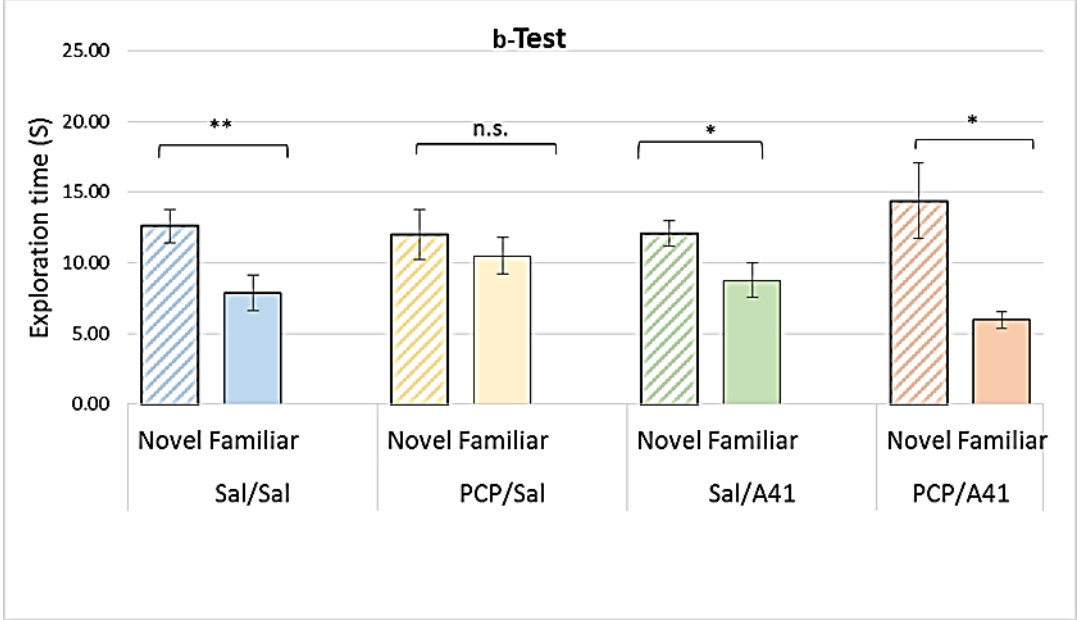


Figure 3.7. The effect of A412997 treatment (5 mg/kg, i.p.) on (a) exploration time of the two identical objects in the acquisition trial of the novel object recognition task; (b) exploration time of a familiar and a novel object in the test phase (retention trial), (* $p < 0.05$, ** $p < 0.01$: significant difference in time spent exploring novel and familiar objects during the test phase; Students paired t-test based on a significant interaction (ANOVA). Data are expressed as mean ± SEM.

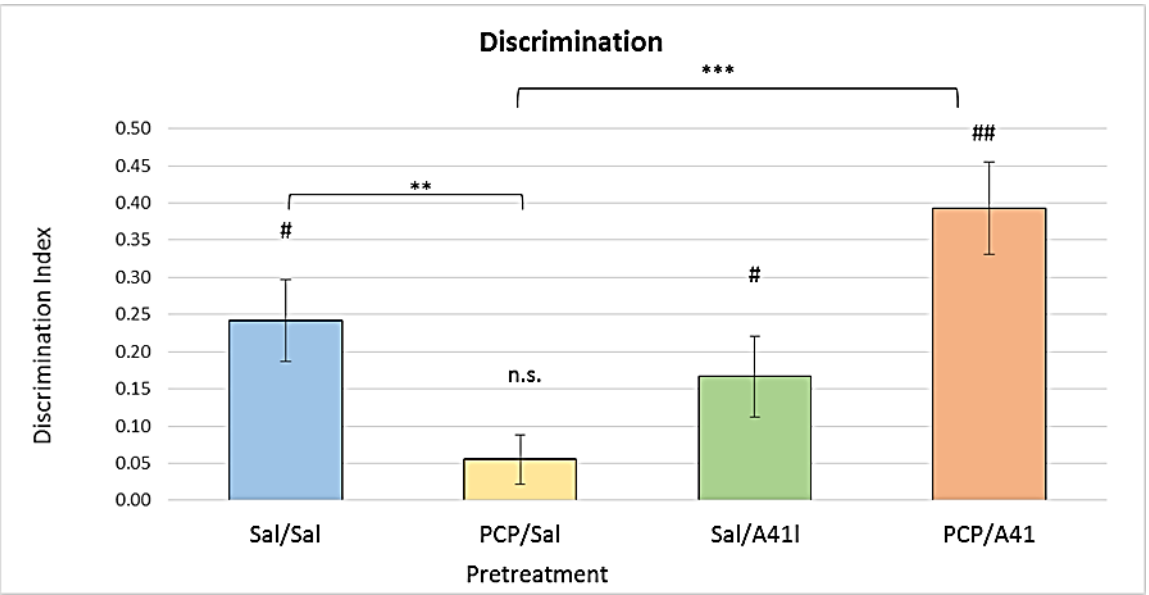


Figure 3.8. The effect of A412997 treatment (5 mg/kg, i.p.). DI measures during the test stage. **P < 0.01; significant difference in exploration time between familiar and a novel object (ANOVA); ** P < 0.01; significant difference in DI between saline and PCP pretreated rats, ***P < 0.001 between PCP pretreated animal treated with saline and PCP animals treated with A412997 (independent sample t-test); # p < 0.05, ## p < 0.01; significant difference from zero (no discrimination: one-sample t-test).

3.4 Discussion

The results of the present experiment demonstrated that sub-chronic PCP pre-treatment disrupts LI. Importantly, testing occurred >10 days after PCP pre-treatment, indicating that in LI, as in other behavioural tasks (Neill *et al*., 2010) the effect of PCP pre-treatment endures beyond the time the drug is present in the brain. The experiment also confirmed what other researchers have found by replicating the disruption of NOR which models behavioural deficits seen in schizophrenia (Neill *et al*., 2010) by PCP pre-treatment: this provided a positive control for the current experiment, confirming that the treatment schedule used was able to replicate previous behavioural findings.

The first major finding of these experiments is that sub-chronic PCP pre-treatment abolished LI, by attenuating the effect of preexposure on conditioned learning: that is in saline pretreated animals preexposure reduced conditioning (i.e. the LI effect), but in PCP pretreated animals there was no effect of preexposure. Animals were also tested in NOR to confirm that the pre-treatments regime was sufficient to replicate the well-documented deficit in NOR (Grayson *et al*., 1997; Sood *et al*., 2011; Neill *et al* 2010). Importantly, previous research has used PCP pre-treatment twice daily for seven days (Grayson *et al*., 2007; Sood et al., 2011): however this study, like other recent study that used juvenile rats (Yavas & Young, 2017; Gupta & Young, 2018), confirmed that PCP pre-treatment for five days is sufficient to cause NOR disruption in adult rats.

It has been reported that PCP or its analogue MK-801 administration to rats evokes many behavioural changes of adult animals, such as hyperlocomotion, impairment information processing and changes of dopaminergic, glutamatergic and GABAergic systems (Bubeníková- Valešová *et al*., 2008; Neill *et al*., 2010). Given acutely, at the time of testing, the effects of these drugs on LI are controversial. There was no effect of acute PCP administration on LI when it was given before preexposure or conditioning, in conditioned emotional response paradigm similar to the one used in the current experiments (Weiner and Feldon ,1992). However MK-801 (0.2 mg/kg) disrupted LI when administered before preexposure, but had no effect of LI when given before aversive conditioning (Traverso *et al*., 2012). This contradicted the previous results which indicated that LI was disrupted when acute MK-801 (0.2 mg/kg) was given after preexposure, but not after conditioning, (Traverso *et al*., 2003). However, acute

administered PCP (2 mg/ kg) prior conditioning but not prior preexposure may potentiate LI (Palsson et al., 2005).

Notably, all these experiments used acute drug administration: that is the drug was present in the brain during different stages of testing. Conversely, a study reported by Turgeon et al., (1998) showed that a low dose (2.5 mg / kg) of PCP 15 minute prior the preexposure and conditioning phases of the experiment did not disrupt drinking behaviour in taste aversion studies, but a high dose (8.6 mg/kg, 20 hour prior the preexposure and conditioning phase) did disrupt LI. This finding dose not eliminate the possibility that the delayed effect of higher dose PCP could affect LI.

In addition, although acute PCP in people causes transitory behavioural changes which resemble some aspects of schizophrenia, people who take PCP frequently exhibit a symptom spectrum and time course which much more strongly resembles schizophrenia itself (Allen and Young, 1978; Pearlson, 1981;). Furthermore, in rats, subchronic but not acute PCP administration induces sensitisation of the mesolimbic dopamine function (Jentsch *et al*, 1998), similar to those reported in schizophrenia sufferers (Breier *et al*., 1997). Moreover, it has been reported that the persistent effect produced by sub-chronic PCP withdrawal provides a viable model of schizophrenia, especially following long-term exposure. (Jentsch and Roth, 1999).

In the present study a subchronic treatment regime was used and a 7- day washout period was selected to ensure that no residual drug remain in the brain at test. This, in turn means that the behavioural effect which were measured during the test phase were due to long term physiological and/or neurochemical changes in brain as a result of having been exposed to PCP, rather than short-term temporary psychopharmacological effects due to the presence of the drug in the brain at the time of testing. Hence, long–term PCP exposure has advantages over acute treatment as a model relevant to schizophrenia, where no residual drug remain in the animal brain at test (Jentsch and Roth, 1999; Neill *et al*., 2010).

There were no effects of either drug or exposure condition on baseline licking, shown by comparison of Time A (licks 80 to 90, before the onset of the tone) and time B (licks 90 to 100, during the tone presentation), indicating there was no evidence of non-specific effects on lick rate, or of contextual conditioning. Looking at the basic learning effect, in

NPE animals, on Test Day 1, PCP pretreated animals showed a lower SR than saline pretreated animals. Although the difference was not statistically significant, a change in basic learning cannot be ruled out due to a floor effect – that is both groups were close to maximum suppression (SR = 0.0). However, during extinction (Test Days 2 to 5) there was no significant differences in SR between saline and PCP pretreated NPE animals, indicating that there was no effect of drug treatment on basic learning, since stronger learning would extinguish more slowly (Joseph et al., 2000). Similarly, in experiment 2, although there was some indication of changes in basic learning (saline NPE vs PCP NPE) on Test Day 1, over the course of the extinction trials, SRs were very similar in both groups. This provides further evidence that basic learning was unaffected by PCP pre-treatment, although the data from experiment 2 must be treated with caution because of the lack of power in the experiment (see below).

In terms of LI, the results also revealed that preexposure has more effect on saline PE than PCP PE animals: that is PCP PE groups showed high suppression (low SR) despite receiving the same preexposure (40 x 5 sec tone presentations at 1 min intervals), as saline PE animals, which showed significantly reduced suppression (high SR) (Figure 3.3). Palsson *et al* (2005) also found that PCP impairs associative learning and potentiates LI in taste aversion conditioning, Importantly, LI was abolished in PCP animal due to attenuating the effect of preexposure on conditioned learning where LI effect was observed in saline pretreated animals but not on PCP pretreated animals

Thus PCP pre-treatment abolished LI, by attenuating the effect of preexposure on conditioned learning: that is in saline pretreated animals preexposure reduced conditioning (i.e. the LI effect), but in PCP pretreated animals there was no effect of preexposure.

Following on from the demonstration that subchronic PCP disrupts LI, the current study examined the effect of the D4 receptor agonist A412997 on the PCP induced disruption of LI and also on PCP induced disruption of NOR. Treatment with A412997 (5 mg / kg, i.p.), twenty minutes prior the LI experiment failed to reverse the PCP-induced disruption of LI. However, importantly it did reverse the disruption of recognition memory in NOR task.

Previous work in our labs showed that the D4 agonist PD168077 restored the attenuation of NOR in animals pretreated with PCP (Sood *et al.* 2011). However, reports that PD168077 may have partial agonist properties (Newman -Tancredi *et al*., 2008), raised the possibility that it was not the agonist action that was important in this effect. Therefore the present study aimed to replicate this finding with a pure D4 agonist, A412997. A dose of (5 mg / kg, i.p.) was selected, based on research showing that this dose of A412997 improved the cognitive performance in behavioural studies on rats (Woolley *et al*., 2008). The results confirmed that the pure agonist, A412997, had a similar effect of restoring PCP-disrupted NOR as did the partial agonist PD168077, confirming that it is indeed a D4 agonist action which is important in their restorative effect.

However, the preliminary data from these experiments show that A412997 did not reverse the effect of PCP pre-treatment on LI as expected at a dose which did restore NOR in PCP pretreated animals. Due to time constraints, this was not a fully balanced design, but was aimed as a preliminary experiment to test whether there were effects on the PE groups, to ascertain whether a full study with a fully balanced design was appropriate. In addition, the group sizes are low, because the study was done as two replicates, but there were technical problems with the first replicate, in that they did not show a PCP disruption in either LI or NOR, which we believe was due to incorrect PCP dosing: the data for this group of animals has not been presented. Based on detection of differences in means of 1.5 SD or more, group sizes on n = 8 per group are required in order to provide power < 0.8. Thus, with n = 4 per group, this experiment was substantially underpowered, and the outcomes need to be treated with caution. Despite these shortcomings the data produced showed no evidence of any effect of A412997 on PCP-induced disruption of LI, giving no clear justification for running the full experiment in the future.

The results demonstrated that D4 receptor activation has an important role in animal model relevant to schizophrenia that improve cognitive dysfunction, a finding which is consistent with findings of past studies by Browman *et al* (2005), which indicated the improved cognitive function observed after A412997 administration to rats in the 5-trial inhibitory avoidance test and social recognition model. Similarly in NOR task, it also indicated the potential role for D4 receptor agonist in improving cognitive dysfunction in schizophrenia (Woolly *et al*., 2008; Sood *et al*., 2011), consistent with the suggestion that

D4 receptor agonist could be a useful tool for treating cognitive symptoms of psychiatric diseases such as schizophrenia (Bernaerts and Tirelli , 2003; Powell *et al*., 2003; Miyauchi *et al*., 2017), although perhaps less effective in treating positive symptoms, since our preliminary data suggest that it does not reverse the effects of PCP on LI.

In contrast, Jentsch et al (1999) concluded that D4 receptor antagonist such as NGD94-1 reversed the sub-chronic PCP induced cognitive dysfunction in vervet monkeys, Moreover, in one-trial step-through inhibitory avoidance task or delayed alternation task in rat, it was found that D4R antagonist (L745870) impaired memory performance (Bernaerts and Tirelli, 2003; Zhang *et al*., 2004). However, intraperitoneal administration of both D4R agonist (PD168077) and D4 antagonist lurasidone, which is an atypical antipsychotic drug reversed NOR deficit in sub chronically PCP treated rats as a model of cognitive impairment associated with schizophrenia, which they suggested that D4R agonist can be used in schizophrenia to enhance cognitive impairment (Huang *et al*., 2017).

Therefore several studies have revealed that D4R agonists have an improving effect on cognition, while D4R antagonist improve it at low doses and worsening it at higher doses (Zhang *et al*., 2004; Woolley *et al*., 2008; Sood *et al*., 2011; Miyauchi *et al*., 2017).

A potential difficulty with assessing LI in this paradigm is that group differences can be masked by a floor effect on the SR scores: that is the SR cannot go below 0.0, so where all groups show high suppression, it is impossible to discriminate degrees of learning. For this reason, in this study we used repeated testing in extinction in order to separate the levels of learning in these animals, on the basis that the stronger the conditioned association, the more extinction trials it will take to extinguish the conditioned association (Joseph *et al*., 2000).

3.5 Conclusion

The present study showed that twice daily subchronic PCP pre-treatment for five days disrupted LI, with little or no effect on basic learning, indicating that this PCP pre-treatment regime disturbs behaviours modelling positive symptoms of schizophrenia. Importantly, also, the results showed that five days of PCP pre-treatment is sufficient to achieve behavioural deficits in NOR, replicating previous findings using seven days pre-treatment (Grayson *et al*, 2007).

Furthermore, the D4 receptor agonist A412997 reversed the deficit in NOR induced by subchronic PCP pre-treatment, but did not reverse the PCP-induced disruption of LI. This supports evidence of involvement of the D4 receptor in memory processing aspects, and suggest that using agonists at D4R, such as A412997, could be useful in improving cognitive symptoms of schizophrenia.

Chapter Four

Chapter 4 - Effect of sub-chronic phencyclidine treatment on dopamine receptor gene expression in the rat brain: a link to schizophrenia

4.1 Introduction

Despite intensive studies, the molecular aetiology of schizophrenia remains ambiguous, because there is no single molecular event that can explain the pathophysiology of schizophrenia, (Liu et *al*., 2011). In patients with schizophrenia, the class of drugs that ameliorate the associated psychotic symptoms are dopamine receptor blockers, so the dopamine hypothesis of schizophrenia has accordingly attracted considerable attention (Olney and Farber, 1995), where several studies on twins, adoptions and families have suggested the importance of the role of genetic factors in aetiology of schizophrenia (Tsuang, 2000; Sullivan *et al*., 2003; Gejman *et al*., 2010).

Increased dopaminergic activity has a role in psychotic symptoms of schizophrenia which is based primarily on pharmacological observations (Anden *et al*., 1970; Creese *et al*., 1976; Arinami *et al*., 1997), and these psychotic symptoms are exacerbated by dopamine agonists (Lieberman *et al*., 1987). The antipsychotic activity of therapeutically used drugs shows a direct correlation with the ability to inhibit D2R (Creese *et al*., 1976). In addition, studies have found that the density of D2R and the D2-like receptors, D3R and D4R, is increased in post-mortem brains taken from schizophrenic patients who had been free from antipsychotic medication for a long time before death (Seeman, 1992; Cui *et al*., 2015).

Because of the dopamine hypothesis of schizophrenia, D4R has received specific attention (Okuyama *et al*., 1999). The atypical antipsychotic clozapine, which treats schizophrenic symptoms without causing motor side effects, has a higher affinity for D4R than D2R and D3R (van Tol *et al*., 1991). In 1993, Seeman *et al.* demonstrated that D4R was elevated in schizophrenic brains in comparison to control. This was found in a pharmacological study to compare clozapine and spiperone, where a higher density of D4R was found in schizophrenics' brains that had a greater sensitivity to clozapine. These observations suggest that D4R is a possible target to moderate the antipsychotic effects of clozapine (Okuyama *et al*., 1999).

However, the association between the schizophrenic process and the elevation in the number of dopamine receptors is controversial (Knable *et al*., 1994) and it is not yet

known which D2 receptors are elevated in the post-mortem brains of schizophrenics (Seeman *et al*., 1992; Reynolds and Sarah., 1995).

It is hypothesised that PCP pre-treatment has downstream effects on dopamine systems, particularly in the mesolimbic pathway, perhaps mediated through changes in the expression of dopamine receptors. Quantitative polymerase chain reaction (qPCR) was used as a method to investigate the expression of genes coding for dopamine receptors in brain tissue taken from rats which had been pretreated with PCP to model schizophrenia.

In a DNA microarray study by (Toyooka et al., 2002), it was found that the expression of genes coding for dopamine receptors from brains of control and PCP-injected mice was different, which was due to the influence of PCP on gene expression levels. Several studies have revealed that extracellular dopamine levels can be increased by PCP injection into the prefrontal cortex and NAc (McCullough and Salamone 1992; Hondo et al. 1994) due to PCP's ability to inhibit dopamine reuptake (Jentsch *et al*., 1998). In addition (Kaiser et al., 2004) noted the influence of PCP treatment on mRNA expression detected in adult rats' prefrontal cortex brain area was related to cognitive dysfunction in schizophrenia.

Dopamine receptors belong to a G-protein coupled receptor (Tan et al., 2003) and consist of five subtypes (D1R, D2R, D3R, D4R, and D5R) which are grouped into two classes according to their biochemical and pharmacological properties, the D1-like family receptors (D1R and D5R) and D2-like family receptors (D2R, D3R and D4R), (Beaulieu et al., 2007).

Previous studies have concentrated on dopamine D2-like receptors genes because they have been linked to many kinds of psychosis disorders, such as schizophrenia (Beaulieu et al., 2007; Rondou et al., 2010). In addition previously, research has shown that dopamine receptor binding and expression are abnormal in post-mortem tissues of patients with schizophrenia (Seeman et al., 2007; Seeman et al., 2005) and have reported increased mRNA and protein expression of D2R in both the medial preoptic area and the paraventricular nucleus after 3 weeks risperidone (D2R antagonist) administration (Zhang et al., 2012). Moreover the affinity of most antipsychotic drugs to D2R relates to their clinical efficacy, although some antipsychotics, principally clozapine, showed a high affinity for D4 receptors (Van Tol et al., 1991; Kapur and Remington, 2001).

Peiyan et al (2015) provided evidence by real-time PCR method that the dopamine D2-like receptors are widely expressed in rat hippocampus, frontal cortex and temporal lobe, and they found that D2-like receptors expression was not the same in different brain regions, which is consistent with findings of this study. Previous studies have reported that D2R expression was found in cortical areas (Seeman et al., 2006; Sillivan and Konradi, 2011; Missale et al., 1998) and D3R in various cortical areas (Sokoloff et al., 2006; Sokoloff et al., 1992), while in frontal cortex it was documented D4R expression (Rondou et al., 2010).

There have been several studies in the literature reporting the two major midbrain SN and VTA and their rostral projections to numerous structures which consists of the largest of the dopamine systems, including the limbic cortex, neostriatum and other limbic regions such as nucleus accumbens, amygdala, olfactory tubercle, by using autoradiography for utilizing D2R (Boyson et al., 1986; Weide *et al*., 1987 and Dubois *et al*., 1986). In addition D2R mRNA also found in cerebellum and hippocampus by Northern blot analysis of these tissues (Boyson et al., 1986, Dawson et al., 1986).

Studies have found that D2R have been implicated in neurological and psychiatric conditions, including schizophrenia and Parkinson disease (Seeman et al., 1987; Meador et al., 1996), in addition it is hypothesized that increased DR2 receptor activity in schizophrenia (Crees et al. 1976, Seeman et al., 1984). Thus it is important to determine dopamine receptors location which enhance the performance of preclinical studies and the examination of specific regions in postmortem schizophrenic brains for investigation the possibility of disturbances of dopamine receptors in schizophrenia (Seeman et al., 1987; Meador et al., 1996).

Previous research has shown that neuroanatomical region- specific expression is an important determinant in the expression of function of these receptors and most of understanding of dopamine receptors localization has come from studies in rat brain, basically depending on mRNA level and using specific antibody for each receptors to visualize receptors protein in brain tissue (Meador et al., 1996).

Because the pathogenesis effects of schizophrenia remain unknown, gene expression changes in PCP animal models could be useful to investigate the aetiology of this disease. In the present study, sub-chronically PCP-pretreated rats were used to investigate the expression of dopamine receptors (D1-D5), which could provide further evidence to validate the PCP animal model of schizophrenia.

4.1.1 Overview of qPCR

Reverse transcription polymerase chain reaction (RT-PCR) was used to analyse the expression of dopamine receptors DA-R (D1-D5) mRNA using specific primer sets for these genes. RT-PCR is a technique that allows the collection and measurement of an accumulating PCR product in real time, based upon combining amplification and detection in a single step by using fluorescent compounds that connect PCR product concentration to fluorescence intensity (Higuchi *et al*., 1993).

Reactions are measured in real time (PCR cycle) to detect target amplification. This value is presented as cycle threshold (C^t), which is defined as the number of cycles required to cross the threshold by the fluorescent signal. It has been demonstrated that a greater quantity of target DNA in the starting material leads to a faster indicative raise in fluorescent signal which will presented as a lower C^t value. Thus a C^t value provides an accurate measurement overall gene expression (Heid *et al*., 1996). The advantages of using SYBR green in RT-PCR assays to quantify the mRNA gene expression level, is that it represents a rapid and sensitive technique for quantitative analysis of gene expression in the CNS, where low amounts of mRNA molecules can be detected (Pfaffl , 2001). Therefore it is suggested that RT-PCR using SYBR green offers important physiological insight on the expression level of mRNA.

In addition, the SYBR green assay for primer design and validation needs fewer days than other analytical techniques such as TagMan, which can require 2 -3 weeks for probe synthesis. However the double strand DNA binding characteristic of SYBR green has been suggested to represent a particular disadvantage in comparison to TagMan because of the high sequence identity of mRNA compared to SYBR green, which means nonspecific products might be detected (Bookout and Mangelsdorf, 2003), but this can be avoided by using specified primers design with high efficiency.

SYBR green is a nonspecific dye that binds to any double stranded DNA product so every qPCR reaction required properly designed primers which are available online as primer design software spesifically to get the desirable SYBR green based qPCR primers (Rozen *et al*., 2000; Wrobel *et al*., 2004; Kibbe *et al*., 2007). Therefore it is important to design primers which reduce the dimeization and non specific amplification. In this study specific primers were made for each gene of dopamine receptors .

The basics of primer design depend on two parameters: specifity and efficiency. When poorly designed primers are used, mispriming occurs, which could lead to nonspecific amplification of squences in the template, hence specifity is important. In a SYBR green based qPCR application, SYBR green dye will bind to any cDNA present in the reaction, therfore the amplification of non specific products leads to produces in valid data: thus specificity of the primer is important to ensure that the amplification product contains only the cDNA of interest. The efficiency (how well the primers perform) is also important. Using primers with efficiency as high as 90-100%, increases the sensivity of the quantification and allows for assay reproducibility. Amplicon length and pimer quality are factors that affect the efficiency (Brenda *et al*, 2011).

4.1.1.1 Primer design parameters:

The optimal primer length is accepted as being 18-24 base pairs. The optimal primer melting temperature (T_m), namely the temperature at which 50 % of the primer and its target are hybridized, should be 63-64ºC. Product size should be between 80 and 150 base pairs, and to allow comparison of the relative expression of several genes, amplicons should be equal in length. In SYBR green detection, fluorescence will be more intense in larger products than in smaller. In addition, repeats in the nucleotide sequence, for example (AGAGAGAGAGA), should be avoided because they promote mispriming; four dinucleotides represents the maximum number of such repeats (Brenda *et al*., 2010; Arvidsson *et al*., 2008).

4.1.2 Aim of the Molecular Study:

The aim was to identify changes in the expression of genes coding for the dopamine receptors D1, D2, D3, D4 and D5 in different regions of the brains (NAc, VTA and FCx)

of PCP-pretreated rats compared to control (saline treated) rats: although D4R were the main interest, analysis of the other four dopamine receptors was carried out in parallel. . These changes could translate into changes in protein levels giving an indication of the changes which may occur in the brains of people suffering from schizophrenia.

4.2 Materials and Methods

4.2.1 Drug Administration

10 Lister Hooded rats were injected with 2 mg/kg PCP, and another 10 injected with 0.9% saline, I.P, twice daily for 5 days. The animals were killed after 26 days (during this period they were used for latent inhibition and NOR experiments).

4.2.2 Brain Dissection and Tissue Sampling

Rats (n = 20) were deeply anaesthetised with isoflurane (2.0 l/min) and subsequently killed by decapitation. The brains were removed immediately. Then, 2 mm (approximately) slices of the brain were cut using an ice cold stainless steel brain matrix (calibrated for 1 mm coronal sections; World Precision Instruments, UK). The regions of interest (NAc, VTA, FCx) were dissected with reference to the brain atlas of Paxinos and Watson (2007). Tissue samples were snap-frozen in liquid nitrogen and transferred to -80ºC for storage until RNA extraction.

4.2.3 Reverse-Transcription Quantitative PCR Analysis

Reverse-transcription quantitative PCR (RT-qPCR) analysis was performed to examine the expression of five dopamine receptors (D1-D5) in the brain tissue of female Lister Hooded rats. Primers were supplied from Oligo Perfect ™ Designer and then diluted to form a stock concentration of 100 µM in dH_2O. Primers were designed to be specific for the dopamine receptor genes (RatDrd1-RatDrd5). Primer sequences for the reference primers used to indicate the baseline of genes were AAGCCTTCCACTATCCGAAGAGAT for RAT Rp18_F, GTACAGTTTTGGTTCCACGTAGCC and for RAT Rp18, and for dopamine receptor gene Rat Drd F and R for D1-D5, table 4.1. See appendix 2 for primer design conditions.

4.2.4 RNA extraction

Total RNA was extracted from snap-frozen (-80ºC) brain slices using a Sigma GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) following the manufacturer's instructions (See appendix 3) and then treated with DNase1. To verify the quality of the RNA prior to cDNA synthesis, 5 µl RNA was visualised on a 2.5% agarose gel via electrophoresis followed by ethidium bromide staining (1.4 µl). This confirmed that the RNA had not degraded and was suitable for carrying out cDNA synthesis. First strand cDNA synthesis was performed following the manufacturer's protocol with a Thermo Scientific Revert Aid Kit, using 0.5 µg of RNA per sample and diluted 1:4 [20 µl of the sample in 60 ddH_2O].

4.2.5 Primer design and reverse-transcription qPCR

Primer stocks were made up to 100 µM and a working stock was made up for qPCR, both primers in one tube of 5 µM 180 µl H_2O + 10 µl FP (Forward primer) + 10 µl RP (reverse primer). The five sets of primers were then tested with 5 µl cDNA of PCP and Saline pretreated samples and one water control. This allowed us to test for suitability for use in qPCR to confirm a single product was being amplified. The following were added respectively: 5 µl Red Taq, 2 µl primer mix, 2 µl cDNA template and 1 µl dH2O with the following cycling conditions: 94ºC for 2 min, followed by 35 cycles at 94ºC for 1 min, 62ºC for 30 sec, 72ºC for 30 sec and 72ºC for 5 min. The PCRs were then visualised on a 2.5% agarose gel to check for single band products. 50 ml 1XTAE was melted in a microwave for 1 min. and then left it to cool before adding 2.5 g high definition agarose (Sigma, UK) and heating to dissolve. Agarose gel was left in a gel frame at room temperature for 15-20 min before adding 1.4 µl ethidium bromide and pouring into a gel mould. PCR products were run on a two-rowed gels at 70 V for 60 min.

Once the suitability of the primers had been confirmed, cDNA synthesis was performed for all RNA samples and qPCR was carried out. The RT-qPCR mixture consisted of 10 µl SYBR Green Jump-start Tag Ready Mix (Sigma-Aldrich), 250 nM each of forward and reverse primers, 1 µl diluted cDNA and sterile water to form a total volume of 20 µl. The cycling conditions repeated as described above. This was followed by a melting curve step (50-95ºC) to confirm that only a single product had been amplified in each reaction.

No template and no reverse transcriptase controls were performed for each primer pair and cDNA, respectively. See figure 4.1 for qPCR protocol.

The purity and concentrations of RNA of the samples were assessed using a Nano Drop 2000 (Thermo Fisher Scientific, USA) spectrophotometer which measured absorbance at the wavelength of interest of all molecules in the samples (PCP or saline), using 1.5 µl of the sample and 1.5 µl of Elution solution. The ratio of absorbance at 260/280 nm were used to assess the purity of RNA, where a ratio of 2.1 indicated pure RNA and a ratio between 1.8- 2.1 is generally accepted as pure for RNA (though this ratio is not 'ideal'). Also, the ratio of absorbance at 260/230 nm was measured; it is best if this is greater than 1.5 as a lower ratio could indicate the presence of contamination.

Primer name	Sequence
RAT *Rp18*_F	AAGCCTTCCACTATCCGAAGAGAT
RAT *Rp18*_R	GTACAGTTTTGGTTCCACGTAGCC
RAT Drd1_F	TAGCTAAGCCACTGGAGAAGCTGT
RAT Drd1_R	TGTGTGTGACAGGTTGGATCTTTT
RAT Drd2_F	CAGAAGGAGAAGAAAGCCACACAG
RAT Drd2_R	ATATTCAGGATGTGCGTGATGAAG
RAT Drd3_F	ACGTGGCTAGGCTATGTGTGAACAGT
RAT Drd3_R	ACAGGATCTTGAGGAAGGCTTTG
RAT Drd4_F	CTATGTCAACAGTGCCCTCAACC
RAT Drd4_R	CTTGCGGAAGACACTTCGAAACT
RAT Drd4_F2	AAAGAGAGGCGCCAAGATCACT
RAT Drd4_R2	GAAGAAAGGCGTCCAACACATC
RAT Drd5_F	CACATGTCTCAAATCTCTCCAACG
RAT Drd5_R	GAAACCTCTTCCTCACAGTCAAGC

Table (4.1). The sequences of the primers used, RAT *Rp18* is the reference gene and RAT Drd is for rat dopamine primer.

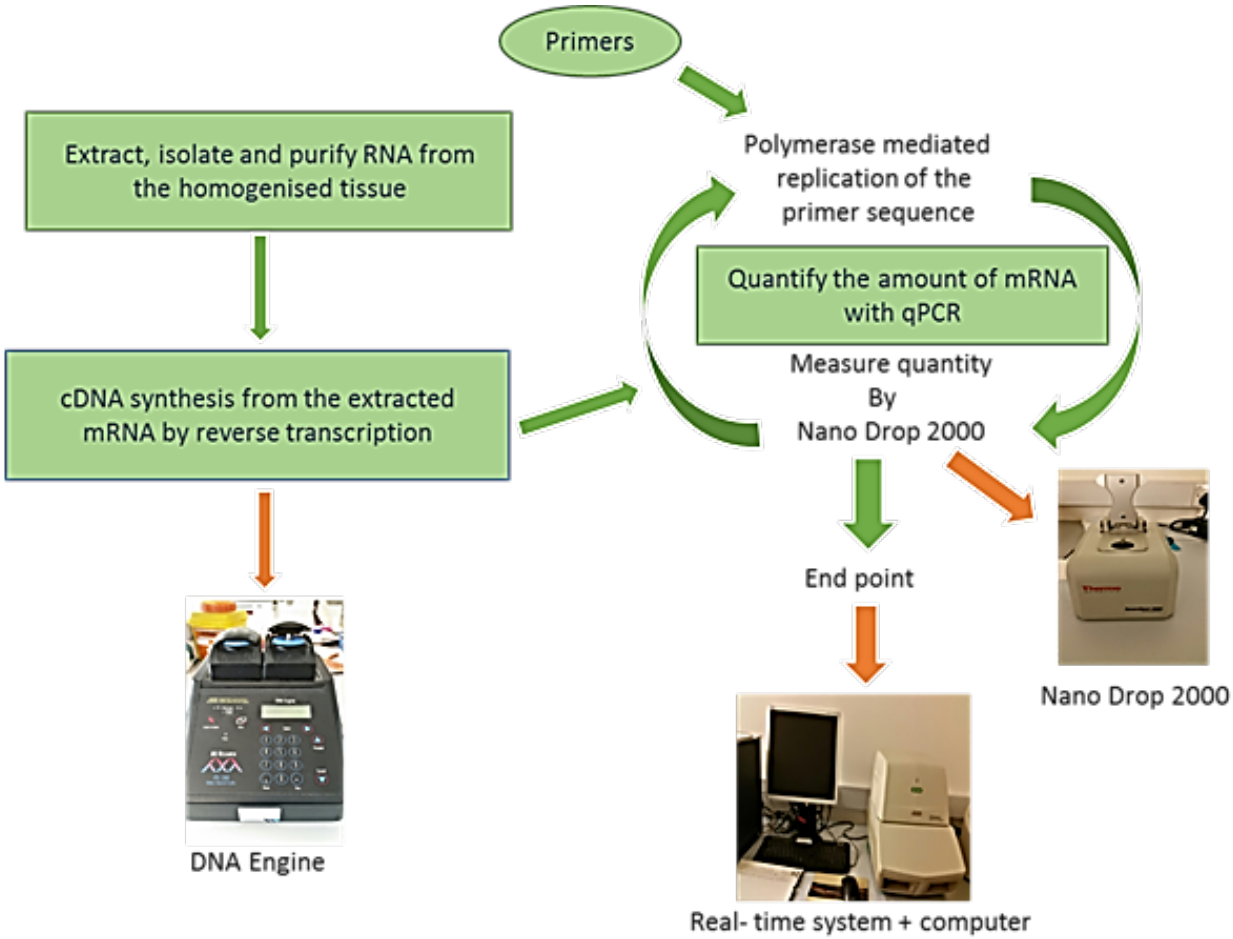


Figure 4.1. Shows Summary of the method for qPCR with equipment's were used for mRNA of dopamine receptor.

4.2.6 Data analysis

To analyse the gene expression data, the ΔCt value was calculated for each example by subtracting the average reference Ct value from the average target Ct (Zhang *et al*., 2010), after which a sample t-test analysis was completed for the ΔC_T values. All statistical analysis were performed using was performed using Graph Pad Prism version 7.00 for Windows, and the significant differences were defined with a p- value smaller than 0.05, n = 10 for each brain area.

4.2.6.1 QPCR analysis $\Delta\Delta C_T$ Method

The C_T value is the threshold cycle: the cycle at which the amplification plot crosses the threshold. Thus a lower C_T equates to higher expression.

-First calculate ΔC_T

ΔC_T (sample) = C_T Target gene – C_T Reference gene

ΔC_T (calibrator) = C_T Target gene – C_T Reference gene

Where: Sample = PCP pre-treatment, calibrator = control (Saline).

Target (gene of interest, DR), Reference gene (housekeeping gene, *Rp18*).

-Second calculate $\Delta\Delta C_T$

$\Delta\Delta C_T$ = ΔC_T (sample) - ΔC_T (calibrator)

- Calculate the normalised target gene expression

Normalised target gene expression = $2^{-\Delta\Delta C_T}$

4.3 Results

The gene expression patterns of dopamine receptors (D1-D2) in different brain regions (NAc, FCx and VTA) are shown in figure 4.2, A, B and C. Due to PCP pre-treatment, there were changes in the gene expression levels of genes for D4R and other dopamine receptors that were different in all tested brain regions. In the NAc (figure 4.2A) the expression of genes coding for Rat Drd1 was significantly increased by PCP pre-treatment [(2.157 ± 0.498); t (9) = 2.323; $p < 0.05$], but there were no significant changes in the expression of genes coding for Rat Drd4 and other receptors.

In the frontal cortex, the expression of genes coding for dopamine Rat Drd2 [(4.724 ± 1.56), t (9) = 2.373; $p < 0.05$], D3 [(3.55 ±1.09), t (9) = 2.339; $p < 0.05$] and Rat Drd5 [(2.42 ± 0.6), t (9) = 2. 339; $p < 0.05$] receptors was significantly increased; however, the expression of genes coding for dopamine Rat Drd4 receptors was significantly decreased [(0.69 ± 0.09), t (8) = 3. 198; $p < 0.01$], (figure 4.2B). Similarly, in the VTA, the expression of the gene coding for dopamine Rat Drd4 receptors was significantly decreased [(0.115 ± 0.188), t (7) = 47. 07; $p < 0.001$], as was expression of the gene coding for dopamine Rat Drd2 receptors [(0.5 ± 0.137), t (7) = 3. 581; $p < 0.01$] and Rat Drd5 [(0.72 ± 0.114), t (9) = 2. 368; $p < 0.05$]; however, there was no apparent change in expression of genes coding for the Rat Drd1 or Rat Drd3 receptors. See figure 4.2C.

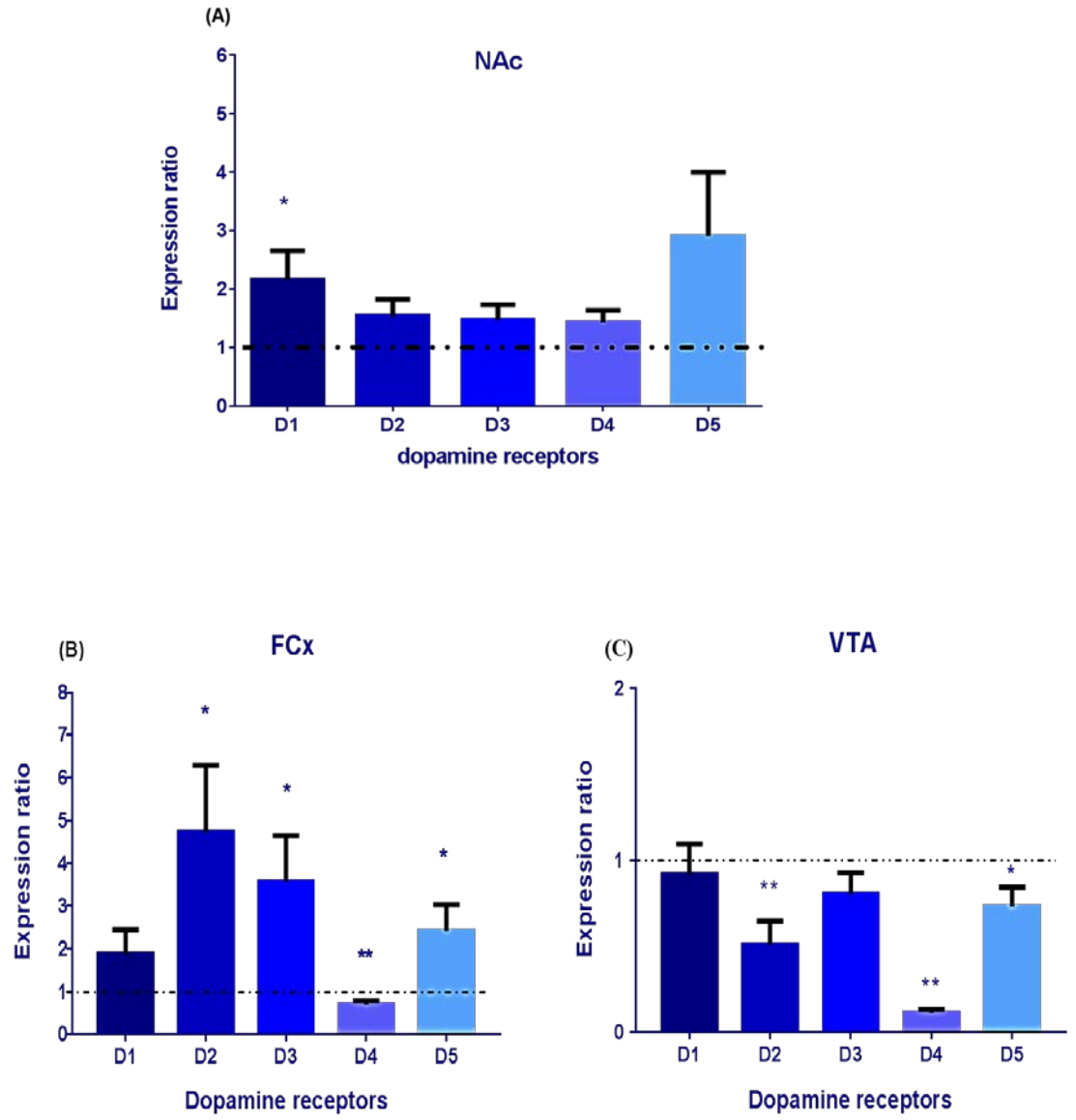


Figure.4.2. Expression profile of dopamine receptors (D1- D5) in NAc (A), FCx (B), and VTA (C). Data are expressed as mean ± SEM for triplicate reverse transcription reactions from each RNA sample according to the equation [ΔC_T (sample) = CT Target gene – CT Reference gene], then a single sample t-test analysis was used to compare ΔC_T values. The dotted line shows and expression ratio of 1.0, equivalent to no change in expression. $p < 0.05$*, $p < 0.01$**.

4.4 Discussion

This study set out to determine changes in expression level of the gene coding for the dopamine receptor D4 as well as D1, D2, D3 and D5 in different brain regions (NAc, VTA and FCx) of PCP pretreated rats. These changes may correlate with protein expression levels, suggesting that gene coding expression can be used as indicator for receptor expression, and leading to identification of molecular changes of schizophrenia. In schizophrenia, there is an association between FCx and cognitive dysfunction (Schijndel and Marten, 2010). Jentsch *et al.* (1998) demonstrated an important role of frontal cortex projections for the effect of PCP, indicating that a lesion in prefrontal cortex could blocked the ability of PCP to induce locomotor stimulation or increase NAc dopamine utilization.

In this experiment, D4R mRNA expression in the FCx was significantly decreased in PCP-pretreated animals compared to the controls, whilst there were significant increases in D2, D3 and D5 receptor gene expression; however, there were no changes in D1 receptor gene expression. Similarly, in experiments using RT-PCR on mice cerebral cortex during pre- and postnatal development, Araki *et al*. (2007) found that D4R mRNA was high during embryonic development, and significantly decreased during the postnatal period and remained low during adulthood. Although, these experiments did not test the effects of PCP pre-treatment, they show that gene expression changes could translate into changes in protein level at least for the D1-receptor, indicating the usefulness of gene coding expression measurements as a substitute for receptor expression which could help for understanding the biological changes of schizophrenics.

In contrast, Stefanis *et al*. (1998) concluded that D4R gene expression was elevated in the FCx of post-mortem brain taken from patients with schizophrenia in comparison with the control in. Conversely, however, a number of studies have been unable to establish any differences in D4R gene expression levels in the post-mortem frontal cortex between schizophrenics and controls using qPCR assay as they claimed that D4R were undetectable in the striatum and FCx because they present at very low levels in this regions (Roberts *et al*., 1996; Mulcrone and Kerwin, 1996), so they failed to found any gene expression changes for these receptors.

Interestingly, the high levels of D4R mRNA found in the frontal cortex and mesolimbic system than in other area of brain (van Tol *et al*., 1991) implied the importance of this receptor because of the apparent significance of this area of brain in schizophrenia, (Okuyama, *et al*., 1997); schizophrenic patients presented clinical signs and symptoms suggestive of frontal cortex dysfunction, including poor insight and impaired performance in problem-solving tasks which indicated that the observed high density of D4R in the frontal cortex and mesolimbic area might be related to the CNS area being of direct interest in schizophrenia (Weinberger, 1988; Tamminga *et al*., 1992).

In NAc, there were no significant changes in the expression of genes coding for D4R and the other receptors (D3 and D5), however there was a significant increase in genes coding for dopamine D1 receptors. In figure 4.2, the expression of genes coding for dopamine D5 seems to be increased: however, this change was not statistically significant, largely due to high variability in the data (large SEMs, 0.18 to 10.25) for fold change ΔC_T. The finding is consistent those of Reynolds and Mason (1994, 1995), which showed no evidence for the presence of D4R in post-mortem putamen from control and schizophrenic subjects using a binding assay to examine D4R by displacing emonapride binding with raclopride, but this experiment was in dorsal striatum of the schizophrenics. However, a number of studies using the same selective ligands (emonapride and raclopride) showed that significant differences do exist, noting a 6-fold elevation of D4R in the putamen of patients with schizophrenia compared to the control group (Schoots *et al*., 1995). This finding was replicated and confirmed by Murray *et al*. (1995), whose own results indicated a 2-fold elevation of D4R in the NAc of schizophrenic patients. Similarly, it was reported by Sumiyoshi *et al*. (1995) that D4R sites are more prevalent in NAc in schizophrenics compared to control subjects. In the VTA, there was a significant decrease in the expression of genes coding for D4R and other receptors (D2 and D5); however, there were no significant changes in genes coding for dopamine D1 and D3 receptors.

In this experiment, it was found that in animals sub-chronically pretreated with PCP, there were changes in gene expression of dopamine D4R, as well as in other dopamine receptors (D1, D2, D3 and D5), in different regions of rat brain. This could provide evidence that PCP pre-treatment has downstream effects on dopamine systems, particularly in the mesolimbic pathway. This might be mediated through changes in the

protein level of dopamine receptors, though it was also found that the changes in expression of genes coding for dopamine receptors varied according to the region of the brain being tested.

In summary, the results of this study have shown an increased expression of D2, D3 and D5 receptors in the FCx, and decreased expression of D4 receptors while in NAc increased expression of D1 was found, but there were no significant changes in the expression of genes coding for the other receptors, In the cell body region in VTA, there were no change in expression of genes coding for D1 or D3 receptors, but there was a decrease in D4, as well as D2 and D5 receptor expression.

Previous reports showed that D1R mRNA declined during pre and postnatal development of mouse corpus striatum and cerebral cortex but there was no change in frontal cortex (Araki *et al*., 2007), This is consistent with the results of this study showing no change of D1R in FCx. In addition they suggested a predominance of D1R in the dorsal forebrain and of D4R in the ventral region which consistent with this study, since D2R and D4R were expressed in VTA. Furthermore previous reports illustrated that the differences in dopamine receptor mRNA expression between ventral and dorsal forebrain could determine the variation in the response of these regions of brain to antipsychotic therapy (Lidow et al., 1997; Lidow and Goldman, 1997). Real -time PCR method by Peiyan et al (2015) provided evidence that D2-like receptors expression was not the same in different brain regions, which is consistent with findings of this study and D2 like receptors are widely expressed in rat frontal cortex and temporal lobe. In NAc, Sumiyoshi *et al*. (1995) reported that D4R expression is more prevalent in NAc from patients with schizophrenia compared to control subjects by using a binding assay to examine D4R by displacing emonapride binding with raclopride. Similarly, previous studies indicated that D2R, D3R and D4R density are increased in post-mortem brains taken from schizophrenic subjects who had been free from antipsychotic medication for a long time before death (Seeman, 1992; Cui *et al*., 2015). Furthermore the finding is consistent with findings of past studies by (Seeman et al., 2006; Sillivan and Konradi, 2011; Missale et al., 1998), which reported that D2R expression was found in cortical areas, and D4R expression was found in frontal cortex (Rondou et al., 2010), while D3R expression in in various cortical areas (Sokoloff et al., 2006; Sokoloff et al., 1992).

The changes in dopamine receptor gene expression shown here presumably equate to changes in protein level in the mesolimbic pathway However, it is unclear where the receptors are expressed. For example changes in presynaptic autoreceptors in NAc, would show as changes in gene expression in the cell body region, that is the VTA. Therefore it is unclear from these experiments whether the changes in gene expression measured relate to local changes in postsynaptic receptors (i.e. in the cell body and/or dendrites) or distant changes in autoreceptors on the terminals. Due to the lack of a selective labelled ligand or antibody for D4R, it was difficult to investigate if RNA expression translated to protein expression and receptor function in the different regions, a hypothesis that remains to be tested (Duan *et al*., 2003). Accurate measurement of dopamine receptor levels and function in specific brain regions is difficult because of the low abundance of receptors subtypes (Araki *et al*., 2007), and the availability of reliable antibodies specific for D4R required for appropriate immunohistochemical experiments. For future work, protein analysis via Western blot and immunohistochemistry are recommended to estimate dopamine receptor protein level in sub-chronically PCP pretreated animals for these three areas of the brain (FCx, NAcS and VTA). In addition, the effects of a D4R agonist A412997 on expression of gene coding for dopamine receptors in the PCP animal model of schizophrenia should be investigated.

The differences in expression of the dopamine receptor types may help to explain the therapeutic efficacy and side effects of current antipsychotic drugs. Classical antipsychotic drugs act primarily on D2 receptors; the increase in D2 expression in frontal cortex may explain why these drugs have limited efficacy in treating negative symptoms.

4.5 Conclusions

This study has shown that at the terminal fields of the mesolimbic pathway, increased expression of D1 was found in the NAc, but there were no significant changes in the expression of genes coding for the other receptors, while there was increased expression of D2, D3 and D5 receptors in the FCx, and decreased expression of D4 receptors. In the cell body region in VTA, there was a decrease in D4, as well as D2 and D5 receptor expression, but no change in expression of genes coding for D1 or D3 receptors.

There were differences in expression of dopamine receptors between saline pre-treated and PCP pre-treated rats in both the cell body and terminal regions of the mesolimbic pathway. These changes may underlie some of the behavioural deficits seen after PCP pre-treatment, and may be important to understand the mechanisms underlying schizophrenia.

The findings of this study suggest that PCP pre-treatment has downstream effects on dopamine systems, particularly in the mesolimbic pathway, perhaps mediated through changes in the expression of dopamine receptors. In addition, changes in dopamine receptor expression in the brains of PCP pre-treated rats, as an animal model of schizophrenia, could help to identify the molecular pathways that could play a role in the aetiology of schizophrenia and could give rise to the pathogenic changes which occur in the brains of people suffering from schizophrenia.

Chapter Five

Effect of PCP pre-treatment on regional brain content of dopamine and its metabolites.

5.1 Introduction

Various dopamine systems in the brain are affected by different types of centrally active drugs, resulting in clear changes of dopamine metabolism and its functional activity (Magnusson *et al*.,1980). Dopamine has also been shown to play an important role in different neuropsychiatric disease such as schizophrenia (Horacek *et al*., 2006; Murray *et al*., 2008), Parkinson's disease (Ferrari *et al*., 2008; Lemke., 2008), Alzheimer's disease (Lanari *et al*., 2006; Geldenhuys and Van, 2008) and depression (Stein, 2008; Belmaker, 2008). One approach to studying these changes is to measure changes in transmitters and metabolites after drug treatment. This can be achieved through a number of separation techniques and detection methods for determination of dopamine and its acid metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), such as gas chromatography, mass spectrometry, and radio-enzymatic methods, (Vogt *et al*., 1980; Magnusson *et al*., 1980): the experiments reported here in used high-performance liquid chromatography (HPLC), with electrochemical detection (HPLC- ED), which offers precise quantitative data. The largest application in neuroscience is the monitoring of dopamine and its metabolites in brain tissue and CSF; in these experiments, different regions of the brain were used, which gives useful regional information about the whole tissue content of neurotransmitter, but it must be remembered that this does not differentiate between the intracellular and extracellular compartments (Syslová *et al*., 2012).

In addition, measurements of monoamine neurotransmitters have led to increase understanding of the association between brain chemistry and the cognitive, emotional and behavioural states of an organism. In human neuropsychiatric research, dopamine and its main metabolites are used as biomarkers in CSF which directly reflect the metabolism process in CNS and contain the highest concentrations of endogenous compounds. Furthermore, changes in dopamine turnover in CSF as well as in discrete regions of the brain provide a useful tool for studying neurochemical changes of neuropsychiatric disorders and for evaluation of pharmacological treatment (Syslová *et al*., 2012).

In clinical studies into the mechanism of the action of clozapine (D2 and 5-HT_2 receptor blockade), it has been suggested that a disruption of the balance of dopamine-mediated neurotransmission occurs in schizophrenia. Therefore, the effect of the NMDA receptor antagonist, PCP, on brain dopaminergic systems is a focal point that has received the particular attention of neuroscience research (Meltzer, 1998; Noda *et al*., 2000).

HPLC-ED is used extensively as a technique for neurotransmitter detection, since it allows concurrent detection of different neurotransmitters and their metabolites in a short period of time (20 minutes), depending on the condition of the separation process (Heidbreder et al., 2001). It is the most common method for measuring biogenic amines because of its sensitivity, specificity, versatility and simplicity: it does not require any pre-treatment of the sample (Cheng and Kuo, 1995).

Although HPLC-ED has been widely used for the separation of dopamine and its metabolites, the samples must be cleaned prior to analysis in order to remove the numerous proteins and lipids contained in brain tissue which would otherwise interfere with the separation by 'sticking' in the column. Therefore, a perchloric acid extraction stage was included to remove these proteins and lipids before injection onto the column (Birgner et al., 2008). Alternative methods for cleaning the sample would be through extraction into solvents such as ethanol (Liu *et al*., 2003) or methanol (Hows *et al*., 2004).

5.1.1 Principles of HPLC - ED:

There are two principle steps in the analysis: separation, where the sample is separated into its constituent compounds; and detection, where the separated compounds are identified and quantified. The mobile phase is pumped through the column under high pressure; the sample is introduced without disrupting the flow or changing the pressure by means of a high-pressure injector valve, either as a component of an autoinjector which is integrated into the autosampler, where the sample can be injected automatically, or manually operated. The autoinjector was used in these experiments as it has the advantage that large number of sample can be analysed in each batch (Figure 1).

Chromatographic separation is achieved in the analytical column. The sample, consisting of a mixture of chemicals, is separated into its component species. Basically, this includes passing the sample into a solvent (the mobile phase) over a solid material (the stationary phase), the latter consisting of a very fine silica powder tightly packed into a column. As the sample goes through the column, different chemical components pass through at different speeds, depending on the size, shape and charge of the discrete molecules, thus separating the mixture into its constituents.

Perfect separation can be achieved by adjusting the composition of the buffer used; for instance, changing the pH will change the charge (ionisation) or the different chemicals consequently will change the component retention in the column, so they can pass the column quickly. In addition, use of ion-pairing agents changes the retention of different compounds depending on their ionisation. For the studies reported here, octane sulfonic acid (OSA), an ion-pairing agents with negative charges was used, which acted to speed up negatively charged ions (e.g., acid metabolites) while slowing down positively charged ions (e.g., amines). In the mobile phase, increasing the percentage of organic solvent leads to a decrease in the retention of all species on the column, thus affecting the run time but not the separation (Hanai,1999; Young,2010).

Electrochemical detection is based on the redox equilibrium reaction of the molecules concerned, where the oxidation reaction is produced by application of a positive potential, reduction by the application of a negative potential, and the current induced is measured at the working electrode. For the experiments described here, quantification was achieved through measuring oxidation, with the electrode potential set at 800 mV. At this potential many compounds will oxidize, including neurotransmitters like dopamine and its metabolites (Cheng and Kuo, 1995).

The aim of the present study was to test the effects of sub-chronic PCP pre-treatment, modelling schizophrenia, on the basal level of dopamine and its metabolites in different brain areas (FCx, NAc, and VTA).

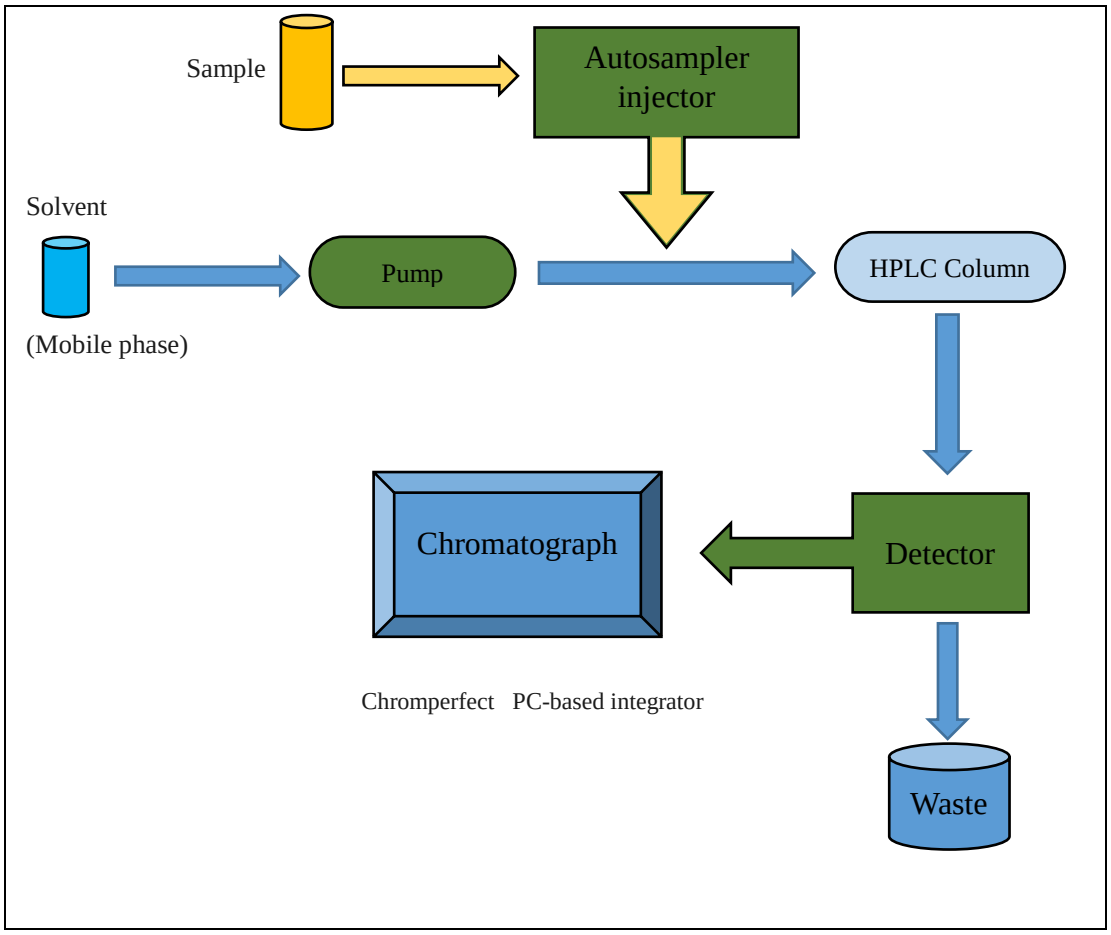


Figure 5.1. Schematic configuration of the experimental layout

5.2 Materials and method

5.2.1 Drug administration

14 Lister-hooded rats were injected with PCP (2 mg/kg, i.p.) and another 14 with 0.9% saline (1 ml/kg, i.p.) twice daily for 5 days. The animals were killed after 26 days (during this period they were used for latent inhibition and NOR experiments: see chapter 3). Animal were killed on the same day in pairs – PCP pretreated and saline pretreated, such that all animals were killed within a three hour period during the morning.

5.2.2 Brain dissection and Tissue sampling

After completion of the behavioural testing, animals were housed in groups of 4 (as previously) under standard conditions with food and water available ad libitum for a further 7 days. On the final day, animals were killed by cervical dislocation under isoflurane anaesthesia (5% at 2.0 l/min; schedule 1), and the brain was immediately removed and placed in an ice-cold stainless steel brain matrix (calibrated for 1 mm coronal sections; World Precision Instruments, UK). Cuts were made 2 mm from the rostral pole, and at 2 mm intervals caudally, to provide separate slices from which the regions of interest (NAc, VTA, FCx) were dissected on an ice-clod glass block, with reference to the brain atlas of Paxinos and Watson (2007). Immediately after dissection, tissue samples were placed in an Eppendorf tube, snap-frozen in liquid nitrogen and transferred to –80ºC for storage until HPLC analysis.

5.2.3 Tissue homogenates

Tissue samples were weighed, and homogenised for 150 seconds (to ensure the tissue was well dispersed) in 0.1 N perchloric acid (200 µl), in a 1.5 ml Eppendorf tube using 'Squishers' (Cambridge Biosciences Ltd). The homogenates were then centrifuged in a cold room (4ºC) for 30 minutes, and the supernatants drawn off carefully. At this stage the supernatants could be frozen (-80ºC) or taken directly for analysis in duplicate by HPLC-ED.

5.2.4. Preparation of standards

Stock solutions of dopamine, DOPAC and HVA, each at a concentration of 10 mM, and a mixture of these bioamines, each at a concentration of 1 mM, were prepared in 0.1 M

H_3PO_4. The stock solutions were aliquoted (50 µl aliquots) and stored at −80 °C until required. A working standard (50 nM with respect to each component) was prepared on the day of analysis by serial dilution of the stock solutions with 0.1 M H_3PO_4.

5.2.5 HPLC analysis

The amount of dopamine and its metabolites in the samples were measured by HPLC-ED. Samples (20 µl) were injected onto the column using autosampler (AS-950-10; Jasco USA). The mobile phase consisted of buffer (75 mM H_3PO_4, 1 mM EDTA, 0.6 mM OSA, 10 % methanol, pH 3.7; vacuum degassed), which was pumped using a pump (Rheos 4000A pump, Switzerland) at 300 µl/min. Separation was achieved with a 150 mm x 2.0 mm LUNA 5 µm column (Phenomenex UK).

Dopamine, with a retention time of 5.6 min, was clearly separated from the other main constituents of the samples which had the following retention times (min): DOPAC, 2.5; 5-HIAA, 7.9; HVA, 11.7. The concentrations were measured using an ANTEC electrochemical detector including a VT-03 flowcell with glassy carbon working electrode set at 800 mV relative to an Ag/AgCl reference electrode (ANTEC Instruments, Holland). Data were collected and analysed using the ChromPerfect PC-based integrator software (Justice Laboratory Software). Although the serotonin metabolite, 5HIAA, was detected using these parameters, these data are not presented as the focus of the study was on dopaminergic systems.

The peak area of dopamine, DOPAC and HVA in each sample was extracted using ChromPerfect, and the concentration of each was calculated using the calibration standard (50 nM). Mean concentrations for each brain region homogenate were calculated from the duplicates, normalised to the weight of tissue used, and expressed as fmol/µg of tissue. Under the conditions employed, the detection limit for the analytes was 2 nM, representing 40 fmol per injection.

In addition, the utilisation ratio (the ratio of tissue concentration in ng/mg protein of primary metabolite to the parent amine) was calculated by the ratio of the tissue level of DOPAC to dopamine in the three brain regions tested (NAc, VTA, and FCx).

5.2.6 Data analysis

Statistical analysis was performed using Graph Pad Prism 7. Prior to analysis, statistical outliers were removed using robust regression followed by outlier identification (ROUT,

Q = 1%), Graph Pad Prism 7). Separate unpaired t-test were then performed to compare the mean concentrations of dopamine and its metabolites in the three different brain regions (FCx, NAc and VTA, n = (8 - 12), between the two independent pre-treatment groups (Saline and PCP). Data are presented as mean ± SEM.

5.3 Results

The basal level of dopamine in the supernatant from the NAc homogenates (Figure.2a) showed a significant decrease in dopamine concentration in tissue taken from PCP pretreated rats (n = 11) compared to saline pretreated (n = 10) ($t(19) = 2.672$, $p < 0.01$). For HVA concentrations, there was a trend towards a significant decrease between saline and PCP groups ($t(22) = 1.818$, $p = 0.08$). However, there was no differences in DOPAC concentrations between pre-treatment groups ($t(19) = 0.8106$), $p > 0.05$).

In FCx, (Figure.2b) The level of DOPAC showed a significant increase between the PCP (n = 11) and saline (n = 9) pretreated groups ($t(18) = 2.203$, $p < 0.05$). However, neither dopamine level ($t(20) = 1.697$, $p > 0.1$) nor HVA ($t(15) = 1.615$, $p > 0.1$) showed any significant variation between the pre-treatment groups.

Levels of dopamine were also measured in VTA (Figure.3) during these experiments. Although there were no significant differences in dopamine concentrations between the PCP (n = 11) and saline (n = 12) pre-treatment groups ($t(21) = 0.839$, $p > 0.1$), the concentrations of DOPAC ($t(21) = 1.771$, $p = 0.09$) and HVA ($t(18) = 1.881$, $p = 0.07$) showed significant differences between the pretreated groups (see table.1 for more details).

Utilisation ratio (DOPAC/dopamine)

The effect of sub-chronic PCP pre-treatment on the utilisation ratio was measured in FCx, NAc and VTA using the unpaired t-test. After removal of statistical outliers (ROUT; Q = 1%), there were no changes in dopamine utilisation between saline and PCP pre-treatment groups in FCx ($t(17) = 0.0144$, $p > 0.05$, in NAc ($t(23) = 1.626$, $P > 0.05$) or in VTA ($t(16) = 1.126$, $p > 0.05$) (Figure. 4).

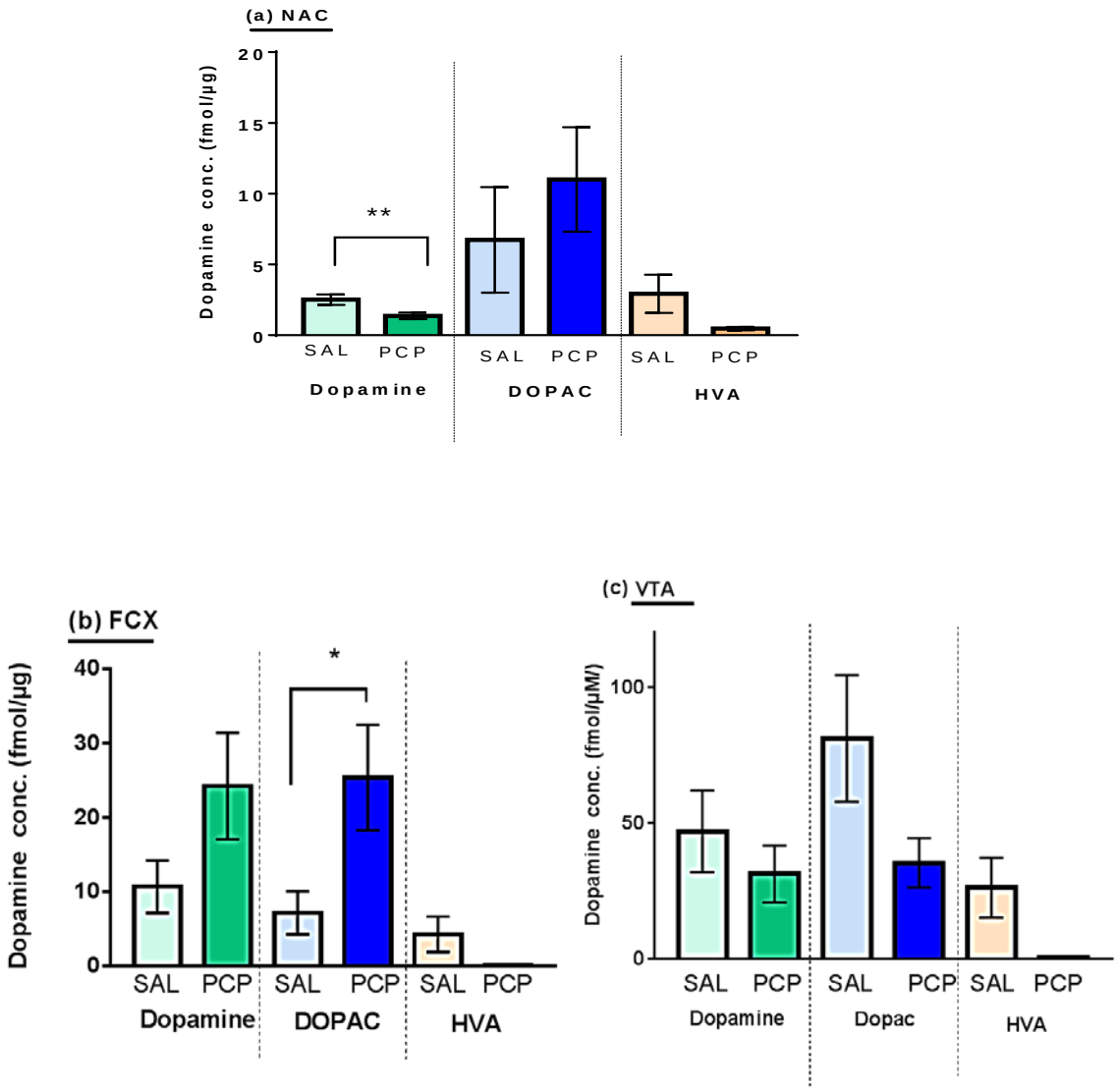


Figure 5.2 Supernatant concentrations of dopamine, DOPAC and HVA in homogenates of (a) NAc, (b) FCx and (c) VTA, in sub-chronic saline or PCP pretreated rats. Data are presented as mean ± SEM. Other details are shown in table 1. * $P < 0.05$; ** $P < 0.01$; significant differences between treatment groups; independent samples t-test.

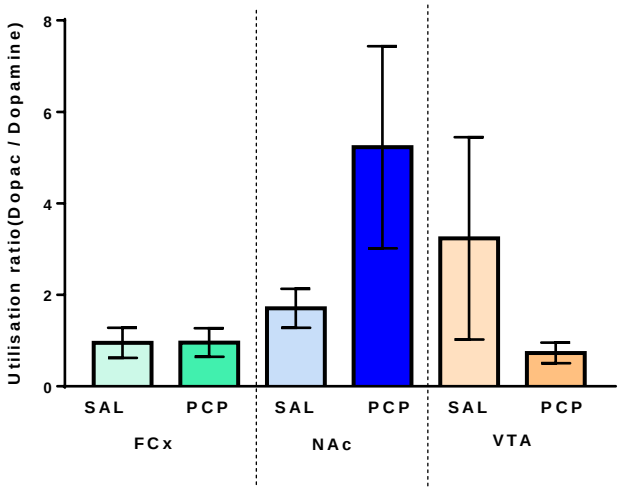


Figure 5.2. Dopamine utilisation ratios (ratio of DOPAC to dopamine) in FCx, NAc and VTA of rats sub-chronically pretreated with saline or PCP. Values are presented as mean ± SEM. There was no significant difference in the utilisation ratios of the pre-treatment groups in any of the three regions.

Region	Pre-treatment	DA	n	DOAPC	n	HVA	n
FCx	SAL	10.68 ± 3.55	11	7.165 ±2.874	9	4.279 ± 2.39	9
	PCP	24.21 ±7.136	11	25.37 ± 7.063*	11	0.1585 ± 0.01	8
NAC	SAL	2.513 ± 0.369**	10	6.739 ± 3.728	10	2.928 ± 1.349	12
	PCP	1.368 ±0.232	11	11 ± 3.69	10	0.4659 ± 0.1	12
VTA	SAL	46.74 ± 15.o3	12	81.04 ± 23.29	12	26.14 ± 11.04	12
	PCP	31.08 ±10.52	11	35.26 ± 9.047	11	0.474 ± 0.1247	8

Table 5.1. Summary of concentrations of dopamine (DA) and its metabolites, DOPAC and HVA in the FCx, NAc and VTA of rats sub-chronically pretreated with saline and PCP. Data are presented as mean ± SEM, after removal of outliers (ROUT, Q = 1%), p < 0.05*, P < 0.01**.

5.4 Discussion

This study set out to investigate the effect of sub-chronic PCP pre-treatment on whole tissue concentrations of dopamine and its metabolites (DOPAC and HVA) in the three localised brain areas which form the mesocorticolimbic pathway. In addition, as a measure of functional activity, the dopamine utilisation ratio (DOPAC/dopamine) was assessed. Thus, the data from these experiments offers a biochemical perspective of the natural substrate underlying the PCP effect that may induce changes in dopaminergic systems, as hypothesized in schizophrenia. HPLC–ED was used to measure whole tissue concentrations of dopamine and its metabolites in the supernatant from brain homogenates.

The results of the present experiment demonstrated that there were changes in dopamine levels in NAc, and in its metabolites (DOPAC and HVA) in FCx but there were no changes in VTA. In addition, there were no changes in dopamine function as assessed by the dopamine utilisation ratio in each region as a result of sub-chronic PCP pre-treatment.

It is interesting that in these experiments, changes in the basal level of dopamine have been reported such as in the NAc area with no accompanying changes in the metabolites (DOPAC or HVA), although the level of HVA showed a trend towards being significantly decreased. There were declines in the dopamine levels in PCP pretreated animals as an animal model of schizophrenia relative to the saline pretreated group: similar results were also seen in previous experiments' *in vitro* studies using brain homogenates of NAc area from patients with schizophrenia in comparison with a control in which low levels of dopamine but a high level of HVA, suggested an increased turnover rate of dopamine in the dopaminergic nerve ending of schizophrenic groups (Toru *et al*., 1982).

In FCx, unpaired analysis also showed increased DOPAC levels associated with PCP pre-treatment, but no changes in dopamine levels. This is inconsistent with other studies that suggested that increased metabolite levels may reflect a synthesis which responded to high transmitter demand (Zetterstrom *et al*., 1988; Abercrombie *et al*., 1989). It was demonstrated that increases in dopamine levels were accompanied by an increase in its metabolites in previous studies using *in vivo* microdialysis in the NAc of rat brain (Young *et al*., 1992 and 1998), although, there were inconsistent metabolite levels in other studies that used microdialysis to measure extracellular dopamine in the striatum, NAc, and

medial frontal cortex of rats (Abercrombie *et al*., 1989). Furthermore, the preliminary results of the current experiments are consistent with the findings of past studies by Jentsch et al. (1997), which indicated that there were no changes in dopamine utilization ratio in rat NAc as a result of administration of 5 mg/kg PCP, twice a day for 7 days, although there was a significant reduction in the prefrontal cortex after pre-treatment with 10 mg/kg PCP once a day for 14 days .

In spite of the high resolution of HPLC, the data were highly variable; there were also a number of outliers which needed to be removed, but even thereafter the variability was high (large SEMs); therefore, the results should be treated with some caution. Although there were clear indications that differences were present, due to this variability it was difficult to assess what were true differences were and what was just the result of random variability.

It might be that there were a number of reasons that led to variable results. Inconsistencies in dissection may have led to the size of the discrete brain areas dissected being variable. Whilst every effort was made to ensure consistency in the dissection, this is an aspect that could be improved in future experiments. In addition, the Squishers that were used to homogenise the samples could have led to contamination that affected the purity of the sample; all the Squishers were washed by distilled water and sterilized using ethanol before and after use. It is notable that there was also considerable variation between data collected using qPCR, where a similar dissection protocol was used: in these experiments a Nano drop system was used to check the purity of the samples and indicated they were pure and free from contamination (see chapter four). For these reasons, it is unlikely that the variability in the HPLC data came from contamination of the samples. Another possible source of error was when weighing the sample as any associated water would affect the tissue weight, and thus the final calculated figure. In future experiments extreme care should be taken to ensure that the tissue is completely dry before weighing.

5.5 Conclusions

The data from the present experiments demonstrated that sub-chronic PCP pre-treatment in rats as an animal model of schizophrenia led to a reduction in whole tissue levels of dopamine in NAc. These changes were not accompanied by changes in dopamine metabolites; however, there were no changes in dopamine level in FCx but rather an increase in the level of DOPAC due to PCP pre-treatment.

HVA was also affected by PCP pre-treatment, where a reduction in concentrations in NAc, FCx and VTA was observed. Thus, it appears that there were changes in whole tissue dopamine levels and its metabolites as a results of PCP pre-treatment, and that these changes may have been different between the brain regions tests, as consistent with work by Abercrombie *et al*. (1998) showing regional variation in the regulation of dopamine release. However, the degree of variability in the samples makes further interpretation difficult.

The experiments described here offer a simple method for simultaneously measuring whole tissue levels of dopamine and its metabolites in addition to providing a method for the approximation of turnover rates in the dopaminergic transmitter systems. Thus, the findings of this experiments suggest that, in general, there are changes in the turnover rate of dopamine as a result of PCP pre-treatment, modelling schizophrenia, which in turn may suggest changes in schizophrenia itself. Further experimental investigations measuring the effect of the dopamine receptor agonist A412997 on whole tissue dopamine release and its metabolites would be beneficial in the investigation of the role of D4R agonists in reversing PCP-evoked effects, and potentially in the treatment of schizophrenia. However, the variability in the data in the present experiments make firm conclusions difficult, and suggest that replication of the experiments with a number of improvements (see above) would be highly beneficial.

Chapter Six

Chapter 6: Discussion

6.1 Summary of results

6.1.1 Effect of D4 agonist on stimulated dopamine release in nucleus accumbens brain slices *in vitro*

These experiments aimed to assess the effect of PCP pre-treatment on D4R modulation of electrically-stimulated dopamine release in NAcS using FSCV in rat brain slices *in vitro.*

Experiment 1, set out to investigate whether the dopamine D4R agonist, A412997, had an effect on electrically-stimulated dopamine release in NAcS, and to determine optimum dose that has the ability to modulate the dopamine release in after application of electric stimulation. A412997, at the doses tested (50 nM, 100 nM, 500 nM and 1µM, 2 µM), showed concentrations dependent effect, and the significant effect was observed with the two highest doses (1 µM and 2 µM) in non pretreated animals. Experiment 2, aimed to check that the effect of A412997 was indeed mediated through D4Rs, by blocking its action with the specific D4R antagonist, L-741,742. The results demonstrated that (2µM) A412997 caused an attenuation in electrically stimulated dopamine release which was completely reserved by dopamine D4 antagonist L-741,742, in non-pretreated animals at the lower dose (10 µM), which alone produced no effect. However, at a high does (50 µM) the antagonist appeared to have an enhancing effect, suggesting perhaps a non-specific action at this dose. The evidence from this experiment confirmed that the effect of A412997 was mediated via D4Rs. The finding is consistent with findings of past studies by Good *et al*., 2013, that dopamine initiates a depolarizing inward current and rise the spontaneous firing of lateral habenula neurons, which was imitated by A412997 and blocked by D4R antagonist L-741,742. Experiment 3, aimed to ascertain the effect of PCP pre-treatment, modelling schizophrenia, on the D4R-mediated modulation of electrically stimulated dopamine release. First, the results from experiment 1 were replicated in control animals, pretreated with saline (1 ml/kg): A412997 attenuated electrically-stimulated dopamine release in slices from saline pretreated similar to that seen in slices from non-pretreated animals. Second, the results of this experiments show that the effect of A412997 on electrically-stimulated dopamine release was entirely abolished in slices taken from PCP pretreated (2 mg/kg, i.p.) rats: that is A412997 had no

effect on electrically stimulated dopamine release in slices taken from PCP pre-treated animals.

6.1.2 Effects of sub-chronic PCP pre-treatment on latent inhibition and novel object recognition and investigation of reversal by A412997

Behavioural experiments, using NOR and LI, aimed to replicate previous findings with regard to the action of PCP on NOR, to extend these by looking at the effect of A412997, and to assess the effect of PCP pre-treatment and A412997 on LI. The results demonstrated that sub-chronic PCP pre-treatment disrupts LI. Importantly, the PCP pre-treatment was completed in 5 days followed by a 7 days drug washout period (Grayson *et al*., 2007), indicating that in LI, the effects seen are due to enduring consequences of the drug having been present in the brain, rather than an acute effect of the drug in the brain during training/testing.

One of the major important findings to emerge from this study is that saline pretreated animals showed the expected LI effect, with significantly reduced learning to the preexposed stimulus (i.e. the LI effect), however, in animals pretreated with PCP the LI effect was abolished, by an attenuation of the effect of preexposure. In this experiment the NOR test was also used to confirm that the pre-treatment regime was sufficient to replicate the well-documented deficit in NOR (Grayson *et al*., 1997; Sood *et al*., 2011; Neill *et al.,* 2010). Importantly, in the present study PCP pre-treatment twice daily for five days were used which was different than the previous study (Grayson *et al*., 2007; Sood *et al*., 2011) that used PCP pre-treatment for seven days, confirming that the treatment schedule used in this study is sufficient to cause NOR disruption in adult rats, that replicate previous behavioural findings in juvenile rats (Yavas & Young, 2017; Gupta and Young, 2018). This study has shown the advantage of long–term PCP exposure as a model of schizophrenia with brain drug free at test (Jentsch and Roth, 1999; Neill *et al*., 2010).

It was also shown that treatment with D4R agonist A412997 (5 mg / kg, i.p.), 20 minutes prior the LI experiment failed to reverse the PCP-induced disruption of LI but, importantly the disruption of recognition memory in NOR task was reversed, confirming that the dose of A412997 which was selected from Woolley *et al*. (2008) was behaviourally effective. The results also confirmed that A412997 had a similar effect as the partial agonist PD168077 for restoring PCP-disrupted NOR, confirming that the

restorative effect due to their D4 agonist action. Importantly, due to time restriction, the behavioural experiment testing the effect of A412997 on LI was not a fully balanced design, but focused on the effect of the drug on PE animals: thus it must be seen as a preliminary experiment, to indicate whether a full study with a fully balanced design was appropriate (see section 3:4 for full discussion). However, since no effect of A412997 on PCP-induced disruption of LI was evident, there were no reason to replicate the experiments in full design.

6.1.3 Effect of sub-chronic PCP treatment on dopamine receptor gene expression in the rat brain: a link to schizophrenia

The experiments described in chapter 4 investigated whether dopamine receptor gene expression was changed by PCP pre-treatment, using qPCR. There were changes in gene expression of dopamine D4 receptors, as well as in other dopamine receptors (D1, D2, D3 and D5), in different regions of rat brain sub-chronically pretreated with PCP: importantly there were decreases in D4R in FCx and VTA, but there were no change in NAc implying that these changes in expression of genes for dopamine receptor varied according to the region of the brain being tested. This shows that receptor expression changes after PCP pre-treatment, suggesting that perhaps similar changes may occur in schizophrenia. However, it is important to note that local changes in gene expression do not directly tell us about the location of actual receptor expression or function: that is it does not tell us whether the receptors are expressed in the cell bodies and dendrites in the local region, or whether they are expressed distally in the terminals. Further experiments using receptor labelling in different brain regions would be required to ascertain this.

6.1.4 Effect of PCP pre-treatment on regional brain content of dopamine and its metabolites

The experiments described in chapter 5 investigated whether PCP pre-treatment caused changes in concentrations of dopamine and its metabolites DOPAC and HVA in localised brain regions. HPLC–ED was used to measure whole tissue concentrations of dopamine and its metabolites in the supernatant from brain homogenates (i.e. both extracellular and intracellular levels of each. This study showed that there were changes in dopamine levels in NAc and in its metabolites (DOPAC and HVA) in FCx, however in VTA there were no changes, in addition as a result of sub-chronic PCP pre-treatment there were no changes in dopamine function as assessed by the dopamine utilisation ratio in each region.

The findings of this experiments suggest that in general as a results of PCP pre-treatment, there were changes in whole tissue dopamine levels and its metabolites, and that these changes may have been different between the brain regions tests.

One must take into account that, as well as drug treatment, these animals also underwent behavioural experiments, including water restriction and exposure to repeated footshock and to tone, treatments which are known to affect dopamine release and turnover (e.g. Young *et al*., 1998). However, all animals received the same behavioural treatments (with the exception that some received preexposure, while others did not) and so any group differences are likely to be due to the drug treatment rather than the behavioural manipulations.

Thus, these studies have shown behavioural, genetic and neurochemical changes related to mesolimbic dopamine function after PCP pre-treatment. These changes are likely to be relevant to understanding changes occurring in brains of schizophrenia sufferers.

6.2 General Discussion

The main aim of the present study was to investigate the neurochemical changes in dopamine D4 receptor function as a results of sub-chronic PCP pre-treatment, which could be useful to understand changes occurring in schizophrenia.

Sub-chronic, treatment with PCP, has been shown to disrupt behaviours which model cognitive and negative symptoms of schizophrenia in rats. It has been found that the mesocortical pathway is implicated in these symptoms (Masana *et al*., 2012), whereas changes in NAc dopamine are implicated in positive symptoms (Howes and Kapur, 2009; Rolland *et al*., 2015). This behavioural deficit induced by PCP is consistent with mesolimbic dopaminergic system after PCP administration, however it is not clear how PCP pre-treatment effects the dopaminergic system (Jentsch and Roth, 1999).

In addition, for reasons that are not well understood, the dose of subchronic PCP required to show behavioural changes associated with schizophrenia is lower in female rats than in male rats (Neill et al., 2010). At the lower dose (2 mg/kg) used in females little or no behavioural effects are seen during pre-treatment, whereas at the higher dose used for males (5 mg/kg) some hyperlocomotion is seen. Therefore it is preferable to use the lower dose, in females, where no overt behavioural changes occurred during pre-treatment.

In this study a multidisciplinary approach was used for studying different aspects of the neurochemical changes after PCP pretreatment modelling symptoms relevant to schizophrenia, therefore giving indications of changes which may occur in schizophrenia, and to ascertain the actions of A412997. These included testing the effects of sub chronic PCP pretreatment on behaviour (LI and NOR), on stimulated dopamine release using FSCV on rat brain slices in NAc, expression of genes coding for dopamine receptors and HPLC analysis of whole tissue taken from local brain areas. The value of using a multidisciplinary approach is that several different aspects of brain function can be investigated.

6.2.1 D4 agonist and cognitive impairment in schizophrenia

Our study is based upon previous research that shown how the D4 agonist, PD168077 displayed no pro-psychotic action in the NOR task (Sood *et al*., 2011) which models behavioural deficits seen in schizophrenia (Neill *et al*., 2010), as would be predicted from the antipsychotic activity of clozapine, which displays D4 antagonist properties, amongst many other pharmacological actions. Rather PD168077 exhibited an antipsychotic-like profile in NOR in rats pre-treated with PCP (Sood *et al*., 2011) an effect which has been recently replicated by other groups (Huang *et al*., 2017; Miyauchi *et al*., 2017). The observation that clozapine, and other atypical antipsychotic drugs, display some antagonist efficacy at D4 receptors attracted attention to the possible role of D4R dysfunction in schizophrenia. However, clinical trials demonstrated that selective D4 antagonists were ineffective for treatment symptoms of schizophrenia (Kramer *et al*., 1997 Corrigan *et al*., 2004).

Since PD168077 has been reported to act as a partial agonist (Newman -Tancredi *et al*., 2008), in the studies reported here this finding was replicated using a pure D4 agonist, A412997, confirming that the behavioural effect was indeed mediated by a D4 agonist action. Studies such as that conducted by Woolley *et al*. (2008) support this line of research with their reported findings that A412997 improved NOR performance in normal animals. Taken together with earlier evidence of pro-cognitive effects of D4 agonists in behavioural tasks (Powell *et al*., 2003; Bernaerts and Tirelli, 2003; Browman *et al*., 2005) it was proposed that activation of D4Rs, through the use of agonists, may improve cognitive impairment This in turn may provide a novel therapeutic approach for treating schizophrenia, but with an emphasis on an agonist effects rather than an antagonist effect.

In this context, it is important to understand how D4R agonists may exert such effects in a model of schizophrenia. Data from these studies showed that there was a reduction in the gene expression for dopamine receptors in mesolimbic neurones, which may reflect a reduction in accumbal presynaptic autoreceptors. However previous studies have reported the possibility that the changes evoked by D4 agonists may be mediated indirectly

through glutamatergic, GABAergic and/or cholinergic neurones (Wang *et al*., 2002; Yuen and Yan, 2011; Huang *et al*., 2017) which may be dysfunctional in schizophrenia (Lauzon and Laviolette, 2010). Thus there is a need for more of an in depth study of the mechanisms through which D4 agonists exert their pro-cognitive effects.

6.2.2 A412997 attenuated dopamine release in NAcS

Using FSCV in brain slices, the results of our study have shown that superfusion of A412997 on to slices taken from animals pre-treated with saline attenuated electrically-stimulated dopamine release in the NAcS, an effect which is abolished in brain slices taken from animals pretreated with PCP. Importantly, from these data, it is apparent that D4R agonists reduce stimulated dopamine release in NAcS. Mechanistically, previous studies have proposed that to maintain the excitation/inhibition equilibrium provided by glutamatergic and GABAergic drive critical for cognitive control an optimum level of D4R activity may be required (Wang *et al*., 2002; Yuen and Yan, 2011; Furth *et al*., 2013). This would present a reasonable route through which fine control of D4R activation may influence such behaviours. Therefore, it is important to understand the mechanisms by which D4Ragonists act in these pathways.

The data of our study support the concept that D4Rs are important in dopamine-glutamate interactions and that dysregulation in this system is important in schizophrenia. Thus, we have shown that the decrease in stimulated dopamine levels *in vitro* caused by a D4R full agonist was attenuated following PCP pre-treatment. On this basis we hypothesize that a dysfunction in D4Rs leads to abnormal dopamine-glutamate regulation in PCP pre-treated rodents, which may underlie cognitive abnormalities associated with schizophrenia.

6.2.3 D4 agonist and positive symptoms of schizophrenia

As discussed above, there is some evidence that D4R agonists could be useful in reversing cognitive impairments seen in memory related tasks (e.g. NOR), which is related to cognitive symptoms of schizophrenia. The data presented here extend this to show that sub-chronic PCP also induced disruption of LI, a behaviour which is related to positive symptoms of schizophrenia. Interestingly, in NOR, A412997 reversed the effect of PCP-pre-treatment: however at least in preliminary studies, it did not reverse the effects of PCP pre-treatment in LI. It is believed that frontal cortex is particularly important region in mediating cognitive processes modelled by NOR (Neill *et al*., 2010) whereas subcortical

mechanisms including mesoaccumbal dopamine systems in behaviours such as LI appear to be more important (Weiner and Feldon. 1987). It may be therefore that the antipsychotic-like effect of A412997 is mediated at the cortical level, and that changes in function in NAc are less relevant to an antipsychotic-like profile. This would lead to a suggestion that D4R agonists may be useful in treating cognitive symptoms, but less so in treating positive symptoms. Therefore, this remains a potentially fruitful line of research, since a major unmet therapeutic need is for effective treatments for cognitive symptoms.

There is substantial evidence for changes in accumbal dopamine function after PCP pre-treatment in spite of the lack of behavioural effect of A412997 in reversing PCP-mediated changes in LI, indicating a lack of functional effect in NAc. In slices taken from PCP-pretreated animals the abolition of attenuation of electrically stimulated dopamine release in NAc by A412997 may account for the lack of effect of the drug in LI. Two lines of research are suggested following this argument, first, as an addition to the LI experiments it is important to test whether A412997 will enhance LI when administered on its own accord in the absence of PCP, as has been reported for other drugs with antipsychotic action (Peters and Joseph, 1993). Independent of any effects of PCP, this will indicate whether A412997 possesses an antipsychotic profile in LI. Second it is important to investigate the neurochemical effects on dopamine release in frontal cortex slices. Due to low signal levels and the presence of noradrenergic terminals in the frontal cortex however, FSCV measurements in in this region are challenging making it difficult to definitively identify a release product as dopamine or noradrenaline since the two show identical cyclic voltammograms.

6.2.4 Dopamine receptors genes expression

Following on from this argument (see above), changes in dopamine receptor gene expression have been described. In this study the results have shown changes in D4R gene expression in frontal cortex and VTA, but not in NAc. This indicates that post-synaptic D4Rs are affected in the cortex region but not in the NAc, since gene expression relates to the location of cell bodies rather than terminals. Thus the difference in behavioural profiles may be dependent on whether the behaviours are mediated primarily in cortex (NOR) where postsynaptic receptors are affected by PCP, or NAc (LI) where they are not. In these experiments our results have shown a decrease in the VTA which

may reflect changes in post synaptic receptors in the cell bodies and dendrites within VTA, and/or decreases in presynaptic autoreceptors located on terminals in NAc and/or cortex. Considering the role of autoreceptors is to attenuate dopamine release, then the abolition of the A412997-mediated attenuation of dopamine release in slices taken from PCP pre-treated rats would be consistent with autoreceptor reduction in NAc, which would be indicated by a decrease in D4R gene expression in VTA. Further experiments would be required to verify the location of the changes in receptor expression (as opposed to gene expression) to verify this hypothesis. However, due to a lack of specific-labelled ligands or antibodies histochemical experiments to test this hypothesis are not feasible at present.

It was also shown that A412997 attenuated electrically-stimulated dopamine release which was entirely abolished in slices taken from PCP pretreated rats in *vitro* and in behavioural experiments D4R agonist A412997 reversed the disruption of recognition memory in NOR task, confirming that A412997 had a similar effect as the partial D4 agonist PD168077 for restoring PCP-disrupted NOR, and that the restorative effect is due to their D4 agonist action. The mesocortical pathway is implicated in cognitive and negative symptoms (Masana *et al*., 2012) and NAc in positive symptoms (Howes and Kapur, 2009; Rolland *et al*., 2015). Thus this behavioural deficit induced by PCP is consistent with mesolimbic dopaminergic system after PCP administration, however it is not clear how the dopaminergic system is affected by PCP pre-treatment (Jentsch and Roth, 1999). In this study A412997 exhibited an antipsychotic-like profile in NOR in rats pre-treated with PCP. However clozapine, the atypical antipsychotic drug, displays D4 antagonist properties, which is hard to reconcile with these actions of D4R agonists. It is known that clozapine has many pharmacological actions and it may be that the antipsychotic effect is not related to D4R actions. In support of this, clinical trials demonstrated that selective D4 antagonists were ineffective for treatment symptoms of schizophrenia (Kramer *et al*., 1997 Corrigan *et al*., 2004). Another possibility is that an optimum level of D4 activity is required for normal function, and that either both increases and decreases in activity would lead to behavioural abnormality (see below). Thus future research is recommended to investigate the mechanisms through which D4 agonists exert their pro-cognitive effects.

This changes of evoked by D4 agonists could be mediated indirectly through glutamatergic, GABAergic and/or cholinergic neurones (Wang *et al*., 2002; Yuen and Yan, 2011; Huang *et al*., 2017). Previous studies have suggested that to maintain the excitation/inhibition equilibrium provided by glutamatergic and GABAergic drive critical for cognitive control an optimum level of D4R activity may be required (Wang *et al*., 2002; Yuen and Yan, 2011; Furth *et al*., 2013). A412997 attenuated electrically-stimulated dopamine release in the NAcS using FSCV in brain slices, an effect which was abolished in brain slices taken from animals pretreated with PCP. This presents a reasonable route through which fine control of D4R activation may influence behaviours. In addition this is consistent with autoreceptor reduction in NAc, which would be indicated by a decrease in D4R gene expression in VTA, although it is not clear through what mechanisms D4R agonists would act in these pathways.

In addition in schizophrenia there were dysregulation of dopamine-glutamate interactions: the data of our in vitro study have shown that the decrease in stimulated dopamine levels caused by a D4R full agonist was attenuated following PCP pre-treatment. Thus a dysfunction in D4Rs leads to abnormal dopamine-glutamate regulation in PCP pre-treated rats, which may underlie cognitive abnormalities associated with schizophrenia. Furthermore cognitive processes modelled by NOR is mediated by frontal cortex (Neill *et al*., 2010) whereas LI appear to be mediated by mesoaccumbal dopamine systems (Weiner and Feldon. 1987). Therefore the antipsychotic-like effect of A412997 may be useful in treating cognitive symptoms, but less so in treating positive symptoms, which may account for the lack of effect of the drug in LI.

6.3 Conclusions

The influence of D4Rs on stimulated dopamine release, through measuring the effect of A412997 on stimulated dopamine release in slice of NAcS of subchronically PCP animals may have an important role in improvements in the neurochemical basis for D4R modulation and suggest the potential use of D4R agonist for the treatment of schizophrenia such as A412997, which could be useful in improving cognitive symptoms.

The data were presented from the voltammetric study (chapter 2) and the behavioural study (chapter 3) could be useful in presenting a potential novel treatment strategy, as together suggested abnormal D4-receptor mediated regulation of accumbal dopamine release in schizophrenia.

In addition, the findings of this study suggest that changes in dopamine receptor expression and in the turnover rate of dopamine in brains of PCP pre-treated rats, as an animal model of schizophrenia, may be useful in identification the molecular pathways that related to the aetiology of schizophrenia which could be help in investigation the pathophysiological changes of the brain of schizophrenic patients.

6.4 Future studies

-Implications to understanding and treating schizophrenia

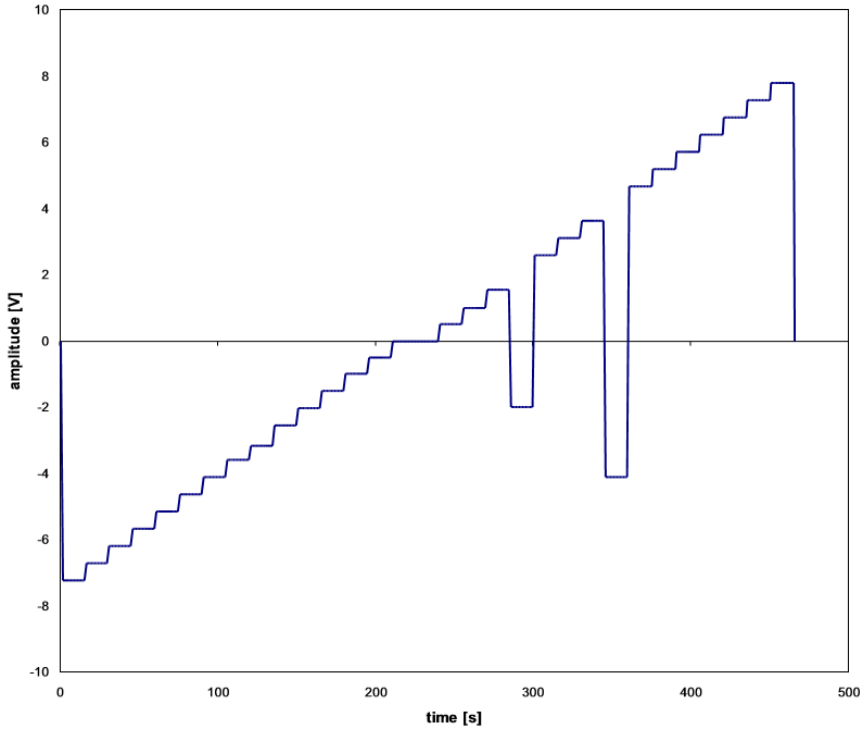
It is recommended that further neurochemical studies be undertaken both *in vivo* and *in vitro* for NAcS which is more associated with the mesolimbic pathway, therefore giving more attention to this area of brain is needed for improve treatment of schizophrenia.
Further experimental investigations for protein analysis such as western blots and immunohistochemistry are needed to estimate dopamine receptor protein level in sub-chronically PCP pretreated animals for identification whether these changes could associated with changes in protein level to gene expression level of dopamine receptors, therefore it is recommended to provide specific-labelled ligands or antibodies for D4R in the future to test this hypothesis

In addition it would be interesting to assess the effects of D4R agonist A412997 on expression of gene coding dopamine receptor in PCP animal model relevant to schizophrenia, as well as its effect on whole tissue dopamine release and its metabolites which could be useful in the investigation of the role of D4R agonists in the treatment of schizophrenia.

Appendix (1)

Silver – Silver Chloride Reference Electrode

NPI Electronics, Tamm, Germany



Acl-01 working scheme

Graphical representation of the in-built voltage ramp sequence applied to the silver wire, to electrolytically coat the surface with AgCl, using an optimised chlorinating procedure developed at the Max-Planck Institute in Gottingen, Germany (NPI Electronics, Germany).

Silver wire was dipped in 2 M KCl solution and cleaned by applying a negative voltage, starting at a potential of -7 V. The voltage then stepped in 0.25 V increments at 15 sec intervals as far as 0 V, where the voltage remained at 0 V for 30 sec. The electrolytic coating then started by applying a regularly incrementing positive voltage (0.25 V every 15 sec), up to a potential of 8 V, with two brief reversals of the potential (- 2 V for 15 sec and -4 V for 15 sec). The potential was held at 8 V for 15 sec, and then returned to 0 V to complete the coating process.

Details taken from:
http://www.npielectronic.de/fileadmin/files/Literature_Brochures/Miscellaneous/ACl-01/Acl01_ad.pdf

Appendix (2)

QPCR primer design conditions:

Condition	MIN	OPT	MAX
PRIMER SIZE	20	24	26
TM	60	63	65
%GC	40	50	60
PRODUCT SIZE	70	100	120
EXP COND	Salt Conc = 50mM	Primer conc = 50 nM	

Oligo Perfect Designer tool from Invitrogen was used to design sets of primers

Details taken from:

https://tools.lifetechnologies.com/content.cfm?pageid=9716&icid=fr-oligo-6?CID=fl-oligoperfect

Appendix (3)

RNA extraction – SIGMA MAMMALIAN KIT PROTOCOL

WASH SOLUTION 2
Dilute Wash solution 2 with 100% EtOH before use – refer to user manual for amount
LYSIS BUFFER

- Require 500µl/Sample
- Add 10µl mercaptoethanol per 1ml Lysis Buffer and mix well.

PROTOCOL

STEP 1 LYSE CELLS

Add 500µl of Lysis/Merc. Solution to each sample, then homogenise with blue stick, Vortex 10secs

1. Then transfer to BLUE FILTRATION COLUMN (1st Collection Tube)
2. Centrifuge for 2 mins at max.(keep supernatant and discard blue column)

STEP 2 BIND

1. Add 500µl of 70% EtOH to filtrate and mix, then transfer 600µl to clear/red binding column (2nd Collection Tube)
2. Spin 15 secs
3. Transfer, then discard and repeat with remaining filtrate

STEP 3 WASH

1. 500µl WASH 1 added to column. Spin for 15secs, then transfer column to new collection tube (3rd collection tube).
2. Then add 500µl WASH 2 to column. Spin for 15secs and discard through floe
3. Add 500µl WASH 2 to column. Spin for 15secs, then transfer to new collection tube (4th collection tube), and centrifuge for 2 mins.

STEP 4 ELUTION

1. transfer to new collection tube (5th Collection Tube)
2. Add Elution Solution (30-60µl depending on expected conc. of RNA and amount), then Stand for 1 min, finally Spin 1 min

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