MECHANISMS UNDERLYING CHANGES AFTER SUB-CHRONIC PCP TREATMENT: CLUES TO SCHIZOPHRENIA

Ersin Yavas

A thesis submitted to the University of Leicester in accordance with the requirements for award of degree of Doctor of Philosophy (PhD) in the Department of Neuroscience, Psychology and Behaviour

2018

THESIS ABSTRACT: MECHANISMS UNDERLYING NEUROCHEMICAL CHANGES AFTER SUB-CHRONIC PHENCYCLIDINE TREATMENT: CLUES TO THE NEUROPATHOLOGY OF SCHIZOPHRENIA

by Ersin Yavas

Dopamine is one of the major neurotransmitters in the mammalian brain and changes in its concentration have been associated with schizophrenia. However, dopamine dysfunction alone cannot account for the genesis of schizophrenia. One unifying theory suggests that there is a disruption of the glutamate-dopamine balance such that glutamate has an effect of increasing dopamine release. Conversely, several studies have reported that the excitatory actions of ionotropic glutamate receptor neurotransmission play an important role in regulating extracellular dopamine levels in the striatum. To understand the potential role of glutamatergic mechanisms in schizophrenia, phencyclidine (PCP), a non-competitive N-methyl-D-aspartic acid receptor (NMDAR) antagonist which models aspects of schizophrenia, was used in the current study. In addition to glutamate, acetyl choline neurotransmission, which is known to modulate dopamine release, also changes after PCP pre-treatment. However, the extent to which PCP modulates cholinergic systems and how such modulation contributes to PCP's psychotomimetic effects are not fully understood. The current study showed that the mechanism of action of PCP involves NMDAR and nicotinic acetyl choline receptor (nAChR) modulation of dopamine function, specifically in the nucleus accumbens shell. The effects of NMDAR and nAChR activation on dopamine release in the nucleus accumbens were assessed using fast scan cyclic voltammetry (FSCV) in brain slices from rats sub-chronically pre-treated with PCP, an animal model of schizophrenia. NMDA increased basal dopamine release in both pre-treated and non-pre-treated striatal slices, but there was no consistent change in potassium-evoked dopamine release. Moreover, NMDA attenuated electrically stimulated dopamine release, a change which was reversed by metabotropic glutamate receptors 2 and 3 (mGluR2/3) antagonism. This suggests that NMDA augments glutamate release via activation of NMDAR on dopaminergic axon terminals, an effect which is dependent on mGluR 2/3 receptors, and that activation of NMDARs, in turn, increases electrically stimulated dopamine release. The nAChR agonist nicotine and the antagonist dihydro-β-erythroidine hydrobromide (DHβE) both modulated dopamine release in brain slices as well. In addition to studies in brain slice preparations, the effects of PCP on cholinergic and dopaminergic mechanisms were assessed through blood-oxygen-level dependent (BOLD) contrast using pharmacological magnetic resonance imaging (phMRI). Specifically, changes in brain-wide BOLD responses to nicotine and amphetamine administration were assessed before and after either saline or PCP pre-treatment. The results indicated that cholinergic and dopaminergic mechanisms were disrupted by PCP treatment. However, resting state MRI showed that brain resting-state functional connectivity was not altered by PCP pre-treatment. Thus, both in vitro and in vivo experiments suggest that cholinergic systems in nucleus accumbens were partially interrupted by subchronic PCP pre-treatment. Therefore, future studies of the role of dopamine and glutamate in schizophrenia should consider the modulatory role of cholinergic systems. Together, these studies suggest that the cholinergic system in nucleus accumbens may serve as an important therapeutic target for treatment of schizophrenia.

ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Dr. Andrew Young for his patience and supportive supervision. I would like to thank my family for always being by myside as well as their enthusiasm and optimism. Especially lots of thanks to my Mum and Dad – the latter sadly passed away during my studies. They were always proud of me and encouraged me. I would like to give special thanks to Dr. James McCutcheon and Dr. Claire Gibson for their helpful comments and advice during my studies. Thank you also to Kate Peters and Dr. Mesut Erzurumluoglu for their company and friendship, and their useful debates on science. Special thanks are also to Dr. Todor Gerdjikov and Dr. Michael Kelly for their patience and invaluable support. Many thanks to all those people who were around at some stage during this project.

Table of Contents

LIST OF FIGURES

List of Abbreviations

ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
Ag/AgCl	Silver/silver chloride
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BOLD	Blood-oxygen-level dependent contrast
CBF	Cerebral blood flow
CBV	Cerebral blood volume
DSM	Diagnostic statistical manual
EAAT	Excitatory amino acid transporter
EPI	Echo-planar imaging
FCx	Frontal cortex
FLIRT	FMRIB's linear image registration tool
FSCV	Fast scan cyclic voltammetry
FSE	Fast spin echo
GABA	γ-Aminobutyric acid
GLAST	Gene deletion
ICA	Independent component analysis
ICD	International classification of disease
IRON	Increased relaxation for optimized neuro-imaging
ITI	Inter-trial interval
KO	Knock out
MCFLIRT	Motion correction linear image registration tool
MELODIC	Multivariate Exploratory Linear Optimized Decomposition into Independent Components
mGluR	Metabotropic glutamate receptor
MION	Monocrystalline iron oxide nanoparticle
MK-801	Dizocilpine
NAc	Nucleus Accumbens
nAChR	Nicotinic acetylcholine receptor
NAcS	Nucleus accumbens shell
NMDA	N-methyl-D-aspartic acid

NMDAR	N-methyl-D-aspartic acid receptor
NOR	Novel object recognition
PET	Positron emission tomography
PCP	Phencyclidine
phMRI	Pharmacologic magnetic resonance imaging
Rbet	Rat brain extraction tool
rCBV	Relative cerebral blood volume
RsMRI	Resting state magnetic resonance imaging
SN	Substantia nigra
VTA	Ventral tegmental area
5-HT	Serotonin

LIST OF PUBLICATIONS

The below are the conference presentations and peer-reviewed journal articles published as a result of this thesis (chronological order):

1- Yavas E., Gupta I., & Young A.M.J. (2015). British Neuroscience Association (BNA): Festival of Neuroscience. Glutamate receptor mediated modulation of dopamine release in nucleus accumbens is not affected by sub-chronic phencyclidine pretreatment, in rat brain slices in vitro, Edinburgh, United Kingdom.

2- Yavas E. & Young A.M.J. (2016). Monitoring Molecules in Neuroscience: 16th International Conference. NMDA receptor modulation of electrically stimulated dopamine release in nucleus accumbens slices (6th ranking out of 118 posters) in vitro. Gothenburg, Sweden.

3- Yavas E., Janus J., Kelly M. & Young A.M.J. (2017). British Neuroscience Association (BNA): Festival of Neuroscience. Effect of phencyclidine pretreatment on amphetamine and nicotine evoked brain activation, measured by pharmacological magnetic resonance imaging, Birmingham, United Kingdom.

4- Yavas E. Janus J., Kelly M. & Young A.M.J. (2017) .FENS SfN Summer School: Chemical Neuromodulation: Neurobiological, Neurocomputational, Behavioural and Clinical Aspects. Effect of phencyclidine pretreatment on brain activation: multiMRI study, Bertinoro, Italy.

5- Yavas E., O'Connor & Young A.M.J. (2018). Monitoring Molecules in Neuroscience: 17th International Conference. Cholinergic mechanisms modulating nucleus accumbens dopamine release: a link to schizophrenia?. Oxford, United Kingdom.

6- O'Connor, Yavas E. & Young A.M.J. (2018). FENS Forum of Neuroscience. Phencyclidine pretreatment disrupts cholinergic modulation of dopamine release in nucleus accumbens in vitro: implications for schizophrenia. Berlin, Germany.

7- Yavas, E., Young, A.M J. (2017). N-Methyl-D-aspartate Modulation of Nucleus Accumbens Dopamine Release by Metabotropic Glutamate Receptors: Fast Cyclic Voltammetry Studies in Rat Brain Slices in Vitro. ACS Chem Neurosci, 10.1021/acschemneuro.6b00397

Author Contributions

1- I wrote the abstract, prepared poster, conducted some experiments, and presented data in conference.
2- I wrote the abstract, prepared poster, conducted experiments, and presented data in conference.
3- I wrote the abstract, prepared poster, conducted experiments, and presented data in conference.
4- I wrote the abstract, prepared poster, conducted experiments, and presented data in conference. I also presented a 3 min "minitalk" on the poster.
5- I wrote the abstract, prepared poster, conducted experiments, and presented data in conference.
6- I carried out all experiments and analysis, and contributed to the preparation of the manuscript.

LIST OF OTHER PEER-REVIEWED PUBLICATIONS

The below are the journal articles and conference papers published during the course of this PhD, not related to this thesis (chronological order):

1- Liu D *et al*. Exome chip meta-analysis elucidates the genetic architecture of rare coding variants in smoking and drinking behavior. Under review.

2- Erzurumluoglu AM *et al*. Meta-analysis of up to 622,409 individuals identifies 40 novel smoking behaviour associated genetic loci. Under review.

My contribution: I was part of the Consortium for the Genetics of Smoking Behaviour (CGSB), led by my colleague Dr. A. Mesut Erzurumluoglu in Leicester (Dept. of Health Sciences). I also contributed to the writing of the discussion section of the 'Erzurumluoglu AM *et al*' paper and carried out a literature review for the genes identified in this study.

CHAPTER 1: NEUROBIOLOGY OF SCHIZOPHRENIA

1.1 Introduction

Schizophrenia is one of the most critical illnesses worldwide, affecting around 1% of the population. Two main hypotheses have been put forward to explain neurochemical changes occurring in schizophrenia; the dopamine hypothesis and the glutamate hypothesis. In the past, the main focus has been on the dopamine theory. However, dopamine dysfunction alone cannot explain all the underlying factors of schizophrenia. On the other hand, glutamate dysfunction seems to offer a more complete explanation of the symptoms, accounting for the full spectrum of behavioural dysfunction seen in schizophrenia. Since, it is known that dopamine function is increased in schizophrenia, it is believed that glutamate could be determinative on dopamine release in the striatal brain regions in schizophrenia. Therefore the research in this project investigated the glutamatergic modulation of dopamine changes in normal animals and in animals pretreated with phencyclidine (PCP), modelling schizophrenia.

1.2 Schizophrenia

1.2.1 Background, symptoms, treatment

Schizophrenia is diagnosed based on criteria set out in either the American Psychiatric Association fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), or the World Health Organization's International Statistical Classification of Diseases and Related Health Problems (ICD-10) (American Psychiatric Association,

2013; World Health Organization, 1992). According to DSM-5 schizophrenia has five sub-classifications which are; paranoid type, disorganized type, catatonic type, undifferentiated type, residual type. The ICD-10 defines two additional types; post-schizophrenic depression and simple schizophrenia. All these types share positive (hallucinations, delusions, thought disorder), negative (deficits in social interaction, emotional expression and motivation) symptoms and cognitive dysfunction (impairments of attention and working memory). Differential diagnosis from other mental disorders and medical conditions is described by DSM-5. A wide variety of mental and medical conditions can manifest with psychotic symptoms that must be considered in the differential diagnosis of schizophreniform disorder. These include psychotic disorder due to another medical condition or its treatment; delirium or major neurocognitive disorder; substance/medication-induced psychotic disorder or delirium; depressive or bipolar disorder with psychotic features; schizoaffective disorder; other specified or unspecified bipolar and related disorder; depressive or bipolar disorder with catatonic features; schizophrenia; brief psychotic disorder; delusional disorder; other specified or unspecified schizophrenia spectrum and other psychotic disorder; schizotypal, schizoid, or paranoid personality disorders; autism spectrum disorder; disorders presenting in childhood with disorganized speech; attention-deficit/hyperactivity disorder; obsessive-compulsive disorder; posttraumatic stress disorder; and traumatic brain injury. Since the diagnostic criteria for schizophreniform disorder and schizophrenia differ primarily in duration of illness, the discussion of the differential diagnosis of schizophrenia also applies to schizophreniform disorder. American, Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders (DSM-5®)

The changes in brain function underlying schizophrenia are not known yet. However, the main focus is on two neurotransmitters, dopamine and glutamate, both of which may be altered in schizophrenia.

Investigations into the mechanism of action of the drugs used to treat schizophrenia have not provided clear understanding of the pathogenesis and physiological mechanisms underlying the disease. Target medications are termed antipsychotics and were introduced serendipitously in 1950s (Ross, *et. al.*, 2006). Chlorpromazine was the first antipsychotic drug which was effective in reducing the positive symptoms of schizophrenia, and its action is now known to be by blocking dopamine D2 receptors (Synder, 2006). In addition to chlorpromazine, haloperidol, perphenazine are the first generation antipsychotics (also known as typical antipsychotics). However, those first generation antipsychotics cause acute and chronic neurological symptoms including tremor, rigidity, dystonia, and dyskinesia (Ross, *et al.,* 2006). Subsequently, second generation antipsychotics (atypical antipsychotics) were developed from clozapine: these include olanzapine and are characterised by good antipsychotic action, but with reduced neurological side-effects. Antipsychotic drugs are reasonably effective in reducing positive symptoms, but have little impact on negative and cognitive symptoms. On the other hand, based on the proposed role of glutamate in schizophrenia (see below), pre-clinical research has suggested that drugs affecting NMDA-type glutamate receptors, including glycine, D-serine, D-cyclosperine, sarcosine, or D-alanine may be beneficial in treatment of negative and cognitive symptoms when these agonists are used in conjunction with either typical or atypical antipsychotics (Coyle, 2006).

1.2.2 Dopamine theory

The dopamine theory of schizophrenia proposes that the condition is due to a disturbed and hyperactive dopaminergic signal transduction. Specifically, the over activation of D2 receptors can lead to global chemical synaptic dysregulation, leading to the positive symptoms of schizophrenia. For example, amphetamine, a drug which induces an increase in extracellular dopamine levels, causes behavioural changes similar to those seen in schizophrenia. Similarly (as above) typical antipsychotic drugs are D2 receptor antagonists. However, second generation antipsychotic drugs, which act at multiple receptor types, are more effective than typical antipsychotic drugs which are targeting D2 receptors. The dopamine theory can explain positive symptoms, but it does not provide an explanation for the negative symptoms and cognitive dysfunction.

1.2.3 Glutamate theory

In contrast to the dopamine theory, the glutamate theory can explain both negative and cognitive symptoms as well as positive symptoms (Javitt 1987). The glutamate theory stems from observations of the effect of mind-altering drugs such as PCP and ketamine (Reynolds *et. al.*, 2005; Javitt, 2007), which are pharmacologically similar, acting as non-competitive antagonists at NMDA-type glutamate receptors. That is, they block the ion channels rather than ligand binding-sites. Thus, the glutamate hypothesis of schizophrenia proposes that an increase in glutamate function underlies the symptoms of the condition. In brief, it is suggested that structural changes in glutamate and glutamate receptors in cortical regions, may underlie schizophrenia.

1.2.4 Glutamate dopamine interactions

Although the importance of dopamine function in the brain is widely recognized, the action of dopamine is still unclear in schizophrenia. A critical component in dopamine actions is its modulation of glutamate transmission that could be different when specific receptors are activated (Tseng and O'Donnell, 2004). There is a complex interaction between glutamate and dopamine in different regions of the brain. For example, D1 receptors potentiate responses mediated by NMDARs, whereas D2 receptors depress AMPA receptor mediated responses (Cepeda, *et al.*, 1993). On the other hand glutamate effects on dopamine modulation have been studied (see section 1.6.3) Dopamine and glutamate interactions are therefore believed to be important in terms of the understanding of the brain dysfunction in schizophrenia

1.3 PCP model of schizophrenia

PCP is a non-competitive NMDAR antagonist (Javitt, 2007). It is also known by its "street name" as angel dust, and is used for recreational purposes. Administration of PCP induces the full spectrum of symptoms seen in people with schizophrenia in all three domains; positive, negative and cognitive. It also causes behavioural changes in animals which mimic schizophrenia like behaviours. Thus, PCP clearly causes changes in the brain activity: however the mechanisms underlying these changes after PCP are as yet unknown. The aim of the research presented here is understanding the mechanism through which subchronic PCP treatment causes these effects, which may give insights into changes in schizophrenia.

1.3.1 Effect of phencyclidine on neurochemistry and behaviour

Core deficits in schizophrenia lie in dysfunctional glutamatergic neurotransmission in the prefrontal cortex, which is mimicked by chronic PCP treatment. Acute PCP treatment causes dopaminergic hypoactivity in the prefrontal cortex and dopaminergic hyperactivity in subcortical regions such as the ventral striatum, but not dorsal striatum (Deutch et al., 1987; Jentsch et al., 1997). To what extent dopamine system hypoactivity, brought on by both acute and chronic PCP treatment, influences cognitive functions remains elusive. It is known, however, that behaviour related to frontal lobe activity such as working memory, visual memory, and response planning is impaired in rats and monkeys after subchronic PCP pre-treatment. For example, rats were pre-treated with PCP and tested in the T-maze test in order to evaluate working memory deficits. In this task, rats have to make the right choice regarding alternating correct entrance (left or right arm) in order to get a reward. PCP pre-treated rats show significantly longer delay to entry into the correct arms compared to control rats (Jentsch and Roth, 1999). Since rats are required to make the right choice in a short period of time, which requires working memory, it is possible that subchronic PCP pretreatment impairs prefrontal cortex function in the T-maze test by decreasing dopaminergic activity in the cortex (Brozoski et al., 1979). Ennaceur and Delacour (1988) introduced the novel object recognition (NOR) test to evaluate memory function. This test is widely used to evaluate specific cognitive components such as working memory, attention, and novelty recognition in rodents (Goulart et al. 2010; Silvers et al. 2007). Since the inter-trial interval (ITI) between familiar and novel object serves as a distraction, animals pre-treated with subchronic PCP are distracted by this short time ITI (Cellard et al., 2007). Since ITI is kept as short as 5min or less, prefrontal dysfunction is likely to be involved in this

distraction. Impaired object recognition usually only occurs 24 hours after hippocampal lesions (Hammond et al., 2004), thus excluding the role of the hippocampus. Instead, it is likely that dysfunctional glutamatergic mechanisms in the prefrontal cortex impairs visual memory and working memory. These tests in the rodents are consistent with the fact that people with schizophrenia show deficits in tests of cognitive function.

Alterations in GABAergic and cholinergic function is also reported in schizophrenia. GABAergic transmission in hippocampus increased by subchronic PCP pretreatment (Nomura et al., 2016. Subchronic PCP pretreatment also change acetylcholine and muscarinic receptor modulation of dopamine release (Yavas and Young; O'Conner, Yavas, Young unpublished data), as well as GABAergic modulation of dopamine release in Nucleus accumbens (NAc) (Ferdinand, Peters, Young unpublished data). The aforementioned studies focused on the effects of PCP. However, other NMDAR antagonists such as MK-801 and ketamine also mimic schizophrenia-like signs in rats, although these drugs are far less potent in evoking psychomimetic schizophrenia-like effects than PCP. One drawback of the PCP model is that PCP effects are gender dependent. For example, in some tests, female subjects are more sensitive to the effects of PCP treatment compared to male, while in other tests, male are more sensitive than female (Snigdha et al., 2011; Elsworth et al., 2015). Thus, more tests are required to validate the PCP model (Mouri et al., 2007). To increase the face and construct validity of the PCP model of schizophrenia, further studies of the effects of PCP modulation of dopaminergic, cholinergic and GABAergic and glutamatergic neurotransmissions are necessary.

1.3.2 Animal models of schizophrenia

Traditional pharmacologically based animal models of schizophrenia (including amphetamine, MK-801, PCP, and ketamine) are used to elucidate underlying mechanisms of symptoms of schizophrenia that are mainly based on dopamine function, such as dopamine receptor sensitization, dopamine turn over, and dopamine hypofunction in the cortex and striatal regions. Although these pharmacological models have face validity in regard to their ability to produce behavioural and neurochemical changes thought to occur in schizophrenia, other models of schizophrenia are available to probe the biological basis of this condition. Early life stress is one of those non-pharmacological models of schizophrenia. Maternal separation (Liu et al., 1997) and early social isolation (Jones et al., 1992) have elucidated a number of neurochemical pathways mediating schizophrenia-like behavioural abnormalities, including maze learning impairments and locomotor hyperactivity in novel environment. Moreover, these deficits can be reversed by antipsychotic treatments (Ellenbroek et al., 1998). Changes brought about by early life stressors are similar to changes observed in schizophrenia. Neurodevelopmental models are utilized to understand how neurodevelopmental malfunctions change brain function and structures at later age. These models are used to explain the delayed onset of schizophrenia. Importantly, they assess whether the early onset of physiological changes in the maturing brain can predict the occurrence of symptoms of schizophrenia in adulthood. For example, neonatal ventral hippocampal lesion in rats give rise to social deficits and hyperlocomotion in adulthood (Sams-Dodd et al., 1997). This developmental animal model of schizophrenia opens a new avenue to understand the impact of hippocampal deficits has on the functioning of other brain regions including the prefrontal cortex, dorsal striatum, and NAc. In fact neonatal ventral hippocampal lesion

can result in schizophrenia-like symptoms in adulthood. For example, rats undergoing such a lesion show amphetamine-induced hyperactivity resembling symptoms shown in schizophrenia - schizophrenia sufferers have attenuated responses to dopaminergic drugs and show hyperactivity to PCP and ketamine treatment (Lipska and Weinberger, 1993). The non-pharmacological models discussed in this section have good face validity in regard to their ability to produce behavioural and neurochemical changes analogous to those seen in people with schizophrenia. These models have moderate construct validity. Genetic models have particularly high construct validity. Knock out mice are widely used model in schizophrenia research. For instance, mice lacking NR1 subunit of the NMDAR are used to understand how impaired glutamatergic transmission affect dopaminergic functioning in schizophrenia (Rodríguez et al., 2017). Neural cell adhesion molecule isoform 180 depleted mice give another genetic model of schizophrenia (Gibbons et al., 2009). This mouse shows abnormal migration of neurons, changes cytoarchitecture in hippocampus, both of which resemble abnormalities seen in people with schizophrenia. However, while genetic models has high construct validity, the functional changes brought about by genetic manipulations still remain largely unknown. Given that the origin and underlying mechanisms of schizophrenia is still controversial, it is unlikely that any single animal models can emulate the spectrum of disorders in schizophrenia. Therefore, future studies should utilize a holistic approach by utilizing multiple animal models to shed light on factors underlying symptoms of schizophrenia.

1.3.3 Phencyclidine evokes dopamine release

Studies have shown that chronic PCP administration changes motor behaviour, cognition, motivation and emotions which are similar to deficits seen in schizophrenia. In our studies subchronic PCP pretreatment was used in which the drug was administered twice a day for 5 days (2 mg/kg). These changes may be mediated via modulating dopamine release in the dorsomedial striatum on left hemisphere in where probe is implanted brain hemisphere (Balla *et al.,* 2001). Moreover, PCP does not seem to directly cause an increase in dopamine release, instead its actions on dopamine release are most likely to be mediated through NMDAR blockade (Balla et al., 2001). It is noteworthy that Balla *et al.* used microdialysis to measure dopamine release, and correlated the changes in dopamine release with the behaviour (Balla *et al.,* 2001). PCP effects on NMDARs may not be directly related to a specific location in the striatal areas rather it may require the whole mesocorticolimbic pathway circuit. Despite the fact that, dopamine changes in dorsal and ventral striatum correlated with behaviour changes, the behavioural effects of PCP may also be mediated in the ventral tegmental area (VTA) and cortical brain areas.

Competitive NMDAR antagonists produce weak behavioural stimulation compared to non-competitive NMDA antagonists such as PCP and its congeners (Bennett et al., 1989; Liljequist, 1991; Svensson *et al.,* 1991; Kretscmer *et al.,* 1999). The differences between competitive and non-competitive NMDAR antagonists may be a result of influence on endogenous excitatory amino acid transporters (EAATs). In fact, PCP enhance more dopamine release in the nucleus accumbens shell (NAcS) than the core in rodents due to the competitive and non-competitive NMDAR antagonists' roles (Marcus *et al.,* 2001). The physiological mechanism underlying the non-competitive effects of PCP may be a consequence of the PCP binding site on the NMDAR. The location of the binding site on

the ion channel is complex so NMDA binds rapidly but dissociates slowly which causes the NMDAR to stay occupied for a long time period. However, competitive receptor antagonists have not been capable of this (Marcus *et al.*, 2001). The studies mentioned above do not give insight into the effects of acute PCP administration and chronic PCP administration on dopamine release: rather they focus mainly the action of PCP on NMDARs themselves.

Acute NMDAR antagonists increase the tonic phase and decrease the phasic phase of dopamine release: phasic dopamine release being described as dopamine release caused by dopamine neuron firing whereas tonic dopamine release is proposed to regulate the intensity of the phasic dopamine response (Grace, 1991). On the other hand, NMDA-enhancement of dopamine release causes an increase in the phasic phase in the NAc when the NMDA agonist is injected in VTA (Ferris, *et al.*, 2014). This study gives an opportunity to ask whether the tonic phase and phasic phase undergo a change after chronic PCP administration rather than acute administration. Ferris and colleagues investigated dopamine synthesis and extracellular dopamine levels measured via microdialysis and they found that chronic PCP administration resulted in NMDAR hypofunction and led to an increase in dopamine release. They claim that dopamine neurons cooperate with presynaptic adaptations to normalize extracellular dopamine tone in during chronic blockade of the NMDAR. This adaptation may lead to a reduction in dopamine signalling leading to cognitive deficiencies and may contribute to psychosis-like symptoms seen after such treatment (Ferris, *et al.*, 2014).

Grayson and colleagues (Grayson *et al.*, 2014) showed that chronic PCP treatment in rats leads to a sensitivity to distraction contributing to impaired cognitive function during a novel object recognition test (NOR). The test measures visual learning and memory (Antunes and Biala, 2012). The inter-trial interval (ITI: the time between learning and

testing recall) was varied between 1 min and 10 min to assess the effect of distraction on NOR (Grayson *et al.,* 2014). Results showed that sub-chronic PCP treatment impaired recognition memory and cognitive function in the presence of a distraction in the experimental group when compared control group (Grayson et al., 2014). Therefore, environmental factors in schizophrenia can enhance the cognitive impairments by spontaneous sounds, which are irrelevant to memory goals. However, the studies mentioned above do not tell the age of subjects and post-natal PCP treatment could differ from adulthood PCP treatment. In present study, cognitive deficits brought by PCP were not the main focus: rather neurochemical changes after subchronic PCP pretreatment were investigated (see details in chapter 1 and chapter 2). However, cognitive deficits in NOR were measured as a positive control to check that the dosing schedules used were effective.

1.4 Dopamine neurotransmission

1.4.1 The mesolimbic dopamine pathway

The main dopamine pathways originate in the substantia nigra (SN) and the VTA of the midbrain, and project to striatal and cortical regions. The main projection from SN terminates in the dorsal striatum, comprising the caudate nucleus and putamen (the nigrostriatal pathway), while projections from VTA terminate in NAc and frontal cortex (FCx). (Figure 1.1).

The NAc is important in the control of motivation-related processes, including reward, stress and arousal. It is separated into two anatomically and functionally distinct subregions, the core and the shell (Zahm and Brog, 1992): the core region connects predominantly with basal ganglia motor structures, whereas the shell connects with more

limbic-related subcortical structures (Sesack and Grace, 2010; Ito *et al.*, 2004). Functionally, the shell subregion is predominantly involved in acquisition and expression of motivated behaviour, while the core region is more concerned with the motor components of such behaviour (Zham and Brog, 1992; Deutch, 1993). Dopaminergic neurones comprising the mesolimbic pathway innervate both regions extensively, and exercise modulatory control of information throughput (Sesack and Grace, 2010). In the context of schizophrenia, the shell region appears to be particularly implicated, with its key involvement in processes such as stimulus salience processing, attentional selection (Deutch, 1993; Berridge and Robinson, 1998) and cognitive flexibility (Zahm, 2000), which are dysfunctional in positively symptomatic schizophrenia sufferers.

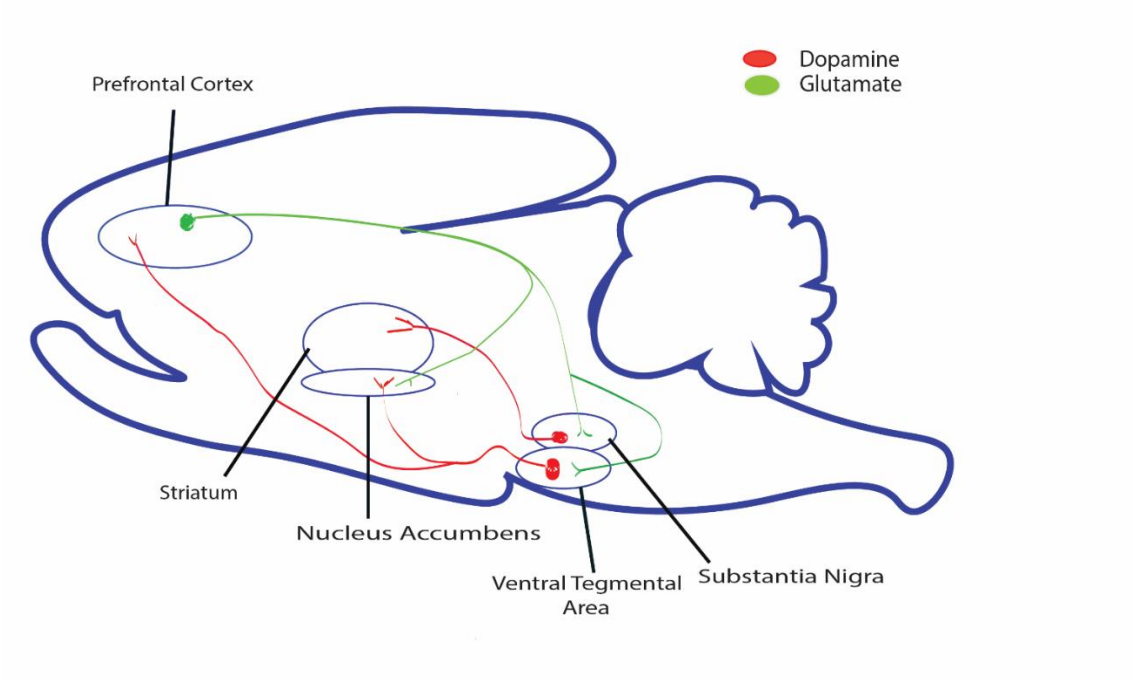


Figure 1.1 Dopaminergic and glutamatergic projections in the rat brain. Modulatory dopaminergic neurons (red) project to the dorsal striatum via the substantia nigra (SN) and the nucleus accumbens (NAc) and prefrontal cortex (PFC) via the ventral tegmental area (VTA) in the rodent brain. Glutamate connections (red) to the striatum, as well as connections to the PFC. Prefrontal cortical efferent excitatory glutamate neurons extend to the striatum, NAc, SN, as well as the VTA.

1.4.2 Receptor composition of Nucleus Accumbens

Since current studies focus on NAc, it is worth discussing what cell types innervate this brain region. NAc receives dopaminergic projections from VTA and glutamatergic afferents from frontal cortex. Medium spiny neurons (MSN) and cholinergic interneurons (Chls) are also located in NAc. Those neurons express different kind of receptors (Fig 1.2). D1R, D2R, mGluRs II/III, α4β2 nAChR, M5R, NMDAR are all expressed on dopaminergic axon terminals (Zhang et al., 2002; Missale et al., 1998; Rondou et al., 2010; Salamone et al., 2014), whereas mGluR I, α7 nAChR, NMDAR are expressed on glutamatergic afferents (Quick et al., 2004; Mitrano et al., 2010; Huang et al., 2011). On the other hand, MSN express GABA-AR, GABA-BR, and D2R (Akiyama et al., 2004; Marcott et al., 2018), whereas ChIs expresses D1R, D2R, α4β2 nAChR, and M4R (Yan et al., 1997; Berendse and Richfield,1993).

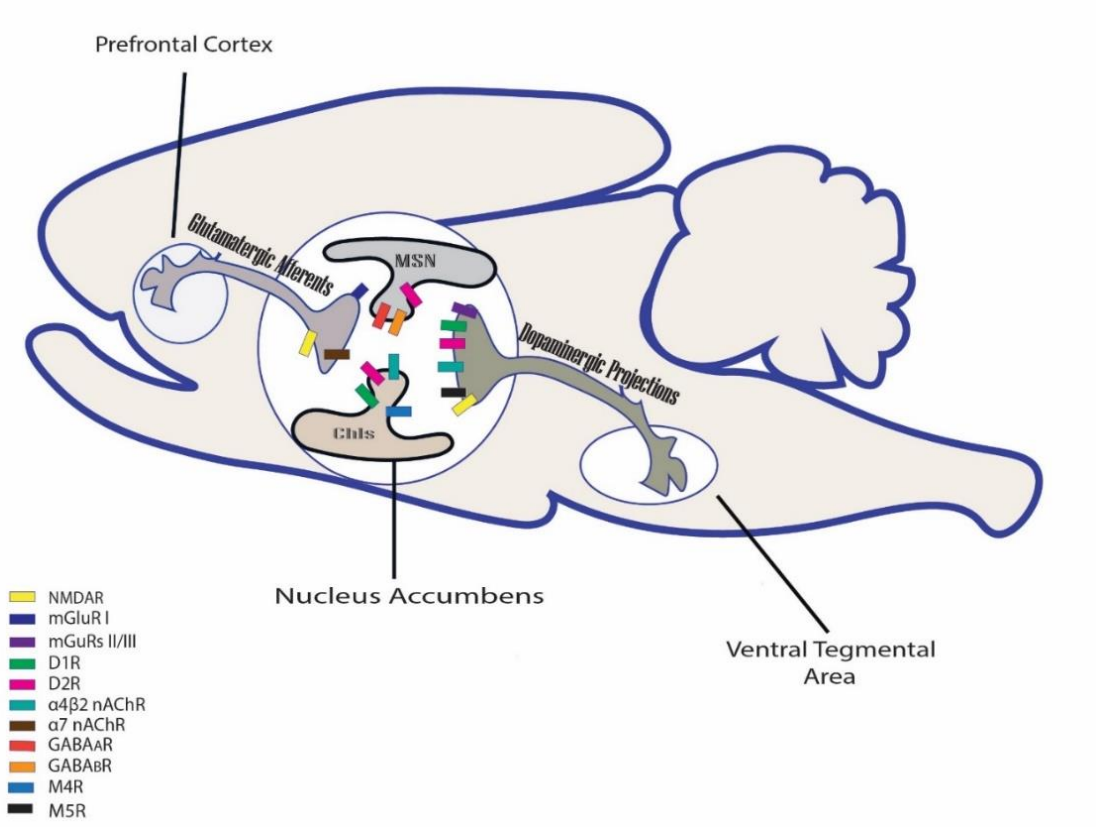


Figure 1.2 Dopaminergic and glutamatergic afferents to NAc. Modulatory dopaminergic neurons project to the NAc from ventral tegmental area (VTA), whereas NAc receive glutamatergic affarents from prefrontal cortex. Medium spiny neurons (MSN) and cholinergic interneurons (Chls) is also located in NAc. Receptors located on those neurons capable of modulating dopamine release in NAc

1.4.3 Dopamine receptor types

Dopamine receptors are G-protein-coupled receptors and divided into two major groups based on biochemical studies (Spano *et al.*, 1978). These studies indicated that D1-like family dopamine receptors positively modulates adenylyl cyclase activity. This finding led to a separation between D1 and D2 type dopamine receptors. There are five main subdivisions of dopamine receptor (D1, D2, D3, D4 and D5), which fall into two main categories; D1-like and D2-like receptors. D1-like receptors include D1 and D5

receptors, and are positively coupled to adenylate cyclase, and are excitatory (Tiberi *et al*., 1991). D2-like receptors include D2, D3 and D4 receptors, and are negatively coupled to adenylate cyclase and are inhibitory (Andersen *et al.,* 1990).

1.4.4 Dopamine receptor localisation

Dopamine receptors also show different location regarding their presynaptic or postsynaptic pattern. For example, D1 receptors are located mainly on the postsynaptic neurons, whereas D2 and D3 receptors are located postsynaptically, but also presynaptically where they act as both heteroreceptors and autoreceptors (Missale *et al.,* 1998; Rondou *et al.,* 2010).

Dopamine autoreceptors are located on dopaminergic presynaptic axon terminals and are responsible for regulating dopamine release by regulating dopamine synthesis by tyrosine hydroxylase and uptake through the dopamine reuptake transporter (DAT) (Zhang and Sulzer, 2012). It is known that dopamine autoreceptors belong to the D2-like family of receptors (Nicola *et al.,* 2000). These receptors activate the Gi/GIRKS protein, and so lead to a decrease in adenylyl cyclase which resulted in change in firing rate (Nicola, *et al.,* 2000). These receptors provide negative feedback on neuronal firing rate, release of neurotransmitters (Wolf and Roth, 1990).

1.4.5 GABA modulation of dopamine in nucleus accumbens

Many addictive drugs seem to regulate dopamine activity by acting upon either dopamine neurons directly or GABA interneurons indirectly (Zhang and Sulzer, 2012). A study by Xi and Stein (1998) showed that GABA-A receptors may be located on the dopamine

neurons and so activation of these receptors will hyperpolarize VTA dopamine neurons or presynaptically depress dopamine release in the NAc. The GABAergic neurons of striatum and NAc project axons to substantia nigra and globus pallidus (Heimer and Wilson, 1975). Therefore, these GABAergic drugs may also regulate the dopamine neurons through D1-like family receptors (D1 & D5) and D2-like family receptors (D2, D3 & D4) receptors in basal ganglia structures such as substantia nigra (D1) and globus pallidus (D2) (Nicola, *et al.,* 2000). In spite of GABAergic medium spiny neurons expressing dopamine receptors, GABA neurons also feedback on to dopamine terminals to modulate dopamine signalling providing an indirect route, via GABA systems, controlling dopamine release (Kohl et al. 1998; Rahman and McBridge, 2002) . On the other hand, infusions of the GABA-A agonists into VTA have been demonstrated to decrease dopamine release in NAc, whereas GABA-A antagonists infusion into VTA increased dopamine release in the NAc. Thus, based on these results the parsimonious explanation is that GABA-A receptors are located on the both dopaminergic projections neurons and GABAergic interneurons (Xi and Stein, 1998). Local administrations have a different impact on dopamine release (Wood, 1982; Akiyama *et al.,* 2004). The different responses may also depend on dopamine receptors such as dopamine autoreceptors and dopamine heteroreceptors in NAc. Therefore, two independent inhibitory mechanisms utilising D2 receptors and GABAergic systems respectively have feedback on dopamine receptors to mediate dopamine signalling in basal ganglia structures. To evaluate GABA activation of electrically stimulated dopamine signalling, the effect of the GABA-A receptor antagonist, picrotoxin, was assessed in brain slices in our experiments.

1.5 Functions of mesolimbic dopamine

Dopaminergic neurons, which are located in the VTA, are activated in response to reward-predictive cues as well as to novel and salient stimuli (Yun *et al.,* 2004). NAc can regulate the motor responses (Mogenson *et al.,* 1980) and NAc neurones have been shown to respond to cues predictive of reward (Yun *et al.*, 2004). Moreover, inactivation of VTA abolished this activity, and also disrupted behavioural responses to the cue. Thus, according to Yun *et al*., cue-evoked activity of NAc neurons is dopamine dependent and is required for behaviour elicited by cues (Yun *et al.,* 2004). D1 receptor antagonists, given locally in NAc, decrease behavioural response to cues, but the D2 antagonist, raclopride, reduced responses to discriminative stimuli; whereas the D1 antagonist, SCH23390, had less effect in reducing the responses than did raclopride (Yun *et al.*, 2004). In contrast to the effects of D1 receptor activation, AMPA and NMDAR antagonists did not decrease behavioural response to the cue stimulus. It is proposed that a combination of NMDA and AMPA receptor antagonists could be used to bypass the activation of NAc neurons (although it is not specified whether this is in core or shell region of NAc) (Yun *et al.,* 2004). Therefore, discriminative stimulus-evoked firing depends on dopamine release in NAc following activation of VTA. NAc has dopaminergic pathways from VTA and glutamatergic pathways from cortex. Both systems modulate dopamine activation and its behavioural outcome may be dysregulated by malfunction glutamate and dopamine mechanisms, which may involve other regulatory transmitters, including acetylcholine. For example, dopamine release is regulated by glutamatergic inputs on cholinergic interneurons in dorsal striatum (Kosillo *et al.,* 2016). An aim of the studies presented here was to test whether glutamate modulation of dopamine release in NAc is dependent also on cholinergic activation.

1.6 Glutamate-dopamine interactions

1.6.1 Glutamate receptor types

Glutamate is the main excitatory neurotransmitter in the central nervous system (Krnjevic and Phillis, 1963). Glutamate receptors can be subdivided into two main categories, ionotropic receptors and metabotropic receptors (Javitt, 2007). There are three separate types of ionotropic receptor based on their pharmacological properties; NMDA, AMPA and kainate. Glutamate agonists stimulate ion channels which enable ions to flow and produce an excitatory postsynaptic depolarizing current and may cause action potential in the postsynaptic neurones (Cotman and Monaghan, 1988). Metabotropic glutamate receptors (mGluR) have eight types which are classified in three groups (Group I [mGluR1 and mGluR5], group II [mGluR2 and 3], and group III [mGluR4, 6, 7, 8]) (Javitt, 2007). However, this pharmacological classification shows variations in different organisms such as C. elegans and Drosophila, hence the need for caution when considering these classifications (Rohrer *et al*., 1994).

Group I metabotropic glutamate receptors, are positively coupled and excitatory in postsynaptic membranes, close to NMDARs and modulate NMDAR function (Rubio *et al.*, 2012). On the other hand, Group II and Group III metabotropic receptors are founded in presynaptic elements; group III mGluRs in the presynaptic active zone act as autoreceptors activated with glutamate release from synapse to which receptors are localized (Shigemoto *et al.*, 1997). The next section will focus on ionotropic receptors due to their excitatory properties and the Group I metabotropic receptors since their excitatory properties which are dependent on an increase in intracellular Ca^{2+} and activation of postsynaptic group I mGluRs, specifically mGluR1 receptors (Linden *et al.*,

1991), the other metabotropic receptors, which are inhibitory, will also be discussed in terms of comparative effects on glutamate function to group I metabotropic receptors.

1.6.2 Glutamate

Glutamate is an excitatory amino acid neurotransmitter in the mammalian brain and plays an important role in mediating learning and memory in the brain (Rezvani, 2006) which is required to keep normal cognitive abilities. Its excitatory role, in instances of excessive activation, can cause neuronal degeneration and decrease in dendritic branching, which are linked to degraded spatial learning (Cortese and Phan, 2005). However, reward sensitivity is also reduced as a result of disrupting glutamate release from synapses (Bechtholt-Gompf *et al.,* 2010). These findings show how glutamate is important in normal brain activation. Excessive release of glutamate in extracellular space, which is linked to some psychiatric disorders, can lead to various changes via its excitatory action on neurotransmitters. Extrasynaptic NMDARs may also be responsible for cell death and mitochondrial dysfunction after activated glutamate spillover through excitotoxicity (Hardingham *et al*., 2002), whereas some studies show that synaptic NMDARs are responsible for excitotoxicity (Papouin *et al*., 2017 ; Wroge *et al*., 2012). However, distinguishing which receptors are synaptic and which are extrasynaptic is not easy, hence it is difficult to be sure of the origin of the excitotocity. One might speculate that this shows the complexity of the glutamate system in the brain, which may explain why, so far, no effective drugs have been developed to regulate glutamate activation. The glutamate hypothesis of schizophrenia posits the assumption that this condition happens as a result of a reduction of glutamate neurotransmission (Javitt, 1990). This hypothesis possibly illustrates how NMDA dysregulation in schizophrenia causes dysfunction in the brain. To support this idea, behavioural studies carried out on animals indicate that PCP

leads to behavioural deficits modelling those seen in schizophrenia; for example, memory dysfunctions in NOR test, which is a task that measures visual memory and episodic memory (Neill *et al*, 2010) and attention (Al-Ali and Young, unpublished data) in experimental animals. In order to develop novel psychopharmacological treatments to ameliorate schizophrenia, the regulatory function of glutamate must be clearly understood. However, control mechanisms at the synapse are highly complex and as yet is not definitely known. For example, GABAergic receptor function and cholinergic receptor function are somehow linked to glutamate function, but it is not clear how these receptor mechanisms change glutamate function and modulation of dopamine signalling in the brain. Therefore, we aimed to investigate interactions between glutamate, GABA and acetylcholine in control of dopamine release in normal brains, and extend this to identify changes in these processes after PCP pretreatment, modelling schizophrenia, which may give indicators of dysfunction occurring in schizophrenic brains.

1.6.3 Glutamatergic modulation of dopamine function

1.6.3.1 Mesocorticolimbic dopaminergic pathway involve acetylcholine modulation

The interaction between glutamate and dopamine is supported by Zmarowski *et al.*, (2005): glutamatergic and dopaminergic inputs in NAc play an important role in selecting motor process responses to discriminative cues (Zmarowski, *et al.*, 2005). When the novel or salient stimuli are applied, dopaminergic inputs from the VTA are activated, whereas glutamatergic inputs from the prefrontal cortex, amygdala, and hippocampus are important in distinguishing between different discriminative cues. The mesolimbic dopamine pathway from VTA to NAc is critically involvd in the control of these behaviours (Zmarowski *et al.*, 2005).

Moreover, glutamate and dopamine receptor activity in NAc can regulate the cortical acetylcholine (ACh) release (Figure 1.3). Thus, higher concentrations of NMDA are sufficient to stimulate NMDAR which, in turn, regulates ACh. However lower concentrations of NMDA need D1 receptors for regulating ACh (Zmarowski *et al.*, 2005). Therefore, these studies show that glutamate and dopamine play an important role in behaviours mediated through projections from VTA to NAc and modulate ACh release in frontal cortex.

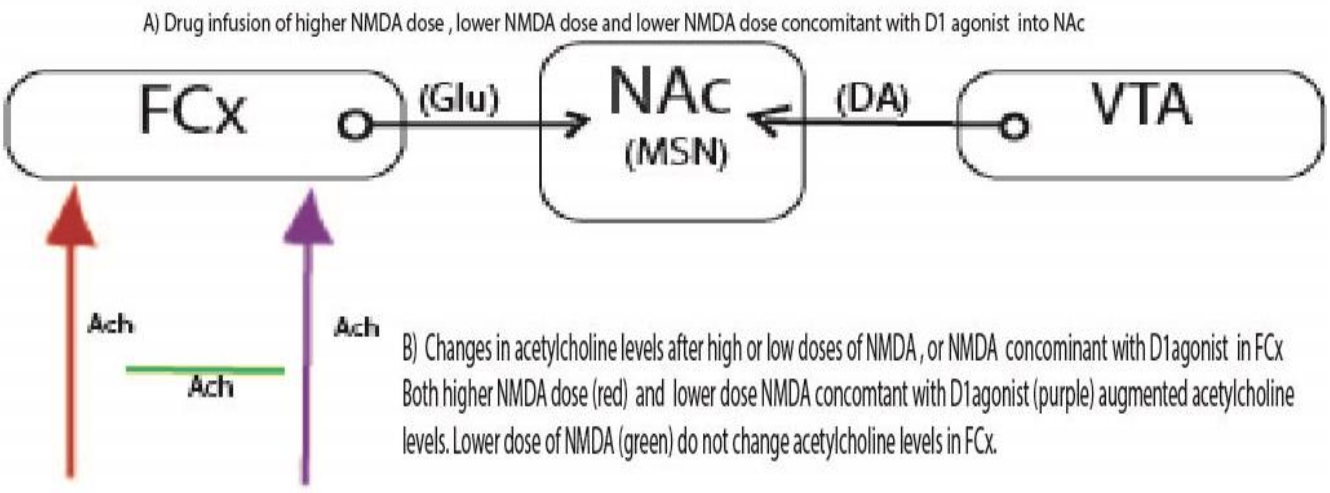


Figure 1.3 NMDA modulation of ACh from NAc to frontal cortex. Higher concentration of NMDA or lower concentration of NMDA concomitant with D1 agonist in NAc enhance acetylcholine release, whereas lower concentration of NMDA does not change ACh release in frontal cortex.

1.6.3.2 Direct application of glutamate does not change dopamine levels in striatum

According to Kulagina *et al.* (2001), extracellular levels of dopamine release are modulated by glutamate in dorsal striatum. Applying intrastriatal injection of kyneurenate, which is an ionotropic glutamate receptor antagonist, caused a decrease in extracellular dopamine release in dorsal striatum. However, intrathalamic infusion of kynurenate did not affect striatal dopamine levels (Kulagina, *et al.,* 2001).

Glutamate injection at a concentration of 1 or 10 mM into striatum did not increase dopamine release but the same concentration of glutamate itself attenuated the kyneurenate-mediated decrease on the resting and increased signals in striatum (Kulagina, *et al.,* 2001). On the other hand, previous studies using microdialysis have shown that intrastriatal glutamate application causes either increase or no change in extracellular dopamine release (Keefe, *et al*., 1992). More importantly, direct application of glutamate into striatum does not change striatal dopamine levels so exogenously applied glutamate agonists could be masked by endogenous glutamate, since the study reports that endogenous glutamate can lead to a tonic excitatory action on dopamine release striatum (Kulagina, *et al.,* 2001). The mechanism of glutamate modulation of dopamine release, hence, involves glutamatergic projections from cortical areas and dopaminergic projections from VTA and substantia nigra in striatal brain regions. However, excitatory inputs to GABAergic and cholinergic interneurons in striatum may mediate glutamate modulation of dopamine release in NAc.

This result shows that glutamate does not affect dopamine release itself in striatum, but that it attenuates the kyneurenate antagonist effect. Therefore, one of the main purposes of glutamate in striatum could be to balance dopamine release, and dysregulation of

glutamate in striatum connected to impairments between dopamine and glutamate balance may underlie some psychiatric illnesses such as schizophrenia (Kulagina, *et al*, 2001). These studies all measured functioning in the dorsal striatum. Although similar processes may occur also in the ventral striatum, we are not aware of any studies which have looked at this specifically. Therefore, an aim of the current work was to investigate glutamatergic modulation of dopamine release in the ventral striatum, specifically NAcS

1.6.3.3 Competetive and non-competitive NMDAR antagonists into striatum

MK801 (dizocilpine) is another non-competitive NMDAR antagonist, similar pharmacologically to PCP and ketamine, which has been shown to increase extracellular dopamine in rat dorsal striatum (i.p. injection) (Miller and Abercrombie, 1996), although a study by Callado *et al.,* (2000) showed no change in NAc dopamine after MK801 in brain slices. However, NMDARs in NAc itself have been shown to play an important role in controlling behaviour. Using the NMDAR antagonist, AP5, Ding *et al.* (2014) showed that injections into NAc led to behavioural impairments in a set-shifting task assessing cognitive flexibility. Specifically, there was an increase in the total number of trials to reach criterion, and the number of errors, indicative of disruption of novel strategy learning or maintenance. However, injections into dorsal-medial or dorsal-lateral striatum had no effect. It could be speculated that the shell may be a richer location in terms of dopamine than core in NAc (Howard *et al.,* 2008). Since impairment of cognitive flexibility is one of the most important symptoms in schizophrenia, this study shows that NMDAR dysfunction in NAc, but not dorsal striatum could be a determinative factor in schizophrenia (Ding *et al.,* 2014). According to Gross and Marshall (2009), dopamine antagonists (SCH23390 (D1), sulpiride (D2)) and glutamate antagonists (AP5 (NMDA), DNQX (AMPA)) reduce the product of the immediate early gene (IEG) c-fos

expression in cortex and striatum (although the authors did not specify which part of striatum), areas which receives major excitatory inputs from other regions of the cortex and from the thalamus. Also, unilateral intrastriatal infusion of dopamine and glutamate antagonists in rats modified methamphetamine-induced behaviours from the characteristic stereotypy to rotating in a direction ipsilateral to the infusion site (Gross and Marshall, 2009). However, when the intrastriatal infusion of AP5, sulpiride and DNQX was applied in striatum, rotational behaviour was observed to be more robust and longer than SCH23390-infused animals (Gross and Marshall, 2009). These results indicate that there is a possible connection between rotation behaviour and IEG expression in striatum which is modified by the antagonist effects at glutamate and dopamine receptors. Hence, dopaminergic afferents from VTA and glutamatergic afferents from cortex are capable of regulating behaviour and gene expression in striatal brain regions (Gross and Marshall, 2009; Zmarowski, *et al.,* 2005).

Synaptic and extrasynaptic NMDARs were shown by using in vitro patch clamp and optical imaging techniques. Garcia-Munoz *et al.* (2014) used *in vitro* patch clamp to study extrasynaptic NMDAR function, in network dynamics. NMDARs located on extrasynaptic membrane and at the synapse also play an important role in excitatory neurotransmission in the regulation of striatal function and contribute to the induction of typical neuronal up and down states in the brain. More precisely, the NMDAR antagonist, AP5 (APV) was used to block NMDAR, and the extrasynaptic NMDAR blocker, memantine (Wu and Johnson, 2015; Savchenko *et al.,* 2016), was used to reduce NMDA activation (Garcia-Munoz *et al.,* 2014). As a consequence, blockade of extrasynaptic NMDARs via memantine reduced overall striatal activity changing striatal network dynamics (Garcia-Munoz *et al.,* 2014). Therefore, extrasynaptic NMDARs should be considered when investigating the NMDAR function since these network dynamics can

be related to dopamine release, and dysfunction of extrasynaptic receptors can play an important role in schizophrenia (Li and Ju, 2012).

Gene deletion of the astrocytic glutamate transporter GLAST (glutamate transporter) cause abnormalities in mice which may be related to schizophrenia (Karlsson *et al.*, 2008). GLAST knockout (KO) mice were used in behavioural tests, such as social and nesting behaviours, anhedonia, sensorimotor gating, instrumental learning and extinction, and discrimination and reversal learning. Results showed that GLAST KO mice displayed deficits in behavioural tests when compared with control mice, which are thought to be related to negative, positive, and cognitive symptoms of schizophrenia (Karlsson *et al.*, 2008). Moreover, LY379268, which is an mGlu2/3 agonist failed to reverse the abnormal effect on GLAST KO mice (Karlsson *et al.*, 2008). Also, chronic administration of LY3414915, another mGlu2/3 antagonist, attenuated the latency on timeout responses in 5-choice serial reaction time test (Amitai and Markou, 2010). Therefore, genetic studies linking abnormal glutamatergic function to behavioural changes which model aspects of schizophrenia also provide support for the view that glutamate is one of the most important neurotransmitters related to schizophrenia.

1.7 Acetylcholine neurotransmission

Cholinergic systems are classified anatomically into three substructures; the first provides innervation from pedunculopontine tegmentum and laterodorsal pontine tegmentum to thalamus and midbrain dopaminergic areas. The second one arises from basal forebrain nuclei projecting throughout the cortex and hippocampus (Dani and Bertrand, 2007), whilst the third is from the cholinergic interneurons in striatum and comprises about 2% of the striatal (ventral and dorsal) neurons (Graybiel, 1990), (Figure 1.4). There are two

main classes of acetylcholine receptor, nicotinic receptors, which are ionotropic, gating sodium, potassium and calcium channels, and muscarinic, which are metabotropic, G-protein coupled receptors. The work described here focusses on the actions of nAChRs.

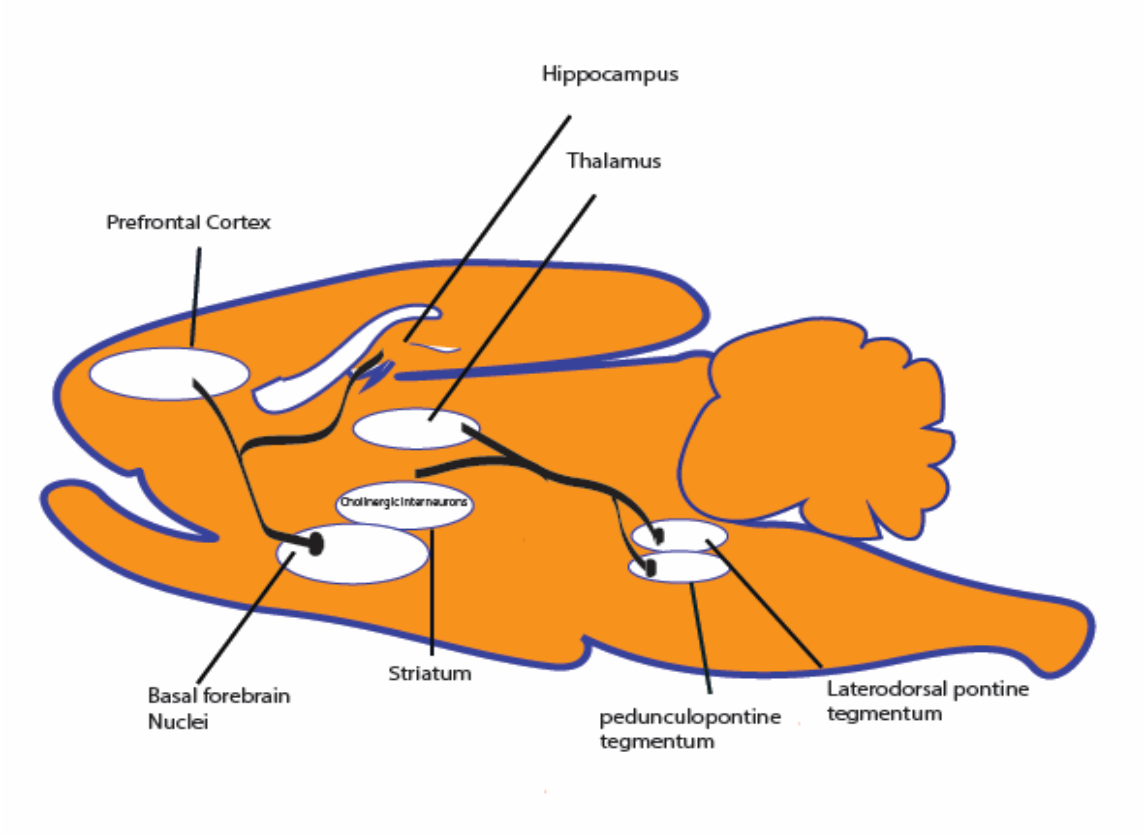


Figure 1.4 Anatomic structure of cholinergic systems. First, innervation from pedunculopontine tegmentum and laterodorsal pontine tegmentum to thalamus and midbrain dopaminergic areas. Second, from basal forebrain nuclei projecting throughout the cortex and hippocampus. Third, cholinergic interneurons in striatum (Dani and Bertrand, 2007; Graybiel, 1990).

1.7.1 Nicotinic acetylcholine receptors (nAChRs)

Nicotinic acetylcholine receptors are a member of the Cys-loop ligand-gated ion channel super-family of receptors (Gotti *et al.*, 2009), made up of five subunits, and possess several subtypes, depending on the subunit composition. The predominant subunits in the central nervous system are the α-subunits (α2 – α6) and the β-subunits (β2 – β4) and

each receptor subtype is classified according to its heteromeric or homomeric compostion: for example α7 are classed as homomeric receptors, while α4β2 nAChRs are classified as heteromeric receptors (Dani and Bertrand, 2007). However, α4β2 nAChRs possess two distinct stoichiometries, (α4)2 (β2)3 and (α4)3 (β2)2, each of which displays functionally different properties, especially with regards to agonist sensitivity, having high (α4)2 (β2)3 and low (α4)3 (β2)2 agonist sensitivity to action of nicotine (Nelson *et al.,* 2003). Moreover, high sensitivity receptors are relevant to volume transmission rather than wired transmission (Nelson *et al*., 2003; Sarter *et al*., 2009). However, it remains elusive which stoichiometry of high (α4)2 (β2)3 and low (α4)3 (β2)2 occurs in striatum (Exley and Cragg, 2008). Although α4β2 nAChRs have distinct stoichometries, they still cooperate between wired and volume transmissions, which indicates that they both work together in the central nervous system (Sarter *et al.,* 2009). Postsynaptic and nonsynaptic receptors modulate excitatory synaptic transmission. However, fast nicotinic transmission in this area is difficult to detect suggesting that cholinergic volume transmission is more prevalent (Dani and Bertrand, 2007). In fact, fast nicotinic transmission is not a major excitatory mechanism in the mammalian brain, occurring only at low densities in neuronal areas (Aramakis and Metherate, 1998). nAChRs play an important role in learning and memory (Ge and Dani, 2005). Even if there is not a major excitatory mechanism, the β2 subunit of nAChRs modulates excitatory activation on midbrain GABA projection neurons and interneurons (Mansvelder *et al.,* 2002). Pharmacological manipulations, especially dose response profiles, are beneficial in the determination of how nicotinic agonists and antagonists, when administered exogenously, alter neurotransmitter activation and its own activation features. α4β2 nAChRs have a higher affinity compared to the α7 subunit because of the kinetics for nAChR activation; desensitization is affected by the exact amino acid sequence of the

α4β2 and α7 subunits: sequences in the M3-4 loop show diversity between receptors which resulted changes in trafficking, protein sorting, and membrane insertion between α4β2 and α7 subunits (Stokes *et al.,* 2015). For example, the α4β2 nAChRs desensitize with slower kinetics even if they have a higher affinity feature than rapidly desensiting α7 nAChRs (Dani and Bertrand, 2007). nAChR expression is quite diffusely spread across brain regions, which makes it more complicated to understand its effect on cognition and behaviour. Dopamine neurons express nAChR subunits such as α6, α5, α4, and β4,β3, B2 in the VTA, with subunits of α3, 4,5 ,6, α7 and β2, b3with β2* nAChRs expressed on dopamine neurons of SN (Azam *et al.,* 2002; Klink *et al*., 2001). However, there is a diversity which is detected between axon terminals and somatodentritic areas of dopaminergic neurons. For example, β4 and α7 are found in VTA, but not on dopamine axon terminals in striatal synaptosomes. However, the β2 subunit of nAChRs is expressed on striatal axon terminals in rodents (Salminen *et al.,* 2004). This anatomical diversity between nAChR subunits on dopaminergic neurons also causes considerable diversity in behavioural situations. Therefore, the subregions of striatum require evaluation by considering these structural differences with regards to dopamine release. The striatum is divided into two main structures; the dorsal striatum is made up of the caudate nucleus and putamen, but the ventral striatum is made up of the ventral conjunction of caudate and putamen, NAc, and part of the olfactory tubercle (Wilson, 1998). Dopaminergic areas in the midbrain receive glutamatergic and GABAergic inputs, with the main cholinergic afferents originating in the pedunculopontine tegmentum and the laterodorsal pontine tegmentum (Woolf, 1991). Therefore, sensitivity and affinity studies of nAChRs provide information about their distinct mechanisms. In addition to this contribution as to their distinct mechanisms, sensitivity and affinity studies also contribute to how such distinct mechanisms regulate the synaptic neurotransmission via influences on direct or indirect

pathways on other neurotransmitter activation mechanisms in the mammalian brain. To sum up, nAChRs have a diverse anatomic and physiological mechanism in midbrain dopaminergic systems. Their subunits shows different distributions between dopaminergic axon terminals in striatal brain areas and VTA. Diverse subunits of different sensitivity and affinity nAChRs, however, work together in central nervous system via wired and volume transmission. Therefore, in our study we assessed whether glutamatergic modulation of dopamine signalling involves β2 and α7 controlled mechanisms, and if so, whether PCP pretreatment changed such modulation.

1.8 Interaction between glutamatergic and cholinergic mechanism in attention

Acetylcholine release is related to attention processes in the mammalian brain (Hahn *et al.,* 2003). As mentioned above, nAChR activation is capable of regulating glutamate transmission. However, it is not known whether glutamate release modulates cholinergic signalling in attention processes and, if so, whether and how they differ among the different subunits of cholinergic receptors. According to Parikh *et al*., (2010) the β2 subunit of nAChRs is necessary for the regulation of glutamate and acetylcholine activity, an activity which is equated to peak amplitudes and signal decay rate obtained by pressure ejections of two doses of α7 nAChR agonist A-582941 (200 pmol and 2 nmol), whilst the amplitude of glutamatergic and cholinergic activity controls the cholinergic activity. However, in the absence of an α7 subunit, it is not necessary to generate maximum signal amplitudes of glutamatergic and acetylcholine transients. Moreover, Parikh *et al.,* (2010) investigated cholinergic regulation to estimate any possible relationship to α7 receptor activation. Results showed that acetylcholine release augmented by α7 agonists, is not changed after 6-OHDA infusion into VTA which removes the dopaminergic inputs from

the VTA to the prefrontal cortex. This shows that different neuronal mechanisms can control dopamine release activation, rather than the α7 subunit of nAChRs. Cholinergic receptor activation is known to play an important role in cue detection (Cragg, 2006). However, it is not clear how glutamate-acetylcholine interaction regulates dopamine release. The α7 subunit does not seem to regulate dopamine release directly after glutamate release. However, the β2 subunit might be a more promising subject of investigation in terms of its effect on dopamine regulation after glutamate release because the dopamine release increased by α7 subunit of nAChRs remained unaffected by the removal of dopaminergic inputs, whilst the β2 subunit did not (Parikh *et al.,* 2010; Dickinson *et al.,* 2008).

The subunit differences in nAChRs described above are important in modulating acetylcholine-glutamate activities which is of vital importance in understanding dopaminergic regulation of attentional processes. However, different nAChR subunits are capable of regulating neurotransmission via distinct cellular mechanisms. Dickinson *et al.,* (2008) investigated α7- and β2-containing nicotinic acetylcholine receptors and showed that both modulate glutamate synaptic transmission in prefrontal cortex synaptosomes via different pathways. Compound A, which is a drug being used as an α7 agonist, augmented endogenous glutamate release triggered by Ca^{2+} influx through the α7 nAChRs, an effect which could not be reversed by DHβE, a β2 subunit selective antagonists. Moreover, voltage open Ca^{2+} channel blocker, Cd^{2+}, did not affect the responses to compound A, which shows that the predominant route of Ca^{2+} entry is via stimulation of the α7 nAChR (Dickinson *et al.,* 2008). These results are contrary to the study by Parikh *et al.,* (2010), mentioned above, in which they showed that the α7 nAChRs subunit did not regulate glutamate activation. However, Dickinson *et al.,* (2008) brought a new debate to the subject of α7 nAChRs by investigating its distinct

mechanisms. In contrast to α7 nAChRS, β2-containing nAChRs use a separate and distinct pathway to modulate glutamatergic synaptic transmission. According to Dicksen *et al.,* (2008), compound 5-I-A-85380, an α4β2 agonist, is not influenced by Ca^{2+} channel blockers, but instead by voltage open Ca^{2+} channels; this is the opposite to what is seen in α7 nAChRs activation, where compound A was not reversed by voltage open Ca^{2+} channel blockers. Thus, these results indicate that different nAChR subunits contribute differently to glutamatergic release through distinct Ca^{2+}-dependant pathways in prefrontal cortex synaptosomes. Therefore, allosteric modulators and ligand studies shed light on the putative glutamate-acetylcholine interaction which represents an important physiological mechanism underlying attention processes which may be relevant for understanding dysfunctions in people with schizophrenia. To what extent dysfunctional glutamatergic mechanisms are brought by subchronic PCP pretreatment changes in endogenous acetylcholine signalling was examined in current study (see chapter 2 and chapter 4). Specifically, whether dysfunctional glutamatergic mechanisms caused by PCP pretreatment could affect acetylcholine modulation of electrically stimulated dopamine signalling in NAc shell region was examined.

1.8.1 Electrophysiological properties of NMDA receptors

NMDA receptor activation is Ca^{2+} dependent and Mg^{2+} sensitive (Takasu, *et al.,* 2002; Momiyama *et al.,* 1996). Mg^{2+} sensitivity of NMDARs has been investigated by Oswald *et al.,* (2015); long-term potentiation, which is related to glutamate release regulation, is achieved by removing Mg^{2+}, which removes the Mg^{2+} block on NMDARs and thus allows Ca^{2+} influx through voltage-gated Ca^{2+} channels. Moreover, Cull-Candy (2004) and Paoletti *et al.,* (2013) investigated the importance of Mg^{2+} in NMDAR mediated

cholinergic regulation. According to Paoletti *et al.,* (2013) cholinergic interneurons express the GluN2D subunit of NMDAR. The presence of the GluN2D subunit at cholinergic neurons contributes to NMDARs with a low conductance state, low sensitivity to Mg^{2+} blocking (Cull-Candy, 2004; Paoletti *et al.,* 2013). Ionic conductance mechanisms may be modulated via a PCP-NMDAR antagonist, and this modulation triggers the release of dopamine and ACh in NAc (Jones *et al.,* 1987). nAChRs also create different inward rectification from other channels, such as nAChRs allowing Ca^{2+} influx at highly negative membrane potentials, leading to a strong driving force for inward current (Jones *et al.,* 1987) which has been attributed to intrinsic voltage dependence, block by internal Mg^{2+}, or a combination of both (Sands and Barish, 1992) . However when it comes to NMDA, Mg^{2+} blocks NMDA until the membrane is depolarized; after that, the depolarizing voltage leads to Mg^{2+} being driven out of the channel. As a result of this process, NMDA administers a current which is followed by Ca^{2+} influx at depolarized potentials (Dani and Bertrand, 2007). However, the effect of PCP pre-treatment on NMDAR activation is, as yet, unknown. According to Jones *et al.,* (1987) dopamine release after NMDA stimulation is Mg^{2+} sensitive. In other words, Mg^{2+} inhibits dopamine release stimulated by NMDA in NAc; moreover, ACh release is also inhibited by Mg^{2+} in NAc. NMDAR subtypes could be related to the inhibition of excitatory amino acid-induced dopamine and ACh release in NAc (Jones *et al.,* 1987). Chronic PCP pre-treatment may cause sensitization of NMDAR activation, which directly affects dopamine release in NAc slices. This study does not examine how long-term PCP administration affects NMDAR activation. In other words, it is not clear how chronic PCP pre-treatment affects Mg^{2+} sensitive NMDAR activation and their subtypes in NAc. These results show that Mg^{2+} sensitivity and Ca^{2+} dependence are both vital to an understanding of the cholinergic-glutamatergic mechanism. However, there are not

many studies which focus on the NMDA subunit in order to estimate cholinergic interaction. Modulation of cholinergic interneurons may be more dependent on the NMDAR subunit than NMDA itself. Whilst NR2D protein distribution is observed in cholinergic interneurons, NR2C is not expressed in those neurons in dorsal and ventral striatum (Bloomfield *et al.,* 2007). Moreover, immunolabeled neurons show ultrastructural characteristics of cholinergic interneurons (Bloomfield *et al.,* 2007). These results are important in terms of understanding how the NRD2 subunit of NMDARs could modulate cholinergic interneurons via glutamatergic afferents.

1.8.2 PCP treatment cause behavioural changes via cholinergic mechanisms

There is evidence that cholinergic mechanisms play a critical role in the modulation of cognitive abilities and motor behaviours, and that changes in ACh signalling may modulate attentional deficits in schizophrenia (Timofeeva and Levin, 2011). It is clear that the brain activity of people with schizophrenia differs from that of normal people. However, there is a conflict amongst studies in this area, some of which claim that the brain activity of people with schizophrenia increases during the attention test (Karch *et al.,* 2009), while another study suggests that brain activity is reduced (Carter *et al.,* 1998). Although glutamate function is reduced in accordance with glutamate theory in schizophrenia, it is not clear whether the interaction between glutamate and ACh regulates attentional processes. ACh signalling acting at nAChRs could be important in the evaluation of cognitive functions that involve attention. The NOR test measures attentional processes in experimental animals which model human attention (Neill, 2010). PCP is an NMDAR antagonists which disturb cognitive abilities have been used in animal studies as these provide a model to investigate cognitive deficits in

schizophrenia after PCP pre-treatment. However, the underlying factors of schizophrenia-like behaviours are unknown after PCP pre-treatment on animals. Studies provide evidence for nAChRs playing a role in cognitive abilities. For example, PCP-induced deficits in NOR are reversed by an α7 nAChRs agonist (A-830) and an α4β2 agonist (PNU-282987) (Miyauchi *et al.,* 2015). However, neither the β2 nAChR antagonist, DHβE, nor the α7 nAChR antagonist, Methyllycaconitine citrate (MLA), affect performance during the NOR test. In fact, it has been shown that DHβE plus lurosidone-AAPD produced a marginal reversal of the PCP effect on discrimination index in the NOR test (Miyauchi *et al.,* 2015).

1.9 Dopamine and acetylcholine modulate synaptic transmission frequency of depolarization-dependant activity

1.9.1 Evidence from FSCV studies

Electrical stimulation is mediated through both dopamine axons and all other passing fibres. (Shin *et al.,* 2015). Electrical stimulation also may activate other neurotransmitters and metabolites, such as nAChRs and muscarinic acetylcholine receptors (mAChRs) which can cause an increase or decrease in dopamine release. ACh has an opposing effect on dopamine regulation through its two main receptor types; nAChR and mAChR. That is dopamine release is enhanced by nAChR activity and reduced by mAChR activity (Kemel, 1992), although mAChR do not express on dopaminergic axon terminals. However, Threlfell *et al.,* (2010) claim that release probability is dependent on the frequency of depolarization of presynaptic activiy. In other words, single pulses leads to inhibition of dopamine release after activation of striatal mAChRs, but burst-like frequency of presynaptic activity which resulted in phasic dopamine activation rather

tonic-like frequency of presynaptic activity which resulted in tonic dopamine activation led to an increase in dopamine release probability. By contrast, reducing nAChR activity can increase dopamine release probability after bursts of dopamine neuron activity (Cragg, 2006). These studies show that pulse-dependant dopamine release is regulated by both mAChRs and nAChRs. Action potential-dependant dopamine release is regulated by cholinergic interneurons via nAChRs. The extent of the importance of the role played by subunits of nAChRs in pulse-dependant dopamine release is uncertain. As mentioned above, α7 and α2β4 homomeric and heteromeric subunits of nAChRs regulate glutamate activation at distinct pathways, and such a condition may also be valid for dopamine regulation. This idea is supported by Zhou *et al.,* (2001), according to whom dopamine release is substantially decreased by using DHβE, a β2 nAChR antagonist in dorsal striatum and NAc using a mutant lacking the β2 subunit. In order to interpret this finding, cholinergic interneuron activation seems important, and ACh release that acts on different subunits of nAChRs may regulate dopamine release in different pathways and brain sub-regions. ACh release is determined by tonic activation of cholinergic interneurons (Aoskia *et al*., 1995), and releasing ACh increases dopamine release (Zhou *et al.*, 2001). However, it is still unknown how endogenous ACh release regulates dopamine activation. Fast scan cyclic voltammetry (FSCV) with carbon-fiber electrodes can be used to monitor pulse-dependant dopamine release in brain slices, hence stimulating brain slices activates other neurotransmitters and increasing dopamine release probability. These results show a depolarisation-frequency dependent cholinergic regulation of dopamine release in striatum at both nicotinic and muscarinic acetylcholine receptors.

1.9.2 Firing modes regulates dopaminergic neurons activation

Striatal tonically active neurons correspond to ACh interneurons which are important to environmental learning, whose interaction happens with midbrain dopamine neurons at the post and presynaptic level (Morris *et al.,* 2004; Calabresi *et al.,* 2000). Cholinergic interneurons constitute about 1-2 % of the neuronal population of the striatum, but their axonal fields are quite comprehensive and have broad dendritic trees compared to projection neurons (Graybiel, 1990; Calabresi *et al.,* 2000). However, it is unknown how ACh is able to modulate dopamine transmission in the striatal brain regions. The striatum is made up of various neurochemicals compared to other brain regions, with medium spiny neurones (MSN) constituting about 90 % of striatal neuron population, and hence the interaction between ACh and dopamine occurs at the MSN dentritic spine. Moreover, MSN are innervated by glutamatergic fibers from cortex and thalamus (Mitrano *et al.,* 2010). Therefore, MSN includes an association between glutamatergic and cholinergic inputs (Calabresi *et al.,* 2000). Ventral striatal regions of the striatum are related to limbic function with dopaminergic afferents from the VTA (Haber *et al.,* 2000) and glutamatergic afferents from the prefrontal cortex, amygdala, and hippocampus (O`Donnell and Grace, 1995). Cognitive and behaviourally-related changes in dopaminergic neurons are dependent on the firing modes, which are tonic firing and burst firing. For example, having rewards and encoded information about the prediction of rewards is accompanied by a change from tonic firing (does not occur in bursts) to burst firing in mesolimbic dopamine neurons (Morris *et al.,* 2004; Schultz, 2002). In addition to dopaminergic neurons, cholinergic interneurons play an important role in learning associated with unexpected events (Schultz, 2002). However, both of these neuron types have opposing effects in terms of excitability of the striatal output neurons (Kemel, 1992; Morris *et al.,* 2004). In order to understand the interaction between cholinergic and

dopaminergic activation, using antagonists whilst applying real-time electrochemical detection on striatal slices could be useful. Studies show that application of nAChR antagonists reduced initial dopamine release probability and subsequently relieved short-term depression, indicating that the short-term depression of the release probability at short interpulse intervals is related to dopamine release (Rice and Cragg 2004; Abeliovich *et al.,* 2000).

1.9.3 The balance between dopamine and acetylcholine

The balance between dopamine and ACh plays an important role in normal behaviour. However, dysregulation of this balance can result in several mental disorders and other behavioural deficits (Hoebel *et al*., 2007). These authors summarised a few situations which have outcomes dependent on the imbalance in dopamine and ACh, such as feeding and satiety, conditioned taste preference and aversion, hypothalamic self-stimulation and stimulation-escape, motivation and depression, drug self-administration and drug withdrawal, sugar bingeing and its opiate withdrawal state. However, it is unknown how disruption of the dopamine and ACh balance after glutamate dysregulation affect schizophrenia. The non-competitive NMDA antagonist, PCP, exacerbates psychotic symptoms in people with schizophrenia and causes schizophrenia-like behaviours in rodents. However, increasing dopamine release after chronic PCP pre-treatment may not target glutamate function in NAc directly; rather, it may act indirectly through cholinergic systems. In order to understand how cholinergic receptor activation is affected by PCP pre-treatment, glutamate and dopamine interaction need to be thoroughly understood. However, it is still not clear how the glutamate-dopamine interaction is affected in schizophrenia. In next section, the glutamate and dopamine interaction will be discussed

to examine how cholinergic receptors may influence dopamine release after PCP pre-treatment in animals

1.10 Dopamine and glutamate interaction

Dopamine receptors are G-protein receptors, with D1 receptors couple to Gs, and adenylyl cyclase activation increases the cAMP which trigger PKA actuation via PKA phosphorylation of DARP-32, but the same process causes the opposite activation of D2-Gi/o receptors (Beaulieu and Gainetdinov, 2011; Greengard, 2001). On the one hand, D1 receptor activation enhances NMDAR responses, which occurs after adenylyl cyclase activation (Synder *et al.,* 1998).

Moreover, the age of the animals used is an important issue that should be taken into consideration in terms of D1-NMDA interaction, which is still controversial in the literature. On the other hand, D2 class dopamine activation decreases NMDA transmission via a platelet-derived growth factor followed by mobilization of intracellular Ca^{2+} and Ca^{2+}-dependant inactivation of NMDARs (Kotecha *et al.,* 2002), indicating that NMDARs are regulated by dopamine activation in the brain. This study indicates that there is an interaction between dopamine and glutamate upon G-protein activation which, as mentioned above, determines dopamine activation in terms of NMDA responses, but it does not indicate how the interaction regulates ACh release or vice versa. Studies which try to determine underlying factors of schizophrenia usually consider either the dopamine theory or glutamate theory, but the evidence presented above suggest that ACh is critically involved in dopamine–glutamate interactions. However, these theories usually fail to account for any effects of ACh activation, which results in some drawbacks in the

investigation of dopamine and glutamate theories, such as in experimental and research design, the design of proper research questions and investigations on animals. However, studies on schizophrenia have still not found any effective pharmacological treatments and the underlying factors of this disease are still, to date, unknown. Therefore, investigations into the role of ACh, in particular the nAChRs subunit of the cholinergic receptors in dopamine-glutamate dysregulation in schizophrenia, are important and still elusive. We proposed that dopamine signalling is modulated by NMDARs and that this modulation involves nAChRs activation. Moreover, we suggest that this modulation is dysregulated through PCP pretreatment. The experiments presented here tested these hypotheses using both *in vitro* and *in vivo* measuring techniques.

1.10.1 Excitatory amino acid dysfunction after NMDA antagonist application - microdialysis study

Excitatory amino acid dysfunction, induced by providing sub-chronic PCP treatment, causes schizophrenia-like behaviour in rodents and changes in brain configuration. N-Acetylaspartic acid (NAA) is the acetylated form of the amino acid aspartate (Moffett *et al*, 2006). Previous magnetic resonance spectroscopy (MRS) studies showed a correlation between the reduction in NAA and neurodegenerative disease (Rowland *et al*., 2001). However, the amount of NAA seems region-dependent in the brain. Reynolds *et al.* (2005) carried out high performance liquid chromatography (HPLC) experiments to show how chronic PCP pretreatment can cause attenuation NAA and its precursor N-acetylaspartylglutamate (NAAG) in rodents. Results show that significant reduction is only found in the temporal cortex after PCP treatment compared to the hippocampus, frontal cortex, and striatum for both the NAA and NAAG. These results show that amino acid changes induced by PCP pretreatment show differences in the rodent brain. The

precise role of NAA and NAAG in the brain are not fully understood, but it has been suggested that they may have a neurotransmitter function by acting on mGluRs (Yan *et al.,* 2003). Therefore, since mGluRs modulate dopamine and glutamate release, we investigated NMDA modulation of dopamine release and assessed as to whether mGluRs are involved in this modulation in NAcS in present study (details in chapter 2).

However, D-serine and glycine can be effective treatments for schizophrenia because the amino acids seem promising in the reversal of increasing dopaminergic function which results from glutamate dysfunction in the brain. To support this idea, Javitt *et al*. (2004) carried out an experiment to assess the effects of long term glycine treatment on dopamine neurotransmitter changes induced by chronic PCP administration by using microdialysis. In these studies they did not see any significant difference between a regular glycine diet and higher (16 %) glycine diet. However, subchronic PCP treatment caused an increase in amphetamine-stimulated dopamine release in both FCx and dorsomedial striatum in animals receiving a regular diet, which was not present in those receiving a high-glycine diet (Javit *et al*., 2004). Moreover, potentiation of amphetamine-stimulated dopamine release was reversed by glycine treatment in both prefrontal cortex (PFC) and dorsomedial striatum (Javit *et al*., 2004). The glycine transport inhibitor, N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)-propyl]sarcosine (NFPS), also reduced amphetamine-induced dopamine release in this study (Javit *et al*., 2004). According to Javitt *et al*., (2004) neither sustained increase in basal dopamine levels nor change in baseline dopamine levels in PFC for NFPS agent. However, the study claims that disturbances in absolute extracellular dopamine levels could occur independently. The time of pretreatment shows different neurochemical and behavioural consequences in animal studies. For example, Castane *et al*. (2015) used in C57BL/6J mice and found that, basal extracellular concentrations of dopamine in dialysate samples of medial PFC

showed significant differences in subchronic PCP pretreated groups when compared to acutely treated and saline-pretreated groups. Moreover, systematic PCP challenge caused an increase in extracellular levels of dopamine in medial PFC in the subchronic PCP pretreated group, but not the acute or saline groups. However, NOR test results showed a significant decrease in discrimination index only in the subchronic PCP treatred group (Castane *et al*., 2015). Preclinical studies show that an NMDA antagonists such as PCP and ketamine potentiate dopamine release in rodents. However, MRI-directed *in vivo* microdialysis studies on monkeys showed that PCP and ketamine did not stimulate dopamine release (Adams *et al*., 2002). These results contrast to rodent studies in terms of use of those drugs on subsequent extracellular dopamine changes. Further, even in the presence of the DAT inhibitor, nomifensin, PCP was ineffective (Adams *et al*., 2002), showing that there is no correlation between dopamine neurotransmission and NMDA antagonism in the ventral striatum in monkeys. It should be keep in mind that different species may give different results. To compare this results, we used rodents in our experiments in order to detect whether measurable dopamine changes occurred in subchronic PCP pretreatment in rats.

1.10.2 Acute phencyclidine change dopamine and its metabolities' activation in the cortex and NAc : microdialysis study

PCP acts as non-competitive NMDA antagonist, but it is not clear that the antagonist mechanism of PCP changes the extracellular dopamine levels. Additionally, PCP increases dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) levels in NAc via action on the VTA dopamine cell bodies (Steinpreis and Salamone, 1993). Studying the interaction between PCP and dopamine and its metabolites it was found that PCP (4.0 mg/kg) significantly increases dopamine and DOPAC and HVA levels;

however, this observation seems to be dose-dependent because PCP (8.0 mg/kg) did not change DOPAC levels (Steinpreis and Salamone, 1993). Moreover, locomotor activation accompanied increases in dopamine and its metabolites in NAc. In order to assess whether dopamine depletion can reverse PCP effects on social behaviour, rats were injected with the catecholamine specific neurotoxin, 6-hydroxydopamine (6-OHDA), or ascorbic acid as control. Although 6-OHDA altered motor behaviours, there was no significant effect of dopamine-depletion on social behaviour, which means 6-OHDA did not reverse PCP-evoked social behaviour (Steinpreis and Salamone, 1993). These results show that PCP changed neurochemical function and behaviour by acting in the NAc. However, it is not clear how PCP changes levels of dopamine and its metabolites in the other brain regions. There are several theories discussed in the literature in an attempt to clarify its effect on the brain, such as that PCP acts on dopamine neurons directly or acts as a non-competitive antagonist on NMDARs. (Doherty *et al*., 1980; Hitzemann *et al*., 1973; Jones *et al.*, 1987; Jones *et al*., 1987). Future studies will bring new approaches to the understanding of the PCP mechanism, which could potentially shed light on the understanding of the physiological mechanisms underlying factors in psychosis.

NMDA mediated cortical dopamine metabolism may involve inhibitory GABAergic interneurons on dopaminergic terminals. PCP blocked NMDAR on GABAergic interneurons which may result in attenuation of GABA inhibitory effect which, in turn, enhanced dopaminergic transmission (Carlsson, 1988). However, variations in dopamine release depend on the administration of PCP and its localisation in the brain. Sershen *et al*. (2008) shows that acute PCP administration does not change dopamine levels in the dorsomedial striatum in microdialysate sample levels compared to the PFC in rats, which could be a result of dopamine reuptake inhibition. Dopamine reuptake inhibition by DAT also occurred in dose-dependent amphetamine, challenging the idea that dose-

dependent amphetamine increases extracellular dopamine release in the PFC and dorsomedial striatum. However, extracellular diffusion and slower clearance in cortex is more significant than in the dorsomedial striatum (Sershen *et al*., 2008). These experimental results show that chronic PCP administration increases amphetamine-induced dopamine release in the prefrontal cortex but not the striatum. They suggest that NMDA agonists modulate dopamine release through an effect on GABAergic feedback and activation of presynaptic GABA receptors. Therefore, we investigated in our experiments whether NMDA modulation of dopamine release activates GABAergic interneurons, which, in turn, change dopamine signalling via giving GABAergic feedback mechanism on dopaminergic axon terminals in nucleus accumbens.

PCP enhanced dopamine release in cortex, but not striatal brain regions is supported by Hondo *et al.* (1994) who investigated the effects of PCP and MK-801(another non-competitive NMDAR antagonist) in the PFC. Systematic injection (7.5 mg/kg) or local perfusion (100 µM and 500 µM) of PCP dose-dependently increased extracellular dopamine levels in medial PFC in HPLC analysis. Local perfusion of PCP also increased dopamine levels but not DOPAC levels. However, local perfusion of PCP caused a biphasic effect of an initial extracellular DOPAC-like increase, followed by a decrease in the NAc (Steinpreis and Salamone, 1993). This result may show a biphasic effect of PCP perfusion into the PFC and NAc. Increasing dopamine levels by local perfusion could be the consequence of its direct action on the terminal field of the mesocortical dopamine neurons (Hondo *et al*., 1994). On the other hand, MK-801 increased dopamine release in a dose-dependent manner after infusion into the PFC (Hondo *et al*., 1994).

This study shows that there is a glutamate dopamine interaction in the frontal cortex, but aforementioned microdialysis studies failed to show acute PCP enhanced dopamine release in striatum. Any dysfunction between these neurotransmitters can cause

hyperlocomotion and stereotyped behaviour in rats, which brings a useful animal model to investigate schizophrenia in humankind. However, with increases in dopamine metabolism, it is not clear whether it is the resultant serotonin effect which can affect the dopamine system. In this study, PCP was administrated via acute intraperitoneal injection and dopamine and its metabolites were analysed using HPLC. Results show that systematic administration of PCP (10 mg/kg) increased DOPAC and HVA but not the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in frontal cortical dialysis. However, tetrodoxin (TTX) reversed PCP-increased dopamine metabolites by blocking the action of PCP in the PFC (Tanii *et al*., 1990). PCP did not change serotonin metabolism, which shows that there is not any significant correlation between PCP and serotonin metabolism on dopamine metabolism in the frontal cortex. Therefore, PCP action on dopamine metabolism should rely on other mechanisms rather than serotonin. NMDA could be a suitable candidate to investigate the relationship between PCP and doapmine metabolism because PCP is well known as an NMDAR antagonist, and PCP can reduce excitatory amino acid function in the frontal cortex, which can cause an increase in dopamine metabolism activity mediated by a PCP-NMDA antagonist (which causes uptake inhibition and maintains dopamine release). These studies indicate that acute PCP injection causes changes in dopamine levels: however, chronic PCP treatment and its possible effects are not mentioned in this study. Therefore an aim of our studies was to assess whether dopamine and its metabolites changed after sub-chronic PCP, using brain microdialysis, and HPLC analysis of brain homogenates.

D2 receptor activation can decrease extracellular dopamine concentrations. The D2 receptor agonist, quinpirole in PFC, caused a decrease in spontaneous motor activity in a dose-dependent manner, but its effect was blocked by the D2 antagonist, raclopride (Del Arco *et al*., 2007). Moreover, PCP-induced motor behaviour effects were reduced by

quinpirole in PFC. These results showed that motor behaviour can be changed via D2 receptor activation in PFC. In addition to motor activity, neurochemical activity can also be disturbed via D2 receptor activation. HPLC analysis of analyte samples collected from the NAc of acute PCP (5 mg/kg)-treated rats, when treated with microinjections of quinpirole in PFC showed increases of extracellular concentrations of ACh and the dopamine metabolites, DOPAC and HVA, but not of dopamine itself, an effect of which was reversed by quinpirole on HVA, DOPAC, and acetylcholine levels in the NAc (Del Arco *et al.*, 2007). Escobar *et al*., (2015) showed that horizontal locomotor activation was induced in quinpirole pretreated rats by quinpirole challenging. However, microdialysis studies show that extracellular dopamine decreased in NAc by quinpirole treatment, which indicated that quinpirole-increased locomotor activation by repeated activation of the D2 receptor was also accompanied by an attenuation of dopamine levels. In addition to microdialysis studies, phasic dopamine levels were also assessed in quinpirole treated rats. Acute quinpirole administration caused a decrease in electrically evoked dopamine release both in control and quinpirole-sensitized rats, but quinpirole injection induced an immediate decrease in dopamine levels in quinpirole-sensitized rats (Escobar *et al*., 2015). These contradictory results obtained from quinpirole challenged microdialysis experiments show that antagonism of D2 receptors attenuated dopamine levels in normal animals, but not in PCP pretreated animals, adding further support to the idea that PCP treatment is capable of changing dopamine signalling.

In addition to decreased dopamine levels in the NAc, glutamate levels were also investigated in this study. According to Escobar *et al*., (2015) glutamate levels as well as dopamine levels decreased after pretreatment of quinpirole. In order to prevent $GABA_B$ receptor activation, the $GABA_B$ antagonist, CGP 52432, was used and basal and K^+ (70 mM)-stimulated extracellular glutamate levels were assessed in both presence and the

absence of CGP 52432. Results showed that glutamate levels in either the presence or absence of CGP 52432 were unchanged after perfusion of 70 mM K^+ into the medial PFC. However, perfusion of 110 mM K^+ into the medial medial increased glutamate levels in saline pretreated rats compared to challenge with quinpirole into NAc. (Escobar *et al*., 2015). These two study using microdialysis and FSCV show that repeated activation of D2 receptors by injection or perfusion of quinpirole cause a significant decrease in dopamine and medial PFC glutamate neurotransmission to the NAc. More generally, it seems that PCP has an effect on dopamine and its metabolites, and on motor behaviours, by activating D2 receptors. However, it is not clear how PCP causes changes in dopamine systems as yet.

Given the body of evidence for dopaminergic systems dysregulation in schizophrenia, current pharmacological treatment focus on dopaminergic receptors. However, antipsychotic drugs which target D2 receptors only relieve positive symptoms and also cause substantial side effects. Hence, the dopamine theory does not explain whole picture of this disease as it fails to account for negative and cognitive symptoms. On the other hand, glutamate dysfunction, mimicked by the NMDAR antagonist, PCP, seems to account for the full spectrum of core deficits in schizophrenia leading to the view that perhaps dysregulation of glutamate-dopamine signalling is important in the origin of schizophrenia. In fact, acetylcholine, which is known to modulate dopamine release in dorsal and ventral striatum (Threlfell *et al.*, 2012) also shows changes after PCP pretreatment. However, it still remains elusive whether glutamate modulation of dopamine release by cholinergic systems is dysfunctional in schizophrenia. Therefore, in this project the research question focused on studying the mechanisms through which NMDAR modulation of dopamine release is mediated, with a particular focus on the role of nicotinic cholinergic systems, and how this may be altered after sub-chronic PCP

pretreatment, in a rat model of schizophrenia. Since the mesolimbic pathway projecting from VTA to NAc, has been implicated in many of the behaviours which are disrupted in schizophrenia, studies focused on mainly NAc and aimed to measure changes in processes modulating dopaminergic signalling after PCP pretreatment, with a view to understanding neurochemical changes underlying schizophrenia.

1.11 Aims and objectives

The specific aims addressed by the experiments presented in this thesis are:

- To characterise neurochemical mechanisms controlling dopamine release in NAc, focussing on NMDAR and nAChR mediated processes
- To assess the effect of PCP pretreatment, modelling schizophrenia, on these mechanisms
- To measure the effect sub-chronic PCP on overall changes in brain activation due to increased dopamine function and to nAChR activation

To achieve these aims, the studies employed a multidisciplinary approach using FSCV, brain microdialysis, HPLC analysis of post mortem tissue, NOR testing and pharmacological magnetic resonance imaging (phMRI) in non-treated and PCP pre-treated rats.

In chapter 2, experiments measured NMDA modulation of potassium-stimulated dopamine release in brain slices *in vitro*, using FSCV. Although changes were seen, technical issues causing substantial variability in measurements, even in control (non drug treated) slices made interpretation difficult.

The studies described in Chapter 3 followed on from those in Chapter 2, and used a more focussed electrical stimulation (instead of potassium-stimulation) to measure the effect of NMDA modulation of stimulated dopamine release. Electrical stimulation has a number of advantages over potassium stimulation which are discussed. Having characterised NMDAR modulation of electrically stimulated dopamine release further experiments assessed other neurochemical mechanisms involved in the modulation,

including GABAergic and, particularly, nAChR-mediated processes. These studies then assessed whether such modulatory processes were dysfunctional after subchronic PCP pretreatment. To confirm the efficacy of the PCP pretreatment, prior to obtaining the tissue for *in vitro* testing, animals underwent NOR behaviour testing to ascertain that the expected behavioural effects were present.

CHAPTER 2: IN VITRO FSCV WITH HIGH POTASSIUM STIMULATION

2.1 Measurement of neurotransmitter release by FSCV

2.1.1 History of FSCV

Electrochemistry is a discipline that is interested in the interaction between electricity and chemical change; measuring the current flow of electroactive species, when chemicals oxidise and reduce following the application of a potential. Application of an external voltage leads to electron transfer between molecules which called a redox reaction and this results in the generation of a current. There are a few electrochemistry techniques such as amperommetry and fast scan cyclic voltammetry (FSCV) used in neuroscience research. In amperommetry, the electrode is held at a constant potential which oxidizes neurotransmitters near to the electrode, but electrode is not immerged into cell rather brought close to the cell (Mosharov and Sulzer, 2005). All electroactive compounds which oxidise below the applied potential will produce a signal. Therefore it is difficult to discriminate between chemical species using amperommetry alone. Rather it is used in conjunction with enzymatic biosensors, where the chemical specificity is provided by the enzyme, and a biproduct of the enzymatic reaction, usually H_2O_2, is measured amperommetrically at the electrode (O'Neill and Lowry, 1995; O'Neill *et al.,* 2004). Cyclic voltammetry is another electrochemistry technique. In this technique, the potential between the working electrode and reference electrode cycles typically from a negative potential to a positive potential, then back to the negative potential. When the electrode potential reaches the oxidation potential of individual compounds, they oxidise, generating a current, which is measured at the working electrode. At low scan rates this

is called linear sweep voltammetry. Although linear sweep voltammetry does give some separation of chemical compounds, it still does not allow separation and identification of individual compounds (Stamford *et al.*, 1992). In the case of FSCV, as the name suggests, the entire voltage sweep occurs very rapidly (normally < 10 ms): this gives a reasonable degree of chemical specificity derived from each compound's unique oxidation and reduction profile seen in the cyclic voltammogram.

FSCV allows measurement of oxidisible and reducible species at reasonable sensitivity (Kissinger and Heineman, 1983). The oxidation and reduction of an analyte depends on potential of the working electrode relative to a reference electrode (usually Ag/AgCl): thus, during oxidation the working electrode acts as the "anode" and anodic (oxidation) currents are measured but during reduction it acts as the "cathode" and cathodic (reduction) currents occur (Lacourse and Olson, 2004). At low scan rates (e.g. linear sweep voltammetry), it is difficult to separate the anodic and cathodic currents of different chemical compounds, but at high scan rates (FSCV) some chemical separation can be achieved.

Thus, FSCV is able to measure electro-oxidisible chemical compounds and this technique has many advantages for studying neurotransmitters as well as its good chemical specificity, the small size of the electrodes allows good spatial resolution and different areas of the brain to be probed (Olson and Lacourse, 2004). Therefore, it is a productive tool for analysing electro-active chemical species such as catecholamines with good sensitivity and reasonable selectivity (Keithley *et al.*, 2011).

In FSCV, a voltage ramp is applied to a carbon fibre electrode and oxidation and reduction of the chemical species occurs at the electrode surface (Stamford *et al.*, 1992). Applying a voltage ramp, induces a relatively large, but stable non-faradaic background current (Olson and Lacourse, 2004), sometimes referred to as the charging current.

In addition to the non-Faradaic (charging) current, a Faradaic current is generated due to the oxidation and reduction of chemical compound: this is the current which enables us to measure the chemicals present. Since the charging current is relatively stable, at least over the short time periods normally used for FSCV recordings, it can be subtracted from the full signal, the residual being the Faradaic current: this is often termed the background subtracted signal. The cyclic voltammogram, produced by plotting the Faradaic current against applied voltage gives a characteristic 'finger-print' for different chemical compounds. The majority of studies using FSCV have focussed on measuring dopamine release, although other chemicals can also be measured (e.g. 5HT : Michael *et al.,* 2007, Jones *et al.,* 2015), and it is also possible to monitor pH changes using FSCV (Stamford *et al.,* 1986; Runnels *et al.,* 1999; Cheer *et al.,* 2006).

The recording electrode characteristics are of vital importance for measuring neurotransmitters. For linear sweep voltammetry carbon microdisk , carbon paste, glassy carbon or platinum-based spherical mercury microelectrodes were used (Olson and Adams, 1960; Baldo *et al.,* 1995; Baldo *et al.,* 1998; Zittel and Miller, 1965). However, these electrodes were rarely used for in vivo experiments due to their limited resolving power (Broxterman and Mos, 1980; Mos *et al.,* 1981). Therefore, the carbon fibre microelectrodes are of critical importance in making good stable FSCV measurements. These comprise a carbon fibre, recording tip, typically 100 to 200 µm in length and less than 10 µm diameter, and enable measurements of µM concentrations of dopamine in localised brain areas. Originally these fibres were mounted in a pulled glass capillary, but more recently, Clark *et al.,* (2010) described a design of electrode in which the carbon fibre was glued into a silica micro-capillary. Although these electrodes were originally designed for detection of neurotransmitters over a long period of time in long term, chronic studies *in vivo* (Clarke *et al.,* 2010), they can also be employed for short term *in*

vitro studies described here. Irrespective of whether measurements use these 'chronic' electrodes or the more traditional pulled glass electrodes, the actual carbon fibre recording tip is essentially the same, and provides equivalent recordings. Due to the size of the electrode, damage to the tissue is kept to a minimum. In addition, because whole the voltage sweep lasts only 8 to 10 ms, it is normal to record at 10 sweeps per second (i.e. 10 Hz), thus giving a subsecond time resolution. FSCV has been applied to studies in brain slices *in vitro* (Toner and Stamford, 1997; Cragg, 2006), anaesthetised rats (Stamford *et al.*, 1985), and freely moving, behaving animals (Robinson *et al.*, 2001).

2.1.2 Theory of FSCV

Oxidation and reduction currents are measured in FSCV. The potential at the microelectrodes is sufficient to oxidize dopamine. A potential of -0.4 V vs a Ag/AgCl reference electrode is delivered and then linearly ramped to a potential +1.3V and back at high scan rate (typically, 400 V/s: figure 2a). Thus, each entire scan lasts only 8 ms, and so scans can be applied multiple times per second: in the experiments described in this chapter, a recording frequency of 10 Hz (i.e. 10 scans per second) was used. When the potential applied to the electrode reaches the oxidation potential for dopamine (approximately 600 mV), dopamine is oxidized to dopamine-O-quinone at the electrode surface, giving up electrons, which are measured as an oxidation current (figure 2b). As the electrode potential then returns to -0.4 V the O-quinone is reduced back to dopamine at the reduction potential (approximately -200 mV). Thus, the extracellular concentration of dopamine can be quantified by measuring the size of the oxidation peak in the background-subtracted (i.e. Faradaic – see above) current (figure 2c). The cyclic voltammogram, achieved by plotting the background subtracted current vs applied voltage (figure 2d) provides the characteristic 'finger-print' for dopamine allowing

identification of the release. (Clark *et al.*, 2010; Stamford *et al.*, 1992). Prior to experiments dopamine calibration was done each experiment day. Dopamine was calibrated by adding dopamine at a known concentration, which is 5µM in our experiments, into the superfusion medium thereby we can generate this figure in 2.1 from this calibration method (details in chapter 2 and 3 methods section) and and exemplify of calibration in figure 2.2 (details in chapter 2 and 3 methods section). However, it should be mentioned here, dopamine and noradrenaline exhibit very similar cyclic voltammograms. Therefore, in brain regions where both transmitters are present (e.g. cortex) it is difficult to distinguish between the two simply on the basis of their electrochemical signal, and further anatomical and pharmacological manipulations would be required to distinguish between dopamine and noradrenaline. However, in striatal brain regions where dopamine predominates, the signal can be attributed to dopamine.

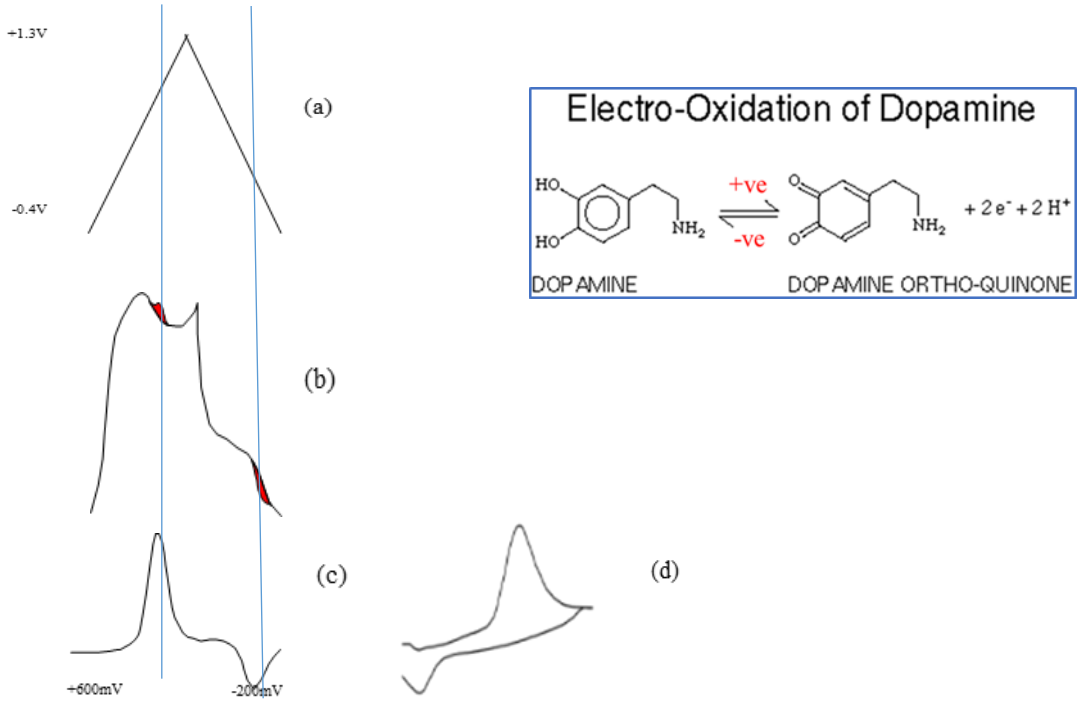


Figure 2.1 : Example traces from FSCV. (a) Input voltage waveform applied; (b) the current flow at the carbon fibre electrode (signals shown in red represent the Faradaic current generated by dopamine oxidation and reduction on top of the background waveform); (c) the background subtracted Faradaic current; (d) the cyclic voltammogram.

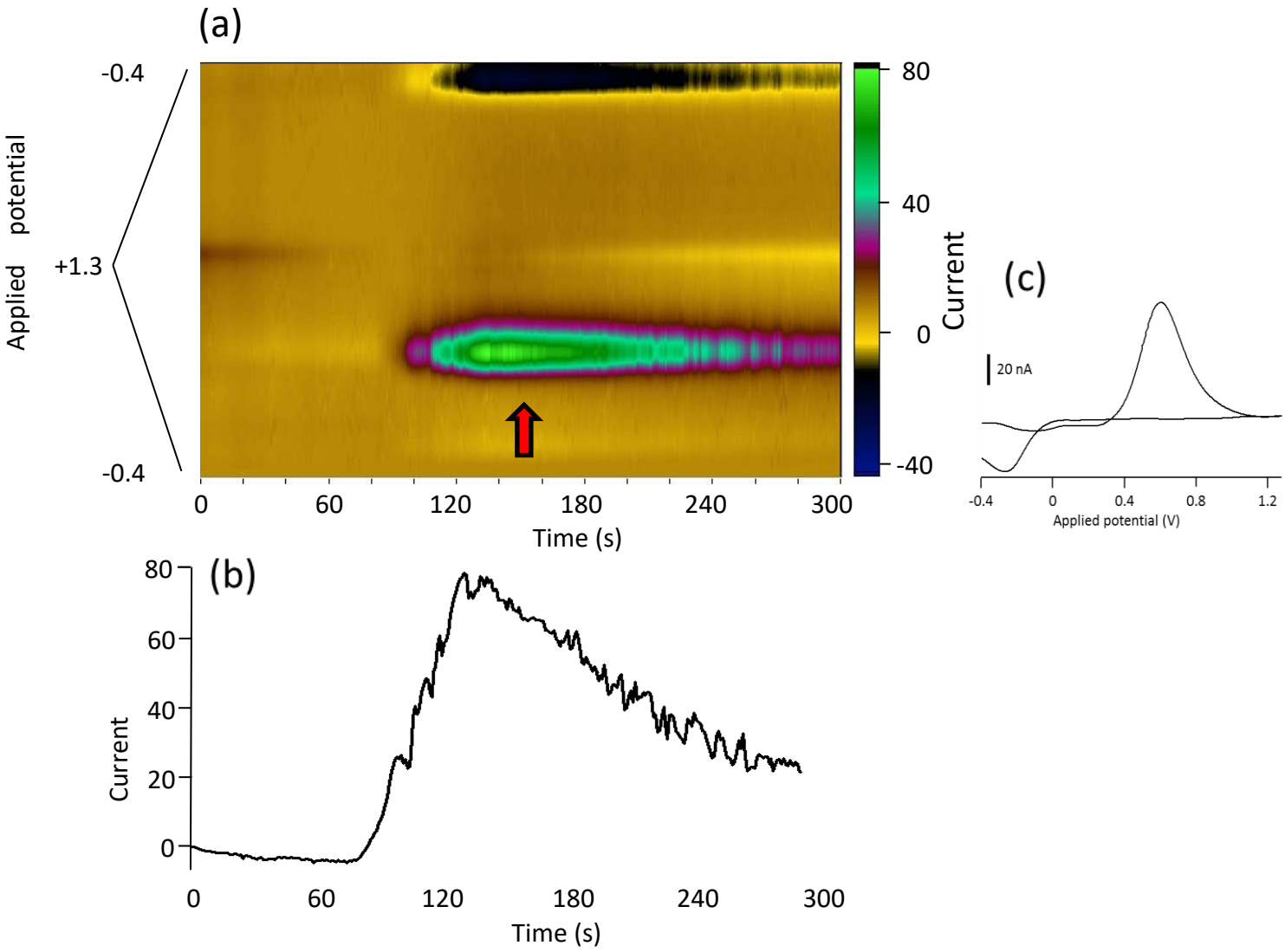


Figure 2.2 : Example calibration of 5µM dopamine. (a)Colour plot obtained by inpit voltage waveform; (b) the current flow at the carbon fibre electrode measured as nA; (c) the cyclic voltammogram. The red arrow on the colour plot indicates the time when the cyclic voltammogram is shown.

2.1.3 Advantages and disadvantages: alternative approaches

FSCV enables measurement of electrochemically active species such as catecholamines (Keithley, *et al.,* 2011). Since each sweep is less than 10 ms in duration, recordings can be made at 10 Hz, enabling real-time measurements of changes in extracellular dopamine concentrations *in vivo* and *in vitro* via its subsecond time resolution and good chemical selectivity. In addition, due to the dimensions of the recording electrode tip (typically 7 µm x approx. 120 µm), it also gives very good spatial resolution (Keithley *et. al.,* 2011). Therefore, FSCV is the most suitable technique currently available to measure transient changes in the concentration of dopamine (Robinson *et al.,* 2003).

On the other hand FSCV has few disadvantages when compared other techniques. It is not suited to long term chronic experiment conditions (Clark *et al.,* 2010). For chronic experiments replaceable electrode systems are required (Cespuglio *et al.,* 1984). Local activation in brain slices can be measured in *in vitro* studies using FSCV. Although *in vitro* slice voltammetry has the advantage localised circuits, and eliminating long loop circuits *in vitro* studies require brain slices instead of whole brains, which leads to the cutting of brain connectivity. Although we can gain important insights about local microcircuitry, it is impossible to know how other neural circuits or pathways interact with each other as a result of pharmacological applications on specific locations in the brain. Therefore, whole brain neurotransmitter activation is not being measured.

Brain microdialysis is a sampling technique which enables measurement of a range of neurotransmitter, metabolite and drugs, over extended recording periods (Total recording time is 160 min. See appendix). It also allows us to measure baseline levels. However, it has poorer spatial and temporal resolution than FSCV.

In vivo imaging like functional magnetic resonance imaging (fMRI) is an important intermediary step between *in vitro* research and clinical trials as it allows us to obtain information about how physiological conditions are changing in a living organism (Jenkins, 2012). It also has the advantage of being able to track changes in the same group animals over time, which is particularly important in drug evaluation studies.

The experiments presented in this chapter aimed to investigate changes in mesolimbic function after subchronic PCP pretreatment focussing on dopamine release in the NAc shell (NAcS), and to assess whether changes in mesolimbic dopamine release in NAcS after subchronic PCP pretreatment was mediated by interaction with NMDAR receptors., Importantly, all testing was carried out at least 1 week after the end of PCP administration. This ensured that the effects being measured were due to enduring mechanistic adaptations caused by the drug treatment, rather than by acute effects of the drug while it was present within the brain as this is a widely used task in which PCP pretreated animals show behavioural deficits (Neill, *et al.*, 2010). In the experiments described in this chapter, potassium stimulation was used to stimulate dopamine neurotransmitter, using protocols previously validated in the lab (Gupta and Young, unpublished).

2.1.4 Aims

1. To show that FSCV *in vitro* can detect the effects of NMDAR activation on potassium-stimulated dopamine release in the NAcS.
2. To investigate the effect of subchronic PCP pretreatment on dopamine release in the NAcS.
3. To investigate whether the effect of subchronic PCP pretreatment on dopamine release in the NAcS is mediated by presynaptic interaction with glutamate receptors.

In addition, as a positive control, to demonstrate that the PCP pretreatment used was effective under the conditions used in these experiments, PCP and saline pretreated animals (aims 2 and 3) were also tested behaviourally using NOR.

2.2 Methods

2.2.1 FSCV

2.2.1.1 Carbon fibre working electrodes

Carbon fibre microelectrodes were fabricated in the lab, as described by Clark *et al*, (2010). Briefly, a 20 mm length of carbon fibre was threaded into a 10 mm length of fused silica (OD 90 μm, ID 20 μm; CM Scientific, Cambridge, UK.) under ethanol. After drying overnight, the fibre was sealed into one end of the capillary with two component epoxy (Devcon, 5 min, 2-part epoxy) and left to dry overnight. Once dry, the tip was cut to a length of 130 ± 20 μm using iris scissors, under microscopic guidance. Next the opposite end of the capillary was attached to a micro-socket (MillMax, 0667; id 0.6 mm; Farnell Electronics, Leeds, U.K), with silver (conducting) epoxy (Circuitworks CW2400,

Farnell Electronics, Leeds, UK.), ensuring that the protruding carbon fibre made good electrical contact with the socket: this was then allowed to dry overnight. (Figure 2.3). Once completed the signal from each electrode was checked and calibrated before use (see below for details).

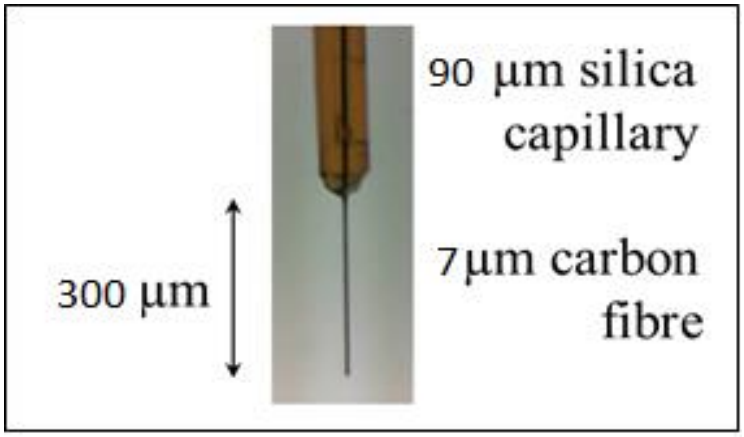


Figure 2.3 Details of the carbon fibre working electrode. Magnified photograph of the electrode tip, showing the carbon fibre recording tip sealed into the fused silica capillary with epoxy.

2.2.1.2 REFERENCE ELECTRODES

Silver chloride coated silver wire (Ag/AgCl) reference electrodes provide a constant reference potential for the voltammetry recordings (Bard *et al.*, 1980). Reference electrodes were constructed in the lab. A 10 mm length of silver wire (0.5 mm diameter; Sigma, UK) was inserted into the socket end of a micro pin plug (individual pin from a 40-way DIL socket, Farnell Electronics, Leeds, UK) and the joint was secured with silver epoxy (Circuitworks CW2400, Farnell Electronics, Leeds, UK). Once dried, the wire was dipped in 2 mM KCl, and electrolytically coated with AgCl using a voltage ramp sequence applied using an NPI Electronics chlorider unit (Scientifica, UK: see appendix A). The reference electrode was rinsed thoroughly with potassium chloride and stored at the end of the experiment.

2.2.1.3 FSCV RECORDING

Recordings were made in a diamond shaped plexiglass tissue chamber (approximate volume 1 ml), mounted on an electrically isolated stand. The tissue chamber was perfused continuously with artificial cerebrospinal fluid (aCSF), comprising (mM) NaCl (126.0), KCl (2.0), KH_2PO_4 (1.4), $MgSO_4$ (2.0), $NaHCO_3$ (26.0), $CaCl_2$ (2.4), glucose (4.0), heated to 32 ± 2 °C by a thermostatically controlled flow-through peltier heater unit mounted adjacent to the tissue chamber, at a flow rate of 1.5 ml/min, provided by a Gilson Minipuls3 peristaltic pump (or in preliminary experiments by gravity feed – see Results section). The tip of the reference electrode was immersed in to a depth of approximately 1 mm close to the edge of the tissue chamber. The reference electrode and the carbon fibre working electrode were connected to a potentiostat via a high impedance headstage (both supplied by Dagan Instruments, Minneapolis, USA). FSCV was achieved by applying a triangular waveform (-0.4 to 1.3 to -0.4 V; scan rate 400 V/sec): application of the voltage waveform and measurement of the resultant current were achieved using Demon voltammetry software (Wake Forest University, USA; Yorgason *et al.*, 2011), delivered from a PC computer. Dopamine was measured as the background subtracted current (nA) occurring at an applied potential of approximately 600 mV.

Following construction, carbon fibre working electrodes were connected to the recording system to check and calibrate the signal. First, they were 'cycled' for 10 min at 60 Hz: that is, the voltage scan was applied at a rate of 60 per second. The frequency was then reduced to the normal recording frequency of 10 Hz and the background current signal was checked to ascertain that it had a good shape and a suitable size (reflecting an appropriate length of exposed carbon fibre). They were then calibrated by introducing dopamine (5 μM) into the superfusate for 1 min, and measuring the peak background

subtracted current flow at the dopamine oxidation potential (approximately 600 mV). This calibration was repeated on each testing day, and the resultant calibration current (nA/ 5 μM) was used to calculate the concentration of dopamine released from brain slices.

2.2.2 Tissue preparation

2.2.2.1 ANIMALS

All experiments reported in this chapter used juvenile female Wistar rats bred within the Division of Biomedical Services, University of Leicester. For experiments using non-pretreated animals (experiment 1 and 2), 4 to 10 days after weaning (age 25 to 31 days), animals were humanely killed by neck dislocation, and the brains were removed and placed in ice cold aCSF. For pretreated animals, drug treatment started 4 to 5 days after weaning (age 25 to 26 days). Animals were injected twice daily with PCP (2 mg/kg; i.p.) or saline vehicle (1 ml/kg; i.p.) for five days, then left drug free for 10 days before the animals were humanely killed as above and the brains were removed and placed in ice cold aCSF. Prior to killing, seven days after the end of pretreatment, animals were tested behaviourally, using NOR (Neill, *et al.*, 2010: see section 2.6.1).

2.2.2.2 BRAIN SLICING

The brain was transferred onto an ice-cold Petri dish and the caudal portion (from the rostral pole of the cerebellum) was removed with a razor blade. The cut surface was then attached to the mounting block of a Vibrotome tissue slicer (NVSLM1 Vibroslice, World Precision Instruments) with cyanoacrylate glue (Loctite Superglue Precision Max). After

the glue had dried (approx. 2 min) the mounting block was placed in the specimen bath and the brain was submerged in ice cold aCSF with crushed, frozen aCSF. Consecutive 400 μm coronal slices were cut and transferred into a slice saver bathed in oxygenated aCSF at room-temperature. The slices were allowed to recover from the trauma of slicing, in oxygenated aCSF at room temperature for at least 1 hour before using for experiments.

All procedures using animals were carried out with appropriate personal and project licence (project number; 60/4390: personal licence; I67DDB722) approval under the Animals (Scientific Procedures) Act, 1986, and with local ethical approval from the University of Leicester Animal Welfare and Ethical Review Body (AWERB). Experimental design and analysis were performed with reference to Experimental Design Assistant (EDA;https://eda.nc3rs.org.uk) (du Sert, *et al.*, 2017).

2.3 Recording

For recording, a slice containing NAc was placed in the slice chamber, and superfused with oxygenated aCSF at 1.5 ml/min. In initial experiments the flow rate was achieved using gravity feed, but in later experiments the flow was delivered by a Gilson Minipulse3 peristaltic pump. The bath temperature was maintained at 32 ± 2°C, using a custom built peltier heater (University of Leicester Biomedical Workshops). The Ag/AgCl reference electrode was placed in the aCSF at the edge of the tissue bath. The slice was held in place by laying a grid over it, and the carbon fibre microelectrode was inserted into NAcS to a depth of about 100 μm using a micromanipulator.

FSCV was started immediately, by applying a triangular waveform (-0.4 to 1.3 to -0.4 V; scan rate 400 V/sec) to the electrode at a frequency of 1 Hz, and dopamine was measured

as the background subtracted current (nA) at the dopamine oxidation potential of approximately 600 mV and the concentration (μM) was calculated by comparison to dopamine calibrations (5 μM) (see section 2.1.3).

2.4 Experimental Procedures

2.4.1 Experiment 1: Potassium Stimulation

Initial experiments tested which concentration of potassium provided the most reproducible stimulated release. The effects of three different concentration of potassium stimulation: 25 mM K^+, 50 mM K^+, and 100 mM K^+ were tested in brain slices from non-pretreated female Wistar rats (n = 7 per group). In each case there was an equivalent reduction in NaCl concentration in the aCSF, to maintain osmolarity. Therefore, when the concentration of KCl was increased the concentration of the NaCl needed to be decreased by an equivalent amount, otherwise the overall ionic content (strength) of the solution would increase. To what extend potassium stimulation changes the resting membrane potential (RMP)? This is an important point that needs to be addressed in order to understand the underlying physiologic mechanisms. RMP is a difference in charge (measured in volts) across a cell membrane at rest, and is approximately -70 mV (Adrian, 1956). This potential is the result of an electrochemical gradient of principally sodium and potassium ions across the membrane. At rest, the membrane is more permeable to potassium ions, thus resulting in the RMP being close to the reversal potential of potassium (-86 mv) (Alger and Nicoll, 1990). In addition to channels open at rest, the RMP is also determined by active transport, where sodium-potassium exchangers pump sodium back into the cell, and potassium back out of the cell against their electrochemical gradients (Adrain 1956).

Changing the concentration of ions in the extracellular space results in a change in resting membrane potential When the extracellular potassium concentrations elevated which result in changes the resting membrane potential, hence, decrease the excitability of the neuron via inactivation of sodium because depolarization brought on by potassium leaves the anode regions unexcitable (Hodgkin and Huxley, 1952; Lin et al., 1999) .

Hodgkin and Huxely demonstrated in their famous experiments (Hodgkin and Huxley, 1952) that the perfusion of artificial cerebral spinal fluid containing high concentrations of potassium ions results in a depolarization of the RMP of cells being recorded. Therefore, the peak size of the second potassium stimulation relative to the first potassium stimulation may be resulted of changes the RMP which resulted in depolarization in brain slices.

It was hypothesized that release of dopamine for 100 mM K^+ would be higher than 50 mM K^+; and 25 mM K^+ (100 mM K^+ > 50 mM K^+ > 25 mM K^+).

2.4.2 Experiment 2 – Slices from non-pretreated animals

Based on the method previously used in the lab (Gupta and Young, unpublished) potassium stimulation was administered for 1 min, starting 10 sec after the start of recording (K1) then superfusate was returned to aCSF. At 30 min a second potassium stimulation (K2) was applied for 1 min, and then the superfusate was returned to aCSF giving a total experiment time of 40 minutes. Thus, peak-dopamine concentration was measured for K1 and K2 in every slice. The four experimental conditions (control, three concentrations of NMDA) were performed on each rat brain, with a different condition in each slice. The order of the experimental conditions was randomized for each experiment. The control condition was designed to ascertain that repeated potassium

stimulation caused consistent dopamine responses: that is the evoked release at the first stimulation (K1) was similar to the evoked release at the second stimulation (K2). In NMDA drug groups, the first potassium stimulation (K1: 1 min, starting at 10 sec) was identical to control. After 15 min, the superfusate was switched to aCSF containing NMDA (15 µM, 30 µM or 60 µM) and the second potassium stimulation (K2) was applied for 1 min starting at 30 min after the start of recording. At the end of the second potassium stimulation, the superfusate was switched back to aCSF, giving a total experiment time of 40 min (Figure 2.4).

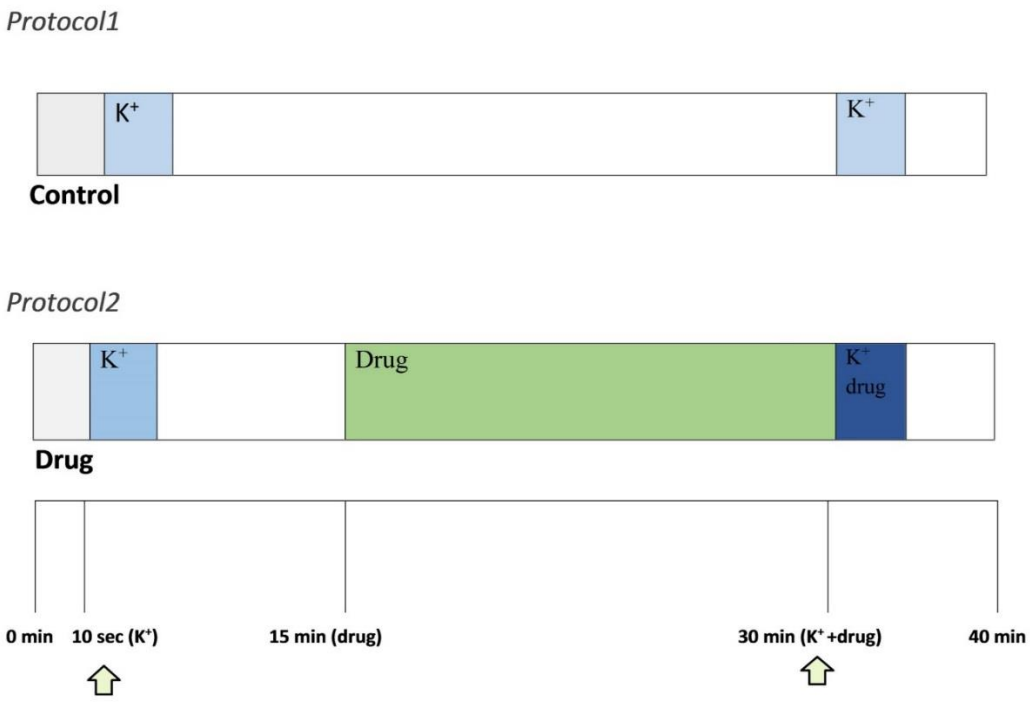


Figure 2.4 Experiment conditions in rat brain slices. Protocol 1 was used for the control condition and protocol 2 was used for NMDA conditions. Dopamine release was stimulated with high potassium aCSF for 1 minute, 30 sec (K1) and 30 minutes (K2) after the start of recording. In control group, slices were continuously perfused with aCSF with K1 and K2 stimulations. In drug group, slices first received K1 stimulation and recovered in maintenance a CSF for 15 minutes, as in control, before inclusion of NMDA (15 µM, 30 µM or 60 µM) in the superfusate. K2 was then applied in the presence of NMDA, before the final 10 min superfusion with normal aCSF. In the drug experiments, K1 served as a control value for dopamine release in each slice and K2 represent the effect of NMDA on stimulated dopamine release.

2.4.3 Experiment 3 – Slices from saline or PCP pretreated animals

In order to test the effects of PCP pretreatment, the NMDA modulation of potassium-evoked dopamine release was tested, as described for experiment 3 (section 2.4.3) in slices taken from rats pretreated with either saline (control) or PCP. As described above, the first potassium stimulation (K1) was without drug in all cases, while the second potassium stimulation (K2), control slices received no drug while test slices received NMDA (15 µM, 30 µM, 60 µM) in the superfusate.

2.4.4 Experiment 4 – Comparison between the K2/K1 ratio and the K3/K2

A separate experiment was carried out to measure the response to a third potassium stimulation (K3) and to ascertain whether the K3/K2 ratio would be more reproducible than the K2/K1 ratio. K1 and K2 were delivered exactly as described in experiments 1 to 3 (i.e. at 1 min and 30 min). After the end of K2, the tissue was superfused for a further 30 min with aCSF, and then a further 1 min superfusion of high potassium (100 mM) aCSF was applied (K3). After that the ratio between K2/K1 and K3/K2 were calculated.

2.5 FSCV Data presentation and analysis

Data analysis was performed using Demon Voltammetry and Analysis Software. The maximum current generated during stimulated dopamine release was measured (peak-dopamine signal, nA), and converted to maximum extracellular dopamine concentration

(peak-dopamine concentration, µM) with reference to the electrode calibration to 5µM dopamine standard.

K1 was compared across saline and PCP pretreatment in order to assess the effect of subchronic PCP treatment on potassium-stimulated dopamine release in NAcS. K1 represented dopamine release after the effects of PCP or saline but before the effects of NMDA drug application, indicating the effect of pretreatment.

The K2/K1 ratios were used to assess the effects of NMDA. We expected to see that the control experiment (slices which did not receive drugs) should not show any significant difference between K1 and K2 (i.e. K2/K1 ratio approx. 1.0). Thus, any significant change in K2/K1 compared to control was attributed to the effect of NMDAR activation. The use of ratio accounted for variability in dopamine release between slices (K1 served as a control value for dopamine release in each slice against the effect of NMDAR activation at K2). The K2/K1 ratio also showed the effects of NMDA to be compared across pretreatment groups to assess for interaction between PCP and NMDAR.

All data are expressed as mean ± SEM. In non-pretreated slices, the mean of each drug concentrations (0 µM, 15 µM, 30 µM, 60 µM) was compared to control group. Statistical analysis of the effect of NMDA on non-pretreated slices was performed on the K2/K1 ratios by using one-way ANOVA followed post-hoc Tukey's test. Statistical analysis of the effects of PCP on NMDA modulation of potassium-evoked release of dopamine was performed by using two-way ANOVA (2 x 4), with pretreatment (saline, PCP) and drug concentration (0 µM, 15 µM, 30 µM, 60 µM) as the factors, followed post-hoc Tukey's analysis. Statistical analysis of the effect of the subchronic PCP pretreatment on NMDA-mediated changes in dopamine release and the ratio between K2/K1 and K3/K2 were performed by t-test. Statistical analysis was performed by using GraphPad Prism 6.

2.6 Behavioural testing

2.6.1 NOR procedure

The NOR testing protocol was described by Grayson *et al.* (2014). Testing was carried out in a black plexiglass arena (52 cm x 52 cm x 40 cm high). Briefly, it was composed of a 3-min acquisition trial, 1-min inter-trial interval (ITI) followed by a 3-min retention trial test. Starting 5 days after the final PCP (or saline) treatment animals were habituated (20 min each) to the test arena on two consecutive days. On the day of behavioural testing, each rat was placed into one corner of the arena and allowed to explore freely for a further 3 min habituation. After 3-min, the animal was removed from the arena, and placed in a holding cage for 1 min inter-trial interval (ITI). The arena was cleaned with 10 % ethanol and two similar objects placed in opposite corners of the box, 10 cm from the sides. For the acquisition trial, the animal was introduced into the arena in one of the corners not containing an object, and allowed to explore for 3 min. They were then placed again in the holding cage for 1 min during which the arena was again cleaned with 10 % ethanol, and one object which was similar to those used in acquisition test and one novel object were placed in the corners of the arena, and the animal was reintroduced to the arena for a further 3 min (retention trial). The objects used were a metal cylinder (5 cm diameter x 10 cm high) and a glass jar (8 cm diameter x 6 cm high). The positions of the novel and familiar objects were counterbalanced across animals. (Figure 2.5).

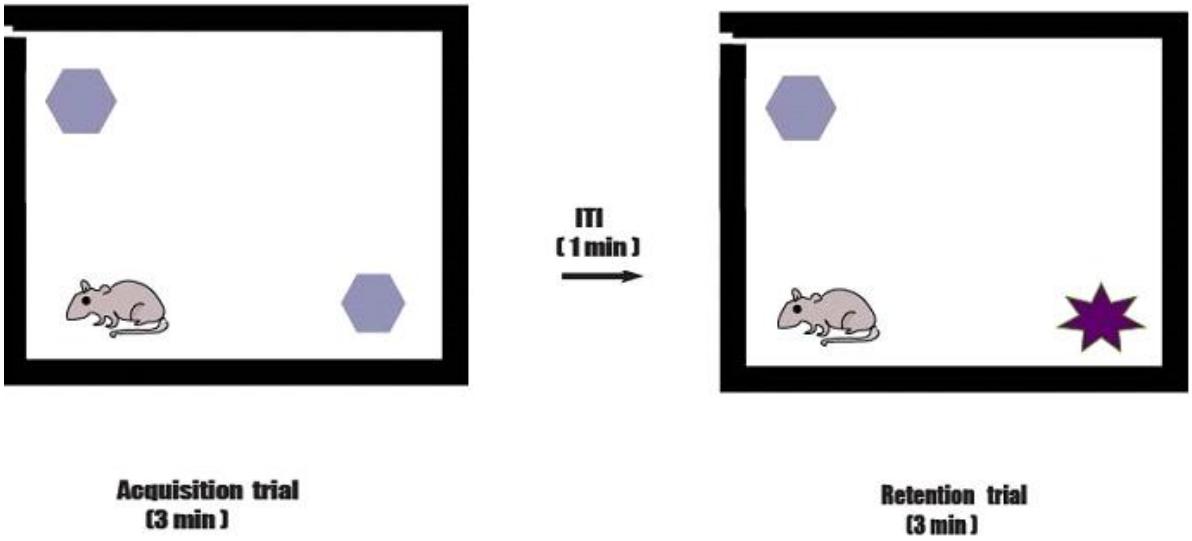


Figure 2.5 Diagramatic representation of the NOR task procedure. In the acquisition trial, two familiar object were introduced into opposite corners of the testing box and animal introduced into one of the unoccupied corners. The animal was given three minutes to explore both familiar object. In the retention trial, after one minute ITI during which one familiar object was replaced with a novel object, the animal was introduced into the same corner of testing box and given three minutes to explore.

2.6.2 Behavioural data presentation and analysis

All experiments were filmed using a webcam attached to a PC and video recorded for off-line behavioural analysis by two independent researchers, blinded to the treatment conditions

The time spent exploring each of two objects in both the acquisition and retention trials were measured using stop-watches. Object exploration was defined as when the animal's head was within 2 cm of the object and it was sniffing or licking the objects, or touching the objects with forepaws while sniffing; this included when the animal was sitting on the object with its head down (Grayson *et al.*, 2007). However, leaning against, circling around, or standing or sitting on the objects with its head up was not deemed to be

exploratory behaviour. The discrimination index (DI: Grayson *et al.*, 2014) was calculated from the times measured during the retention trial, by using the formula;

(time on novel object – time on familiar object) / (time on novel object + time on familiar object).

All data are expressed as mean ± SEM. Statistical analysis of acquisition, testing and DI were performed by using unpaired t-test. Statistical analysis was performed by using GraphPad Prism 6.

2.7 Results

2.7.1 Experiment 1 – Potassium stimulation

The mean concentration of the dopamine released after potassium stimulation was dependant on the potassium concentrations as expected (25 mM K^+ = 0.33 µM, 50 mM K^+= 0.46 µM, and 100 mM K^+ = 1.85 µM). One-way ANOVA showed a significant effect of potassium stimulation (F (2, 39) = 8.193, P = 0.0011). Post hoc analysis revealed that all three concentrations of potassium evoked a significant increase in dopamine release (Tukey's Post hoc Test) on each concentration to ascertain whether each one produce a significant increase. The highest potassium concentration (100 mM) produces a significantly bigger response. (Figure 2.6 and 2.7).

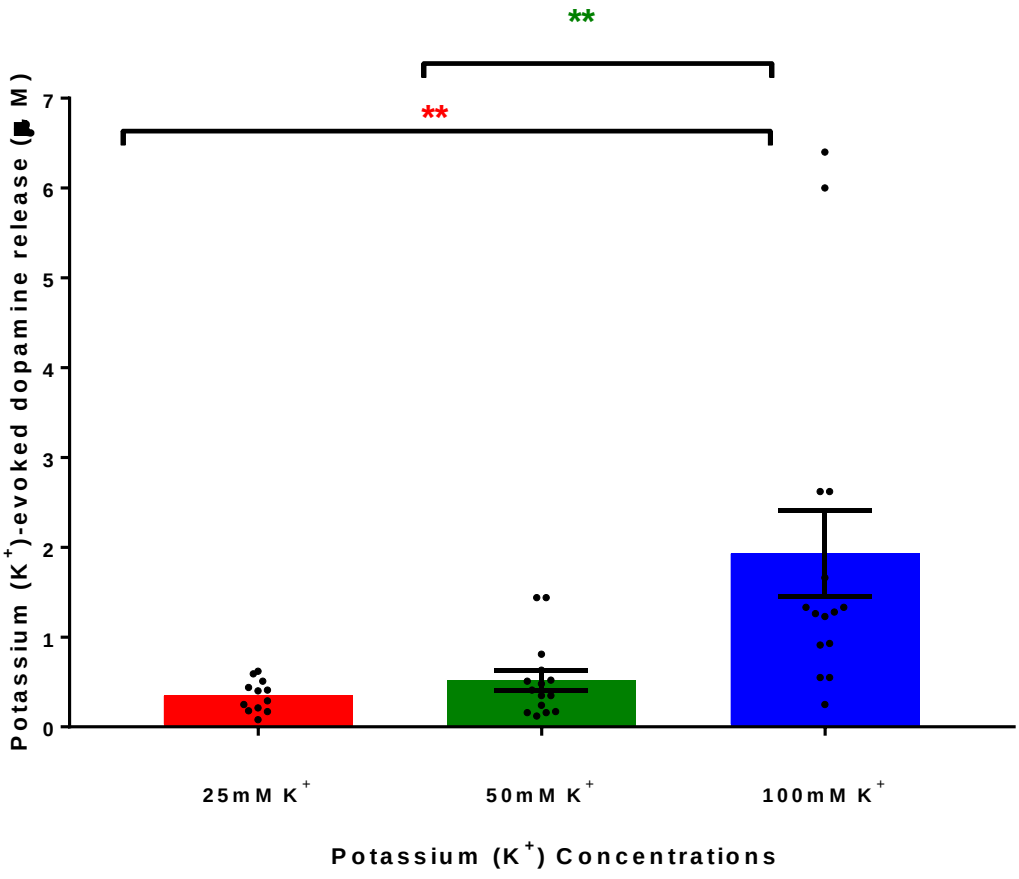


Figure 2.6 Potassium-evoked dopamine release in 25 mM K^+, 50 mM K^+, and 100 mM K^+ on non-treated striatal slices. ** p < 0.001: Tukey's post hoc test, based on a significant one-way ANOVA. Data are mean +/- SEM (n= 14 to 15 per group).

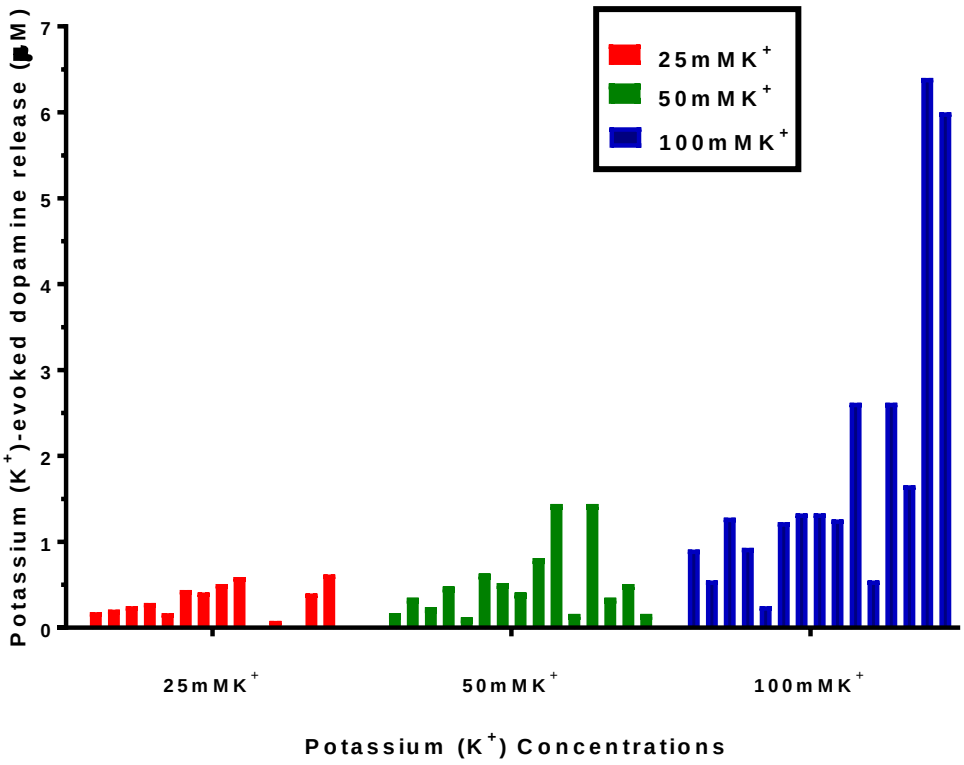


Figure 2.7 Potassium-evoked dopamine release in 25 mM K^+, 50 mM K^+, and 100 mM K^+ on non-treated striatal slices. Individual data points converted to µM and presented in bar graph.

2.7.2 Experiment 2 – Effect of NMDA in slices from non-pretreated animals

In the preliminary experiments, NMDA (15 µM, 30 µM, 60 µM) showed an increase in basal (non-stimulated) dopamine from striatal slices. The basal endogenous dopamine release outflow in rat striatal slices obtained from naïve rats (n = 21) was increased significantly by NMDA administration F (3, 80) = 4.819, P=0.0039 These changes were concentration-dependent since there was a significant difference between the different NMDA concentrations. Tukey's Post hoc analysis revealed that NMDA increased basal dopamine release at all three doses (Figure 2.8 and 2.9).

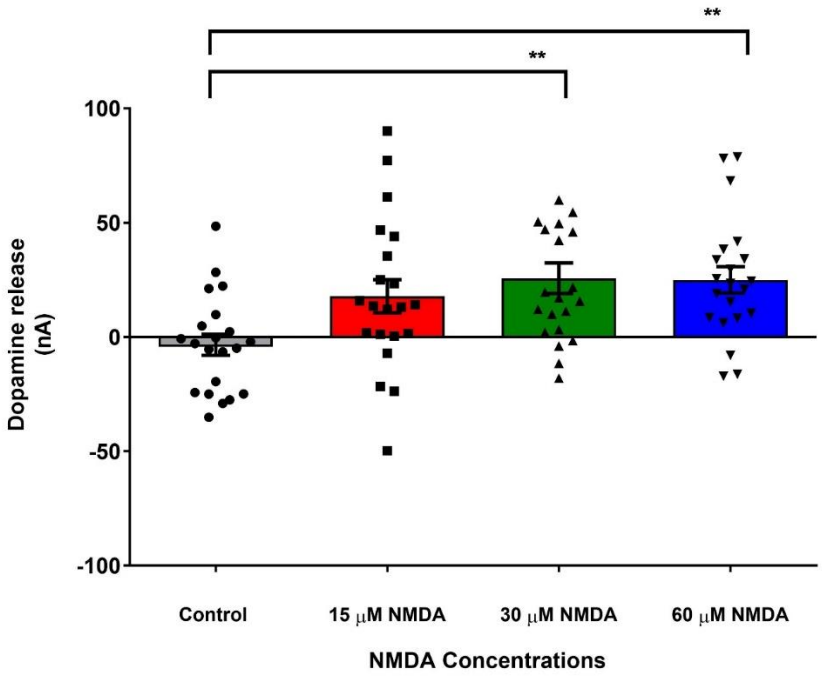


Figure 2.8 NMDA (15 µM, 30MµM, 60µM) effect on basal dopamine release. Release of dopamine from slices of rat striatal tissue was assessed by different concentration of NMDA applied in the superfusate. Statistical analysis was performed by two-way ANOVA. ** P=0.0039 difference from the control. Data are mean +/- SEM (n = 21 per group).

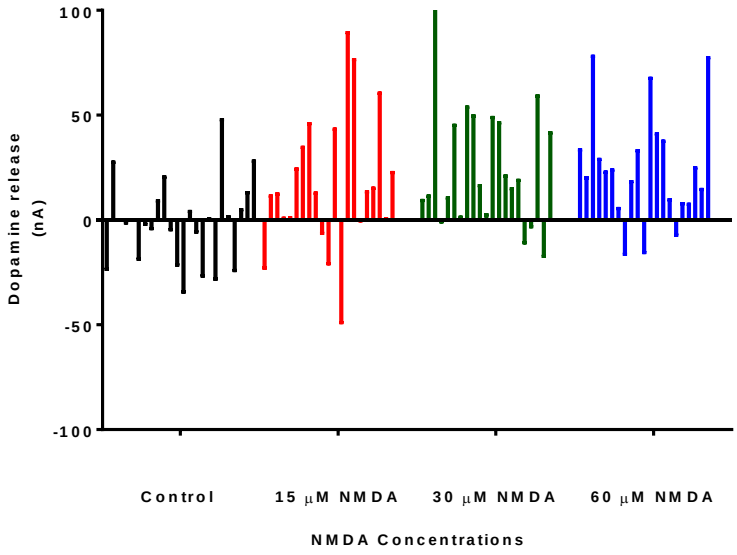


Figure 2.9 NMDA (15 µM, 30MµM, 60µM) effect on basal dopamine release. Individual data points showed in bar graph per subject.

Although there was an indication of an effect of NMDA on potassium stimulated dopamine release, statistical analysis showed that there was no significant difference between the different drug groups and the control group ($F(3,80) = 0.4427$; $p = 0.7231$: Figure 2.10). The study results showed that NMDA at concentrations of 15 µM, 30 µM, 60 µM was ineffective on potassium-stimulated (100 mM) dopamine release in slices obtained from non-treated rats. However, it should be emphasised that there was some indication of an effect, but the substantial variability in the data makes interpretation difficult. It worth noting here that we would normally simply give K2/K1 ratio, since this enables us to look specifically at the effect of the drug on stimulation, irrespective of variations in baseline release between recordings.

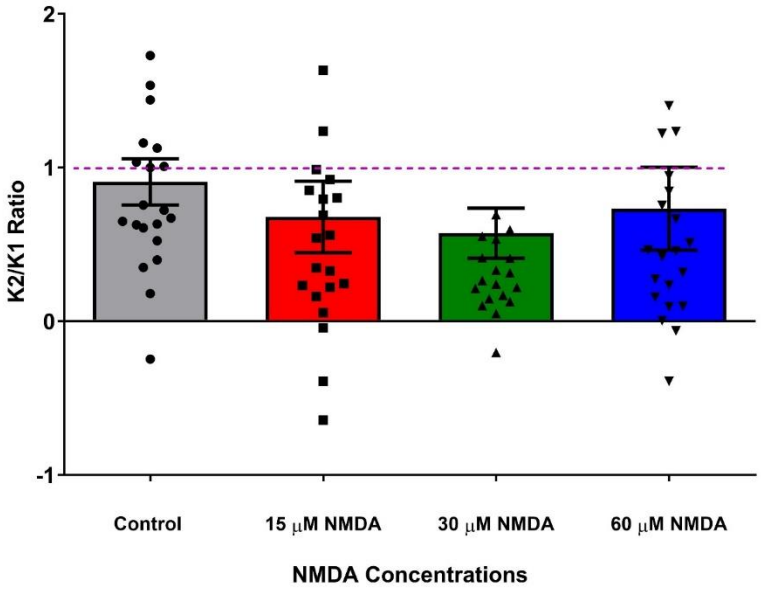


Figure 2.10 NMDA (15 µM, 30MµM, 60µM) effect on potassium-stimulated (100 mM) dopamine release. Data are presented as ratio the ratio of release at K2 (with drug) and K1 (without drug). Dashed line indicates a K2/K1 ratio of 1.0: that is no difference between K1 and K2. Statistical analysis was performed by one-way ANOVA. Data are mean +/- SEM (n = 20 per group).

2.7.3 Experiment 3 – Slices from saline or PCP pretreated animals

NMDA at concentrations of 15 µM, 30 µM, 60 µM showed an increase in baseline dopamine release from striatal slices (Figure 2.11). The baseline endogenous dopamine release outflow was increased significantly by NMDA administration. There was a significant main effect of NMDA (F [2, 42] = 6.342; P=0.0039), but no main effect of drug (F [1, 42] = 0.2576; P = 0.6144) and a significant interaction (F [2, 42] = 3.653; P = 0.0345)

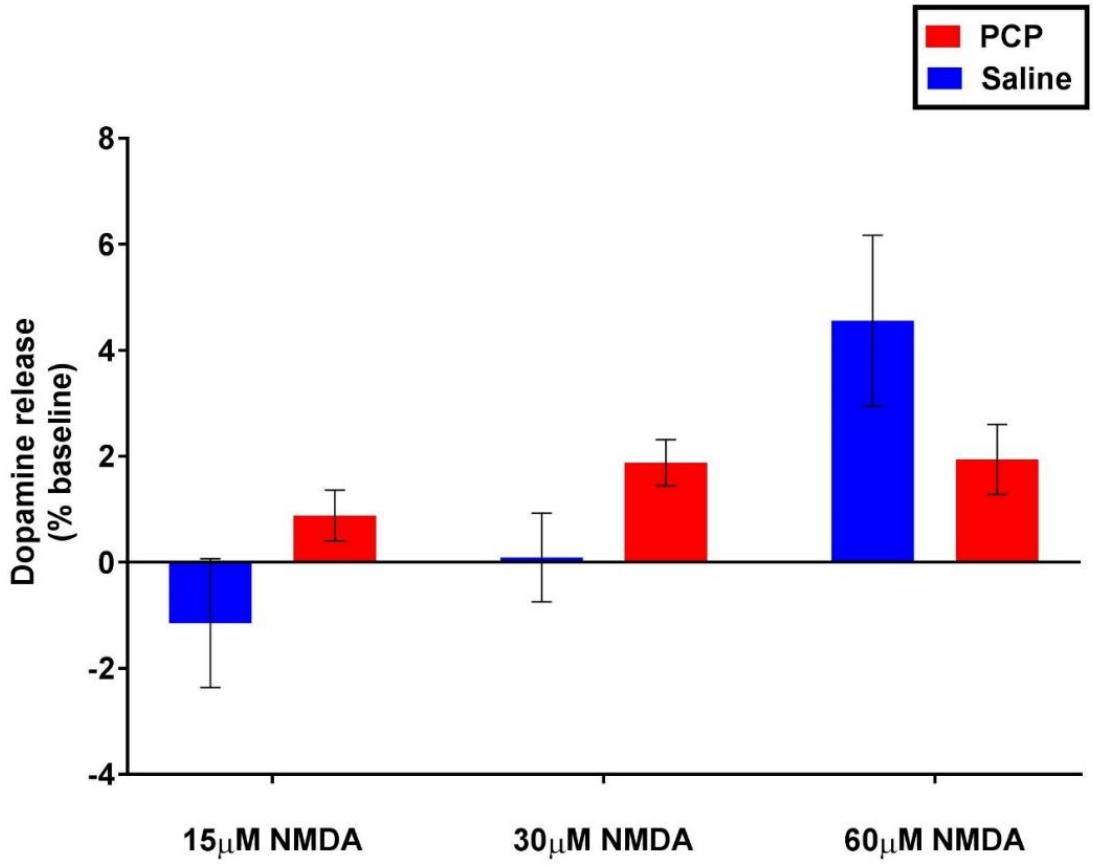


Figure 2.11 Effects of NMDA (15 µM, 30 µM, 60 µM) on release of basal dopamine from slices of rat striatal tissue. Release of dopamine from slices of rat striatal tissue was assessed by different concentration of NMDA applied in the superfusate. Statistical analysis was performed by two-way ANOVA. Data are mean ± SEM (n= 21 per group).

The effect of subchronic PCP pretreatment on peak-dopamine release in the NAcS was assessed by considering the K1 ration between saline and PCP pretreated brain slices (Figure 2.12). There was a significant in increase in peak-dopamine concentration in the PCP pretreatment group when compared to saline ($t(62) = 4.839$; $p<0.001$). Subchronic PCP pretreatment significantly increased potassium-evoked dopamine release (on K1 stimulation) in the NAcS compared to saline pretreatment.

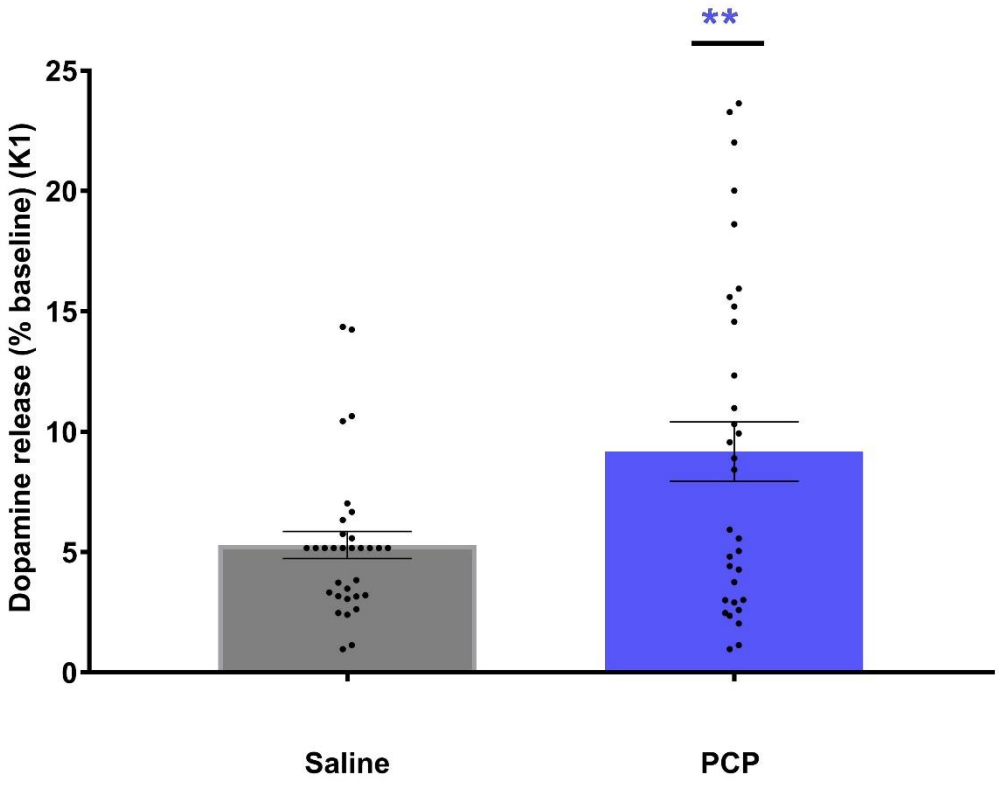


Figure 2.12 Effect of subchronic PCP pretreatment on stimulated dopamine release. Subchronic PCP pretreatment consisted of PCP (2 mg/kg i.p.) administered twice daily for 5 days followed by a 7-day drug free period. ** $p < 0.01$; difference from the control. Statistical analysis was performed by t-test. Data are mean +/- SEM (n= 31 per group).

The effect of potassium-stimulation on peak-dopamine release in pretreated animals was assessed between saline and PCP pretreated brain slices. There was a significant main effect of NMDA ($F [3, 40] = 3.906$; $P=0.0155$), but no significant main effect of PCP ($F [1, 40] = 0.486$; $P=0.4898$), nor a significant interaction ($F [3, 40] = 0.01817$; $P=0.9966$). There was a significant effect of NMDA (15 µM, 30MµM, 60µM) on potassium-stimulated release of dopamine (Figure 2.13). Release of dopamine from slices of rat striatal tissue was stimulated by 100 µM KCl, applied in the superfusate. There was no significant change between control and NMDA groups.

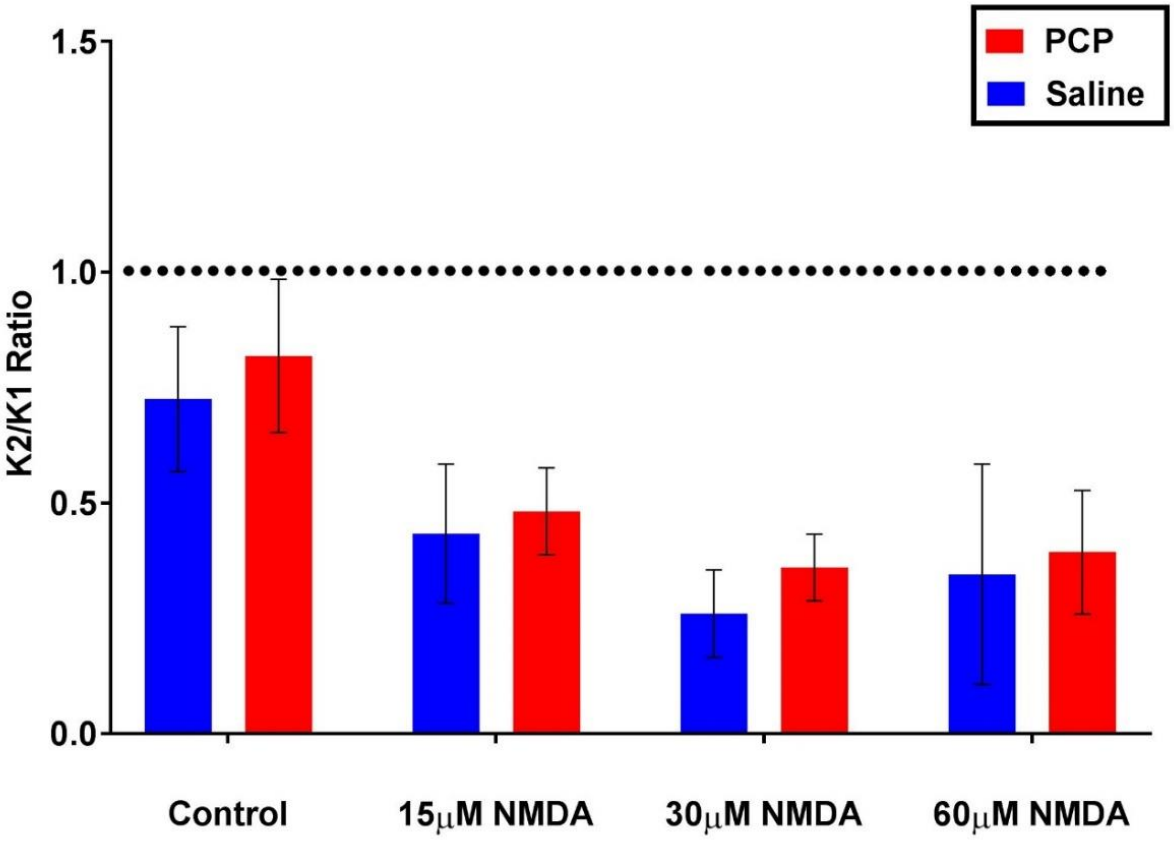


Figure 2.13 Effect of PCP pretreatment on potassium (100 mM) stimulated dopamine release. Data are presented as ratio the ratio of release at K2 (with drug) and K1 (without drug). Dashed line indicates a K2/K1 ratio of 1.0: that is no difference between K1 and K2. Statistical analysis was performed by two-way ANOVA. Data are mean +/- SEM (n = 21 per group).

2.7.4 Experiment 4 – Comparison between the K2/K1 ratio and the K3/K1 ratio

In the drug experiments described above, K1 served as a control value for dopamine release in each slice and K2 represent the effect of the drug on accumbal dopamine release. However, in our experiments almost every time K2 value was substantially lower than K1 which made it difficult to interpret drug effects. In this experiment we compared K2/K1 ratio with K3/K2 ratio, to ascertain whether the latter gave more reproducible stimulated release in non-drug treated slices

Results confirm that the K2/K1 ratio was below 1.0, indicating that K2 was substantially lower than K1. On the other hand the K3/K2 ratio was close to 1.0, indicating a good reproducibility between K2 and K3, and were statistically different from the K2/K1 ratio ($t(18) = 3.456$; $p < 0.001$) n = 10 per group]. (Figure 2.14).

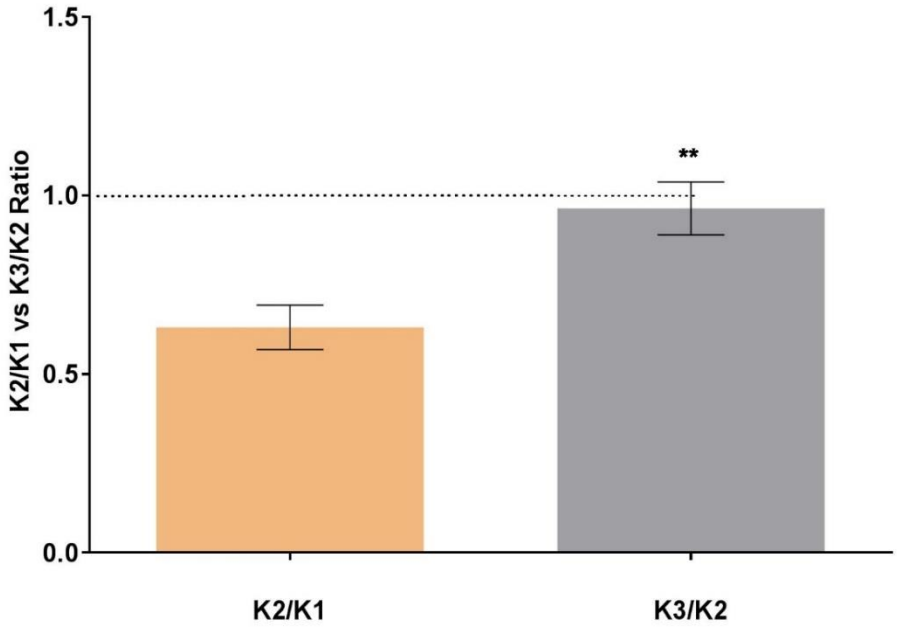


Figure 2.14 Difference between the K2/K1 ratio and the K3/K2. ** P < 0.01; difference from the K2/K1. Statistical analysis was performed by t-test. Data are mean ± SEM (n = 10 per group).

Subchronic PCP pretreatment caused behavioural changes in same group animals. One week after the end of pretreatment, animals were tested in NOR test to confirm the behavioural effect of PCP pretreatment. There was a significant difference in test period between familiar object and novel object in saline pretreated animals ($t(30) = 3.543$; $p = 0.0013$) and a significant difference in DI between saline pretreated animals and PCP pretreated animals ($T(30) = 3.607$; $P = 0.0011$: Figure 2.15).

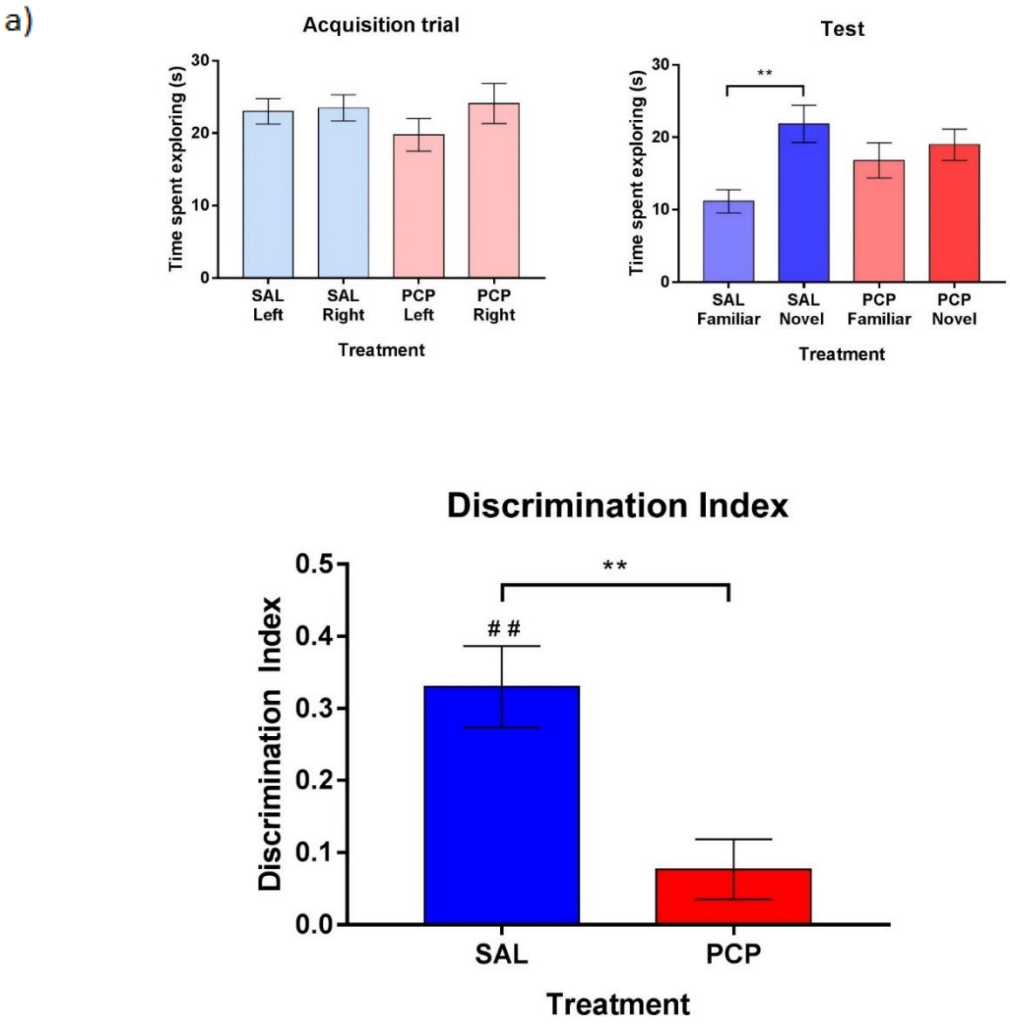


Figure 2.15 Effect of PCP pretreatment on NOR test. (a) Time (s) spent exploring the two familiar objects during the acquisition stage (3 min); (b) time (s) time spent exploring the familiar and novel objects during the test stage (3 min); (c) DI calculated from the time spent exploring familiar and novel objects during the test stage. ** P <0.0013 ; significantly different time spent exploring; one-sample t test. **p < ; 0.0011, difference from saline pretreated group; independent samples t test. Data are mean ± SEM (n= 32 per group.) ## p < 0.01 significant difference from zero (no discrimination) : one-sample t-test

2.8 Discussion

The aim of the current study was to (1) measure the effects of the local activation of NMDAR on potassium stimulated dopamine release in the NAcS by using FSCV on non-pretreated accumbal slices and (2) to measure the effects of PCP pretreatment on this. The results showed that NMDA did not produce any consistent change in the potassium stimulated dopamine release in NAcS.

One problem with the data presented is that there is a great deal of variability between experiments. There are a number of possible reasons for this, which have been explored. It is extremely important to maintain a constant flowrate of perfusate. The syringe pumps which have been used in the past (Gupta and Young, unpublished) did not maintain a reliable and consistent flow rate, and so were replaced with a gravity fed system. This provided better reliability, but it was still difficult to maintain a constant flow rate across the full period of the experiment, particularly when changing solutions. We have therefore moved to using a peristaltic pump (Gilson Munipuls3), which provides a very constant flow rate.

The data suggest that changes are occurring, but it is very difficult to interpret the findings due to the extreme variability in the data which may be down to the problems of using potassium stimulation Perhaps application of potassium stimulation into striatal slices may cause depletion via its strong depolarizing effect in striatal brain slices. Neuronal activity could be decreased by the activation of an ion conductance resulting in membrane depolarization. Therefore, dopamine release could be affected by the process of NMDAR inhibition. The size of the stimulus, the fact that it affects the whole slice (so potentially affecting the health of the slice, but also reducing the specificity of the responses, since everything is activated) and the duration of the stimulus may all lead to high depletion,

which would affect the reproducibility of the responses. There is also a potential problem of spreading depression or spreading depolarization which describe a hyperactivity followed by inhibition (Dodick and Gargus, 2008). Hence, cells are 'shut down' by potassium stimulation. Increasing extracellular potassium ion concentration and excitatory glutamate contribute to the depression (Richter *et al.,* 2008). Therefore, this methodology with potassium stimulation is not sufficiently reproducible to definitively assess dopamine activation in NAc brain region. Due to these potential problems with using potassium stimulation, we established and validated an electrical stimulation procedure for further studies, as it provides more localised stimulation: the data from these experiments are presented in Chapter 3.

The results of our study demonstrate that although NMDA did not cause significant changes in potassium-stimulated dopamine release on non-treated striatal slices, baseline dopamine release was changed after NMDA bath application. Therefore, the proposal that NMDA modulates dopamine release cannot be ruled out in view of the baseline dopamine changes, which suggest that dopamine regulation is related to glutamate function in subcortical areas. Thus, the effects of NMDA modulation on dopamine modulation remain elusive. For example, a direct application of glutamate into the striatum does not change the dopamine-related electrochemical signals so glutamate activation which regulates the dopamine flux could be masked by endogenous glutamate procedure (Kulagina *et al.,* 2001). In addition to endogenous glutamate activation, desensitisation of NMDARs is another possibility, which needs to be taken into consideration when evaluating the results. Studies carried out using voltammetry by applying electrical stimulation show that activation of NMDARs inhibits dopamine release (Iravani and Kruk, 1996; Wu *et al.,* 2000) and that the effect appears to be impulse-dependent. These studies indicate that NMDA has effects which are both

inhibitory and facilitatory on dopamine release in the striatum because NMDARs could be located post-synaptically on GABA neurons (Schofflemeer, *et al.,* 2000).

Thus, we decided to move to electrical stimulation which overcomes many of the problems encountered with potassium stimulation (see chapter 3) is discrete and stimulates just the target area, in addition, electrically stimulated peaks return to baseline levels in a short time period and can be delivered repeatedly without degrading release.

2.9 Conclusion

This study is showing that NMDAR activation moderates dopamine function in NAcS. We have used FSCV in rat brain slices in vitro to measure dopamine release in nucleus accumbens shell to assess the effect of NMDAR activation. NMDA is an NMDAR agonist: it increased basal dopamine release in both non-pretreated and pretreated striatal slices, but there was no consistent change in potassium-evoked dopamine release. Slices from non pretreated animals did not show consistent effects of NMDA on potassium stimulated dopamine release, whereas PCP pretreated slices did show some effect of NMDA. Since potassium is a strong depolarizing agent, dopamine release by drug manipulations after potassium stimulation gave a high variation which makes it difficult to interpret with or without potassium stimulation on dopamine changes. Results show that NMDA did not produce any consistent change in the potassium stimulated dopamine release. This result may be due to the high concentration of potassium stimulation used. However, concentration dependent results indicate that the higher concentration is necessary to evoke consistent dopamine release although the lower doses did produced dopamine release. In our studies we showed that potassium stimulated dopamine release

was concentration dependant and 100 mM potassium was chosen as target stimulation because it gave clearer dopamine release. However, the duration of the response was such that the dopamine levels had not always returned to baseline before drug application.

To assess potassium-evoked dopamine release the K2/K1 ratio was taken and expected to get the ratio ~ 1.0 for both non-pretreated and PCP pretreated brain slices. However, the ratio levels were found to be lower than 1.0 because the second potassium stimulation was always lower than the first stimulation. On the other hand K3/K2 ratio obtained from non-pretreated slices ratio was close to 1.0 which showed stable release levels with multiple stimulations. Thus, it may be preferable to use the K3/K2 ratio to investigate drug effects on potassium-evoked dopamine release. However, (1) the stimuli are sub-maximal, as they are considerabley lower than the maximum obtained at K1, and (2) this risks exposing the slices to excessive potassium stimulation which may change the true effect of the target drug because of slice would be stimulated three times by higher concentration of potassium stimulation since we have measured the second and the third-time potassium stimulation gave a better ratio compared the ratio of the first and the second-time potassium stimulation.

CHAPTER 3: IN VITRO FSCV WITH ELECTRICAL STIMULATION

3.1 NMDA modulation of electrically stimulated dopamine release

Dysfunctional glutamate systems underlie the pathology of schizophrenia. Studies usually focus on the mesolimbic dopamine pathway, from VTA to NAc since this is the pathway which has been most implicated in mediating the at least the positive symptoms of schizophrenia. However, the suggestion that a core deficit in glutamatergic neurotransmission may underlie schizophrenia has led to speculation that interactions between glutamate and dopamine may underlie the behavioural changes (Javitt, 1987). There is no direct synaptic connection between glutamate and dopamine in NAc suggesting that glutamate exerts its influence on dopamine through extrasynaptic and volumetric transmission (Segovia and Mora, 2001). As mentioned above glutamate receptors are divided into two groups: ionotropic and metabotropic glutamate receptors. In particular NMDARs are implicated because drugs such as PCP, a non-competitive antagonist at NMDARs, induces schizophrenia-like symptoms in normal people and exacerbates symptoms in patients with schizophrenia (Javitt, 2007). However, it is still controversial where NMDARs are located in NAc (Salamone *et al.*, 2014; Gracy and Pickel, 1996). On the other hand, metabotropic glutamate receptors may have an important role in schizophrenia. Metabotropic group-II (type2 and 3) glutamate receptors are well known as having inhibitory properties in glutamate regulation. Karasawa *et al.* carried out a microdialysis study that showed that MGS0039, a group- II (type 2 and 3) metabotropic receptor antagonist, caused increased extracellular dopamine levels in NAcS. However, the results are not conclusive, since (1) the antagonist is relatively non-

selective and (2) the microdialysis probe position was not restricted in NAcS: it was also sampling also from NAc core (Karasawa *et al.,* 2006). Metabotropic glutamate receptors also interact with NMDARs which and this interaction may be important in modulating dopamine release in striatum. However, the mechanism of interaction between ionotropic and metabotropic glutamate receptor activation, which, in turn, alter dopamine signalling, remains elusive. Group-II (type 2 and 3) metabotropic receptors have been shown to be inhibitory, and reduce dopamine release (Conn and Pin, 1997; Karasawa *et al.,* 2006) and group-II (type 2 and 3) metabotropic receptor antagonism facilitates glutamate transmission by NMDARs. The interaction between group-II and NMDA glutamate receptors relies on endogenous dopamine because dopamine depletion in striatum abolished the interaction between NMDA and group-II glutamate receptors (Mela *et al.,* 2006). When group-II metabotropic receptor activation was disrupted, striatal ACh release was enhanced via facilitating NMDAR activation (Mela *et al.,* 2006). This supports the view that group-II (type 2 and 3) metabotropic receptors have an inhibitory effect on NMDA-augmented glutamate release in striatum. On the other hand, group-II metabotropic receptors can stimulate protein kinase C (PKC) to phosphorylate cortical NMDARs and increase NMDA currents (Tyszkiewicz *et al.,* 2004). Glutamate release may be enhanced by nAChRs activation. These nAChRs are a member of the Cys-loop ligand-gated ion channel super-family (Gotti *et al.,* 2009) of receptors, and possess several subtypes, with each subtype classified according to their heteromeric or homomeric composition, such as their α and β subunit ratio include α2 to α6 and β2 to β4 combinations (see chapter 1 for more detail). However, it is still unknown whether the different stoichiometry of α4β2 lead to differences in the effect of glutamate activation of α4β2. Allosteric (positive and negative) modulation studies could help in the understanding of the interaction between acetylcholine at nAChRs and glutamate in

attention processes. Grupe *et al.,* (2013) showed that NS9283, which is a positive allosteric modulator at the low sensitivity α4β2, enhances cholinergic regulation of glutamatergic neurotransmission in the cortex. This study shows that nAChR receptor activation regulates glutamate release in the cortex. Moreover, cholinergic interneurons in striatum also regulate dopamine release via cortical glutamatergic afferents (Kosillo *et al.,* 2016). Both mAChR and nAChR contribute to NMDA mediated control of dopamine release in striatum (Zhang *et al.,* 2014)

Therefore, behavioural and neurochemical studies suggest that abnormal excitatory amino acid transmission is a critical determinant of schizophrenia and that many of the behavioural changes seen may be mediated through interactions with dopamine systems, particularly in the mesolimbic pathway under the regulatory control of cholinergic mechanisms.

3.1.1 Methodological approach to electrical stimulation

In the experiments reported previously (chapter 2) potassium stimulation was used: the tissue was stimulated by bath application of high-potassium artificial cerebrospinal fluid (aCSF) for 1 minute. However, there were a number of problems with this approach: bath application of potassium gave a very large stimulus, which activated the whole slice and produced a release that lasted several minutes. This meant that a gap of 30 min was needed between stimuli, and even then the dopamine levels had not always returned to baseline before application of the next stimulus. In addition, the long inter-stimulus interval, meant that each recording session lasted over an hour: over this period of time there was some evidence of tissue deterioration, and from a technical point of view, the

FSCV recordings needed to be run at 1 Hz (as opposed to the more common 10 Hz) because of limitations in the size of the data buffer in the software. Recording at 1 Hz over prolonged periods of time tends to produce changes in the background voltammetry signal, which can obscure the dopamine signal, making interpretation of the data difficult.

Therefore for the experiments described in this chapter, local electrical stimulation was used. Initial studies aimed to assess the optimal stimulation conditions, after which these parameters were used to measure the effects of bath applied drugs. The advantages of this approach were that the stimuli were short and discrete, and responses generally returned to baseline within 20 sec; stimuli could be repeated every 3 min; repeated stimulations, in the absence of drug produced reproducible responses; the experiments were shorter duration, allowing FSCV scans to be made at 10 Hz, with accompanying improvement in the stability of the signal. The data for the validation of the technique, and the effects of drugs, are presented in this chapter.

3.1.2 Aims

- To establish methodology for local electrical stimulation of the slices, to ascertain the optimal stimulation and measurement parameters for repeated electrical stimulation, and to validate the methodology for measuring the effects of bath applied drugs.
- To investigate NMDAR modulation of dopamine release in NAcS of rat brain slices by applying FSCV, and to ascertain the receptors involved.

- To assess the role of different transmitter systems (GABAergic systems, mGluR2/3, nAChR) in modulating electrically-stimulated accumbal dopamine release in rat brain slices using FSCV.
- To assess the effect of PCP pretreatment on this NMDAR mediated regulation of dopamine function.
- To investigate whether the effect of subchronic PCP pretreatment on dopamine release in the NAcS is mediated by cholinergic interaction with glutamate receptors.
- To confirm that the subchronic PCP pretreatment used was sufficient to bring about behavioural deficits in novel object recognition test.

3.2 Methods

3.2.1 Fast scan cyclic voltammetry (FSCV)

Extracellular dopamine concentrations were measured in NAcS by applying FSCV: the working and reference electrode construction and calibration and recording procedures were as described in chapter 2. However, electrical stimulation was used to investigate extracellular dopamine concentrations rather than potassium stimulation. Therefore, the stimulator system and stimulation electrodes are described in this section.

3.2.2 Stimulation electrode

Parallel tungsten wire stimulating electrodes were constructed in the lab. Two 20 cm lengths of tungsten wire (0.075 mm) were inserted into a two-chamber (ϴ-section) glass capillary (the width of the septum is approximately 0.2 mm and wall thickness is

approximately 0.2 mm), such that the two wires were separated (and so electrically insulated) by the septum running the length of the glass. The glass was then pulled to a narrow tip (approx. 0.5 mm) over a gas flame, and the tungsten wire was cut as it protruded from the glass. The ends of the glass were sealed by heating over a flame, such that approximately 0.5 mm of tungsten protruded. Electrical connectors were attached to the wires at the other end of the tubing, to enable connection to the stimulator (Figure 3.1).

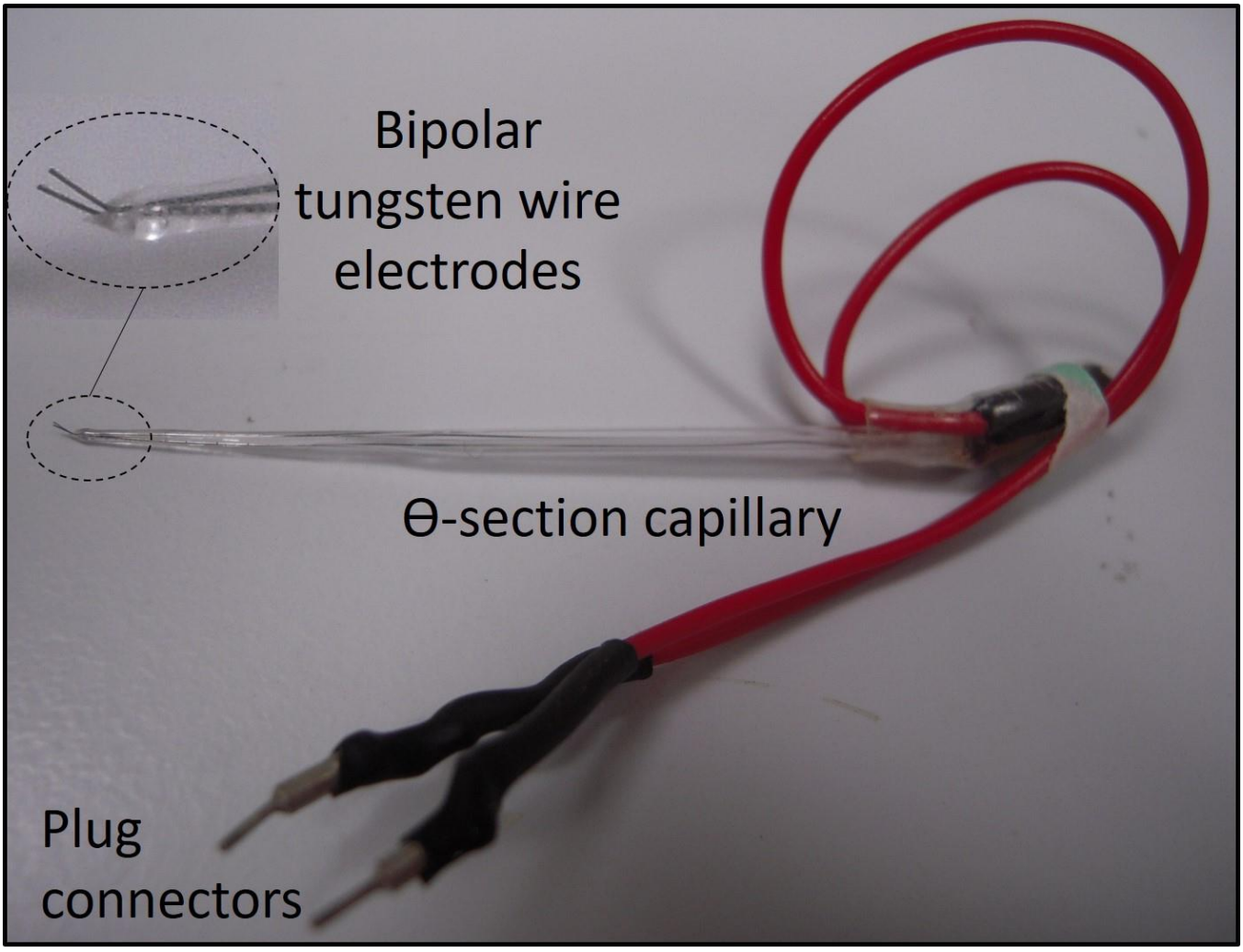


Figure 3. 1 Bipolar tungsten wire stimulation electrode.

3.3 Tissue preparation

3.3.1 Animals

For experiments using non-pretreated animals, female Wistar rats (Division of Biomedical Services, University of Leicester) were humanely killed 4 to 10 days after weaning (age 25 to 31 days), and the brains were removed and placed in ice cold artificial cerebrospinal fluid (aCSF: (mM), NaCl (126.0), KCI (2.0), KH_2PO_4 (1.4), $MgSO_4$ (2.0), $NaHCO_3$ (26.0), $CaCl_2$ (2.4), glucose (4.0). For pretreated animals, drug treatment started 4 to 5 days after weaning (age 25 to 26 days). Animals were injected twice daily with PCP (2 mg/kg; i.p.) or saline vehicle (1 ml/kg; i.p.) for five days, then left drug free for 10 days before they were humanely killed and the brain removed, as described above. Prior to killing, seven days after the end of pretreatment, animals were tested behaviourally, using a novel object recognition task.

All procedures using animals were carried out with appropriate personal and project licence (project number; 60/4390 personal licence; I67DDB722) approval under the Animals (Scientific Procedures) Act, 1986, and with local ethical approval from the University of Leicester Animal Welfare and Ethical Review Body (AWERB).

3.3.2 Brain slicing

The brains were transferred from aCSF to a tissue slicer (NVSLM1 Vibroslice, World Precision Instruments) and consecutive 400 μm slices were cut. Each slice was cut in half along the mid-line, and placed in a tissue saver, comprising a wire mesh suspended in continuously oxygenated aCSF at room temperature, for at least one hour to recover from slicing.

For recording, a unilateral slice containing NAcS was transferred to the tissue chamber of the recording apparatus, and superfused with continuously oxygenated aCSF at 33° ± 1°C, flow rate 2 ml/min, for 60 minutes, using a peristaltic pump (Gilson Minipuls3), before the start of recording. Each brain yielded 2 or 3 bilateral slices containing NAcS giving sufficient tissue for 4 to 6 treatments per brain. In each case, different drug conditions were used on each slice from any one brain, with the order of testing randomised across brains, meaning that the n-values quoted are the number of slices, each from different brains.

3.3.3 Chemicals and drugs

PCP hydrochloride, AP-5 and MSPG were purchased from Tocris, UK. All other drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK). Stock solutions of drugs (10 mM) were made up as follows, aliquoted, and stored at -80°C: MSPG was dissolved in 10 mM NaOH; Picrotoxin was dissolved in 100 mM in DMSO; PCP was dissolved in physiological saline; NMDA was dissolved in water. Working concentrations of the drugs were made freshly on the day of each experiment by appropriate dilutions of the stock solutions in aCSF.

3.4 Experimental Procedures

3.4.1 Electrical Stimulation

The bipolar stimulating electrodes were positioned in NAcS, and the carbon fibre working electrode was placed in the tissue in approximately 0.5mm to the stimulating electrodes (Figure 3.2). A Ag/AgCl reference electrode was placed remotely in the tissue chamber.

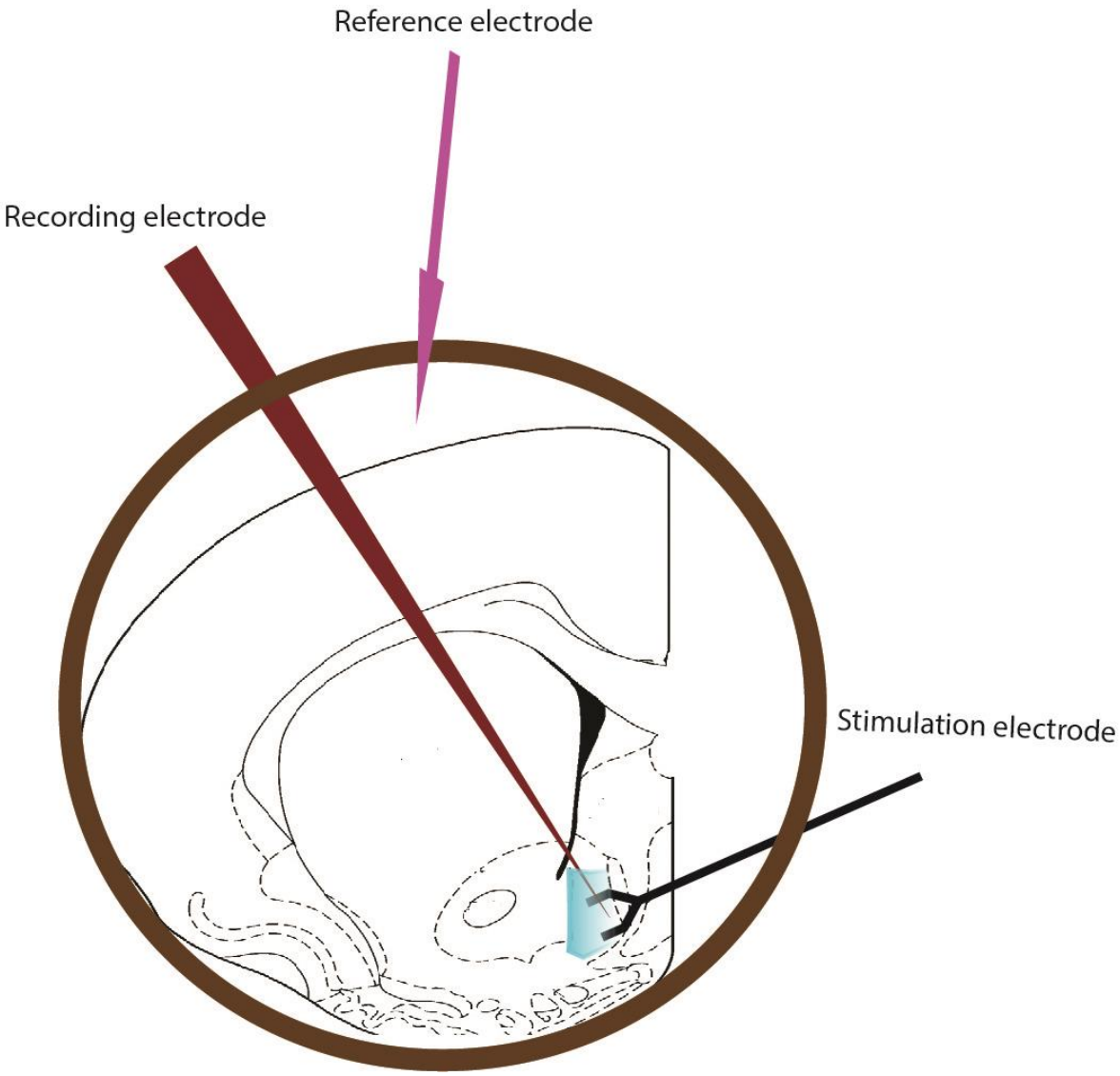


Figure 3.2 Setup of tissue chamber composed of three electrodes; working electrode (left), stimulation electrode (right), and reference electrode.

FSCV was performed as previously described (see chapter 2). Briefly a triangular waveform (-0.4 to +1.3 to -0.4 V; 400 V/sec relative to Ag/AgCl reference electrode) was applied to the carbon fibre working electrode, and the dopamine concentrations were assessed by measuring the oxidation current at 600 mV in the background subtracted signal, using Demon Voltammetry software (Wake Forest, USA: see chapter 2 for detail).

Electrical stimulation was delivered to the stimulating electrodes: pulses were generated from the Demon voltammetry software and delivered to the electrodes through a constant current stimulus isolator (Iso-Flex; AMP Instruments). For validation experiments the stimulation parameters were systematically varied to ascertain the optimum parameters for the main experiments (see below). Based on these results, experiments assessing the effects of drugs used 12 stimulus trains (30 pulses; 500 µA, 4 ms, 60 Hz) applied at 3 min intervals. Repeated 15 sec current recordings were made, with the stimulation occurring 5 sec into each recording, and the current generated by dopamine oxidation (+600 mV) was measured as described above. The concentration of dopamine release was calculated from the oxidation current, by comparison with a dopamine standard calibration (5 µM) carried out prior to each experiment session. Example of FSCV (Figure 3.3). See figure 2.1 and 2.2.

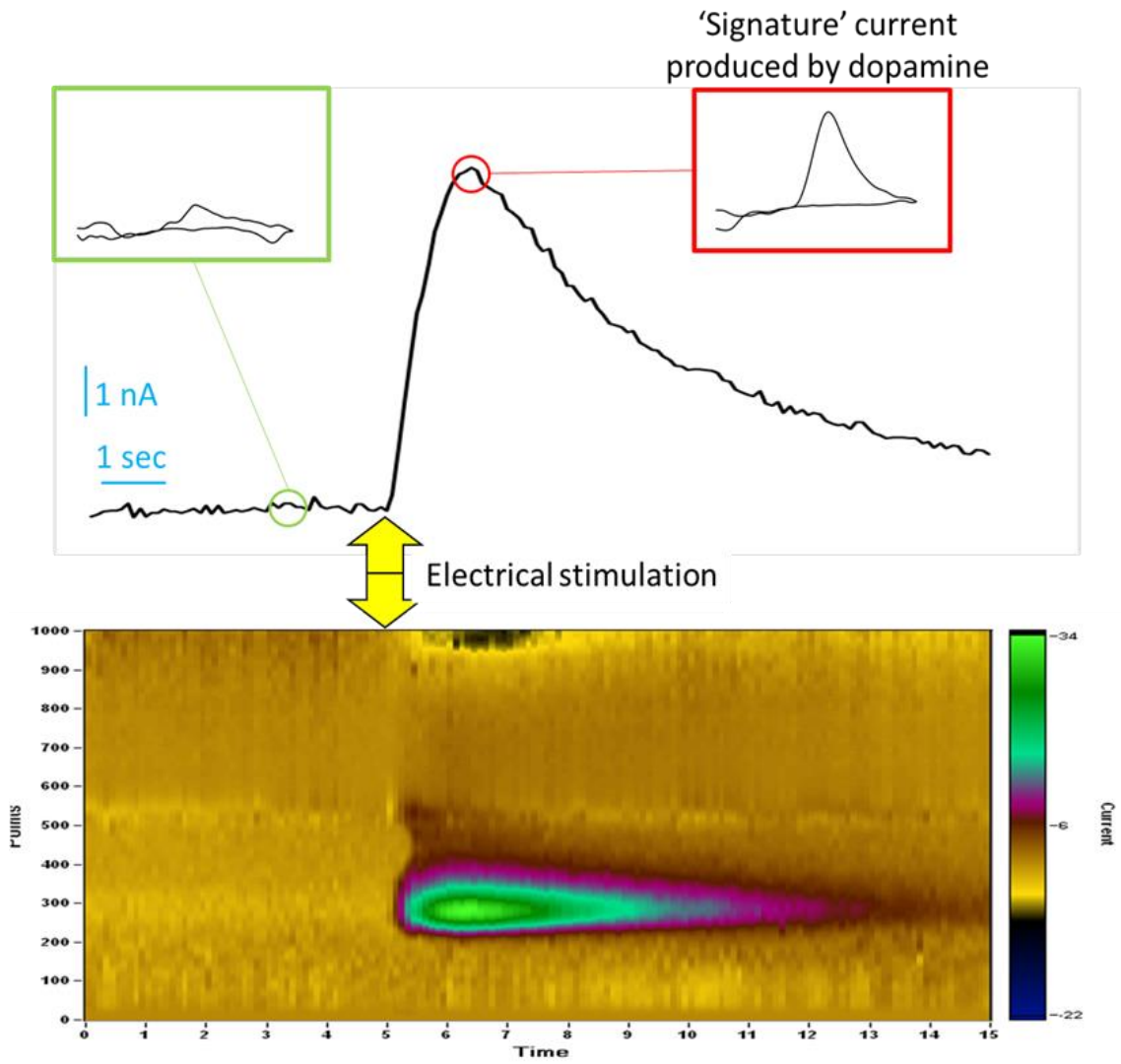


Figure 3.3 Example FSCV recording and colour plot. The top panel shows the current vs time plot at the dopamine oxidation potential, shown as the horizontal white line on the colour plot (bottom panel).Electrical stimulation was given 5 sec after the start of the 15 sec recording (inset shows the cyclic voltammogram measured at the peak release).

3.4.2 Experiment 1: Validation of electrical stimulation

In previous experiments all recordings were carried out using potassium stimulation. The experiments described in this chapter used electrical stimulation. Before using electrical stimulation to study pharmacological effects, preliminary experiments were carried out to ascertain the optimum stimulation parameters to use. Pulse width (4 ms), current (500 μA), and frequency (60 Hz) were kept constant and the number of pulses (5, 10, 20, 30) was varied. To decide the optimum parameters we considered (1) size of response, (2) the clarity of the dopamine signal in the cyclic voltammogram, and (3) reproducibility of response. The order of the experimental conditions was randomized for each experiment. In each case, twelve stimulus trains were applied at 3 min intervals, with the stimulus train applied 5 sec into each recording with the whole experiment lasting 39 min. No drugs were applied in these experiments. Statistical analysis was carried out by t-test, using GraphPad Prism 6.

3.4.3 Experiment 2: TTC Staining

In order to ascertain whether drug treatments used affected the 'health' of the slices (e.g. whether there were any excitotoxic effects), the mitochondrial function marker, triphenyl tetrazolium chloride (TTC) was used. TTC is an indicator of mitochondrial function: active mitochondria display a coloured red. Therefore tissue with active mitochondrial function stains red. After the completion of recording, a representative sample of untreated slices and slices treated with NMDA (15, 30, and 60 μM; n = 4 of each) were stained with TTC, to ascertain the viability of the slices. Slices were transferred to 1 cm diameter dishes (3 ml), and 1 ml of 2% TTC in aCSF was added and incubated at room temperature, and in darkness, for 30 min. After staining, TTC was aspirated off and

replaced with 1 ml of formal saline (10% formalin in 0.9% saline solution). The degree of staining (showing functional mitochondria, indicative of living tissue) was assessed qualitatively in each slice under low magnification.

3.4.4 Experiment 2: Tetrodotoxin (TTX) dependence of stimulated release

Tetrodoxin (TTX) is a sodium channel blocker, hence it prevents action potentials. In present study, TTX was applied during stimulation to check whether the electrically stimulated dopamine release was action potential dependent. TTX (1 µM) was applied in the superfusate after baseline stimulations (S1 to S4). At the end of the drug stimulation period (S8) the superfusion medium was returned to aCSF for the remainder of the recording session.

3.4.5 Experiment 3: Effects of drug treatment on electrically stimulated release of dopamine in slices from non-pretreated animals

The effects of NMDA (15 µM, 30 µM, and 60 µM), the non-competitive GABA-A receptor channel blocker picrotoxin (100 µM), α4β2 subunit of nAChR antagonist DhβE (1 µM), and 2/3 type metabotropic receptor antagonist MSPG (200 µM) on baseline and electrically-stimulated dopamine release were tested in slices from non-pretreated animals by applying FSCV in *in vitro* brain slices. The order of the experimental conditions was randomized for each experiment. Total experiment time was 39 minutes. Statistical analysis was carried out by two-way ANOVA, using GraphPad Prism 6

Slices were superfused continuously with oxygenated aCSF (flow rate 1.5 ml/min; Gilson Minipulse3), and repeated 15 sec FSCV recording were made at 3 min intervals. A

stimulation train (30 pulse 4 ms; 500µA; 60Hz) was delivered 5 sec into each recording period. After the first four stimuli (S1 to S4, labelled Baseline) the perfusion was switched to oxygenated aCSF containing the appropriate drugs, and superfusion continued for a further four stimulations (S5 to S8, labelled Drug). The superfusate was then returned to oxygenated aCSF without drug for a further four stimulations (S9 to S12, labelled Washout). In control (drug-free) experiments tissue (in slices from either non-pretreated, saline pretreated or PCP pretreated animals) was superfused with oxygenated aCSF at 1.5 ml/min throughout the twelve stimulation, without drug application. The control (non drug treated slices) ascertained that the electrically-stimulated release remained consistent across the full duration of the experiment (12 stimulations). The effect of drugs was determined by comparing electrically stimulated release during the drug application period (S5 to S8) with release during the baseline stimulation period (S1 to S4), and by comparison between the drug treated slices and the equivalent stimulation number in control (non-drug treated) slices. Recovery to baseline stimulated levels after the end of drug treatment was assessed by measuring the responses during the washout period. Data were normalised to the mean baseline concentrations of stimulated release during the control period, and presented as percentages of control.

3.4.6 Experiment 4: Saline/PCP pretreated

The effect of pretreatment with PCP on NMDA modulation of electrical stimulation-evoked release of dopamine was performed and further investigation of the mechanism used concomitant application of DhβE (α4β2 nAChR antagonist; 1µM). PCP (2 mg/kg) or vehicle (0.9% saline) (volume 1 ml/kg) was administered twice daily for five days, and the animals were then left for at least five days (drug free) before testing. Full details

of the procedure were as previously described (chapter 2). Following the drug-free period, the animals were humanely killed, the brains removed, 400 µm slices containing NAcS were cut, slices were placed in the recording chamber, and after equilibration (60 min) electrical stimulation were applied (12 pulse trains at 3 min intervals: 30 pulses, 4 ms, 500 µA, 60 Hz) all as previously described. Drugs (NMDA and DHβE) were applied in the superfusion medium during stimulations 5 to 8. Baseline, Drug, and Washout periods were compared between slices taken from saline and PCP pretreatment animals in order to assess the effect of subchronic PCP pretreatment on dopamine release. Superfusion was carried out as described non-pretreated experiments above.

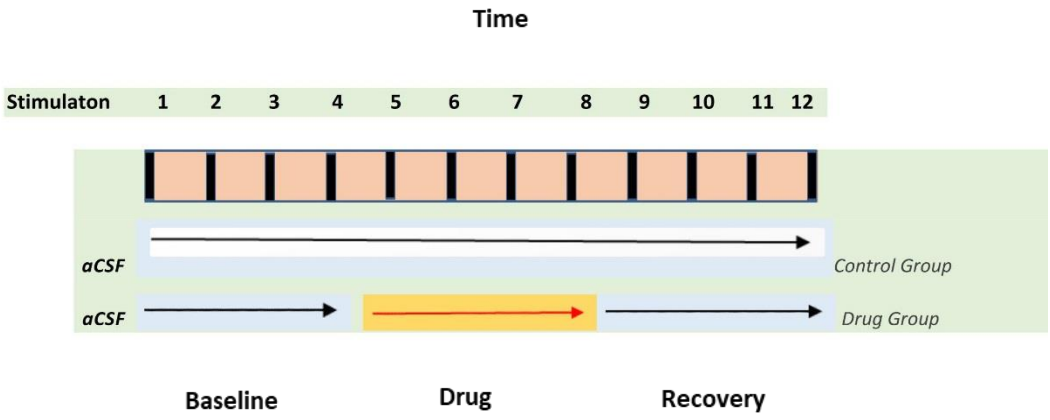


Figure 3.4 Schematic of the experimental schedule. Baseline is 1 to 4 recordings. Related drug was applied during recording 5 to 8 (red arrow), washout is 8 to 12 recordings. In control, slices were not supefused drug rather aCSF superfused acroos 12 stimulation. In drug group, slices were superfused aCSF as seen control group, drug was switch on between 4 and 8 stimulations period, drug was switch off at last 4 stimulation period (8 to 12).

3.4.7 Measurement of dopamine reuptake

To ensure nicotine does not change DAT activation, tau which is a measure of exponential decay from peak to baseline was measured (Yorgason, *et al.*, 2011). Tau, the rate of slope decay, is considered an indication of dopamine reuptake changes (Yorgason, *et al.*, 2011), hence tau values were calculated from nicotine perfused slices (Figure 3.5). In fact, tau is more accurate than half-life because it is a measure of the whole slope of decay and a more appropriate measure of reuptake. Baseline, drug, and washout periods were considered in this calculation. There were no obvious differences of peak levels between PCP and saline pretreated slices. Long-term changes evoked by PCP pretreatment may cause dysregulation of cholinergic systems which modulate dopamine release in NAc

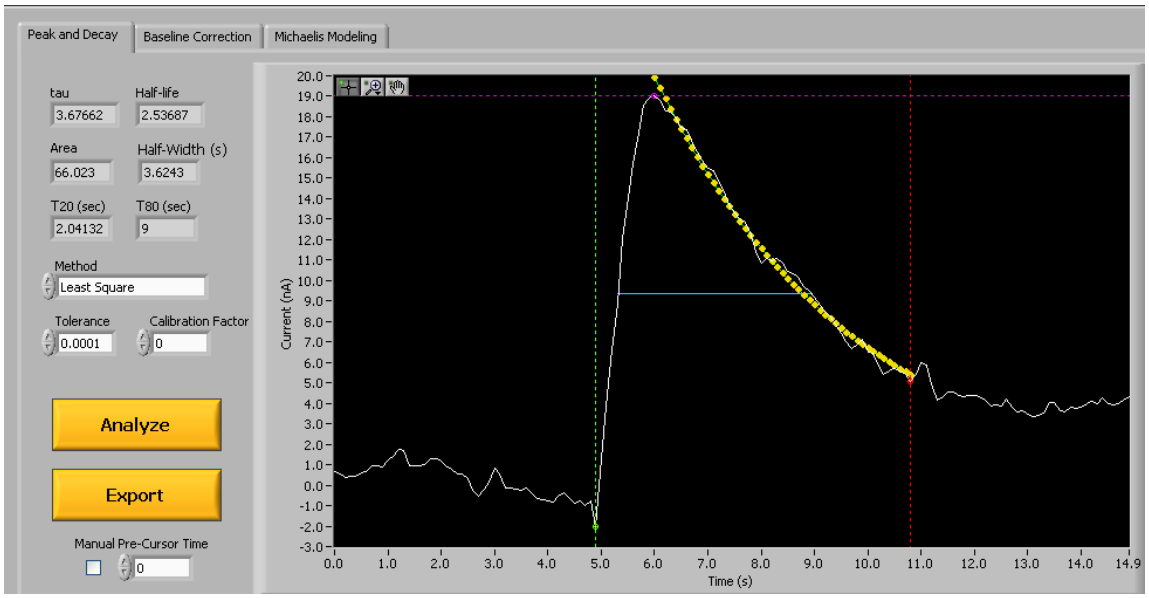


Figure 3.5 Measurement of dopamine reuptake. Red line shows peak and post stimulation cursors. Blue line shows full width at half width. Yellow line shows tau or half line.

3.5 FSCV Data Presentation and Analysis

The maximum extracellular concentration (peak-dopamine concentration, µM) was calculated from the background-subtracted voltammogram (Demon Voltammetry software), and defined as the maximum current generated during dopamine release (peak-dopamine signal, nA), by comparison with a calibration to 5µM dopamine standard. The mean baseline stimulation of the four baseline stimulations (S1 to S4) was calculated for each slice and the data for each slice were expressed as a percentage of the mean baseline for that slice. All data are expressed as mean ± SEM percentages of the baseline stimulated levels. Statistical analysis was carried out by t-test (experiment 1); and two-way ANOVA, followed by appropriate post-hoc analysis (experiments 2 and 3). DAT analysis calculated tau based changes. It is recommended tau measuring dopamine uptake (Yorgason et al., 2011). (details in discussion section) Statistical analysis was performed by using GraphPad Prism 6.

3.6 Results

3.6.1 Experiment 1: Validation of electrical stimulation

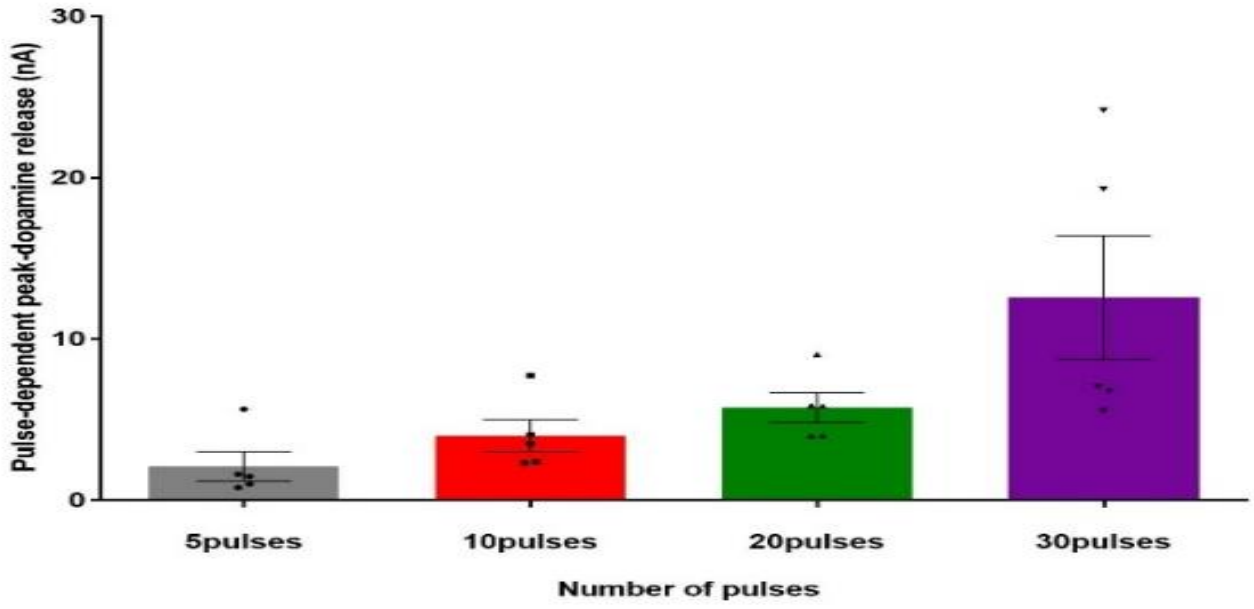


Figure 3.6 Effect of different pulses on electrically stimulated dopamine release. Peak dopamine release was dependent on the number of pulses delivered; Dunnett's post hoc test, (n = 5 per group). Data represent **nA dopamine release.**

In characterisation experiments, diffirent pulses has been used to characterise dopamine release behaviour. Therefore, 5, 10, 20, and 30 pulses applied to electrode while the parameters were kept at 60Hz, 4ms, 500 µA. Results show that 30 pulses gave obvious and definitive response regarding clear colour plot and voltammogram, hence we chose and carried out experiments with 30 pulses. Significant interaction between pulses ($F [12, 178] = 378.9$; $P<0.0001$). The other pulses did not give such a nearly clear dopamine response. Qualitative data from obtained the colour plot and analysis of voltammogram

(Figure 3.7) showed that 30 pulses gave the clearest dopamine response when compared to other used pulses.

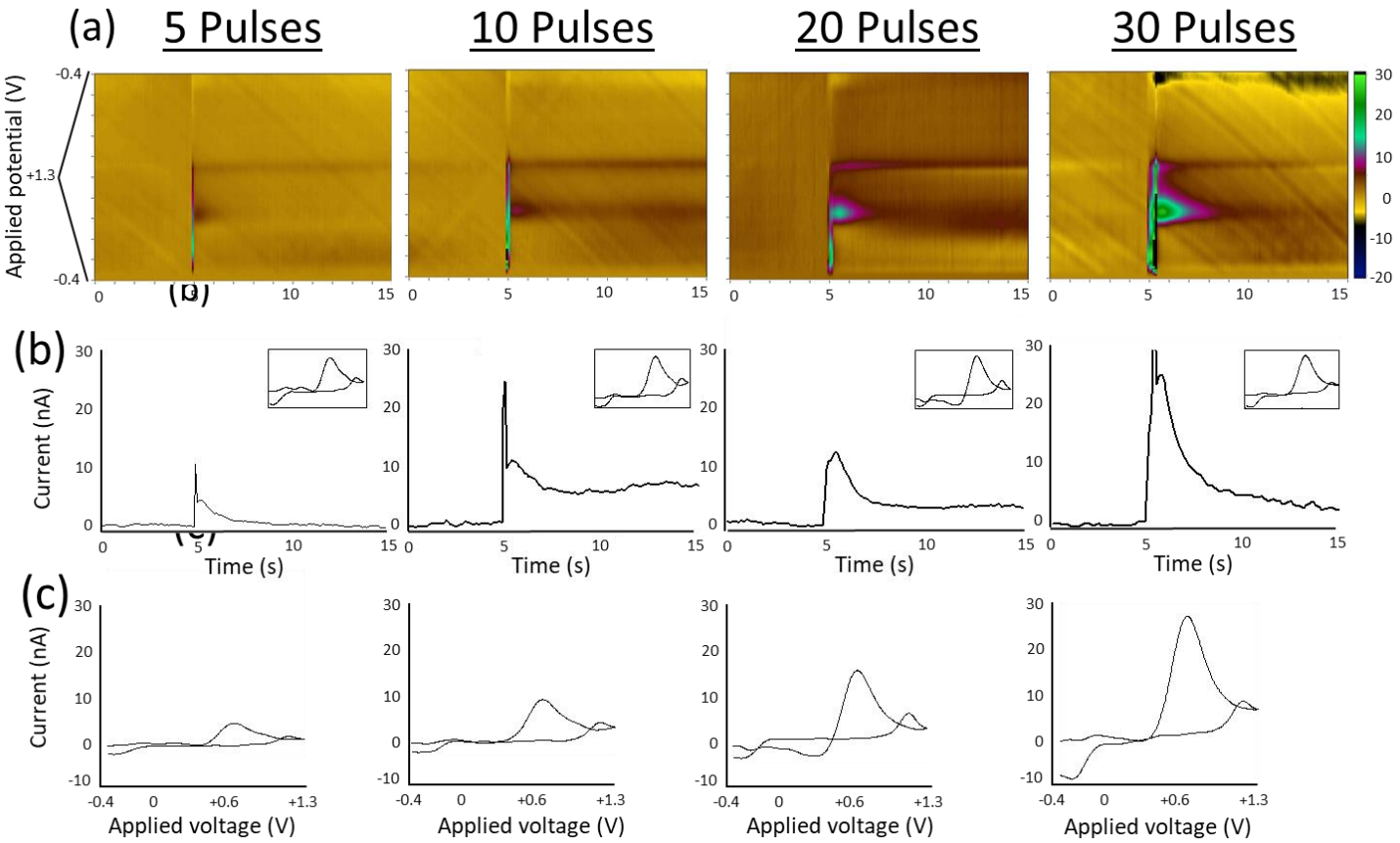


Figure 3.7 Example FSCV recordings of electrically stimulated dopamine release using 5, 10, 20 or 30 pulses. (a) Colour plots, (b) Current vs time plots (inset shows the amplified cyclic voltammogram at peak dopamine release to aid identification); (c) scaled cyclic voltammograms (current vs voltage plots).

3.6.2 Experiment 2: Slices from non-pretreated animals

NMDA at concentrations of 15 µM, 30 µM, and 60 µM in the superfusate caused a significant dose-dependent decrease in electrically stimulated dopamine release NAcS in slices from naïve rats (n = 12; Figure 3.8 and figure 3.9). Two-way ANOVA showed a main effect of stimulus number ($F[11,132] = 8.32$; $p < 0.0001$) and of drug ($F[3,396] = 72.91$; $p < 0.0001$) and a significant interaction ($F[33,396] = 3.69$; $p < 0.0001$). Moreover, the changes were concentration-dependant since there was significant difference between the different NMDA concentrations. Post hoc analysis revealed that NMDA decreased electrically stimulated dopamine release at concentrations of 30 µM and 60 µM: although there was an indication of a decrease also at 15 µM, this was not significant. However, concentration dependence was confirmed by a significant linear trend in the drug effect over the doses of NMDA ($R^2 = .3573$; $p < .0001$).

Following the end of NMDA superfusion, the stimulated release of dopamine showed limited, dose-dependent recovery over the subsequent four stimulations. After 15 µM there was almost full recovery, while in 30 µM there was partial recovery. However, in 60 µM, there was little sign of recovery. On this basis 30 µM was chosen as the most appropriate dose for further studies, as it gave a consistent and significant attenuation of the stimulated release, which was not maximal (and so gave scope for observing modulation in either direction). Example FSCV data obtained from 30µM NMDA showed in figure 3.10.

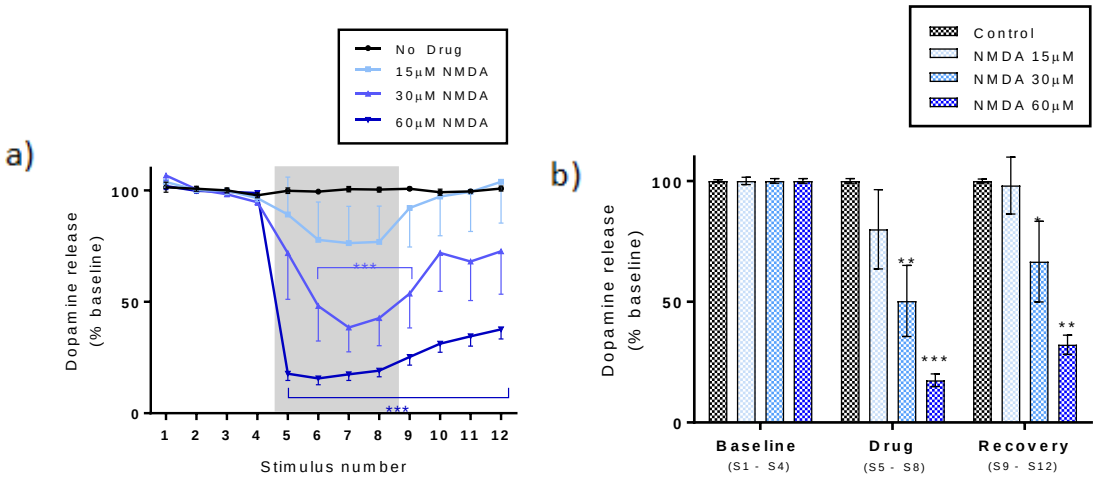


Figure 3.8 Effect of different doses of NMDA on electrically stimulated dopamine release. (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$; *** $p < .001$; Significant difference from baseline stimulated levels; Dunnett's post hoc test, based on a significant interaction from ANOVA: n=12 per group.

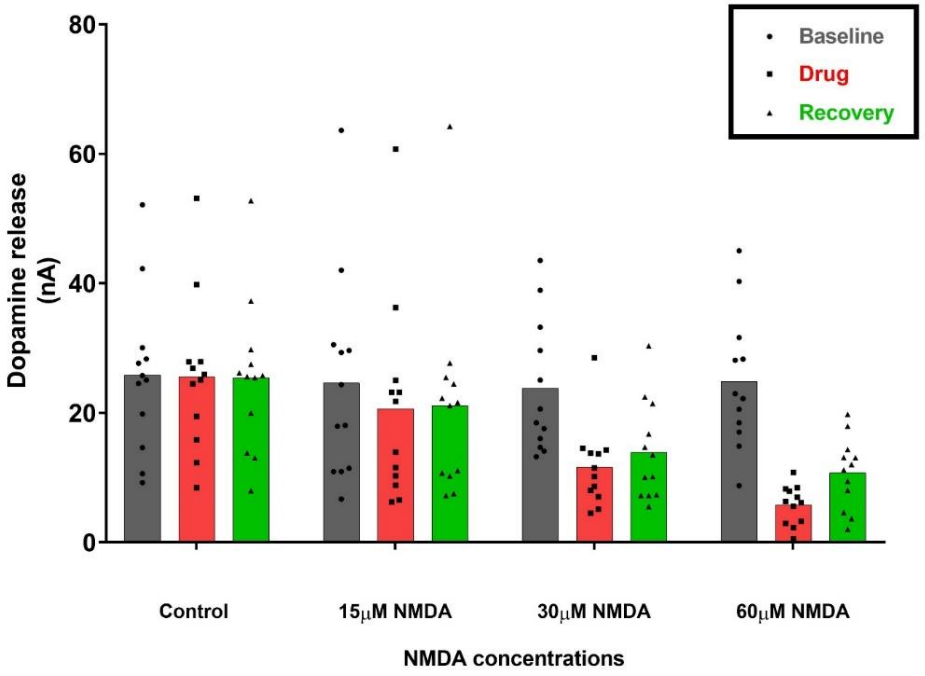


Figure 3.9 Effect of different doses of NMDA on electrically stimulated dopamine release. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine release** from control, 15 µM NMDA, 30 µM NMDA, 60 µM NMDA.

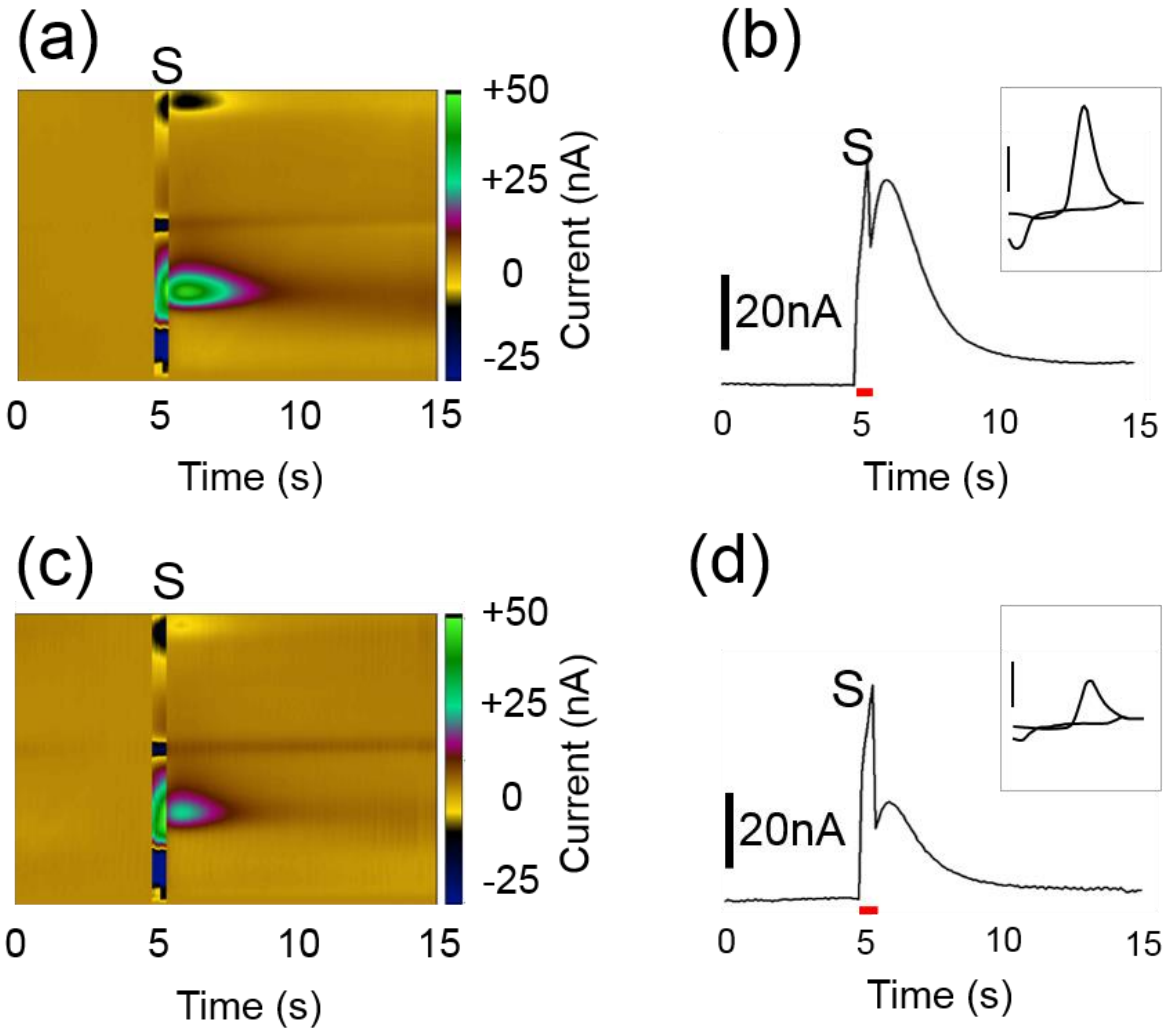


Figure 3.10. Example FCV data from a single slice showing effect of NMDA (30 µM). (a) Colour plot and (b) current vs time plots of electrically stimulated (red bar) dopamine release during baseline period (S1). (c) Colour plot and (d) current vs time plots of electrically stimulated (red bar) dopamine release during bath application of 30 µM NMDA (S8). In each case "S" indicates the stimulus artefact. Insets in (c) and (d) indicate cyclic voltammorgams measured during the release, confirming the identity of dopamine (scale bar = 20 nA).

The effect of NMDA (30 µM) was entirely blocked by the specific NMDAR antagonist AP5 (50 µM). Statistical analysis using two-way ANOVA confirmed a significant main effect of drug ($F[3,240] = 166.0$; $p < 0.0001$) and of stimulus number ($F[11,240] = 11.48$; $p < 0.0001$), and a significant interaction ($F[33,240] = 9.844$; $p < 0.0001$). Post hoc analysis showed a significant decrease by NMDA, which was abolished by AP5, while AP5 had no effect on stimulated release when applied alone (Figure 3.11).

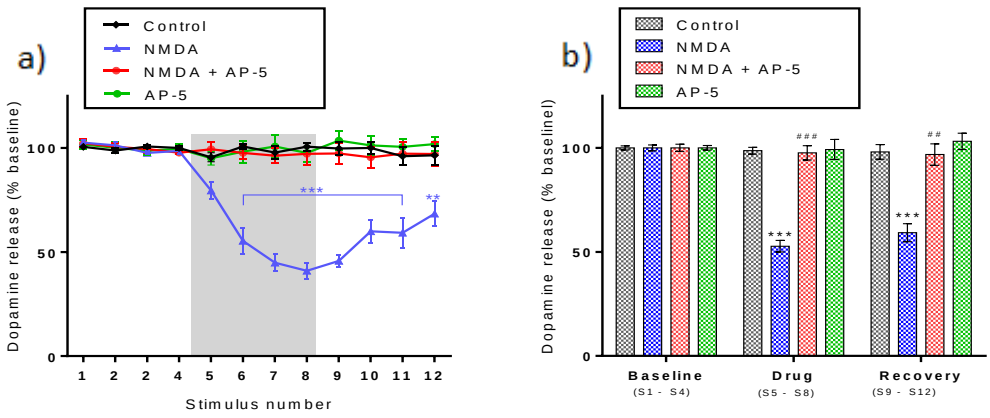


Figure 3.11 Reversal of the effect of NMDA (30 µM), by AP-5 (50 µM) (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods.

*** $p < .0001$; Significant difference from non drug treated slices; ## $p < 0.01$; ### $p < 0.001$; significant difference from NMDA alone; Dunnett's post hoc test, based on a significant interaction from ANOVA: n=6 per group.

It is unlikely that the depressant effect of NMDA was due to a direct action on dopamine neurones, since (1) histological evidence shows that NMDARs are sparsely located on

dopamine terminals, and (2) any direct effect of NMDA would be expected to be excitatory. Three possible indirect routes for this action were considered; (1) via GABAergic systems, given the abundance of GABAergic medium spiny neurones and interneurons in NAc: (2) through mGluR-mediated mechanisms, given the evidence that mGluR group 2 and 3 receptors have been shown to be inhibitory, and reduce dopamine release (Conn and Pin, 1997; Karasawa *et al.*, 2006); or (3) via cholinergic systems, since cholinergic interneurons constitute of 2 % of striatal neurons and nAChRs are capable of regulating dopamine release in those regions (Threlfell *et al.*, 2012).

3.6.3 Experiment 3: TTC Staining Results

NMDA caused dose dependent and receptor specific depression dopamine release in NAcS region. It is possible that these changes reflected excitotoxic damage. However, arguing against that, we have seen some degree of recovery during washout (i.e. S9 to S12). To assess whether decreasing dopamine release brought by NMDA resulted from excititocity we stained slices with TTC after completion of recording. Results showed substantial staining in NMDA treated slices indicating that they were still alive, thus implying that the drug treatment did not cause excitotoxic damage. Staining of representative sample from obtained TTC is shown Figure 3.12

Since NMDA is excitotoxic, we considered whether NMDA has an excitotoxicity effect on the tissue, which could account for the attenuation of stimulated dopamine release. TTC staining of rat brain slices following FCV recording and superfusion with (a) aCSF alone, or with NMDA, (b) 15 μM; (c) 30 μM or (d) 60 μM. staining of are presentative sample (n = 4 for each concentration) of slices with the mitochondrial function marker TTC after completion of recording showed strong staining in all cases, indicating a

healthy slice (intact mitochondrial function): indeed the level of staining was similar to that of nondrug treated slices. The degree of staining (showing functional mitochondria, indicative of living tissue) was assessed qualitatively in each slice.

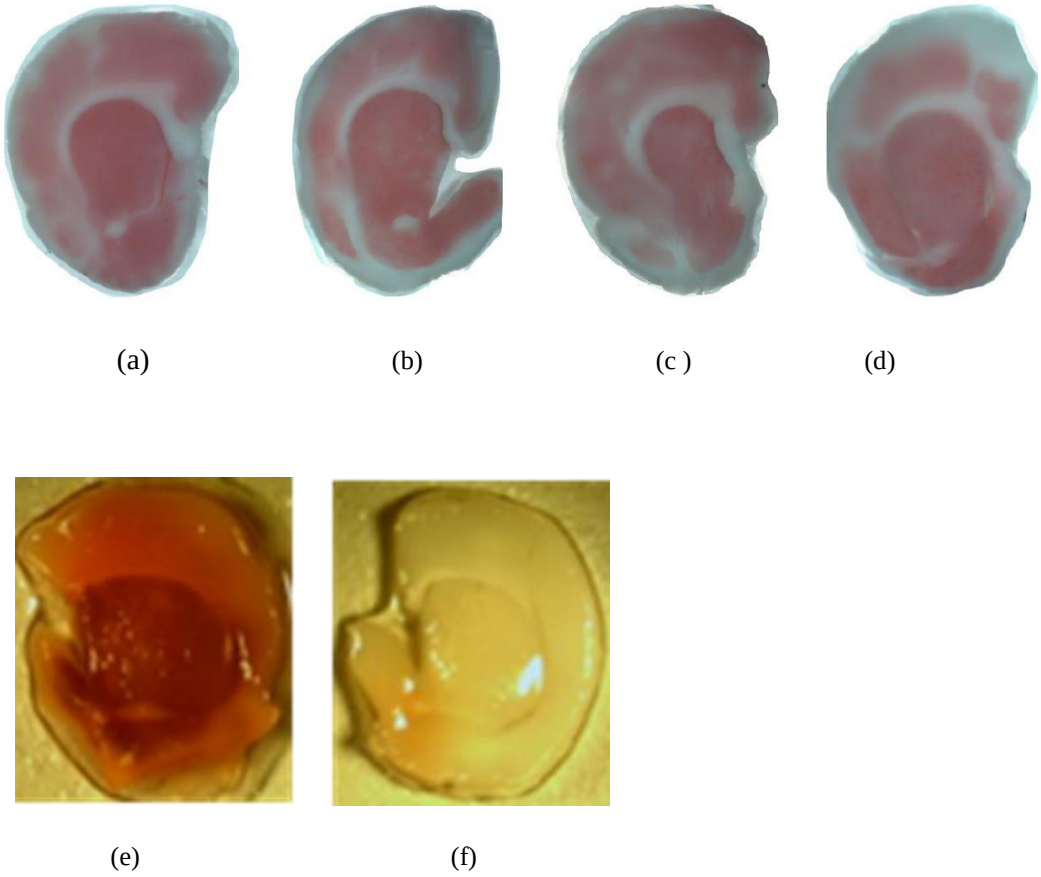


Figure 3.12 TTC staining of rat brain slices following FSCV recording and superfusion with (a) aCSF alone, or with NMDA, (b) 15µM; (c) 30 µM or (d) 60 µM. For comparison, (e) a healthy slice, and (f) a slice that has been exposed to oxygen and glucose depletion are shown (from Peters, Gibson & Young, unpublished).

Stain intensity correlates with the number and functional activity of mitochondria (Goldlust et al., 1996), since it is active mitochondria that convert TTC to the coloured product, TPF (1,3,5-triphenylformazan): thus any stain observed indicates active mitochondria. Where a slice is not metabolically active no stain is visible (see fig 3.12e). However, TTC is staining is not always predictable technique to measure cell viability. In fact, in vivo and in vitro studies apply different TTC staining method both of which gives different results regarding cell viability assessment (Dettmers et al., 1994, Park et al., 1988). One consideration is that transient impairment of dehydrogenase activity may result from false detection of TTC staining (Li et al., 1997, Rehncrona et al., 1979). Therefore, results shown in our experiments need caution to evaluate NMDA toxicity. Although we have compared three concentration of NMDA to control slices to assess NMDA toxicity it worth also comparing those concentrations with a dead slice as positive control which might give a better assessment of TTC staining detection on cell viability.

3.6.4 Experiment 3: TTX experiment results

Since electrical stimulation may have non-specific effects, it was important to check that the dopamine changes seen after stimulation did reflect neurotransmitter release. This was ascertained by testing the TTX-dependence of the changes. The result showed that electrically stimulated dopamine release was TTX-dependent in NAc brain slices. Although there was a small decrease in response during the first stimulation after the application of TTX (S5), from the second stimulus after drug application (S6) stimulated release was entirely. As would be expected, recovery was slow after the switching back to aCSF without TTX, but 45 min after the end of TTX application (S24) there was an indication of a small recovery of the dopamine signal. This therefore confirmed that the electrically stimulated release was action potential dependent dopamine release.

Experiment protocol for TTX and TTX inactivated dopamine release showed in figure 3.13 and 3.14.

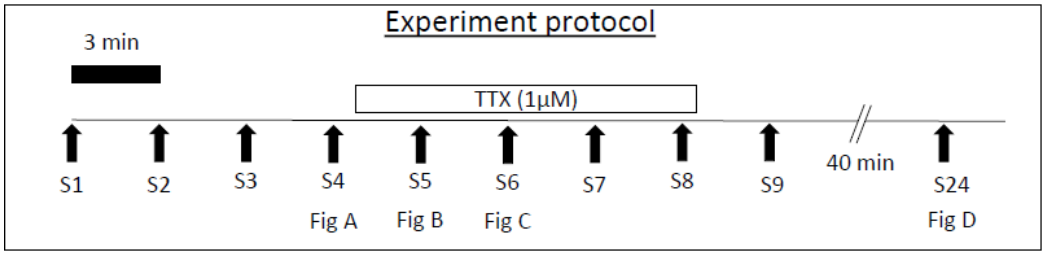


Figure 3.13 Experimental protocol for determining TTX-dependence of electrically stimulated dopamine release.

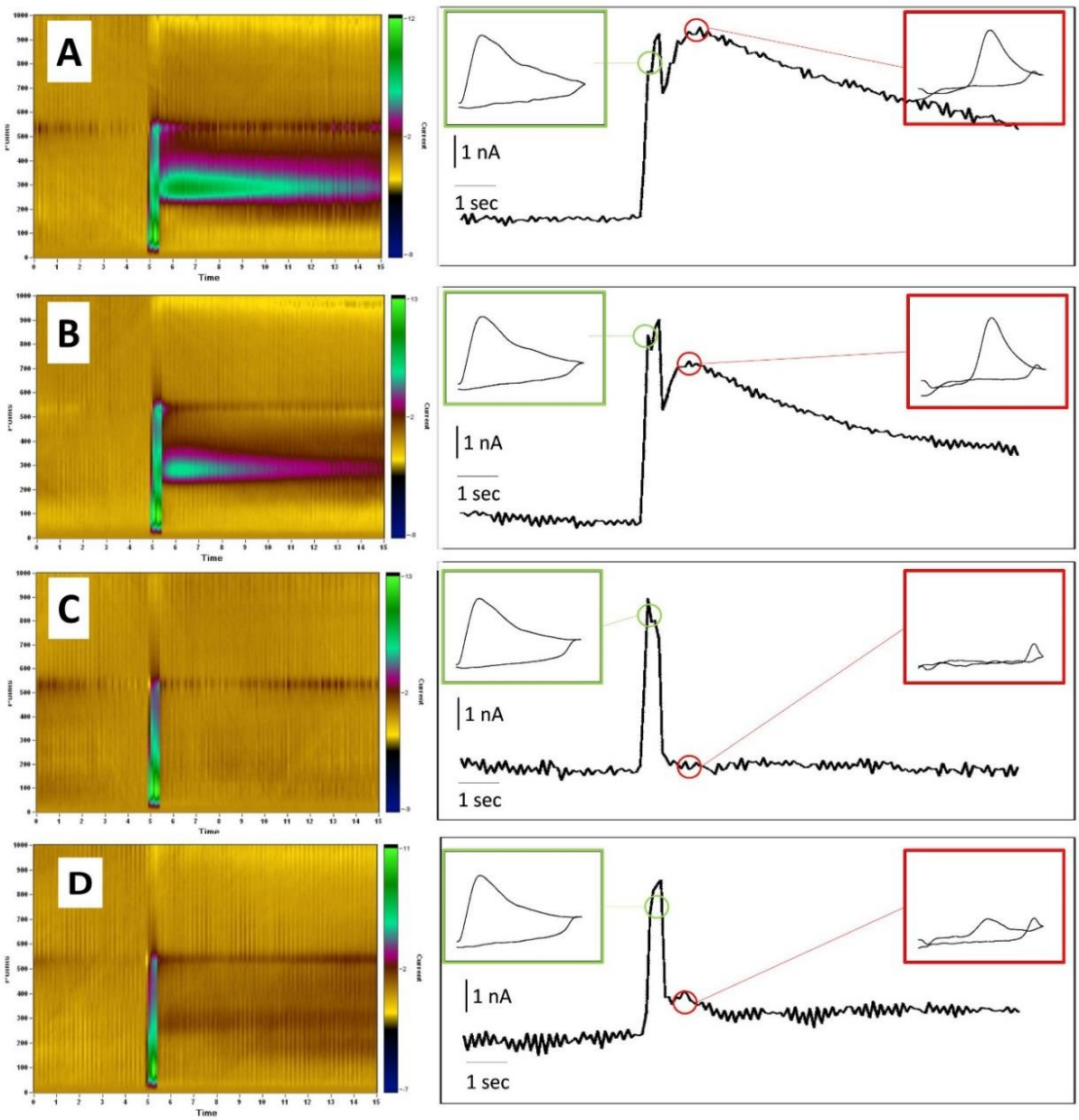


Figure 3.14 TTX inactivated electrically stimulated dopamine release. A) Baseline stimulation (S4), B) The first stimulation after TTX application (S5), C) The second stimulation after TTX application (S6), D) Stimulation 45 min after the end of TTX application (S24).

3.6.5 Experiment 3: Effects of drug treatment on electrically stimulated release of dopamine in slices from non-pretreated animals

The NMDA receptor agonist NMDA (30 µM), $GABA_A$ receptor antagonist picrotoxin (100µM), and 200 µM MSPG were seen to have different effects on dopamine release. To investigate whether the inhibitory effect of NMDA was mediated through GABA systems, the GABA-A receptor antagonist, picrotoxin (100 µM) was applied concomitantly with NMDA (30µM). However, contrary to the prediction, picrotoxin applied concomitantly with NMDA did not reverse the effect of NMDA, but rather showed some signs of enhancing it. There was a main effect of stimulus number ($F [11,324] = 9.31$; $p < 0.0001$) and of drug ($F [2,324] = 48.15$; $p < 0.0001$) and a significant interaction ($F [22,324] = 3.00$; $p < 0.0001$). Post hoc analysis (Dunnett's test) showed that NMDA caused a significant reduction in stimulated release either in the absence or the presence of picrotoxin.

On the other hand, blockade of group 2 and 3 mGluR using the relatively non-specific receptor antagonist MSPG (200 µM) did reverse the effect of NMDA (30 µM) on stimulated dopamine release. There was a main effect of stimulus number ($F [11,228] = 14.45$; $p < 0.0001$) and of drug ($F [3,228] = 149.3$; $p < 0.0001$) and a significant interaction ($F [33,228] = 9.165$; $p < 0.0001$). Post hoc analysis (Dunnett's test) showed that NMDA caused a significant reduction in stimulated release which was abolished in the presence of MSPG, while MSPG alone had no effect. Tukey's HSD test on each time point showed a significant attenuation of the NMDA depression of electrically stimulated dopamine release during, and immediately after the drug application period (n = 5 to 6: Figure 3.15).

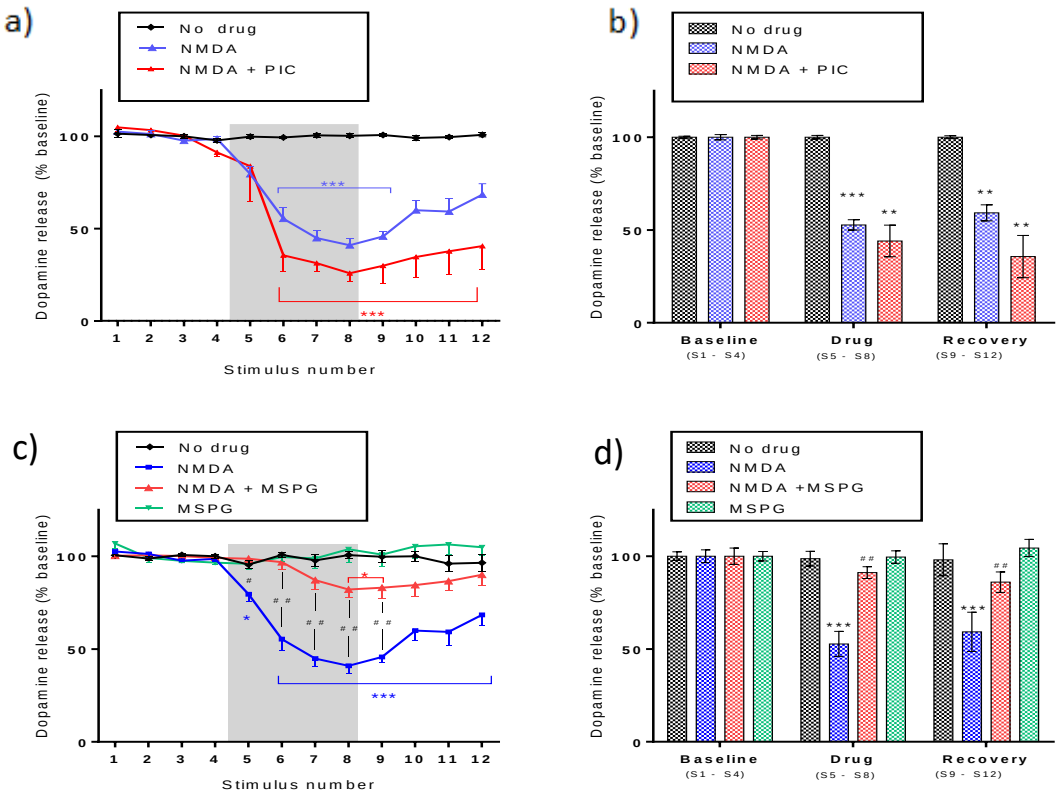


Figure 3.15 Effect of picrotoxin (100 µM; a,b) or MSPG (200 µM; c,d) on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM). (a,c) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b,d) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$; *** $p < .001$: significant difference from control (Dunnett's post hoc test). # $p < .05$; ## $p < .01$; ### $p < .001$: significant difference between NMDA alone and (a) NMDA + picrotoxin or (c,d) NMDA + MSPG (Tukey's HSD test): n = 5 to 7.

In addition, there was a significant nAChRs regulation on endogenous dopamine activation in non-pretreated slices. The β2 nAChRs subunit antagonist, DHβE (1 µM)

when administrated with 30 µM NMDA showed a significant reversal of the decrease in stimulated release caused by 30 µM NMDA administration, although it did not fully reverse it. There was a main effect of stimulus number ($F [11,252] = 4.276$; $p < 0.0001$) and of drug ($F [3,252] = 323.8$; $p < 0.0001$) and a significant interaction ($F [33,252] = 15.82$; $p < 0.0001$: Figure 3.16).

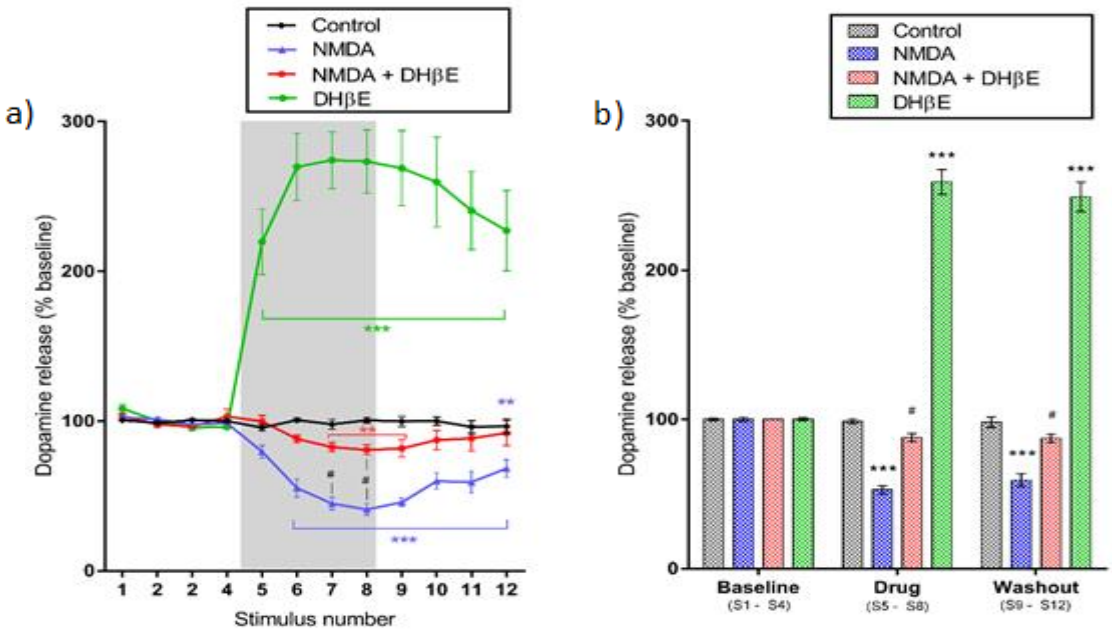


Figure 3.16 Effect of DHβE on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM).(a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$; *** $p < .001$; Dunnett's post hoc test, based on a significant interaction from ANOVA: n=12 per group.

Previous studies have shown show that both mAChRs and nAChRs contribute to NMDA mediated control of dopamine release. DHβE applied with concomitant NMDA attenuated NMDA-depression of dopamine release in NAcS. On the other hand, DHβE

caused a substantial and significant increase in stimulated dopamine release when applied alone. It is likely therefore, the co-occurrence of two independent process one depressant modulated NMDARs and one facilitatory which is mediated through α4β2 receptors, rather than the NMDA effect being mediated through cholinergic mechanism. In order to investigate cholinergic modulation of dopamine with or without PCP pretreated slices, we investigated the effect of different doses of DHβE on electrically stimulated dopamine release in NAcS.

The nAChRs receptor antagonist, DHβE at concentrations of 100 nM, 300 nM, 1 μM, in the superfusate caused a dose-dependent increase in electrically stimulated dopamine release from accumbal slices (Figure 3.17 and 3.18). The stimulus-evoked dopamine release in slices obtained from naïve rats (n = 7) was increased significantly by DHβE administration of 300 nM and 1 μM , but not 100 nM. Two-way ANOVA showed a main effect of stimulus number ($F [11,288] = 21.91$; $p < 0.0001$) and of drug ($F [3,288] = 130.9$; $p < 0.0001$) and a significant interaction ($F [33,288] = 6.583$; $p < 0.0001$). Dunnett's post hoc analysis revealed that DHβE significantly increased electrically stimulated dopamine release at 300 nM and 1 μM concentrations.

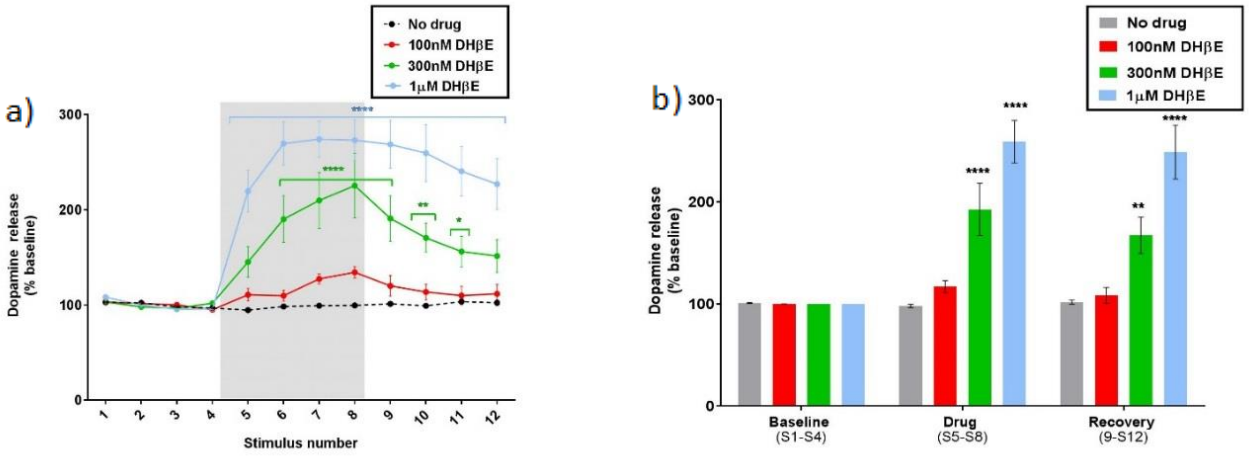


Figure 3.17 Effect of different doses of DHβE on electrically stimulated dopamine release. (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. ** $p < 0.01$; *** $p < 0.001$; Dunnett's post hoc test, based on a significant interaction from ANOVA: n = 12 per group.

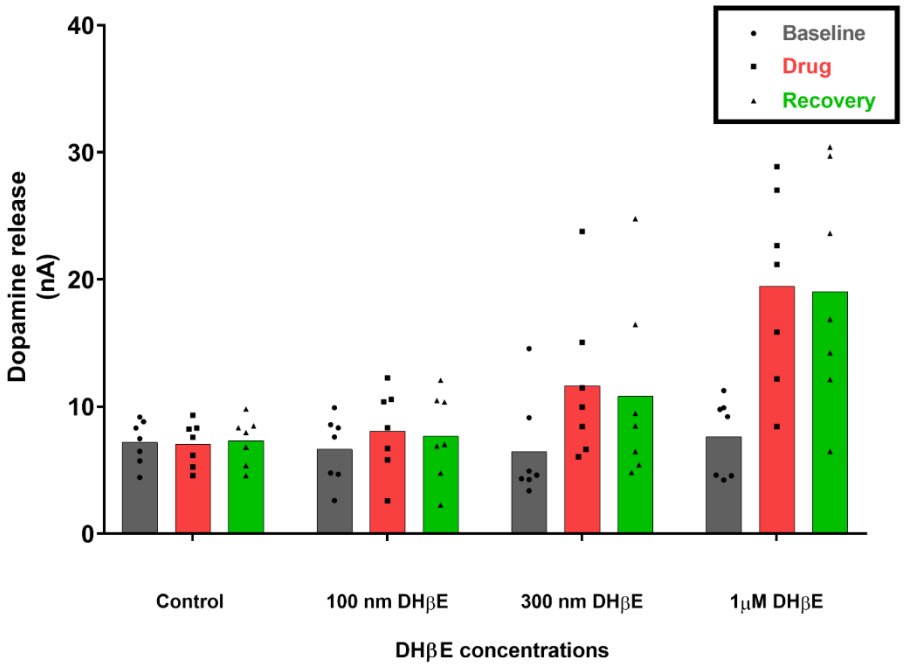


Figure 3.18 Effect of different doses of DHβE on electrically stimulated dopamine release. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine release** from control, 100 nM DHβE, 300 nM DHβE, 1 μM DHβE

Cholinergic interneurons comprise 2% of striatal neurons and modulate dopamine in release by activation of nAChRs and mAChRs located on dopaminergic axon terminals in NAc. The dose dependent results show that blocking α4β2 subunit of nAhRs increased dopamine release in NAcS. The lowest concentration did not change significant dopamine release, but two the two high doses (300 nM and 1 µM) did cause significant increases. Whereas the lowest dose returned fully to baseline levels in washout, starting in the stimulation after the drug was switched off (S9), the higher doses did not, although they did show a sign of returning baseline levels: 300 nM DHβE showed partial, but consistent return to baseline levels compared to highest doses.

3.6.6 Acetylcholine modulation of dopamine and glutamate release

Nicotinic acetylcholine receptors (nAChRs) are expressed on both dopaminergic axon terminals and cholinergic interneurons in NAc. In the current study, antagonism of β2 subunit of nAChRs by DhβE dose-dependently enhanced dopamine release in slices obtained from drug-naïve and saline pretreated rats. On the other hand, subchronic PCP pretreatment somewhat interrupted dose dependent dopamine release brought on by DHβE. The literature shows that phasic and tonic cholinergic signalling modifies dopamine and glutamate release. Further, high frequency stimulation of presynaptic β2 subunit of nAChRs, which mimics phasic ACh signalling, evokes endogenous ACh release from synaptic vesicles (Zhou et al., 2001), which in turn attenuates glutamate transmission efficiency (Pakhotin and Bracci, 2007; Rice and Cragg, 2004). Phasic nAChR stimulation desensitizes tonic acetylcholine mediated neurotransmission by modulating calcium release from internal vesicular stores (Emptage et al., 2001; Zhou et al., 2015).

To what extent does phasic nAChR activation reducing glutamate signalling in NAc. One possibility is that nAChR activation potentiate activation of mGlURs II/III receptors expressed on dopaminergic axon terminals in NAc. Since mGluRs II/III is known to inhibit excitatory post synaptic currents, endogenous Ach may indirectly attenuate glutamate transmission by modulating glutamatergic receptors. Another possible mechanism for ACh reducing glutamate transmission in NAc is through an indirect mechanism involving activation of muscarinic AChRs; striatal glutamatergic transmission is modulated by ACh modulation of muscarinic receptors (Sugita et al., 1991). For example, ACh activates M5 receptors located on dopaminergic axon terminals M4 receptors on GABAergic interneurons, the effects of which is to modulate glutamate transmission.

Behavioural studies showed that endogenous acetylcholine elicits dopamine release from dopamine terminals by activating α4β2 nicotinic acetylcholine receptors located on dopamine neurons in the VTA and α4β2 nicotinic acetylcholine receptors located on dopamine terminals in the nucleus accumbens (NAc) (Xi et al., 2009).

In addition to α4β2 nicotinic acetylcholine receptor modulation of glutamate transmission, β2 subunit of nAChRs also modulates dopamine release in dorsal striatum and NAc. DHβE has bidirectional effect on tonic and phasic dopamine release (Rice and Cragg, 2004). DHβE augments dopamine release induced by high frequency stimulation, whereas low frequency stimulation attenuates dopamine release in striatal brain slices. The mechanisms for enhancement of dopamine release involves the release of endogenous ACh and subsequent desensitization of presynaptic β2 subunit by application of DHβE. Rice and Cragg (2004) suggested that desensitization of nAChRs on dopamine terminals has an effect of increasing phasic dopamine neuronal firing. However, nicotinic receptors can bidirectionally regulate both phasic and tonic dopamine release in dorsal

striatum (Rice and Cragg, 2004). As discussed above, dopamine release is enhanced by high frequency stimulation of dopamine terminals, whereas low frequency stimulation reduces dopamine release. Studies in current chapter extend these findings by confirming that high frequency stimulation releases dopamine through a cholinergic mechanism involving acetylcholine release and activation of nAChRs. Moreover, nicotine can enhance acetylcholine release by potentiating allosteric cholinergic receptors. This may in turn desensitize the efficacy of acetylcholine to activate β2 subunit of nAChRs, resulting in reduces potentiated dopamine release or increased levels of Ach supress dopamine release. Therefore, high acetylcholine signalling can reduce dopamine release indirectly in two ways: 1) by reducing the efficacy of dopaminergic receptors on dopaminergic axon terminals in NAc, and 2) by modulating mGluRs II/III to reduce glutamate release, which in turn reduces dopamine release. Additionally, DHβE desensitizes endogenous acetylcholine release and disinhibits dopamine release, whereas nicotine potentiates acetylcholine release via activation of allosteric receptors (e.g., by HEPES) which reduces dopamine release in NAc. Finally, subchronic PCP pretreatment tends to affect the ability of acetylcholine to modulate electrically stimulated dopamine release. PCP may do this in two ways: 1) directly through modulation of glutamate function or 2) indirectly through modulation of cholinergic transmission.

3.6.7 Experiment 4: Saline/PCP pretreated

Experiment 4 tested whether NMDA modulation of dopamine release was affected by subchronic PCP pretreatment. Based on the observation that the effect of NMDA on stimulated dopamine release in non-pretreated slices appeared to be reversed by DHβE

(see Figure 3.16), we also investigated whether cholinergic mechanisms were involved in dopaminergic modulation affected by PCP pretreatment as seen in non-pretreated slices. Animals were pretreated in vivo with PCP (2 mg/kg, i.p.) or saline vehicle (2 ml/kg, i.p.), twice daily for 5 days and remained drug-free for the remainder of the experiment, and so had been drug free for at least 10 days prior to FSCV experiemnts. In tissue taken from either saline or PCP pretreated animals, NMDA produced a similar decrease in electrically stimulated dopamine release as taken from rats non-pretreated animals, providing a good replication of the effect seen in slices from non-pretreatedanimals.

Three-way ANOVA showed a main effect of stimulus number ($F [11, 240] = 19.57$; $p < 0.0001$) and of NMDA ($F [1, 240] = 531.7$; $p < 0.0001$), but no main effect of PCP ($F [1, 240] = 0.518$; $p = 0.472$) and a significant stimulus number x NMDA interaction ($F [11, 240] = 26.98$; $p <0 .0001$): no other interactions were significant. Post hoc analysis (Tukey's test) showed that PCP pretreatment had no effect on stimulated dopamine release in control (No drug) slices or NMDA treated slices. Specifically, NMDA caused a significant decrease in stimulated release in both PCP pretreated and saline (vehicle) pretreated animals ($n = 5$ to 6). (Figure 3.19 and 3.20).

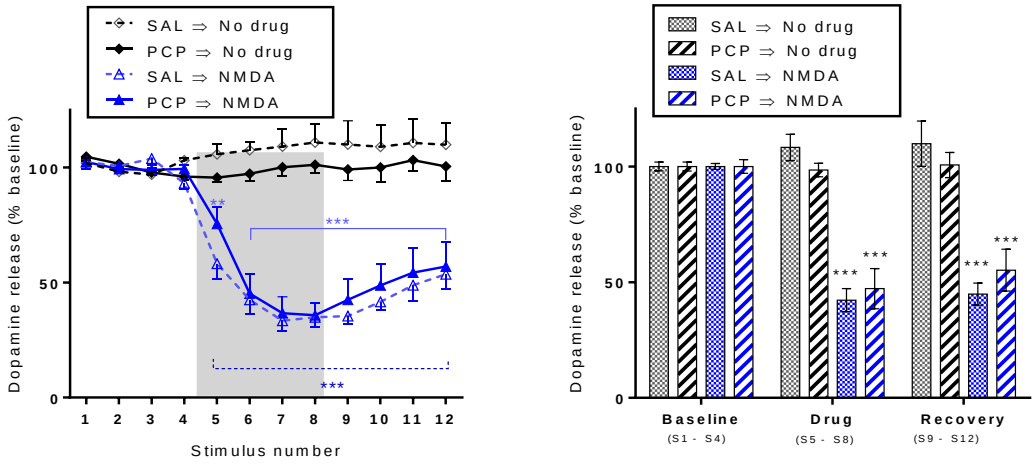


Figure 3.19 Effect of PCP (2 mg/kg, i.p., twice daily for 5 days) or saline vehicle (1 ml/kg, i.p., twice daily for five days) on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM). (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$; *** $p < .001$: significant difference from baseline period (Tukey's post hoc test), and from the appropriate saline pretreated groups (Tukey's test): n = 5 to 6.

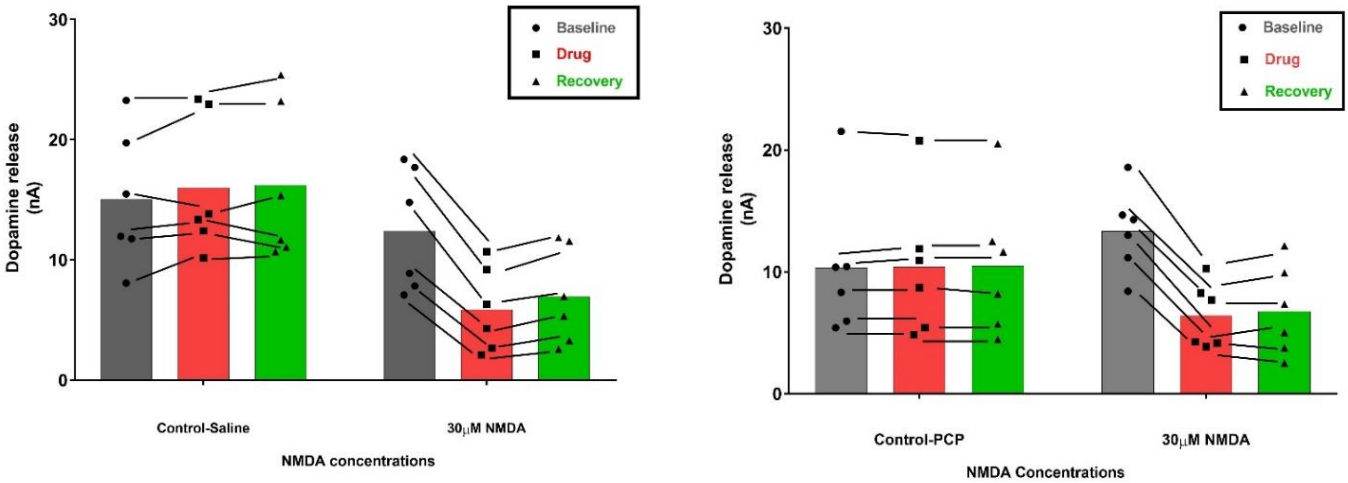


Figure 3.20 Effect of PCP (2 mg/kg, i.p., twice daily for 5 days) or saline vehicle (1 ml/kg, i.p., twice daily for five days) on attenuation of electrically stimulated dopamine release brought about by NMDA (30µM). Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

Animals were pretreated *in vivo* with PCP (2 mg/kg, twice daily for five days), then remained drug-free for the remained of the experiment. One week after the end of pretreatment they were tested in the NOR task (Grayson *et al.,* 2007) to confirm the behavioural effect of the PCP pretreatment. Both saline pretreated and PCP pretreated animals showed similar exploration of both objects during the acquisition stage. During the test stage, animals pretreated with saline showed the expected preference to explore the novel object over the familiar one. However, animals pretreated with PCP did not show this preference, but instead spent similar amounts of time exploring each object. Statistical analysis showed a main effect of object type ($F [1, 20] = 6.508$, $p = 0.02$), but not of drug treatment ($F [1, 20] = .10$; $p = 0.75$) nor any interaction ($F [1, 20] = 3.16$; $p = 0.09$). (Table 3.1). Discrimination index (DI) is a measure of how much animals discriminate between the two objects, with a value of zero indicating no discrimination. The saline pretreated group showed, as expected, a DI significantly above zero ($T [5] = 5.26$; $p = 0.003$), an effect that was significantly reduced after PCP pretreatment ($T [10] = 3.686$; $p = 0.004$): indeed PCP pretreated animals, the DI did not differ significantly from zero ($T [5] = 2.334$; $p = 0.067$).

	(a) Acquisition		(b) Test		(c) DI
	Left	Right	Familiar	Novel	
Saline	23.9 ± 2.5	23.2 ± 2.1	10.6 ± 2.3	25.8 ± 3.7	0.43 ± 0.10 **
PCP	21.3 ± 2.1	22.4 ± 4.0	17.9 ± 4.0	20.6 ± 3.7	0.10 ± 0.04 ##

Table 3.1 Results from the NOR test. (a) time (s) time spent exploring the two familiar objects during the Acquisition stage (3 min); (b) time (s) time spent exploring the familiar and novel objects during the Test stage (3 min); (c) DI calculated from the time spent exploring familiar and novel objects during the Test stage. * $p < .01$; significantly different from zero; one-sample t-test; ## $p < .01$; significantly different from saline pretreated group; independent samples t-test). Data are mean ± SEM; n = 6 per group.

The results from the behaviour testing showed the expected reduction of NOR after PCP pretreatment (Grayson et al.,2007), confirming that the pretreatment was effective: for more detail see chapter 2).

To test whether cholinergic modulation of dopamine release was capable of reversing NMDA-depression of dopamine release DHβE was applied concomitant with NMDA in slices taken from either saline pretreated or PCP pretreated animals. Since 1 μM DHβE gave a consistent augmentation of dopamine release, we used this concentration in pretreated slices. In this experiment we chose the highest doses of DHβE (1 μM) as it the most consistent change in dopamine in slices from non-pretreated animals.

Three-way ANOVA showed a main effect of stimulus number ($F [11,240] = 8.199$; $p < 0.0001$) and of NMDA + DHβE ($F [1,240] = 236.9$; $p < 0.0001$) and of PCP ($F [1,240] = 126.6$; $p < 0.0001$) and a significant stimulus number x NMDA + DHβE interaction ($F [11,240] = 13.19$; $p < 0.0001$). Post hoc analysis (Tukey's HSD test) showed that PCP pretreatment had no effect on stimulated dopamine release in control (No drug) slices or NMDA treated slices. Specifically, NMDA caused a significant decrease in stimulated release in both PCP pretreated and saline (vehicle) pretreated animals (Figure 3.21 and 3.22).

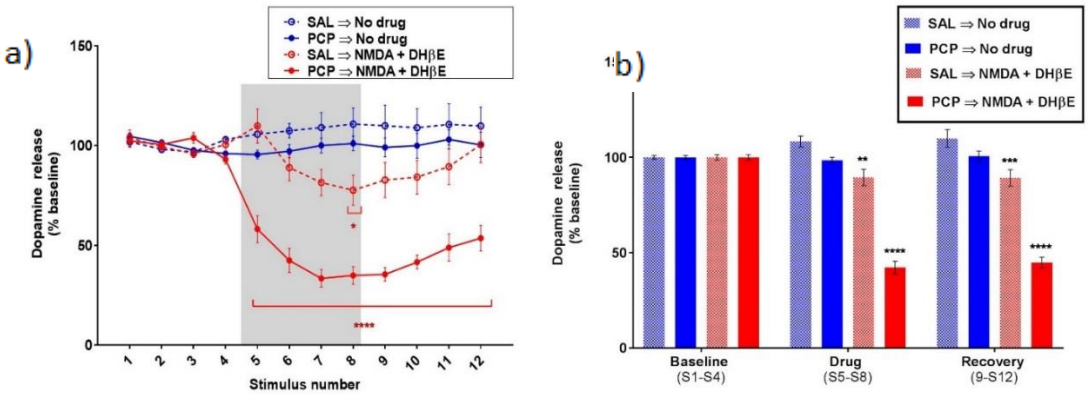


Figure 3.21 Effect of PCP (2 mg/kg, i.p., twice daily for 5 days) or saline vehicle (1 ml/kg, i.p., twice daily for five days) on attenuation of electrically stimulated dopamine release brought about by NMDA (30μM) and reversed by DhβE (1μM). (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. ** $p < .01$; *** $p < .001$; **** $p < .0001$: significant difference from baseline period (Tukey's post hoc test), and from the appropriate saline pretreated groups (Tukey's test): n = 6.

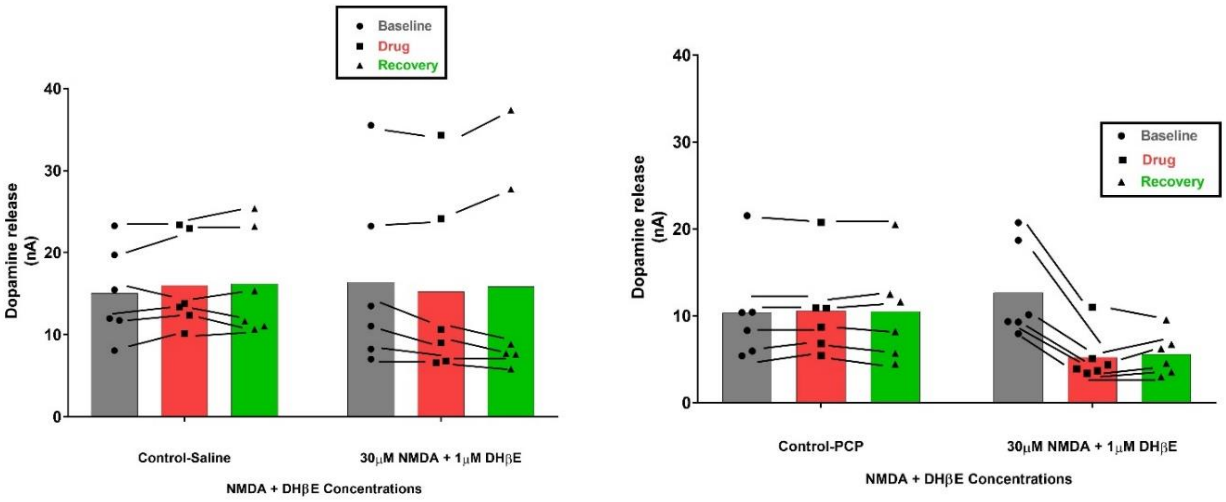


Figure 3.22 Effect of PCP (2 mg/kg, i.p., twice daily for 5 days) or saline vehicle (1 ml/kg, i.p., twice daily for five days) on attenuation of electrically stimulated dopamine release brought about by NMDA (30μM) and reversed by DhβE (1μM). Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

The nAChR antagonist, DHβE at concentrations of 100 nM, 300 nM, and 1 μM in the superfusate caused a dose-dependent increase in electrically stimulated dopamine release in NAcS. Data were obtained from saline pretreated animals (Figure 3.23 and 3.24). The stimulus-evoked dopamine release in slices obtained from saline pretreated rats (n = 7) was increase significantly by DHβE administration at all three concentrations. Two-way ANOVA showed a main effect of stimulus number ($F [11,240] = 40.73$; $p < 0.0001$) and of drug ($F [3,240] = 199.1$; $p < 0.0001$) and a significant interaction ($F [33,240] = 9.173$; $p < 0.0001$). Post hoc Dunnett's analysis revealed that DHβE significantly increased electrically stimulated dopamine release at all three concentration concentrations.

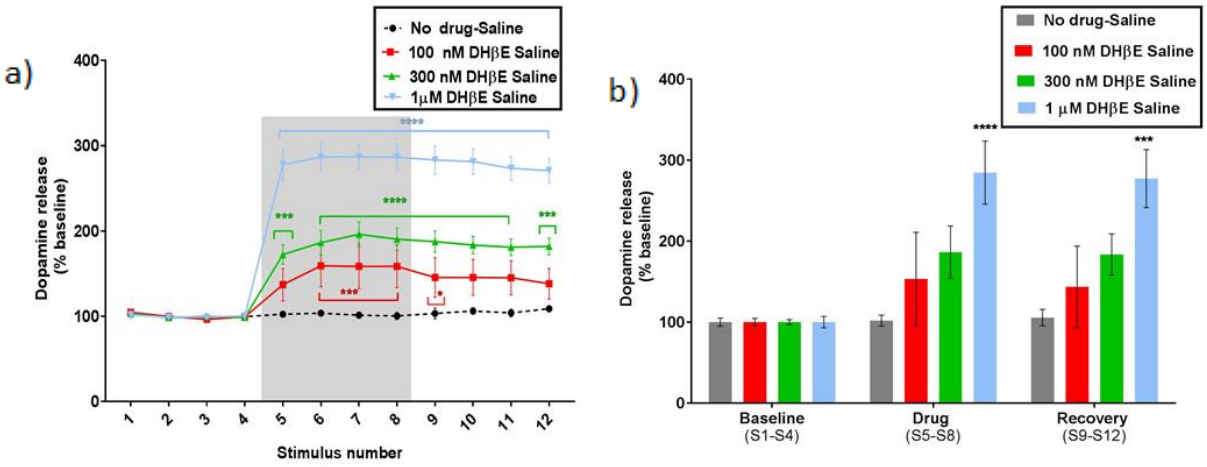


Figure 3.23 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of DHβE. (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; *** $p < .001$; **** $p < .0001$ significant difference from baseline period. Dunnett's post hoc test, based on a significant interaction from ANOVA: n = 6 per group.

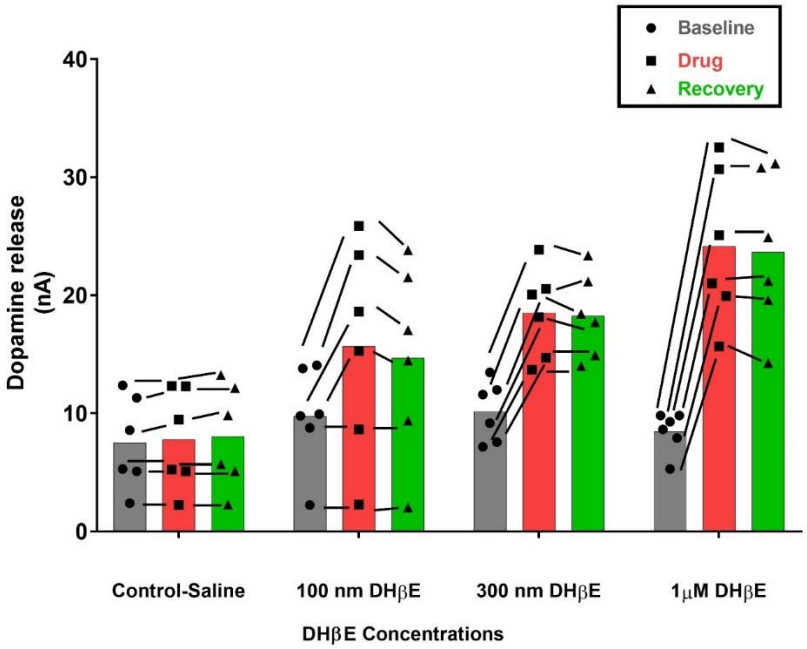


Figure 3.24 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of DHβE. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

DHβE at concentrations of 100 nM, 300 nM, and 1 μM in the superfusate caused a dose-dependent increase in electrically stimulated dopamine release in accumbel slices from saline pretreated animals (Figure 3.22 and 3.23). However, in slices taken from PCP pretreated animals, the the DHβE did cause an increase in stimulus-evoked dopamine release but this was not dose-dependent (Figure 3.25 and 3.26). ANOVA showed a main effect of stimulus number ($F [11,276] = 39.22$; $p < 0.0001$) and of drug ($F [3,276] = 94.16$; $p < 0.0001$) and a significant interaction ($F [33,276] = 4.403$; $p < 0.0001$). Post hoc analysis revealed that DHβE- significantly increased electrically stimulated dopamine release at 100 nM, 300 nM and 1 μM, concentrations.

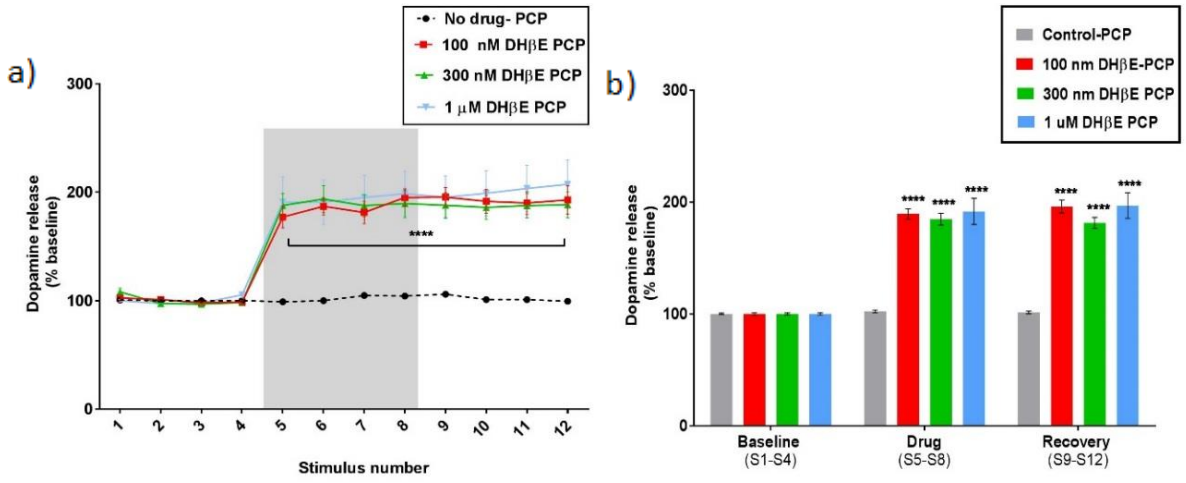


Figure 3.25 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of DhβE (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. **** P < .0001 significant difference from baseline period. Dunnett's post hoc test, based on a significant interaction from ANOVA: n=7 per group

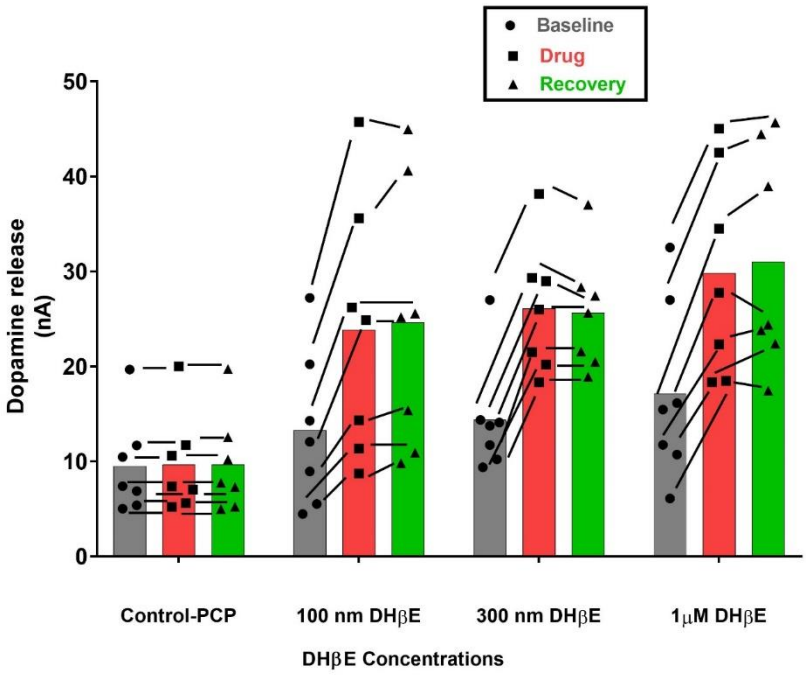


Figure 3.26 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of DHβE. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

Although, DHβE enhanced dopamine release in slices taken from both PCP and saline pretreated rats, in slices from PCP pretreated animals brain slices the effect was not dose-dependent, whereas in slices from saline pretreated animals the drug effect was dose-dependent. Moreover, in slices from both saline and PCP pretreated animals, the stimulated release did not return baseline levels during the washout period. The 100 mM DHβE showed almost full recovery in saline group, but not in the PCP pretreated group. In similar experiments conducted on slices from non-pretretead rats the stimulated dopamine release returned to baseline levels during the washout period. These results may indicate age dependent endogenous dopamine release changes by blocking α4β2 subunit of nAchRs in NAcS. In further experiments acetylcholine was activated by application of nicotine in NAc brain slices which may help to elucidate the whole story of specifically how the α4β2 subunit of nAChRs activation shapes endogenous dopamine release (details in chapter 3).

The nAChRs receptor agonist, nicotine at concentrations of 300 nM, 500 nM, and 1 µM in the superfusate caused a dose-dependent increase in electrically stimulated dopamine release from accumbal slices obtained from saline pretreated animals (Figure 3.27 and 3.28). The stimulus-evoked dopamine release in slices obtained from saline pretreated rats (n = 4) was increased significantly by nicotine administration in 500 nM, 1 µM, but not 300 nM. Two-way ANOVA showed a main effect of stimulus number ($F [11,144] = 6.951$; $p < 0.0001$) and of drug ($F [3,144] = 30.68$; $p < 0.0001$) and a significant interaction ($F [33,144] = 1.574$; $p = 0.036$). Post hoc analysis revealed that nicotine significantly increased electrically stimulated dopamine release at 500 nM and 1 µM, but not 300 nM concentrations.

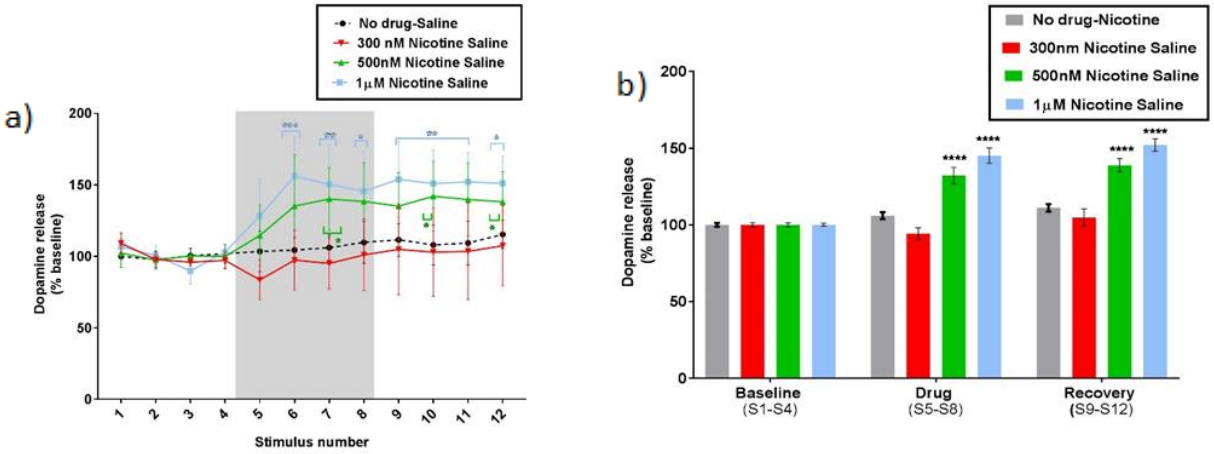


Figure 3.27 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of nicotine. (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * p < .05; ** p < .01; *** p < .001; ****p < .0001 significant difference from baseline period. Dunnett's post hoc test, based on a significant interaction from ANOVA: n=4 per group.

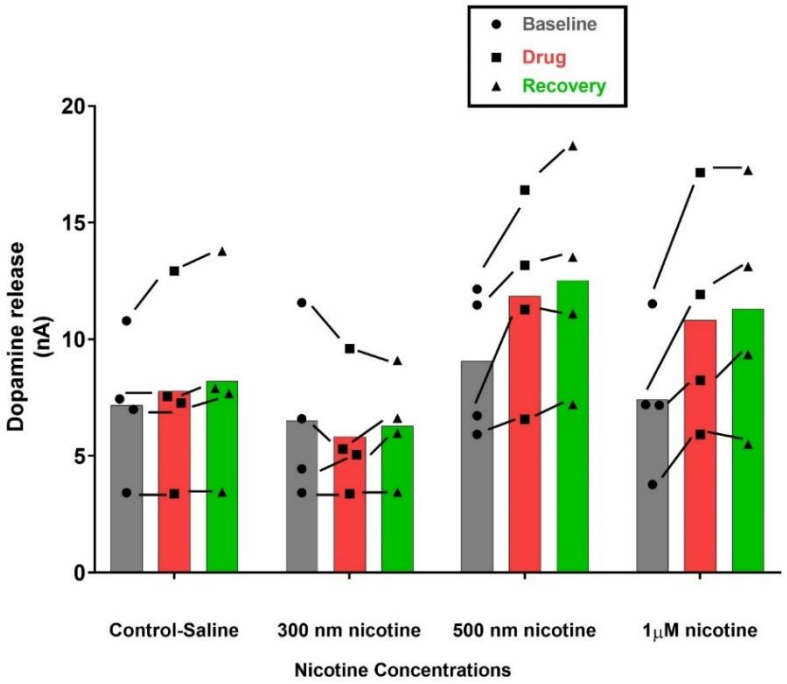


Figure 3.28 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of nicotine. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

The stimulus-evoked dopamine release in slices obtained from PCP pretreated rats (n=4) was increased significantly by nicotine administration in 500 nM, and 1µM, but not 300 nM (Figure 3.29 and 3.30). Two-way ANOVA showed a main effect of stimulus number ($F [11,132] = 3.971$; $p < 0.0001$) and of drug ($F [3,132] = 21.65$; $p < 0.0001$), but no significant interaction ($F [33,132] = 1.222$; $p = 0.213$). Post hoc analysis revealed that there is nicotine significantly increased electrically stimulated dopamine release at 1µM and 500nM, but not 300nM concentrations.

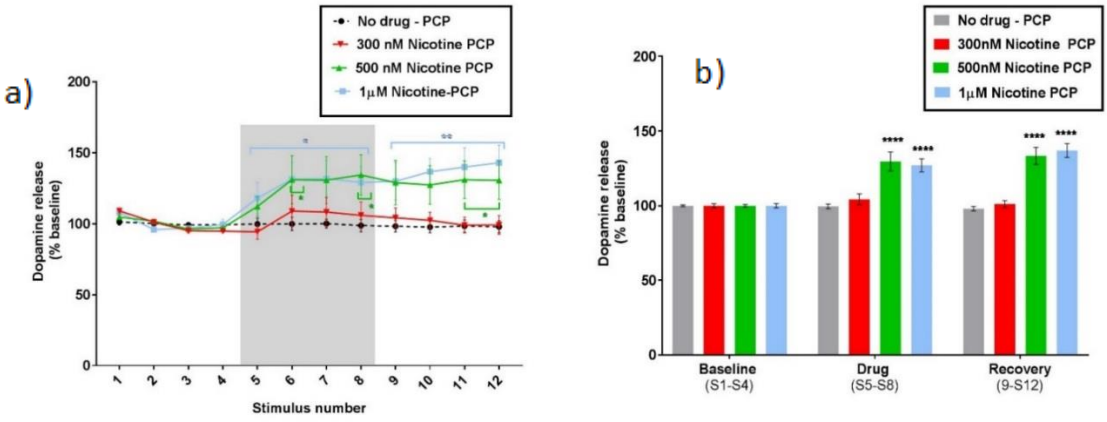


Figure 3.29 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of nicotine.(a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$; **** $p < .0001$; Dunnett's post hoc test, based on a significant interaction from ANOVA: n=4 per group.

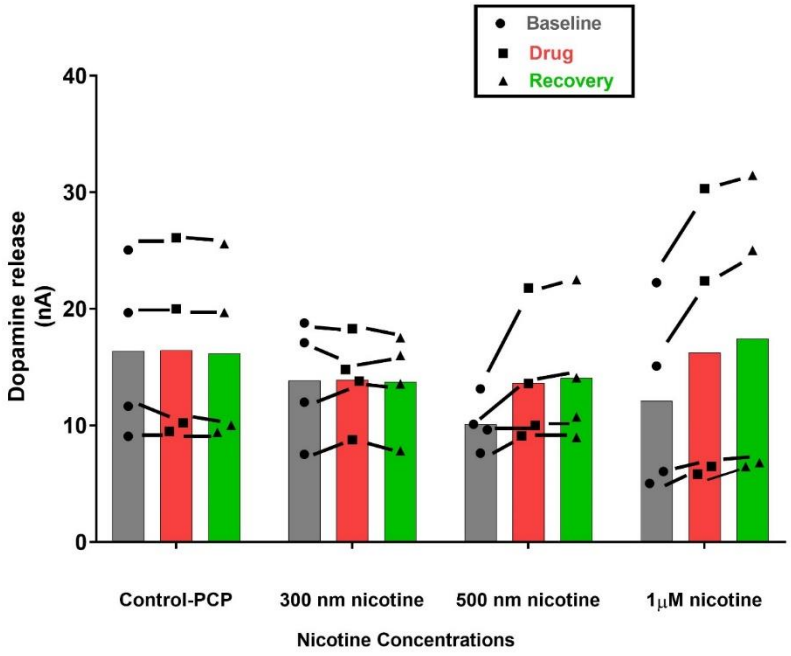


Figure 3. 30 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of nicotine. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

Activation of nicotinic receptors by nicotine inhibits evoked dopamine release probably through desensitization of acetylcholine (Goutier *et al.*, 2016). Nicotine supresses striatal dopamine release in response to low-frequency stimuli (Rice and Cragg, 2004; Goutier *et al*., 2016), but nicotine modulated electrically stimulated dopamine release is calcium dependent facilitation (Zhang and Sulzer, 2004). Therefore, presynaptic nicotinic receptors likely facilitate calcium entry in dopamine terminals which regulates dopamine release probability in synaptic vesicles. Subchronic PCP pretreatment did not change nicotine enhanced dopamine release, hence PCP is unlikely to have changed calcium channels which resulted similar dopamine signalling by nicotine through calcium entry through the presynaptic receptors. The other possibility is nicotine-enhanced dopamine release may be regulated by presynaptic sodium channels because inward current activation shows differences to nicotine (Melis *et al.*, 2008; Mathie *et al.*, 1990) and PCP

treatment may change the sodium channels, but not calcium channels. Initial nicotine application enhanced dopamine release, but the increased dopamine release by nicotine showed attenuation after a while due to desensitization by nicotine in mesolimbic dopamine system. However, the aforementioned studies indicate that the augmentation of dopamine release is frequency of action potential and calcium dependent (Rice and Sulzer, 2004; Zhang and Sulzer, 2004). In the present study, high frequency stimulation was applied to measure dopamine release, stimulation parameters mimicking phasic dopamine activation. Nicotine did not change phasic dopamine signalling in NAcS as nicotine showed similar changes in electrically stimulated dopamine release in saline and PCP pretreated animals' brain slices in NAcS. In DHβE the dose dependent profile also showed similar releasing pattern between non-pretreated and saline pretreated animals' brain slices in previous experiment. However, DHβE modulated electrically stimulated dopamine release was changed by subchronic PCP pretreatment. These results indicate that cholinergic systems are affected by PCP pretreatment. We suggest that PCP changes acetylcholine activation which shapes dopamine release in accumbal brain regions. Altered dopamine function after PCP may be modulated through disruption of cholinergic signalling either indirectly via glutamatergic control of cholinergic activity or through a direct effect of PCP on cholinergic receptors. Previous studies indicate that PCP is an ion channel blocker (Edson *et al.*, 1980), hence nicotinic ion channel receptors may also be blocked by PCP pretreatment which may explain these effects on endogenous dopamine signalling. Since a week washout time is enough time to make sure there is no enduring drug effect on receptors it is unlikely PCP blocked ion channels in current study. However, it is still uncertain why both nicotinic agonists and antagonists show similar results on saline and PCP pretreated slices. These results raise questions regarding nicotinic receptor modulation of dopamine release after subchronic PCP pretreatment. To

further investigate this, experiments were conducted using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES) to potentiate acetylcholine activation and compared between saline and PCP treated brain slices. HEPES is known as a zwitterionic biological buffer and is one of Good's buffers (www.sigmaaldrich.com). A zwitterion or ammonium ion is a molecule with two or more functional groups such as one has a positive and one has a negative electrical charge, hence net charge of zwitterion is zero (http://www.aqion.de/site/zwitterions).

Nicotine at concentrations of 500 nM or 1 µM applied concomitant with HEPES at 100 µM concentration in the superfusate caused an increase in electrically stimulated dopamine release from accumbal slices (Figure 3.21). The stimulus-evoked dopamine release in slices obtained from saline pretreated rats (n = 6) was increase significantly by nicotine concomitant with HEPES administration at concentrations of 500 nM or 1 µM, but HEPES alone did not cause changes in dopamine release. Two-way ANOVA showed no main effect of stimulus number ($F [11,168] = 0.9041$; $p = 0.5378$) and of drug ($F [2,168] = 3.545$; $p = 0.0311$) but no significant interaction ($F [22,168] = 0.1966$; $p > 0.999$). However, mean of the four stimulations for 1 µM nicotine concomitant with 100 µM HEPES in recovery showed a significant difference (Figure 3.31 and 3.32).

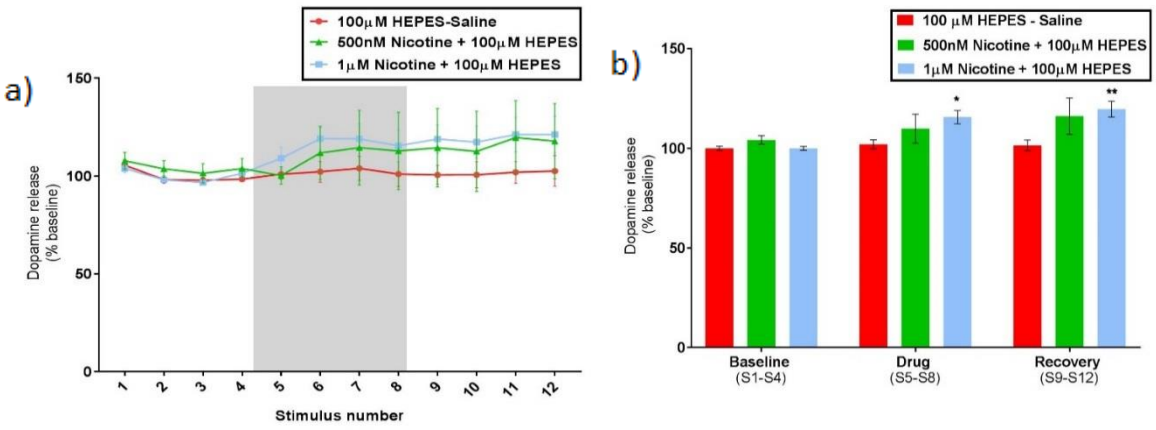


Figure 3.31 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$; ** $p < .01$ significant difference from baseline period. Dunnett's post hoc test, based on a significant interaction from ANOVA: n=6 per group.

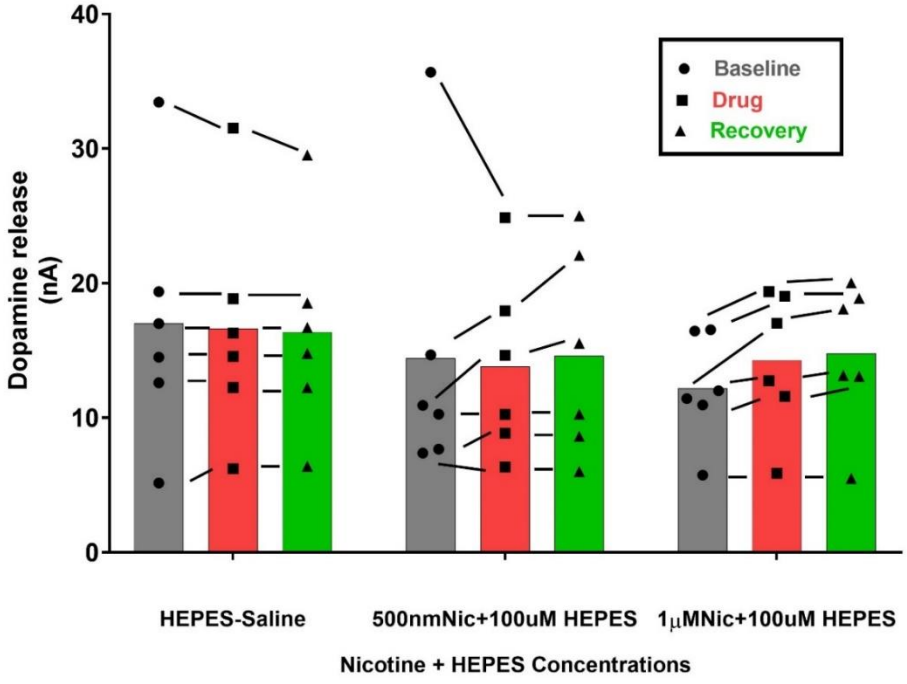


Figure 3.32 Effect of saline pretreatment on electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

In contrast, the stimulus-evoked dopamine release in slices obtained from PCP pretreated rats (n = 6) was increase significantly by nicotine in the presence of HEPES administration in 500 nM or 1µM, but HEPES alone did not cause changes in dopamine release. Two-way ANOVA showed a main effect of drug ($F [2,168] = 8.7$; $p = 0.0003$), and main effect of stimulus number ($F [11,168] = 2.375$; $p = 0.009$) but no significant interaction ($F [22,168] = 0.6131$; $p = 0.91$. On the other hand, saline pretreated slices (n = 6) showed a significant effect of stimulus number ($F [11, 168] = 0.9041$; $P = 0.5378$), but no main effect of drug ($F [2,168] = 3.545$; $p = 0.0311$) and no significant interaction ($F [22,168] = 0.1966$; $P = 0.9999$). (Figure 3.32 and 3.33). Although there is no significant difference mean of the four drug treated and recovery stimulations for both 500 nM and 1 µM applied concomitantly with HEPES showed a significant difference (Figur 3.33 b) and raw data showed in figure 3.34

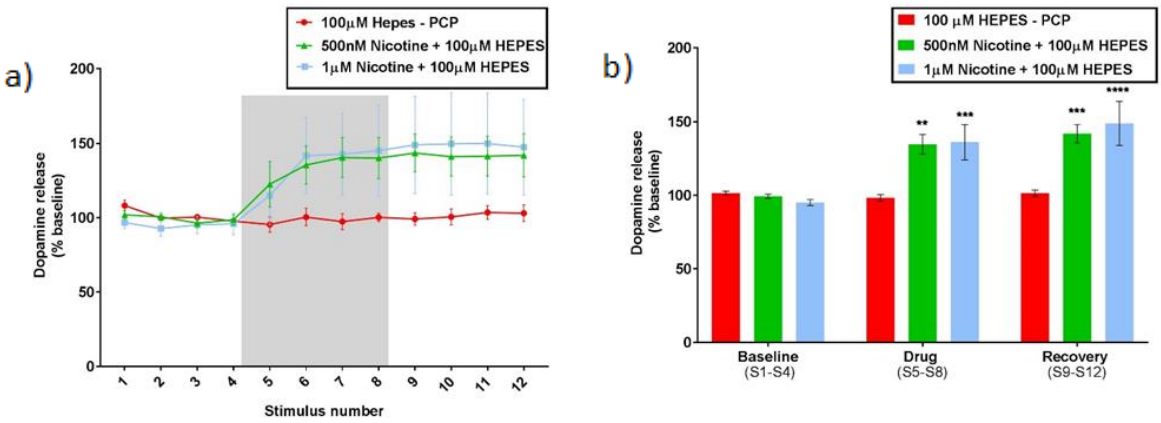


Figure 3.33 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. ** $p < .01$; *** $p < .00$ significant difference from baseline period. Dunnett's post hoc test, based on a significant interaction from ANOVA: n=6 per group.

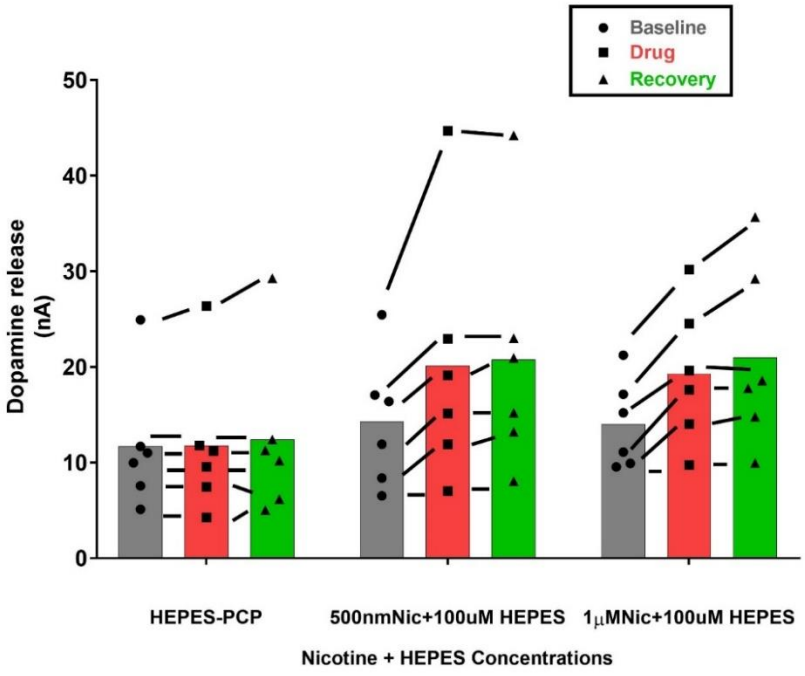


Figure 3.34 Effect of PCP pretreatment on electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly. Mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and recovery (S9 to S12) periods. Data represent **nA dopamine** release.

In previous nicotine experiments, we applied three different nicotine concentrations (300 nM, 500 nM, 1 µM). Doses of 500 nM and 1µM gave a dose dependent augmentation of electrically-stimulated dopamine release, whereas a concentration of 300 nM did not show significant increase in slices taken from either saline or PCP pretreated rats. Therefore, we concluded that the low concentration of nicotine did not affect electrically-stimulated dopamine release in NAcS. Therefore, the 300 nM concentration was not used in the subsequent experiments.

In this experiment, we applied HEPES to potentiate acetylcholine activation. HEPES used as a positive allosteric modulator (PAM) of the α4β2 subunit of nAChRs (Schulte *et al.,* 2016). On its own the PAM does not change neurotransmitter activation (Schulte *et al.,* 2016) which is supported by the results from the present experiments: the PAM by itself did not change electrically stimulated dopamine release, hence HEPES was used as control group for both saline and PCP pretreated slices in order to check stability and reproducibility of the stimulated responses over the 12 stimulations. However, when we applied HEPES concomitantly with nicotine it caused a dose dependent increase in dopamine release in slices from saline pretreated animals, but the increase was not dose dependent in slices from PCP pretreated animals. Interestingly, nicotine plus HEPES potentiation of electrically-stimulated dopamine release in the saline group compared to nicotine without HEPES experiments in saline group. We pointed out that potentiated nicotine agonism effect of acetylcholine release by HEPES attenuated electrically stimulated dopamine release. In slices taken from both PCP and saline pretreated animals there was a significant attenuation of nicotine electrically stimulated dopamine release. Mean release show that there was a decrease in dopamine release in both groups, in saline group, HEPES + 500 nM diminished mean release. In these experiments, peak dopamine release continued to be augmented through the recovery period (S8 to S12) (i.e. after the

end of drug superfusion) in both saline and PCP pretreated brains' slices. Although there was no drug in recovery period (S8 to S12) it is possible that age dependent changes lead to continuation of enhanced peak dopamine release because non-pretreated slices shows expected recovery process after drug switched off over S8 to S12. In addition to age-dependent difference, DAT activation may also be important. Therefore, we decided to test DAT activation in nicotine plus HEPES experiments. DATs may be changed in maturing brain and result in differences in reuptake and diffusion changes, which, in turn, mean that dopamine remains in the synaptic cleft for longer after the end of DHβE application (i.e. washout period). Age-dependent brain changes may enhance DAT activation, which, in turn, would change dopamine reuptake and decrease the rate of released dopamine returning to baseline levels. It is proposed that dopamine reuptake for nicotine after PCP pretreatment does not occur because DAT cannot gate synaptic dopamine spillover in striatum (Cragg and Rice, 2004). To ensure nicotine does not change DAT activation, tau which is a measure of exponential decay from peak to baseline, was measured (Yorgason, *et al.,* 2011) (details in experimental procedures).

Nicotine at concentrations of 500 nM and 1µM applied concomitant with HEPES at 100 µM concentration in the superfusated. Nicotine + HEPES unlikely activated DAT activation in PCP pretreated brain slices, since stimulated responses over the 12 stimulation did not show significant difference. However, mean release data showed significant difference at washout period. Two-way ANOVA showed a main effect of stimulus number ($F [11, 84] = 2.569$; $p=0.0074$), but no significant drug effect ($F [1, 84] = 0.347$; $p=0.5574$) and no significant interaction ($F [11, 84] = 2.569$; $p=0.7865$). (Figure 3.35). Post hoc analysis revealed that nicotine plus HEPES did not significant increase electrically stimulated dopamine release by NMDA (500 nM, 1 µM).

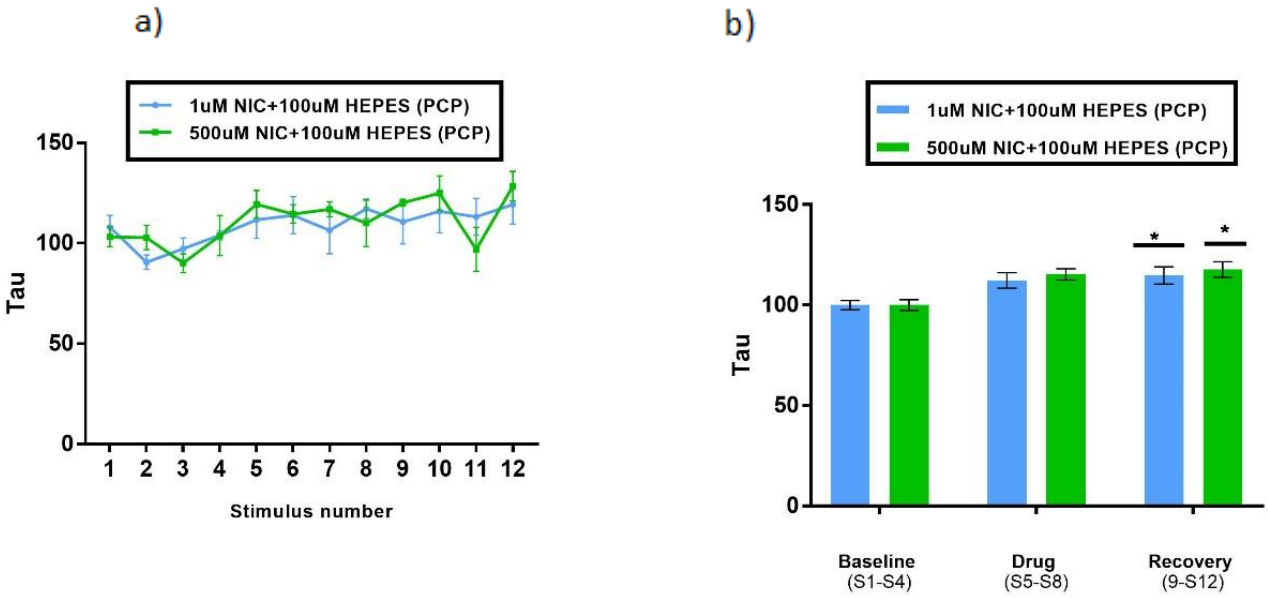
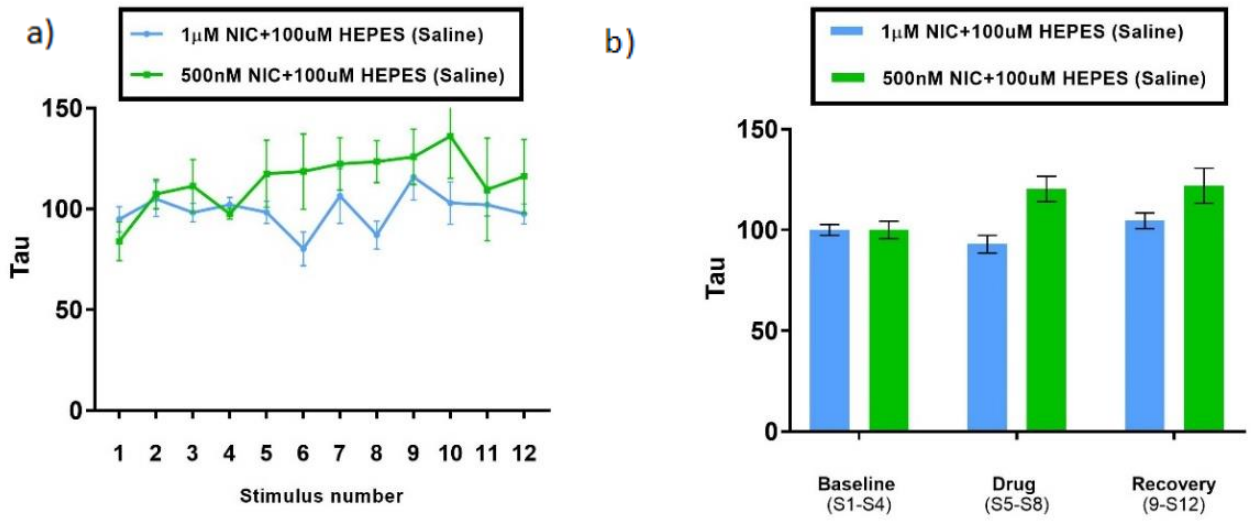


Figure 3.35 Effect of PCP pretreatment on DAT activation after electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. * $p < .05$ significant difference from baseline period. n=5 per group.

On the other hand, in slices from saline pretreated animals there was no significant difference in stimulated responses over the 12 stimulations after nicotine (500 nM, 1 µM) concomitant HEPES (100 µM). Two-way ANOVA showed a significant main effect of drug ($F [1, 96] = 8.883$; $p=0.0036$) but no main effect of stimulus number ($F [11, 96] = 1.00$; $p = 0.4466$), and no significant interaction ($F [11, 96] = 0.8039$; $p = 0.6359$). (Figure 3.36).



Figur 3.36 Effect of saline pretreatment on DAT activation after electrically stimulated dopamine release brought by different doses of Nicotine + HEPES concomitantly (a) Time course of the effects over twelve stimulations at 3 min intervals: drugs were applied in the superfusate during stimulations S5 to S8, indicated by the grey panel; (b) mean electrically-stimulated dopamine release during baseline (S1 to S4), drug (S5 to S8) and post-drug (S9 to S12) periods. n= 5.

3.7 Discussion

3.7.1 Electrical stimulation and potassium stimulation

Although both electrical and potassium stimulation activates neurones by causing depolarization of brain slices (Hillman and Mcllwain, 1961; Hillman *et al.*, 1963), the mechanisms by which they achieve this are different, leading to advantages and disadvantages of each technique used in combination with FSCV recording. In addition to neurotransmitter stimulation, both of these techniques stimulate cell metabolism (Mcllwain, 1955). However, the release of neurotransmitter evoked by electrical stimulation and potassium stimulation is not only as a result of metabolic changes in the tissue (Mcllwain, Synder, 1970). Potassium stimulation activates the whole slice, which leads to a large, long lasting release, with a likelihood of depleting the tissue, whereas

electrical stimulation gives a smaller, more localised and more rapid response. In addition to those advantages of electrical stimulation, it is more reproducible which gave very reproducible responses over 12 consecutive stimulations, hence allowing testing of the effects of drugs throughout stimulation conditions. However, both electrical stimulation and potassium stimulation are capable of increasing efflux of all the amino acids which is the common characteristics of stimulations in *in vitro* brain slices (Chase and Katz, 1969), but release amplitudes and time-courses of stimulation responses show differences between electrical stimulation and potassium stimulation. An advantage of electrical stimulation is that the responses are pulse and frequency dependant. This makes it possible to manipulate electrical stimulation parameters to measure different aspects of release (Cragg, 2006). Although, potassium stimulation is also concentration dependent, there is much less scope for manipulation than with electrical stimulation. In our studies, we have shown that different potassium concentrations (25 µM, 50 µM, 100 µM) give different stimulated release, but that the responses are very variable at lower concentrations (see chapter 2). In addition, dopamine responses, particularly to stimulation with higher concentrations of potassium were slow to return to baseline, and, indeed, in some cases did not fully return to baseline. On the other hand, electrical stimulation produced rapid dopamine responses which returned to the baseline level within a few seconds after stimulation. On the basis of these results, we chose to use electrical stimulation in subsequent experiments, since (1) the stimulation-evoked release was rapid in onset, (2) levels returned to baseline within a few seconds and (3) repeated stimulation (3 min apart) produced very reproducible responses, allowing repeated electrical stimulation for testing of the effects of drugs.

3.7.2 NMDA modulation of dopamine release

Using repeated electrical stimulation, we were able to measure the effect of NMDA receptor activation on stimulated dopamine release, and to investigate the mechanism of the interaction.

In these experiments, NMDA itself caused a dose-dependent decreased in electrically stimulated dopamine release in slices from both non-pretreated and pre-treated animals. It is unlikely that this effect was due to a direct action of NMDA on dopamine terminals, since NMDAR activation generally causes excitation and would resulted in electrically stimulated dopamine efflux. In addition, NMDARs on dopaminergic axon terminals in NAcS are sparse; hence, a direct effect of NMDA on dopamine terminals is unlikely. Rather, the effect is likely to be mediated through inhibitory intermediate neurones in the NAc. One possibility would be GABA: however, the decrease in stimulated dopamine release seen during NMDA (30 μM) application was not reversed by the GABA-A receptor antagonist, picrotoxin, indicating that the action of NMDA on stimulated dopamine release was not mediated through GABAergic activation. Although, GABA neurons express nAChRs with α4 and β2 subunits (Mansvelder *et al.,* 2002) and striatal MSNs constitute about 90% of striatal neurons, GABAergic receptor inactivation by picrotoxin did not have an effect on dopamine release in our experiments. On the other hand, the widespread distribution of mGluR in striatal areas, including NAc has been reported. Eight different mGluR have been identified, which cluster into three main groups (Conn and Pin, 1997). While group I receptors are primarily excitatory, and found postsynaptically, groups II and III receptors (mGluR-2 and mGluR-3 respectively) are inhibitory and are located extrasynaptically (mGluR-2) and presynaptically (mGluR-2, mGluR-3) as both autoreceptors and heteroreceptors (Conn and Pin, 1997). They are

therefore capable of the modulating release of glutamate itself, and other neurotransmitters, and several studies have reported an inhibitory effect of mGluR-2/3 activation on dopamine release in NAc (Conn and Pin, 1997; Karasawa *et al*., 2006). The present experiments showed that concomitant application of the non-specific group-II (mGluR 2 and 3) metabotropic receptor antagonist MSPG reversed the NMDA-mediated decrease in stimulated dopamine release. From this we may conclude that the NMDA effect is mediated via group-II (mGluR 2 and 3) metabotropic receptor. The most likely location for these receptors would be on the axons or terminals of the afferent mesolimbic dopamine neurones (Pistillo *et al.,* 2015), providing presynaptic inhibition of dopamine release. One possible mechanism for this may be through extrasynaptic 'volume' transmission by glutamate, which has previously been suggested as one mechanism of action of glutamate in NAc (Garcia-Munoz *et al.,* 2015). Thus, NMDAR activation would lead to increased spill-over of glutamate from the synaptic cleft, allowing activation of inhibitory mGluR located presynaptically and perisynaptically on dopamine terminals, leading to the observed decrease in dopamine release (Zhang and Sulzer, 2003; Fuxe *et al.,* 2013). (Figure 3.37).

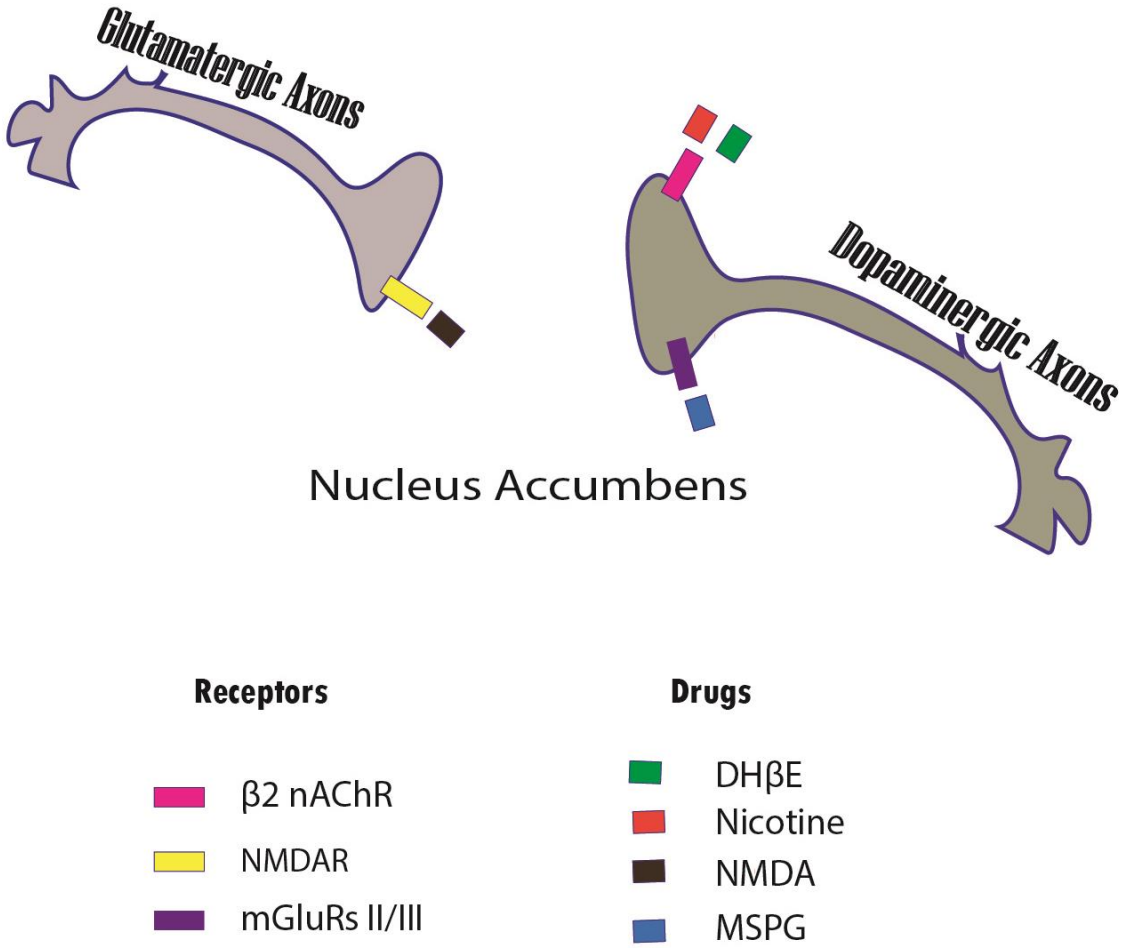


Figure 3.37 Acetylcholine and glutamate modulation of dopamine release. NMDA activates NMDA receptors leading to spillover of postsynaptic glutamate and activation of mGlu2/3 receptors that, in turn, inhibit dopamine release from presynaptic receptors in NAcS region. DhBE and nicotine targeting β2 subunit of nAChRs activated acetylcholine release leading to increase in phasic dopamine and reduce efficiency of glutamate signalling. Abbrevations; β2 nAChRs; β2 nicotinic acetylcholine receptors, NMDAR; N-methyl-D-aspartate receptor, mGluRs II/III; type II/III metabotropic glutamate receptors, DHβE; Dihydro-β-erythroidine hydrobromid, NMDA; N-methyl-D-aspartate, MSPG; (RS)-α-Methyl-4-sulfonophenylglycine.

In contrast to GABAergic mechanism, ACh activation was seen to regulate NMDA-mediated decrease in dopamine release in NAcS slices. The α4β2 selective nAChR antagonist, DHβE, reversed the decrease in stimulated dopamine release caused by NMDA. NAChRs are capable of regulating glutamate and GABA release, but are

uniquely distinguished by different subtypes of nAChRs (Guo *et al.,* 1998). Although DHβE reversed NMDA-depression of dopamine release, it also caused increased dopamine release when given alone. However, it is still controversial whether ACh enhances or reduces glutamate release. In striatum, glutamate and GABA-regulated neurotransmission is reduced by using mAChR agonists (Calabresi *et al.,* 2000). Bernardi *et al.,* (1976) claims that ACh reduced excitatory synaptic potentials in striatum, suggesting that mAChR and nAChR regulation of excitatory amino acid activation are distinct from each other. According to Guo *et al.,* (1998) glutamate release is enhanced after perfusion of the nicotinic agonist, carbachol in the ventral lateral geniculate of chick brain slices, showing that this brain region receives excitatory glutamatergic inputs. In fact, α7 receptor activation modulates dopamine release in VTA (Mameli-Engvall, *et al.,* 2006). In the experiments reported here, the effect of mAChR activation on dopamine release was not investigated, although preliminary experiments do show muscarinic modulation of dopamine release in NAcS (O'Connor, Yavas & Young, unpublished). Rather we measured the effect of nAChRs activation investigated by using the α4β2 antagonist, DhβE, and the α7 and α4β2 agonist, nicotine.

According to literature dopamine is modulated by nicotine through activation of α7 and sensitization of β*2 nAChRs in the midbrain at the somatodentritic level (Mansvelder *et al.,* 2002; Schilstrom *et al.,* 1986). In addition to α7, nicotine also may regulate dopamine release probability with α6 subunit. This subunit activated dopamine release by nicotine in NAc which indicate that α6-nAChRs is expressed in dopaminergic axonal terminals in NAc (Exley, *et al.,* 2008). Nicotine is capable of dopamine regulation via mechanisms involving different subunit of the glutamatergic receptors. *In vitro* application of nicotine has been shown to modulate AMPA receptors by increasing excitatory postsynaptic current amplitude in VTA dopaminergic neurons in a presynaptic and pre-terminal

manner (Mansvelder and McGehee, 2000). In addition to AMPA, the stimulatory action of nicotine on the mesolimbic dopamine system is to a considerable extent mediated via stimulation of NMDARs within the VTA, supporting the view that NMDA participates in the action of nicotine-mediated indirect effects in NAc, and that perhaps different glutamate receptors could be important in glutamate-nicotine interaction in VTA and NAc. In other words, acetylcholine is capable of modulating dopamine by activating acetylcholine receptors in VTA, on neurones projecting to NAc (Gorniet *et al.*, 2000) but also by local activation of acetylcholine on dopamine terminals in NAc (Maex *et al.*, 2014).

To investigate whether acetylcholine without NMDA regulates dopamine release we applied DHβE at different concentrations to see whether it showed dose-dependent effects on electrically stimulated dopamine release in slices taken from both PCP and saline pretreated rats. DHβE dose-dependently enahnced dopamine release in the saline pretreated group, replicating what was seen in the non-pretreated group, but not in the PCP pretreated group. However, dopamine release did not return to baseline levels in drug-free washout process in either saline or PCP pretreated slices, whereas dopamine returned baseline levels in non-pretreated slices following DHβE superfusion in NAcS. This suggests that age-dependent differences may resulted in changing acetylcholine modulation of electrically stimulated dopamine release after subchronic PCP pretreatment. DHβE is an antagonist at the α4β2 subunit of nAChRs. It caused a dose dependent increase in electrically stimulated dopamine release in non-pretreated and saline pretreated brain slices, but not PCP pretreated slices which indicates that blocking acetylcholine activation somehow enhanced dopamine release by higher action potential frequency-dependence electrical stimulation in NAcS. However, this was not seen in slices from PCP pretreated animals. We conclude that maybe phasic dopamine activation

which is related to reward mechanisms in NAc is somehow disrupted following PCP pretreatment supporting the hypothesis that people with schizophrenia show dysregulation of reward mechanisms compared to normal population.

In addition to DHβE, nicotine was also used to characterize ACh modulation of dopamine release which possess advantage in terms of evaluating holistic approach by using agonist and antagonist of nAChRs. Surprisingly, nicotine caused dose dependent dopamine release in both the saline and PCP pretreated groups. This result shows a difference compared to the effect of DHβE, since both the agonist and the antagonist showed a significant dose dependent change in saline group, but DHβE did not gave a dose dependent changes in electrically stimulated dopamine release in PCP group.

The effects of nicotine experiments in slices from saline pretreated animals support the previous findings of how nicotine modulates dopamine release at higher frequency stimulation (Rice and Sulzer, 2004). Further experiments were conducted to investigate whether ACh modulation of dopamine release was changed by PCP pretreatment. ACh activation may somehow activate endogenous glutamate, which, in turn, inhibits dopamine release in NAcS. We decided to potentiate ACh activation to evaluate whether dopamine release was shaped by ACh modulation and if so whether the effects were different between slices taken from saline and PCP pretreated animals. Therefore, HEPES was superfused alongside nicotine at different concentrations in NAcS.

Further experiments looked at nicotine plus HEPES application. The rationale for using HEPES is to check whether potentiated ACh activation shows differences on electrically stimulated dopamine release. Although HEPES is zwitterionic biological buffer, it is also a PAM at the α4β2 subunit of nAChRs (Weltzin and Schulte, 2011; Weltzin *et al.,* 2014; Schulte *et al.,* 2016): notably it does not have activity at the α7 subunit, hence any

activation of ACh modulated dopamine release would be related to α4β2 receptor activation due to HEPES action on allosteric sites on α4β2 receptors which may help to differentiate α7 and α4β2 receptor activation of electrically stimulated dopamine release in NAcS. Pharmacological manipulation to investigate neurochemical activation is a useful way. However, synapse physiology and anatomy of target receptors which is α7 and α4β2 nAChRs in our case further support those pharmacological manipulation to investigate receptor activation.

Nicotine affinity on α4β2 and α7 subunit of nAChRs makes it difficult to specify whether those results obtained from our experiments show nicotine enhancement of dopamine release by activation of α4β2 or α7 subunit. In this respect, we examined NAcS: however, although α7 subunits are found in VTA, they do not transfer to dopamine axon terminals in striatum (Salminen *et al.*, 2004). On the other hand, synaptosome studies show that α7 nAChRs possess a modulatory role on NMDARs modulation of acetylcholine in NAc (Zappettine *et al.*, 2014). In fact, α7 and NMDA co-localization was observed at individual glutamatergic nerve terminals in nAchRs (Zappettine *et al*., 2014). However, it should be noted that the α7b-containing nAChRs have been found to desensitize rapidly in oocyte membrane (Couturier *et al.*, 1990) and can be expressed on glutamatergic nerve terminals in NAc (Zappettine *et al*., 2014). Therefore, the lack of effect of nicotine, superfused onto the slices for 39 min in our experiments, could simply be due to desensitization of the receptors. Moreover, chronic nicotine exposure studies show that α7 is more resistant to desensitization than β2 subunit and α7 subunit compensates β2 desensitization by preventing hypoacivity of dopamine transmission (Mansvelder *et al.* 2002). However, it is unknown whether nicotine shows high affinity α7 or α4β2 at initial nicotine exposure, so it is difficult to distinguish nicotine binding activation on both α7 and α4β2 nAChRs. In our experiments, we studied dopamine transmission in NAc; hence,

we did not measure how α7 activation in VTA modulates dopamine release in NAc region (Klink *et al.,* 2001). Rather the studies focused on NAcS and dopamine signalling changes brought about by nicotine in this brain region. In VTA, α7 subunits modulate dopamine release by activating glutamate (Zappettine *et al.,* 2014). In our experiments slices from PCP pretreated animals showed NMDAR dysfunction and DHβE did not show differences on electrically stimulated dopamine release. One implication is that α4β2 nAChRs may interact with NMDARs and modulatedopamine release in NAc in a similar manner to how α7 nAChRs modulate dopamine release by interacting glutamate in VTA (Zappettine *et al*., 2014; Maex *et al.,* 2014). According to Risso *et al.,* (2004) activation of nAChRs positively modulates the NMDARs. However, in our study, antagonism of nAChRs by DHβE partially reversed the decrease in stimulated dopamine release caused by NMDA on non-pretreated and control slices but not PCP pretreated slices. We have shown that NMDA concomitant with DHβE reversed NMDA-attenuation of dopamine release in saline and non-pretreated slices, whereas DHβE did not reverse NMDA depression dopamine release in PCP pretreated slices. However, DHβE by itself caused substantial augmentation of electrically stimulated dopamine release as well. We conclude that DHβE-mediated electrically stimulated dopamine release may be dependent on the stimulation parameters used. In our experiments, we used multi-pulse stimulation at 60 Hz frequency, parameters which mimic phasic dopamine activation. Dopamine neuron firing frequency in normal physiological mechanism is around 5 Hz (Grace and Bunney, 1983). Frequency of putative dopamine neuron firing in the slice varies whether one uses extracellular (3–8 Hz) (Grace and Onn, 1989), perforated-patch (2–5 Hz) (Neuhoff *et al.,* 2002), or whole-cell recordings (1–3 Hz) (Wanat *et al.,* 2008). Results shows that subchronic PCP pretreatment somewhat disrupted the acetylcholine modulation of phasic dopamine activation seen in saline or non-pretreated slices (See

results in chapter 3; Yavas and Young unpublished data; O'Connor, Yavas, Young unpublished data).

It appears that there is cross talk between ACh and glutamate on dopamine regulation in normal population. However, such a cross talk is somehow disrupted after PCP pretreatment. Given that PCP pretreatment provides a good model for aspects of schizophrenia, a fuller understanding of the mechanisms of the interaction between cholinergic, glutamatergic and dopaminergic systems, and how this interaction is dysregulated after PCP pretreatment, may shed light underlying factors of schizophrenia.

Transporter activation is essential for extracellular neurotransmitter regulation in central nervous system. However, there is a difference between dopamine and glutamate transporters process regarding with their anatomic, physiological characterisation in different brain regions. For example, dopamine transporter density is around 175-350 molecules per μm^3 in striatum at perisynaptic and nonsynaptic sites (Hersch *et al.*, 1997), whereas glutamate transporter density is 15200-22700 molecules per μm^3, mainly on glial cells, at presynaptic and postsynaptic sites (Lehre and Danbolt, 1998). The aforementioned studies imply that DAT is restricted for uptake of spillover dopamine compared to glutamate transporters.

In the present study dopamine reuptake changes were investigated to ascertain whether dopamine transport affected stimulated dopamine levels in the synaptic cleft produced by nicotine. In addition, we investigated possible differences between saline and PCP treated brains. Dopamine release did not return to baseline levels during the washout period after nicotine with or without concomitant HEPES. Those animals which underwent PCP pretreatment may show differences either because of the effect of PCP or the effect of

age, since DAT activation may show differences as a resulte of age difference or pretreatment affect. Although DAT cannot gate synaptic dopamine spillover in striatum we consider that nicotine may in some way activate DAT in saline and PCP pretreated slices. However, neither PCP and saline group showed a significant difference in dopamine reuptake following application of nicotine (500 nM and 1μM) with concomitantly applied HEPES (100 μM), whereas the mean of the four recovery stimulations (Figure 3.24) did show a significant difference in tau numbers.

In our experiments we frequently saw an artefact on the current measurement, caused by electrical stimulation (Figure 3.38). Although the artefact potentially confounded results, electrically stimulated dopamine release was not affected by artefacts (Figure 3.27), and it did not create a problem in interpreting the results, because (1) the stimulus artefact was separable from the peak dopamine release, both temporally and from its cyclic voltammogram; (2) the dopamine release, but not the stimulus artefact was abolished by TTX (1 μM) superfusion; (3) in some recordings in NAc shell, very similar dopamine release was seen, but with no stimulus artefact; (4) electrical stimulation in a non-dopaminergic regions showed a very similar stimulus artefact, but no dopamine signal, Even stimulating with fewer (5 as opposed to 30) and narrower (0.5 msec as opposed to 4 msec) pulses, a stimulus artefact was often seen (Figure 3.37). As with the higher stimulations the artefact was clearly separable from the peak dopamine release, both temporally and from its cyclic voltammogram. Thus, in the present experiments it is showed that the stimulation artefacts did not confound the measurement of dopamine.

Figure S1 Electrical stimulation: 30 pulses, 4 msec, 60 Hz, 500µA
Example recording from Nac shell where no stimulus artefact was seen

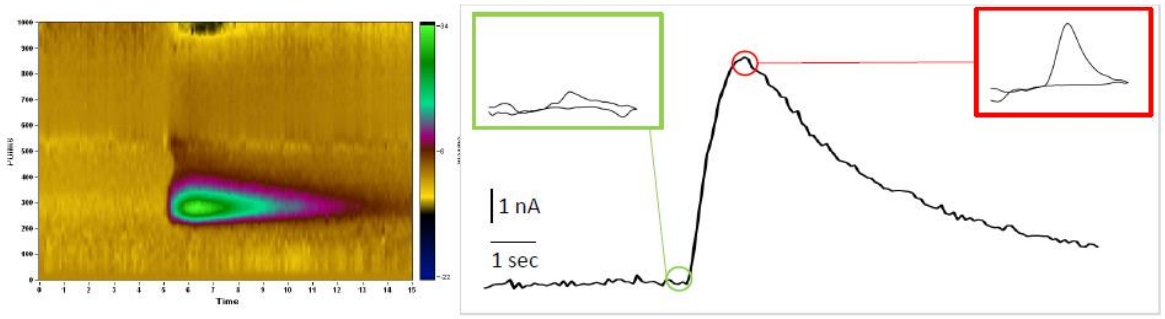


Figure S 2 Electrical stimulation: 30 pulses, 4 msec, 60 Hz, 500µA
Example recording from parietal cortex showing stimulus artefact but no release

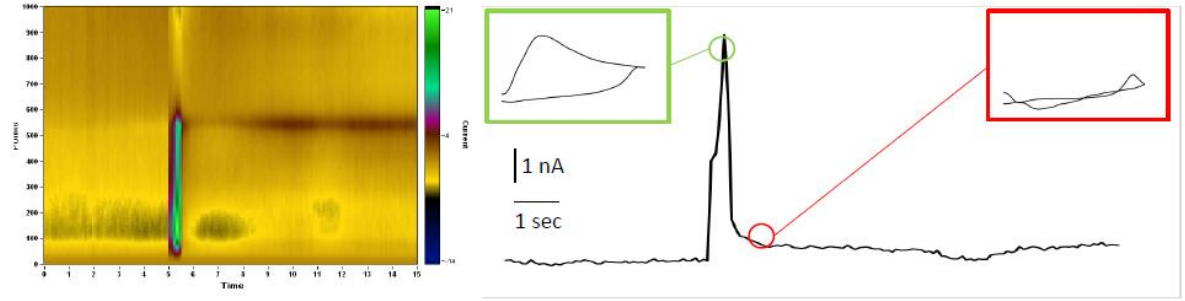


Figure S3: Electrical stimulation: 5 pulses, 0.5 msec, 60 Hz, 400µA
Example recording from Nac shell, showing stimulus artefact even with fewer (5 pulses) and narrower (0.5 ms) pulses

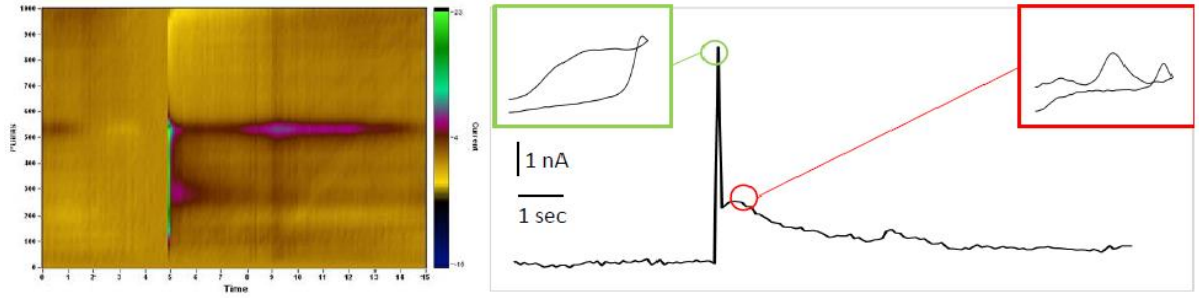


Figure 3.38 Stimulus artefacts seen in different stimulation parameters. S1) 30 pulses, 4 msec, 60Hz, 500 µA in NAcS . S2) 30 pulses, 4 msec, 60Hz, 500 µA in parietal cortex, S3) 30 pulses, 0,5 msec, 60Hz, 400µA in NAcS. In S1, colour plot on right and voltamoggram on the left shows that dopamine release occurred without artefact. In S2, no dopamine release observed, but artefact come off. In S3, dopamine release occurred following by artefact. Release can be separated comparing by oxidation peak on voltamogram (right) and non oxidation peak voltamogram of artefact (right).

3.8 Conclusion

Electrical stimulation possesses many advantages compared to potassium stimulation including rapid onset of dopamine release, returning baseline levels within few seconds, enabling measurement of drug effect over repeated stimulations. Thus, the effects of relevant agonists and antagonists on electrically stimulated dopamine release was used to understand mechanisms involved in NMDA modulation of dopamine release in NAcS. Moreover, in order to try to understand whether these mechanisms may be implicated in schizophrenia, experiments were also carried out in slices taken from PCP pretreated animals, modelling schizophrenia. Our experiments showed NMDA modulation of dopamine release, which presumably required intermediate mechanisms, since there is a paucity of NMDARs on dopaminergic axon terminals in NAcS. We found that mGluR receptor activation was likely to be involved in this mechanism since NMDA-depression of dopamine release was reversed by mGluR2/3 receptor antagonism. Further experiments would be required to fully characterise this effect. In addition, we found that cholinergic receptor activation is also involved in NMDA modulation of dopamine release, but not GABAergic receptor activation. GABAergic receptor antagonism did not reverse NMDA inhibition dopamine release and may even have enhanced NMDA depression of dopamine release. Dopamine release did not return to baseline levels on pretreated brain slices during the washout process which was enough time to see drug recovery on non-pretreated slices in our experiments. We thought that DAT activation may somehow cause to spillover dopamine release in NAcS which, in turn, produced slower recovery due to long lasting uptake process. Analysis revealed that DAT activation does not change electrically stimulated dopamine release either in PCP group or saline group.

CHAPTER 4: EFFECT OF PHENCYCLIDINE PRETREATMENT ON AMPHETAMINE AND NICOTINE EVOKED BRAIN ACTIVATION, MEASURED BY PHARMACOLOGICAL MAGNETIC RESONANCE IMAGING

4.1 Detecting BOLD signalling changes and resting state network changes by PCP treatment

4.1.1 Brain mechanism changes measured by phMRI

The spatial and temporal resolution of pharmacological MRI (phMRI) can help to determine brain responses to drug manipulation. The temporal resolution of phMRI differs from other techniques like microdilaysis (≥ 1min.) and fast scan cyclic voltammetry (sub-second measurement), with approximately 1 second time resolution easily achievable by phMRI. Relative cerebral blood volume (rCBV) time course in the NAc and another brain regions are known to be linked to dopamine release (Ferrari *et al.*, 2002; Rahman, 2003). According to Choi *et al.* (2006), nicotine induced rCBV changes in different brain regions including the infralimbic cortex, visual cortex, interpeduncular regions, NAc, cingulate and thalamus gives a good indication of the region-dependent activation enhanced by nicotine in the brain. Given possible respiratory artefacts, natural fluctuations in blood oxygenation in the MRI technique, the highest peak which is obtained from the infralimbic cortex in this study could be uncertain as to whether it responds directly to nicotine-mediated molecular changes or through other

non-specific mechanisms. In fact, animals in this study underwent IRON; (increased relaxation for optimized neuro-imaging: measures plasma volumes rather than red blood cell volume) scans rather than the blood-oxygen-level dependent contrast (BOLD) technique. BOLD contrast fMRI measures neuronal activity indirectly by way of cerebral blood flow (CBF) and CBV which lead to changes in deoxyhaemoglobin concentration relative to oxyhaemoglobin (Arthus and Boniface, 2002; Friston *et al.*, 2003). Moreover, in IRON technique monocrystalline iron oxide nanoparticle (MION) contrast agent is injected in the femoral vein (Weissleder *et al.*, 1990). The IRON technique gives more spatial localization of active brain regions and possess higher statistical power than BOLD (Weissleder *et al.*, 1990). However, there are no iron oxides approved for usage as a MRI contrast agents in human studies (Mandeville, 2012). Although both of these techniques include nonlinear changes in cerebral blood volume, there are no observable changes on CBV after nicotine injection (Choi *et al.*, 2006). However, it has been shown that NAc has constant activation after nicotine injection, which supports the idea that exposure to nicotine changes the mesolimbic function and neuronal plasticity, as the NAc is related to addiction in the mesolimbic pathway (Lacy *et al.*, 2016; Cao *et al.*, 2016). Thus, while this study indicates that nicotine is capable of activating different brain regions, especially in NAc, there is no discussion of the dosing regime or the time course of the response. The drawback of this study is that the dose regime and the importance of the time course of injection is not mentioned. In the experiments described in this chapter, we aimed to assess nicotine activation based on time and injection procedures and the results obtained were compared within the target brain regions. In addition to nicotine, the effect of amphetamine were also evaluated.

4.1.2 Nicotine modulates BOLD activation and functional connectivity networks is time and dose dependent

According to Bruijnzeel *et al.* (2014), nicotine (3 mg/kg) increased the BOLD signal in the gustatory cortex and motor cortex as early as 1 minute post-injection, whilst the BOLD signal in the dorsal striatum, basolateral amygdala, central amygdala, motor cortex, sensory cortex, NAcS, (but not the NAcC) did not show increases until 5 min post injection. However, no increase was observed in the NAcS and dorsal striatum at 10 minutes post-injection. Surprisingly, the highest dose of nicotine (6 mg/kg) evoked a unique temporal pattern in BOLD signal changes in different regions of the brain such as the dorsal striatum, motor and somatosensory cortices, NAcS, but again not the core. However, the same dose of nicotine did not change the BOLD signal in the NAc and dorsal striatum, or the motor and somatosensory cortices at 10 minutes post-injection. The administration of the nicotinic antagonist, mecamylamine, caused negative BOLD signal changes in the motor cortex and somatosensory cortex, but not in the NAc or striatum (Bruijnzeel *et al.,* 2014). These results show that nicotine's effects in different regions of the brain are time- and dose-dependent. Although nicotine is an α4β2 subunit of nAchRs agonist, the results indicate that nicotine also plays a major role in the mesolimbic pathway (mesolimbic pathway described in chapter 1). It should be noted here that the nicotine antagonist (mecamylamine) did not change the BOLD signal in the NAc area, but nicotine increased the BOLD signal in same region in a dose- and time-dependent manner. Indeed, different anatomical structures that evolved at different times may be characterised by various local network topologies (Schwarz *et al.,* 2012), and which network properties are modulated by active engagement of specific brain circuits has recently begun to receive attention (Deuker, *et al*., 2009). This study shows how different dosages and time courses determine the effects of nicotine in different brain

regions, as is supported by Huang *et al.* (2015), and that different brain regional connectivity is affected by different interval subcutaneous nicotine challenges after chronic nicotine pretreatment in resting-state (rs) fMRI.

In Huang *et al's* study (2015) three groups were compared; saline group, first-time nicotine challenged and nicotine pretreated groups. There was no significant functional connectivity changes between these three groups obtained by rsMRI. On the one hand, in shorter-term intervals of 3 hr to 6 hr, strong rsMRI circuits were observed at the interpeduncular nucleus, NAc, orbitofrontal area, and insula in chronic nicotine pretreated group. On the other hand, weaker rsMRI circuits were found in the hippocampus with SN, and VTA at shorter intervals between doses .Interestingly, there was no significant difference observed between the control group and 24 hr group in the nicotine-challenged group (first dose of nicotine) (Huang *et al.,* 2015). In this experiment, the prefrontal area, NAc, and anterior cingulate cortex are taken as seed regions in which rsMRI changes were observed in response to nicotine challenge. Anterior cingulate connectivity with prefrontal areas and NAc showed decreases after the first nicotine administration, but not in any of the four experimental groups. Moreover, these seed regions showed decreases the strength of resting state functional connectivity in circuits after nicotine in the control groups and 3 hr and 6 hr groups, whereas an increase in strength of resting state functional connectivity circuits was observed after nicotine in the 12 hr and 24 hr groups (Huang *et al.,* 2015). These results show that nicotine and its activation depend on brain region and time, which is related to the desensitization process and the number of nicotinic receptors in different brain regions.

In summary, BOLD signal changes brought on by either acute or chronic nicotine injection can be detected by phMRI in rodents. However, BOLD signal changes following nicotine depend on time of measurement as well as on treatment protocol. For

example, nucleus accumbens and dorsal striatum generated BOLD signal five minutes after nicotine injection, but not after one minutes or ten minutes later. Moreover, BOLD signal also depended on the location as well as the dose of nicotine. For example, somatosensory cortex generates BOLD signal with 6mg/kg nicotine, whereas the amygdala generated BOLD signal with 3mg/kg nicotine five minutes injection. In addition to BOLD activation, functional connectivity networks differentiate by nicotine in which injection maintained either chronic or acute treatment and imaging time.

For instance, animals receiving chronic nicotine treatment showed functional connectivity changes involving nucleus accumbens that were observed during two time-windows: a short time window (3-6 hours) or a longer time window (12-24 hours) post-treatment, whereas animal undergo first-time nicotine injection did not show such changes in functional connectivity. In fact, first-time nicotine injected rats did not differ from saline treated rats regardless of whether when the observations were made (i.e., between 3-6 hours or 12-24 hours).

1 Min *BOLD Signal*	5 Min *BOLD Signal*	10 Min *BOLD Signal*	3-6 Hours *Functional Connectivity*	12-24 Hours *Functional Connectivity*
First-time Nicotine	**First-time Nicotine**	**First-time Nicotine**	**Chronic Nicotine**	**Chronic Nicotine**
Gustatory Cortex **(3mg/kg)**	Dorsal Striatum **(3mg/kg)**	Basolateral Amygdala **(3mg/kg)**	Interpeduncular Nucleus **(4 mg/kg)**	Prefrontal Cortex **(4mg/kg)**
Motor Cortex **(3mg/kg)**	Basolateral Amygdala **(3 mg/kg)**	Central Amygdala **(3mg/kg)**	Nucleus Accumbens **(4mg/kg)**	Nucleus Accumbens **(4mg/kg)**
	Central Amygdala **(3mg/kg)**	Motor Cortex **(3mg/kg)**	Orbitofrontal Cortex **(4mg/kg)**	Anterior Cingulate Cortex **(4mg/kg)**
	Motor Cortex **(3mg/kg)**	Sensory Cortex **(3mg/kg)**	Insula **(4mg/kg)**	
	Nucleus Accumbens Shell **(3mg/kg)**			
First-time Nicotine	**First-time Nicotine**	**First-time Nicotine**	**First-time Nicotine and Saline**	**First-time Nicotine and Saline**
Dorsal Striatum **(6mg/kg)**	Dorsal Striatum **(6mg/kg)**	None	No difference	No difference
Motor Cortex **(6mg/kg)**	Motor Cortex **(6mg/kg)**	None	No difference	No difference
Somatosensory Cortex **(6mg/kg)**	Somatosensory Cortex **(6mg/kg)**	None	No difference	No difference
Nucleus Accumbens Shell **(6mg/kg)**	Nucleus Accumbens Shell **(6mg/kg)**	None	No difference	No difference

Figure 4.1 Nicotine-evoked time dependent BOLD signalling and functional connectivity changes. Data obtained from Bruijnzeel *et al.* (2015) and Huang *et al.* (2015).

4.1.3 Anaesthetic regime confound drug effect

Although clear time- and dose-dependent changes have been seen after nicotine, it is important to take into account the anaesthetic regime when evaluating the nicotine effect. Anaesthetic is a major confound in assessing drug effects on the brain in phMRI because the calcium uptake process can affect results by changing neurotransmitter activation. For example, GABA-A and glycine receptor activation are enhanced, whereas nAChR, serotonin and glutamate receptor activation is inhibited during isoflurane administration (Campagna *et al.*, 2003). Therefore, the optimal concentration of target drug should take

into account which anaesthetic has been used otherwise BOLD signal changes will be affected by physiologic changes resulting from the anaesthesia regime.

Signal changes after acute nicotine administration were measured using different anaesthetics (α-chloralose, medetomidine, thiobutabartibal urethane). Results showed that urethane anaesthesia is a good option for phMRI because of normal coupling between the BOLD contrast, CBF and CBVw, and a good correlation between the BOLD signal and spectral power (13-70 Hz) in rats (Paasonen *et al.,* 2016). However, one of the most important drawbacks of this study is the doses of nicotine. Changes in mean arterial blood pressure (MABP) can be induced by a high dosage of nicotine (Paasonen *et al.,* 2016). Nicotine dosages were kept stable for all the anaesthetics used in this study. High dose nicotine might somehow over activate the cholinergic system after providing the anaesthesia protocol. Although urethane appears to be the optimal anaesthetic for phMRI, subcortical regions of the brain showed smaller BOLD responses than for cortical areas (Paasonen *et al.,* 2016). However, recovery from urethane is very poor, meaning that it is really only suitable for non-recovery experiments, and is not appropriate for use in longitudinal experiments where repeated anaesthesia sessions, with recovery, are required. For these types of experiments, isoflurane is preferable, and this was the anaesthetic chosen for the experiments reported here (chapter 4). Animals showed a quick recovery in their home cage after MRI session in our experiments. Therefore, the isoflurane effect recovered in short time which is why we conducted our imaging experiments by using isoflurane protocol. However, urethane is still used in phMRI studies for preclinical drug discovery where recovery is not required.

The studies mentioned above do not give any indication as to how chronic nicotine pretreatment changes brain activation in different regions, but this question needs to be addressed to take a holistic approach to nicotine's effects in order to understand nicotinic

actions in schizophrenia, and in other conditions including addiction. Subchronic nicotine pretreatment can change underlying neuronal activation which in turn changes the BOLD signal (see below). Therefore MRI measurement of the BOLD signal using a T2*-weighted pulse sequence are useful for investigating pathological conditions (Supprian *et al.,* 1997). Moreover, studies carried out on people with schizophrenia show that the T2 of water protons differed between white and grey matter (Andreasen *et al.,* 1991; Williamson *et al.,* 1992). It should be noted here that the T2 refer to structural MRI, hence BOLD signalling was not investigated in these technique, whereas T2* weighted is a parameter in MRI to investigate BOLD signalling changes. These findings are supported by Calderan *et al.* (2005). According to their study, subchronic nicotine pretreatment significantly changed rCBV values in the mediodorsal thalamus, lateral posterior thalamus, cingulate cortex, and prefrontal cortex. In addition to rCBV changes, nicotine also caused notable increases in T2 values in the cingulate cortex and showed a trend to increase in the prefrontal cortex and NAc in rats. However, these changes in T2 and rCBV could be a result of indirect vascular effects dependent on neuronal activation after chronic nicotine pretreatment (Calderan *et al.,* 2005).

Nicotine is known as being a α4β2 and α7 subunit of the nAChRs agonist. Increases in cortical blood flow after nicotine administration are mainly mediated by the α4β2, rather than α7 subunit (Uchida and Hotta, 2009). As we consider unanswered questions on how the α4β2 subunit of nAChRs plays an important role in schizophrenia, nicotine is a promising probe to use in cojuction with fMRI to enhance our understanding of normal and pathologic brain activation underlying factors of schizophrenia

4.1.4 Changes in glutamatergic mechanisms measured by phMRI

In addition to the cholinergic system, recent evidence suggests that the core deficit in schizophrenia is dysfunctional glutamate systems. The PCP model is used widely in rodents to investigate the underlying factors of schizophrenia (Javitt, 1999: see chapter 1 for detailed discussion). Pharmacological studies have shown that PCP changes neurotransmitter activation by acting on NMDAR, while electrophysiological studies have shown underlying changes in neural network function following subchronic PCP (Asif-Malik *et al.,* 2017). However, PCP-induced changes in brain function can also be examined using phMRI. In the studies reported in this chapter, phMRI was used to investigate the effects of subchronic PCP on the spatio-temporal distribution of the BOLD signal following stimulation by injection of either nicotine or amphetamine. In limbic cortical regions, which include the medial prefrontal, cingulate, orbito-frontal, and retrosplenial cortices, PCP caused significant rCBV changes (Gozzi *et al.,* 2008. In addition to limbic cortical areas, PCP-induced major foci of activation in dorsal striatum and NAc. However, there were no significant rCBV changes in somatosensory, piriform, insular cortices, hypothalamus, superior colliculi, or cerebellum (Gozzi *et al.,* 2008). Acute intraperitoneal preadministration of clozapine, followed by an intravenuous challenge with PCP 30 min later, significantly reversed PCP-evoked rCBV changes in the antero-dorsal and posterior dorsal hippocampus, but not in the NAc (Gozzi *et al.,* 2008). In this experiment, MRI data was acquired at least 20 min following administration of PCP challenge. The mGluR2/3 agonist, LY354740, significantly inhibited the effect of PCP in the striatum, NAc, and ventro-medial thalamus, whereas the D2 receptor antagonist, raclopride, did not reverse PCP-evoked rCBV changes (Gozzi *et al.,* 2008). These result show that PCP challenge has a widespread effect on different brain areas by antagonising the NMDARs which are used as a model in schizophrenia

research. Its activation can be reversed by mGluR activation, which has an inhibitory affect in glutamate release due to these receptors being expressed on dopaminergic axon terminals, providing presynaptic inhibition of dopamine release (Hu *et al.,* 1999). However, D2 receptor activation is unlikely to be involved in PCP effects as it does not affect the attenuation of rCBV changes after PCP administration. It should be mention that PCP in this experiment was administrated on the day of the experiment meaning that the drug was present in the brain at the time of recording. However, in our experiments animals underwent PCP treatment (twice a day for five days) and animals underwent phMRI scans after a week washout: therefore the PCP was not present at the time of MRI testing (see methodology below). Although PCP is a NMDAR antagonist, the study mentioned above does not describe how PCP pretreatment changes the subunit composition of NMDARs. Subunits of NMDAR degenerated in schizophrenia (Kantrowitz and Javitt, 2010). The study by Shatillo *et al.* (2015) showed how NMDAR subunit composition could be investigated by fMRI which is promising in the understanding of glutamate theory in schizophrenia. NMDAR composition is widely spread in the CNS and their activation plays a critical role in synaptic plasticity which may be compromised in neurologic and psychiatric disorders (Paoletti *et al.,* 2013; Cortese and Phan, 2005). However, the subunit composition of NMDAR differs regarding physiological brain processes in different regions. For example, fMRI studies show that ifenprodil, a NR2B subunit antagonist, reduced cortical spreading depression, which is a phenomenon implicated in migraine, and reduced BOLD signal. However, such a change was not observed in the NR2A subunit of NMDAR after pretreatment with an NR2A selective antagonist TCN-201, though the BOLD signal itself was changed (Shatillo *et al.*, 2015). These results indicate that manipulations of NMDAR subunits can be detected in fMRI by changing their BOLD signal, but their pharmacological effects

are different to each other regarding subunit differences of NMDAR; therefore further study is needed to confirm molecular changes, such as HPLC or PCR analysis.

In addition to nicotine, amphetamine is also a suitable subject to understand dopamine function in fMRI studies. Previous studies (Dixon *et al.,* 2005; Febo and Pira, 2011) have demonstrated amphetamine-evoked brain activation using phMRI. The studies reported here extend this to investigate changes in amphetamine-evoked (i.e., dopamine-mediated) brain activation following PCP treatment to try to elucidate changes in dopaminergic mechanisms after PCP, modelling schizophrenia.

4.1.5 phMRI and Methodological Considerations

phMRI can be used to measure drug-induced changes in the BOLD signal over the entire brain, which makes it a popular technique in drug discovery where the drug interacts with a range of molecules and molecular sites depending on drug action (Chen *et al.,* 2005a; Chen *et al.,* 2005b; Upadhyay *et al.,* 2011). phMRI is an important intermediary step between *in vitro* research and clinical trials as it allows the determination of information about how physiological conditions can change in a living organism. The other prominent advantage is being able to track variations in the same group of animals over time, which is particularly important in drug evaluation studies.

BOLD contrast fMRI can be applied to pharmacological models, and has a number of advantages compared to other methods. Molecular changes are capable of being measured by BOLD-contrast fMRI whereby whole-brain changes induced after drug administration can be visualised in a single animal, or group of animals, over time. When a brain is challenged by psychoactive compounds that can potentially disrupt

neurovascular coupling, this, in turn, can render results difficult to interpret as unknown drug effects on neurovascular coupling affect the BOLD signal and may mask true neuronal activity at neurotransmitter receptors. For this reason, in some cases it has been reported that a target drug may not match the expected functional sites (Ogawa *et al.,* 1993). A few explanations have been given in an attempt to clarify this sort of mismatch, such as distributions of receptors and drug affinities for these receptor and excitatory and inhibitory receptors' feedback (Iannetti and Wise, 2007; Breiter *et al.,* 1997). Studies show that there is a correlation between BOLD response and synaptic activity more than a correlation in between BOLD response and firing activity (Arthus and Boniface, 2002). Therefore, BOLD changes are more related to neurovascular and neurometabolic coupling. In other words, BOLD fMRI only measures the vascular response to neuronal activation. It is therefore a secondary measure of brain activation. Krimer's study (1998) support the evidence that sometimes BOLD changes existing drug-evoked changes in neuronal processing independently. In other words, a target drug may not match the functional sites (Herkenham, 1987). Interpretation of BOLD responses therefore requires caution. There are some techniques can be applied in phMRI experiments to help obtain precise interpretable results such as vascular masks; minimising the contamination of surrounding parenchyma with spatial smoothing (Hlustik *et al.,* 1998), controlling global drift effects (Lowe *et al.,* 2008). Physiological changes with or without drugs can also interfere with the interpretation of BOLD changes (Verberne and Owens, 1998). Arterial blood pH, pO_2, PCO_2 also influence BOLD responses (Sicard *et al.,* 2003). Minimising possible physiological factors is vital to get the correct BOLD signal in phMRI. The drug administration route may also affect cardiovascular and respiratory changes, thereby choosing the optimal path of administration, and ensuring drug dose is stable over the experiments is also critical to ensure stable, functional brain changes by the target drugs.

General anaesthetics like isoflurane have certain advantages compared to other anaesthetics. For example, recovery from anaesthesia is fast and less hepatotoxic than for halothane (Flecknell, 1987). However, it causes hypotension, respiratory depression and potent cerebral vasodilators (Hansen *et al.*, 1988), and may change neurotransmitter activation by increasing dopamine release and decreasing acetylcholine release (Opacka-Juffry, *et al.*, 1991; Shichino *et al.*, 1997). However, isoflurane does not negate the BOLD response in rat brain (Steward *et al.,* 2004). All in all, general anaesthetics cause an enhancement in inhibitory transmission and a reduction in excitatory synaptic transmission (Richards, 1998).

4.1.6 Resting state MRI

In addition to phMRI measurements, functional connectivity changes between pre- and post- PCP/saline treatment were assessed by applying "resting state" MRI (rsMRI) to examine how PCP treatment changes functional connectivity (see methodology section). Resting state connectivity focuses on connectivity assessed across individual BOLD time points during resting conditions (Friston, 2009). Resting-state networks consist of anatomically separated, but functionally connected, regions displaying a high level of correlated BOLD signal activity. Resting state functional connectivity analysis provides information about asymptotic and pathological conditions in human and animal research. However, some confounding factors such as head motion, anaesthesia, and physiological factors may change the haemodynamic responses, which result in changes in functional connectivity. According to Vincent *et al.* (2007), functional connectivity networks can be detected under general anaesthesia. There are still core functional connections known as default mode networks which seems stable to provide reliable information (Sforazzini

et al., 2014). Those functional connections that are seen in the resting state indirectly predict the cognitive process and its changes due to pathological conditions (Hutchison *et al.*, 2011). It worth bearing in mind that due to a lack of sensory tasks, it is difficult to detect direct cognitive changes in rodents, but rsMRI allows measurement of the connectivity brain in cognition related regions. Several analysis methods have been used to attempt to analyse functional connectivity, including the Pearson correlation coefficient, which is used to measure the region connectivity of slow BOLD activation and provide definition functional connectivity networks (Wang *et al.*, 2012) , and data-driven independent component analysis (Iraji *et al.*, 2016). Both techniques are applied to investigate functional connectivity organisation. Basically, images should provide spatial localization; however, the brain's cortical folding differs between individuals even within the same species (van Essen, 2013), resulting in misalignment and spatial blurring of functional connectivity gradients (Robinson *et al.*, 2013). Normalization followed by registration is achieved by co-registration of all images using relevant algorithms which enable comparison between subjects or groups.

4.1.7 rsMRI and methodological considerations

The analysis relies on information obtained from slow BOLD activation responses in human and animals. However, this information is somewhat confounded by the anaesthesia regime which can change the neural activity and neurovascular coupling, motion, cardiac responses, and target drugs that are either excitatory or inhibitory (see introduction above). Although fMRI can be denoised using post processing steps such as motion correction, resampling, removing non-physiological signals, temporal filtering, spatial smoothing, these approaches do not take into account the external confounder of

a preclinical rsMRI study such as injection route, anaesthesia regime and duration of the drug in the system. Therefore, the analysis of functional connectivity networks still remains elusive (Caballero-Gaudes and Reynolds, 2017), though there are some core topological principles which remain stable (Liang *et al.*, 2013). In other words, some functional changes brought about by pharmacological manipulations do not change internal or external factors.

4.2 Background

Acetylcholine, which is known to modulate dopamine release in striatum. Altered dopamine function after PCP may be mediated through disruption of cholinergic signalling, either indirectly via glutamatergic control of cholinergic activity, or through a direct effect of PCP on cholinergic receptors. The experiments presented in this chapter aimed to investigate BOLD signalling changes in cholinergic and dopaminergic activation of the brain for amphetamine and dopamine after subchronic PCP treatment by phMRI. In addition to BOLD signalling changes, resting state functional connectivity changes for PCP is measured by performing rsMRI. Since acetylcholine (chapter 3) and glutamate (chapter 2 and chapter 3) modulation of dopamine release changed by subchronic PCP pretreatment data obtained from *in vitro* brain slices by FSV experiments current study aimed to assess BOLD signal changes for amphetamine and nicotine in order to understand dopaminergic and cholinergic mechanisms whether disrupted by subchronic PCP treatment. To achieve this, phMRI *in vivo* technique is applied. In the experiments described in this chapter, longitudinal phMRI used on same group animals.

4.2.1 Aims

1. To assess whether amphetamine and nicotine produced a detectable BOLD response in baseline scans, before and after subchronic PCP treatment.

2. To assess changes in amphetamine- and nicotine-evoked BOLD signalling after subchronic PCP treatment.

3. To assess the change in resting state functional connectivity networks after subchronic PCP treatment.

4.3 Method

4.3.1 Animals

All experiments reported in this chapter used female Wistar rats bred within the Division of Biomedical Services, University of Leicester. For experiments before subchronic PCP treatment animals 50 to 60 days after weaning (age 25 to 31 days), animals were challenged by amphetamine and nicotine one week interval and taking to MRI scanner to assess BOLD activation. For treated animals, drug treatment started 60 to 70 days after weaning (age 81 to 90 days). Animals were injected twice daily with PCP (2 mg/kg; i.p.) or saline vehicle (1 ml/kg; i.p.) for five days, then left drug free for 10 days. Following by washout period, animals were taken to MRI experiments to assess BOLD signal changes brought by on amphetamine and nicotine like before subchronic PCP treatment.

4.3.2 Chemicals and drugs

PCP hydrochloride was purchased from Tocris, UK. All other drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK). Stock solutions of drugs (10 mM) were made up by dissolving in physiological saline, then aliquoted, and stored at -80°C. Working concentrations of the drugs were made freshly on the day of each experiment.

4.3.3 PCP treatment

PCP (2 mg/kg) or vehicle (0.9% saline) (volume 1 ml/kg) was administered twice daily for five days, and the animals were then left for at least five days (drug free) before testing. Full details of the procedure was as previously described (see above and chapter 2).

4.4 Procedure

MRI scanning was performed using a 9.4 T preclinical MRI scanner (Agilent Technologies, Santa Clara, CA, USA) with a 310 mm bore diameter and 12 cm inner-diameter gradient (600 mT/m maximum gradient strength), interfaced with a DirectDrive Console.

Rats were placed in a dedicated cradle with a bite bar, ear inserts and an anaesthetic nose cone with continuous anaesthetic scavenging. All scans were performed during the light cycle under anaesthesia using 1-2% isoflurane in oxygen. Respiration and body temperature were monitored continuously during scanning using a custom monitoring and gating system (SA Instruments, Stony Brook, NY, USA). Respiration was measured by a pneumatic respiration pillow while body temperature was measured via a rectal

temperature probe. Body temperature was maintained at 37 ± 1^{o}C using a heater fan controlled via a thermostatic feedback mechanism. Radiofrequency transmission and reception was achieved with a 72 mm volume coil and 2-channel surface receiver coil specifically designed for rat brain imaging, respectively (RAPID Biomedical, Rimpar, Germany).

The rat brain was positioned at the isocenter of the magnet and located with a fast gradient echo scan. 3D gradient echo shimming of first and second-order shims was performed on a whole-brain voxel and shim quality was confirmed using point resolved spectroscopy (PRESS) of the water peak. The timing of the phMRI experiment is outlined in Figure 4.1 below. A BOLD-weighted echo-planar imaging (EPI) scan (TR/TE = 2000/8 ms, FOV = 30 x 30 mm, 128 x 128 matrix, 32 repetitions, scan time = 17 mins 40 secs) was used to acquire a baseline functional scan prior to injection of either amphetamine or nicotine drug challenge. A T2-weighted structural scan was collected to enable registration of functional BOLD images to a higher resolution structural image enabling all datasets to be coregistered. A fast spin echo (FSE) sequence with TR/TE = 3000/400 ms, FOV = 28 x 28 mm (256 x 256 matrix), number of signal averages = 3, scan time = 9 mins 42 secs, was used to acquire whole-brain, T2-weighted structural images. Before running the baseline scan, a single repetition of the EPI sequence was acquired to guarantee image quality post shimming. Amphetamine (1 mg/kg. i.p) or nicotine (0.6 mg/kg, i.p) challenges were delivered after the baseline EPI scan: the animal remained in the cradle to minimise head movement, but the cradle was removed from the scanner for the injections. On replacing the rat in the scanner, location and shimming were again optimised. This was followed immediately by the structural (FSE) MRI scan. The EPI scan was repeated 20 min after the drug injections following an initial single repetition scan to check image quality. Following scanning, animals were returned to their home

cage and allowed to recover from the anaesthetic. After two scans, one week apart, they were treated with PCP (2 mg/kg, i.p, twice daily for five days) and then left drug free for ten days which is sufficient washout time to ensure no drug remains at the receptors. The two scans were then repeated as above, at one-week intervals, as shown in Figure 4.2, 3 and 4.

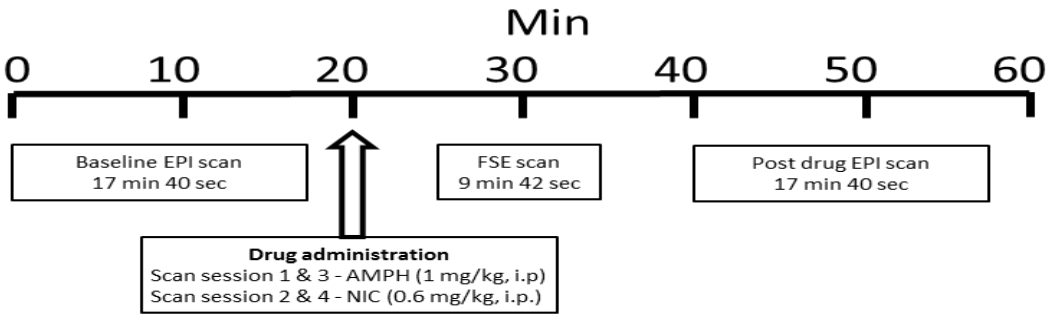


Figure 4.2 MRI method. **Summary of timings of recordings within each scanning session.** Animal is taken to echo planar imaging (EPI) for 17 min 40 that provides a baseline scan to compare drug effect. Flowing by baseline imaging animals are injected either amphetamine (AMPH) or nicotine (NIC) challenging and then fast spin echo imaging aooled to obtain structural imaging for alignment. Finally, EPI is repeated to compare baseline EPI. Total imaging time is 60 min per animal.

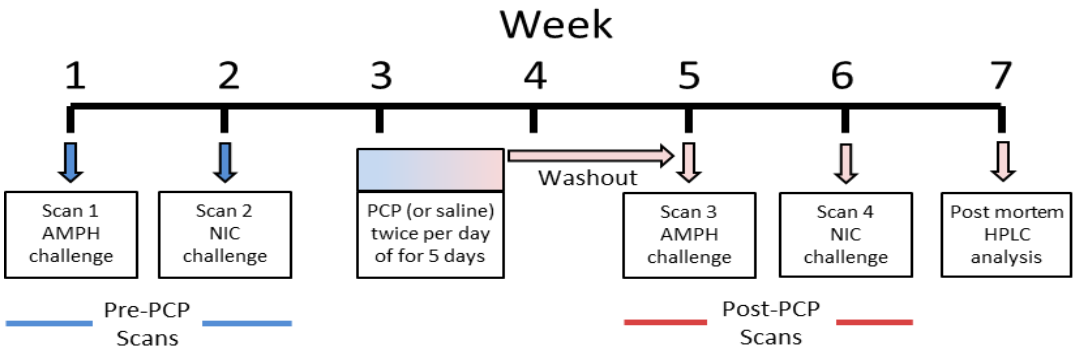


Figure 4.3 MRI method. Summary of experiment schedule, showing time frame of repeated scanning sessions relative to PCP (or saline vehicle) treatment. Animal is challenged by amphetemaine (AMPH) and undergo scanner in first week. Following by the first week, animal is challenged by nicotine (NIC) and scanned in the second week both week of these called pre PCP scans. In third week, animals undergo to subchronic PCP treatment or saline treatment twice per day for 5 days and given a week time to washout to ensure no drug on receptors. Following up to washout, nicotine and amphetamine challenges repeated as before PCP treatment and finally animals culled and brain removed for further analysis by using high performance liquid chromatography (HPLC).

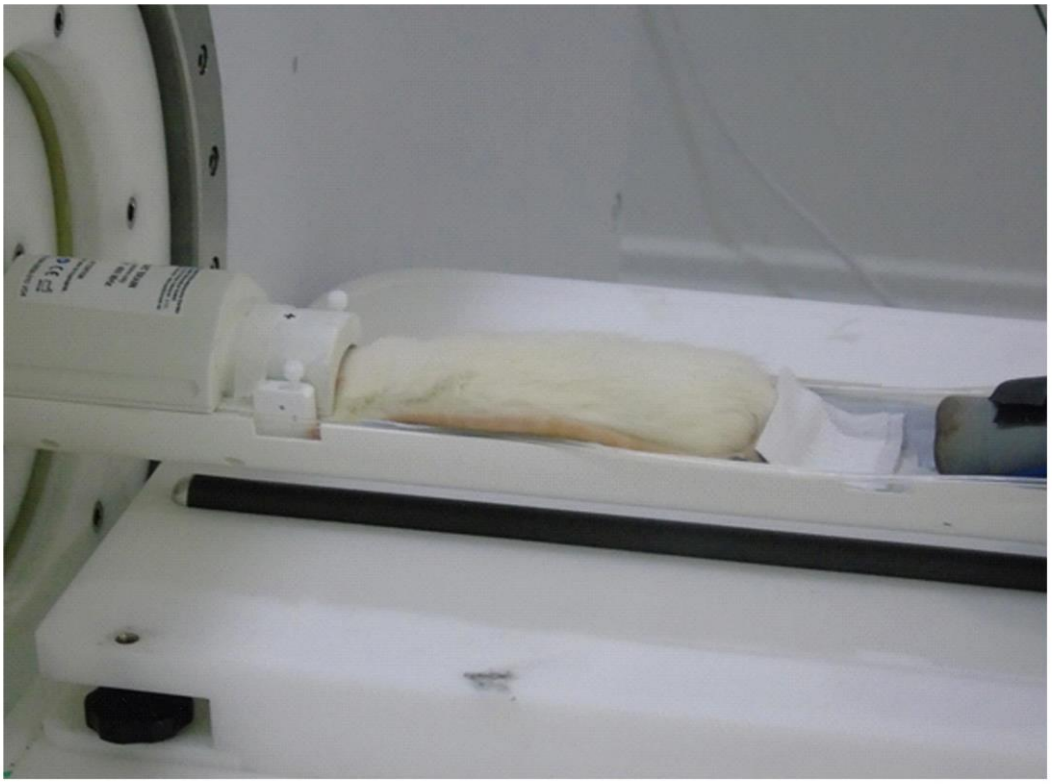


Figure 4.4 MRI method. Rat cradle and equipment used for MRI experiments comprising, rat brain surface coil, anaesthetic nosepiece, anaesthetic scavenger system, head holder, thoracic transducer, respiration pillow, heater system and rectal probe.

Analysis of phMRI scans performed in FSL (FMRIB, Oxford) were as follows: motion correction using the motion correction linear image registration tool (MCFLIRT) to correct for motion between repititions, brain extraction using an optimised rodent brain extraction tool (rBET) to remove non-brain tissue from all images. It is worth noting that the reason behind using rBET is the BET programme for human fMRI data is not suitable in rodents due to differences in dimension and shape between human and rat brains, hence extraction by BET brain mask does not give an accurate brain extraction (Wood *et al.*, 2013). This was followed by bias field correction using FAST (tissue-type segmentation) to correct for the sensitivity profile of the surface receive coil; independent component

analysis (ICA) to identify and remove noise components from phMRI data using Multivariate Exploratory Linear Optimized Decomposition into Independent Components (MELODIC); FEAT general linear modelling to fit the phMRI data to an off-on drug stimulus model for first level fMRI analysis; FMRIB's linear image registration tool (FLIRT) linear registration for registration of functional datasets to structural scans followed by registration of individual structural scans to an average study structural image to align all functional scans in the same space for subsequent group-level analysis(Jenkinson and Smith, 2001) ; group-level analysis using FEAT to identify differences between pre- and post-PCP and pre- and post-saline phMRI maps (mixed effects model, paired groups, Z (Gaussianised T/F) statistic images were thresholded using clusters determined by $Z > 2.3$ and a connected cluster significance threshold of $P = 0.05$). Analysis for rsMRI was similar to phMRI, but FEAT analysis was not applied to rsMRI analysis. Instead, MELODIC ICA was run after the dual regression analysis run which is main part of rsMRI analysis in order to a group-average ICA.

Analysis of phMRI data was performed in FSL (FMRIB, Oxford) as followed below (Woolrich et al., 2009; Smith et al., 2004; Jenkinson et al., 2012);

4.4.1 Motion Correction

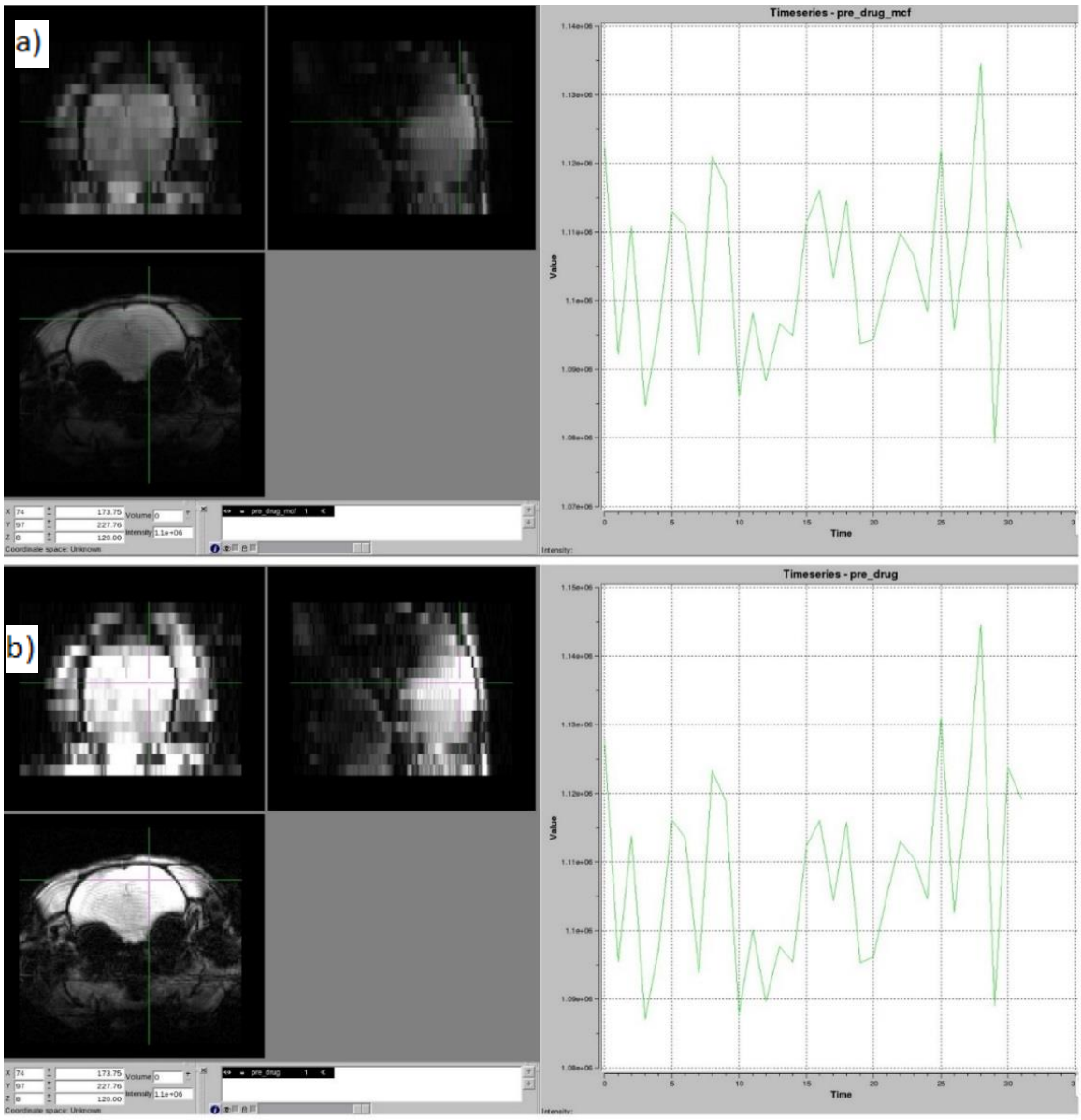


Figure 4.5 Pre- and post-motion correction (image + time-course). Temporal and spatial changes compared before and after motion correction. On the left side. Sagittal, axial and coronal brain slices are shown. On the right side, the time-course of the MRI signal is shown. Between EPI scans the rat cradle was taken out of scanner in order to challenge the animal either by amphetamine or nicotine. Since this procedure is a potential source of motion-related artefacts. Data shows there is no difference between before and after motion correction. On the left side, time shows time-course of the EPI signal for the voxel selected on the RHS (i.e. where the crosshairs are placed). The signal intensity between figure a and b different. This is simply due to the way the motion correction works. The values in the images are scaled slightly during the motion correction step.

4.4.2 Rat brain extraction (rBET)

Figure 4.6 Post-brain extraction. To remove non-brain tissue from all images. Sagittal, axial and coronal slices are represent above. rBET provided improved extraction of rat brains compared to conventional human brain extraction methods (BET). This step facilitates upcoming steps such as bias field correction and linear registration.

4.4.3 FAST (Bias Field Correction)

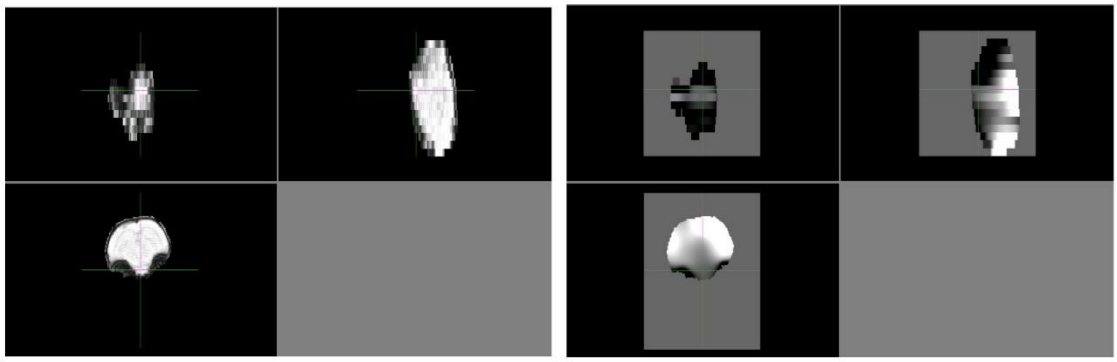


Figure 4.7 Post-bias field correction. To correct for the sensitivity profile of the surface receive coil (on the right hand side, a coil sensitivity profile image that was used to normalise the brain images is shown). The surface receiver coil, which is used to receive the MRI signal from the rat brain, has higher sensitivity at the cortex compared to sub-cortical regions. This introduces and artificial sensitivity profile in the images. To deal with this problem, bias field correction was applied in preprocessing of data analysis using the FAST tool in FSL.

4.4.4 Independent Component Analysis (ICA) (Identity and remove noise components)

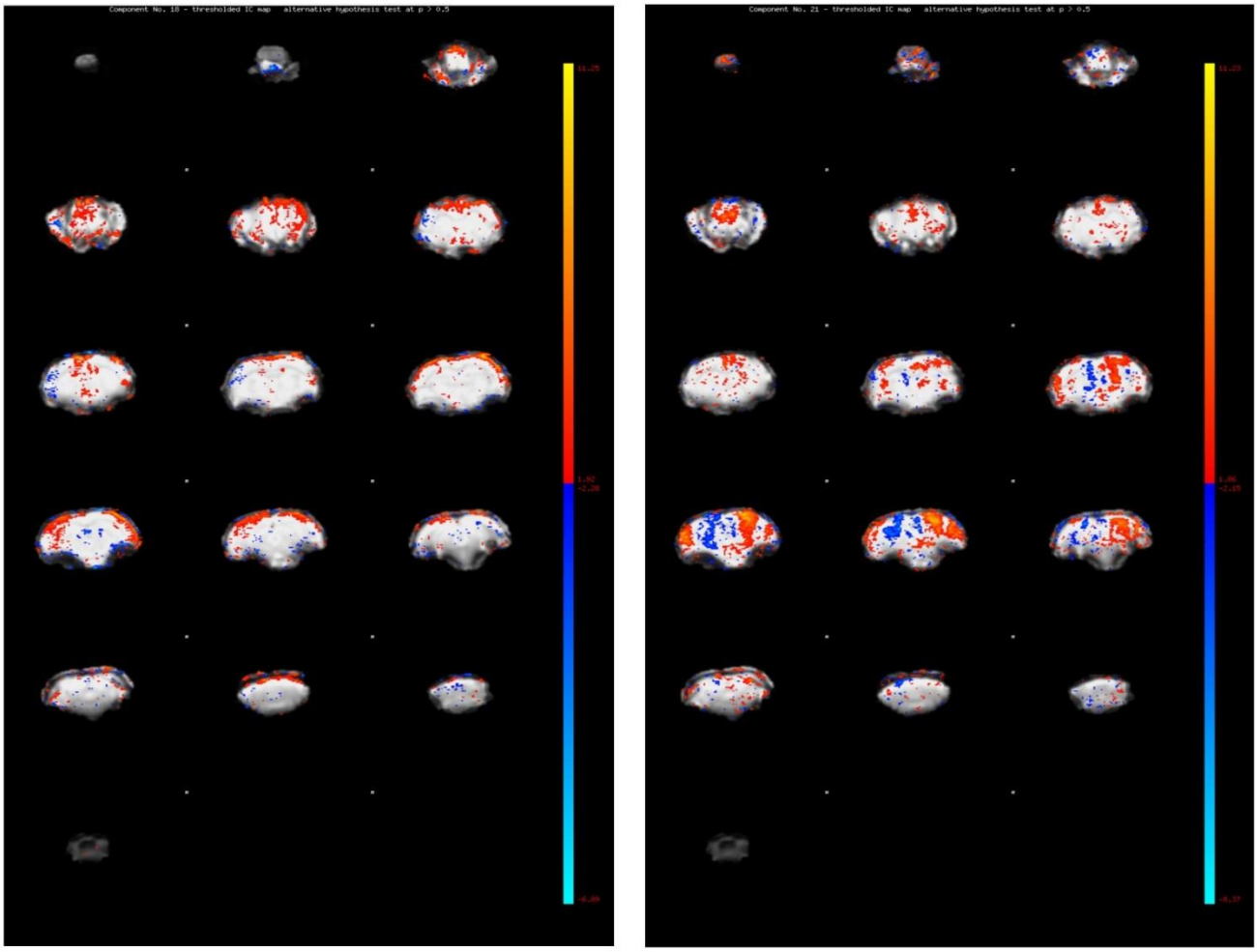


Figure 4.8 Example of "good" and "bad" ICA component. On the left hand side, a typical noise component associated with motion artefact is shown, with motion-related correlation around the edge of the brain. On the right hand side, a non-artefactual component is represented, with meaningful physiological signal inside the brain. Without ICA analysis, artefact resulted from either physiological process such as heart rate, respiration or non-physiological process such as movement of the rat between injections can confound the BOLD signal and subsequent analysis. ICA was used to identify and remove all artefactual components from each dataset.

4.4.5 FEAT Design (First level MRI using FEAT to model the off-on drug stimulation)

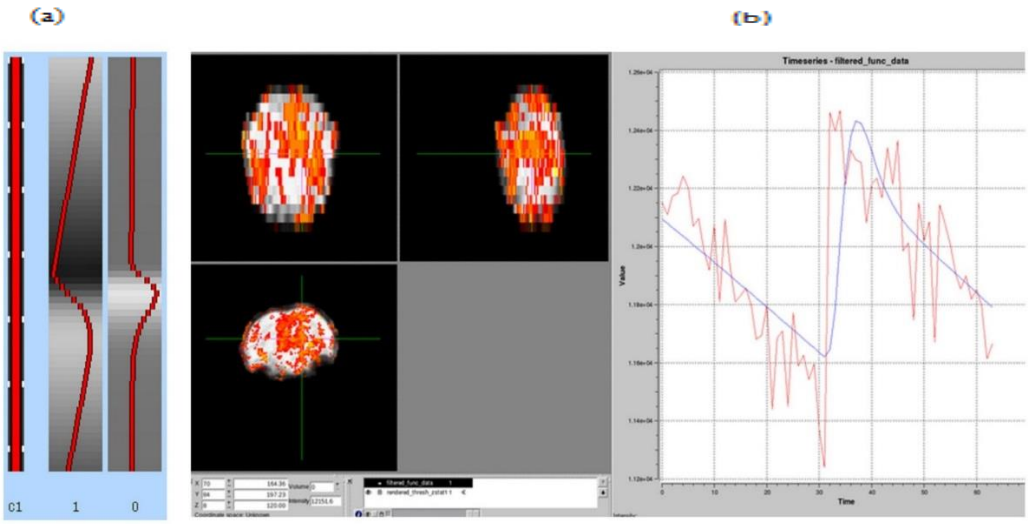


Figure 4.9 a) FEAT General Linear Model (GLM) design and b) example of phMRI time course fitted to GLM. Figure a) off-on general linear model represented. Drug injection and non-injection means on and off respectively. Figure b) heat maps obtained by applying this off-on model shows how the drug, either nicotine or amphetamine, changes the BOLD signal in sagittal, axial and coronel slices. On the right hand side, a sample fit of the time-course from a single voxel to the GLM is shown. This process is repeated for every voxel in the brain to generate drug activity maps.

4.4.6 Flirt (Specific Template)

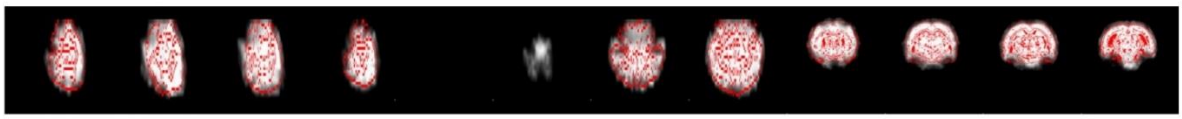


Figure 4.10 Registration for each subject to average structural image. A high-resolution Structural scan was used to align functional data from all subjects thereby facilitating a group level, statistical analysis of all subjects. The red colour shows the functional data aligned on structural imaging.

4.4.7 FEAT (Higher level analysis)

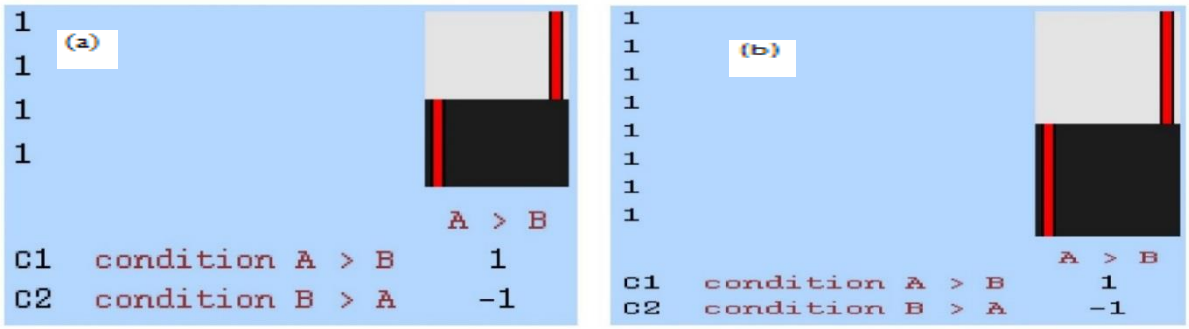


Figure 4.11 FEAT group level analysis design for both a) Saline and b) PCP analysis. Group-level analysis of c-registered datasets using FEAT to identify differences between pre- and post-PCP and pre- and post-saline phMRI maps (mixed effects model, paired groups, Z (Gaussianised T/F) statistic images were thresholded using clusters determined by Z > 2.3 and a connected cluster significance threshold of P = 0.05).

4.5 Results

Results obtained from these experiments are presented as follows: (1) effect of amphetamine on brain activity (2) effect of nicotine on brain activity (3) effect of PCP on amphetamine- and nicotine-induced brain activity (4) changes in resting-state brain activity.

4.5.1 Effect of amphetamine and nicotine on brain activity using pharmacological MRI

First-level MRI analysis using FEAT shows amphetamine- and nicotine-evoked BOLD signal that is region dependent. BOLD signal was obtained before and after PCP pretreatment for amphetamine and nicotine challenges, the results of which show that

both drugs produce increases in BOLD signals in a number of brain region. Figures 4.12 and 4.13.

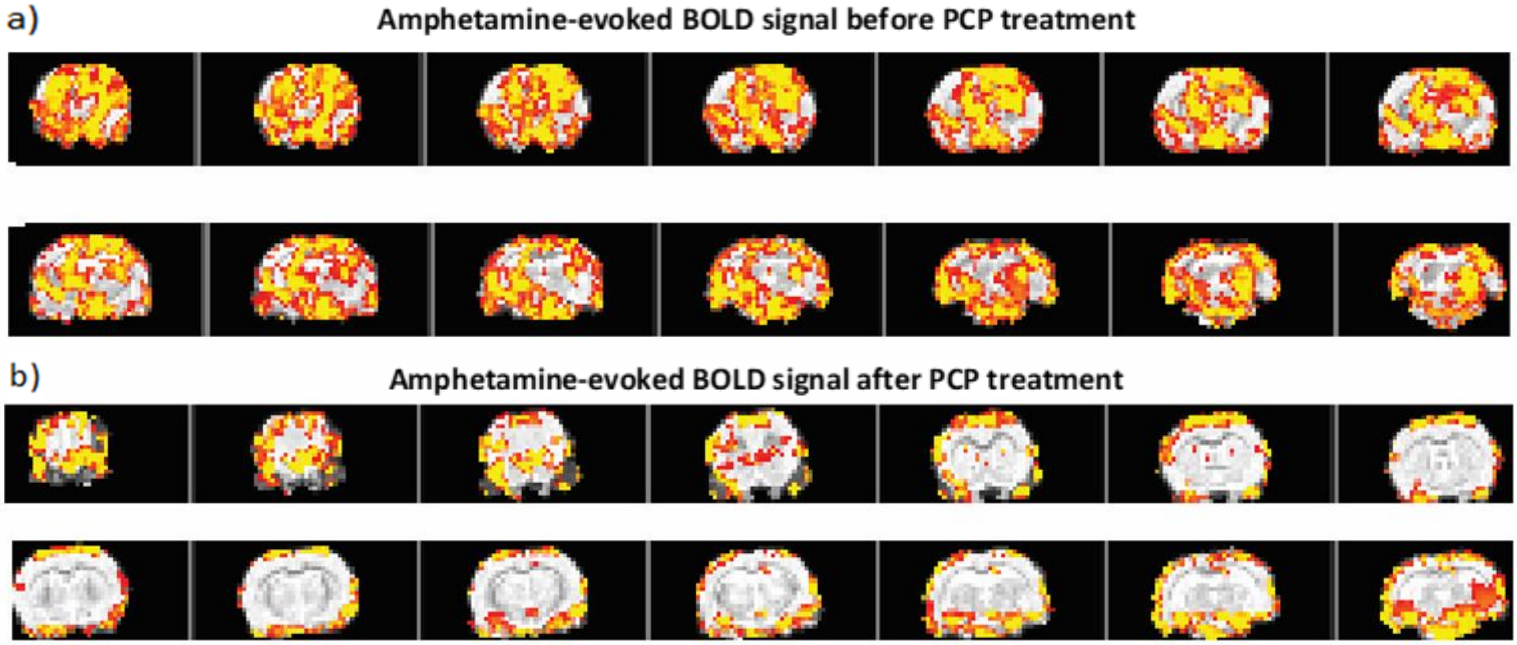


Figure 4.12 Exemplar phMRI maps from 1 subject for (a) amphetamine pre-PCP, (b) amphetamine post-PCP. Nicotine enhanced BOLD signal in cortices, dorsal and ventral striatum, thalamus, hypothalamus and hippocampus. However, BOLD signal intensity attenuated mainly in striatal brain regions following by subchronic PCP treatment.

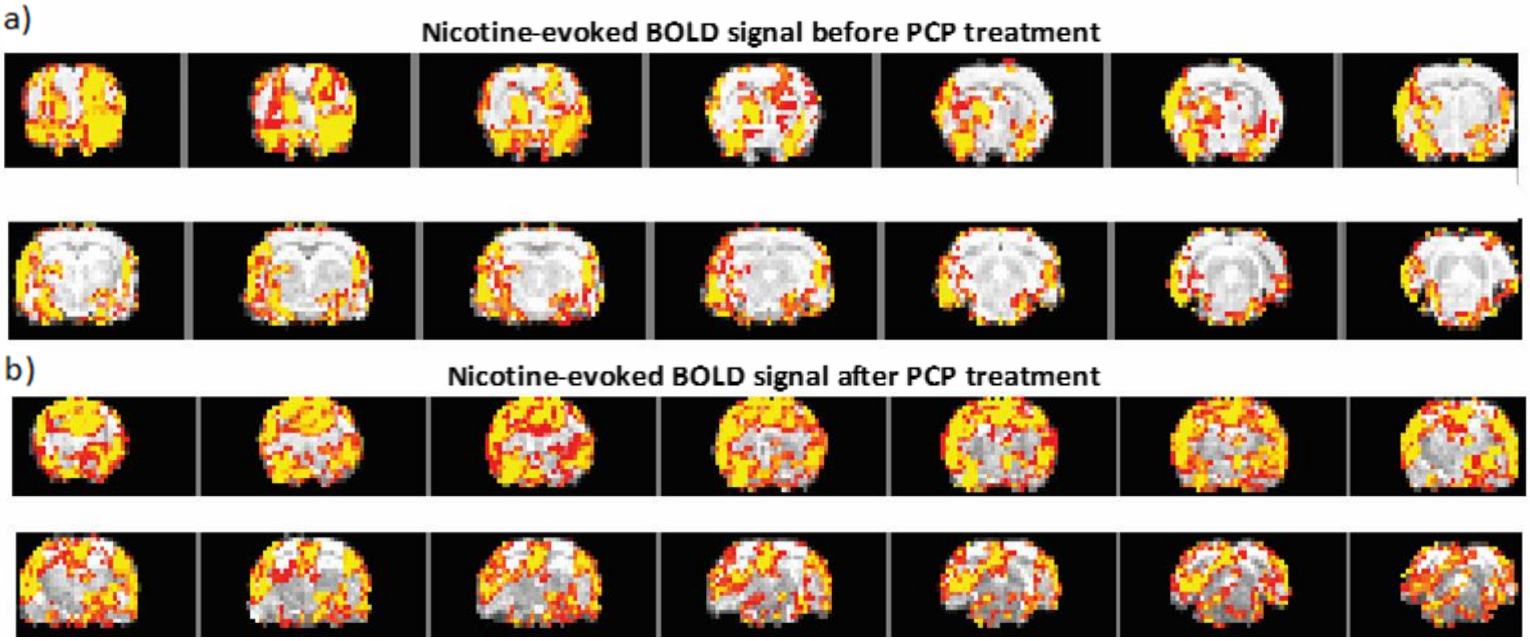


Figure 4.13 Exemplar phMRI maps from 1 subject for (a) nicotine pre-PCP, (b) nicotine post-PCP. Nicotine enhanced BOLD signal in cortices, thalamus, and ventral hippocampus. However, BOLD signal intensity increased and showed wide spread activation across the brain following by subchronic PCP treatment for nicotine challenge.

Amphetamine in our experiments activated specific brain regions including the cortex, striatum, VTA and substantia nigra.. Dixon *et al.,* (2005) supported the idea that amphetamine has its own activation-specific brain regions. According to the dopamine hypothesis of schizophrenia dopamine over activation underlies core deficits. In our experiment, different brain regions were activated by a single injection of amphetamine before PCP treatment.

In addition to amphetamine, nicotine evoked BOLD signal in specific brain regions both before PCP treatment. The first-level analysis confirmed nicotine activation on cholinergic mechanisms (Figure 4.13) the cholinergic system is somewhat changed by PCP pretreatment in our previous *in vitro* experiments, hence further experiments were conducted by using longitudinal phMRI. Data obtained from MRI experiments confirmed that nicotine evoked BOLD activation, especially in regions where cholinergic neurons are known to be located such as the dorsal striatum, NAc, cortex, SN, hippocampus, thalamus, VTA (Lapper and Bolam, 1992; Meredith *et al.,* 1989; Eckenstein and Thoenen, 1983; Clarke *et al.,* 1987; Melander *et al.,* 1985; Sofroniew *et al.,* 1985; Oakman *et al.,* 1995). Cholinergic systems may be mediated by PCP pretreatment and those changes brought by PCP on cholinergic mechanisms may somewhat leads to changes on dopaminergic mechanisms. However, phMRI does not allow us to measure neurotransmitter signalling changes rather BOLD signalling changes by molecular activation can be measured. Therefore in further experiments PCP evoked changes for amphetamine and nicotine challenges were assessed by longitudinal MRI.

4.5.2 Effect of PCP on amphetamine and nicotine using phMRI

phMRI data obtained from nicotine- and amphetamine-challenged brain activation pre- and post-PCP pretreatment is useful to evaluate BOLD signalling for specific brain regions. However, those individual heat maps do not tell the whole story of PCP activation on brain mechanisms. An off-on drug stimulus model by applying first-level analysis does not enable measurement of the PCP effect on dopaminergic and cholinergic brain activation. Therefore, we used group-level analysis using FEAT to analyse PCP activation for dopaminergic and cholinergic brain mechanisms by challenging with amphetamine and nicotine, respectively. We used a mixed effects model and paired group analysis to evaluate the PCP affect. This approach relies on accurate coregistration of EPI scans from all subjects, which was achieved through use of rBET and FLIRT. Once all datasets were aligned in the same space, statistical comparison of groups was possible.

Results show that downregulated activation for both amphetamine and nicotine by PCP pretreatment, but no upregulated activation was seen for either nicotine amphetamine by PCP pretreatment (Figure 4.14, 4.15). In other words, PCP leads to attenuation of the amphetamine and nicotine-induced brain activity on dopaminergic and cholinergic mechanisms respectively, but not leads to enhancement. On the other hand, in the control experiment, saline-pretreated animals did not show either upregulation or downregulation for amphetamine and nicotine challenges.

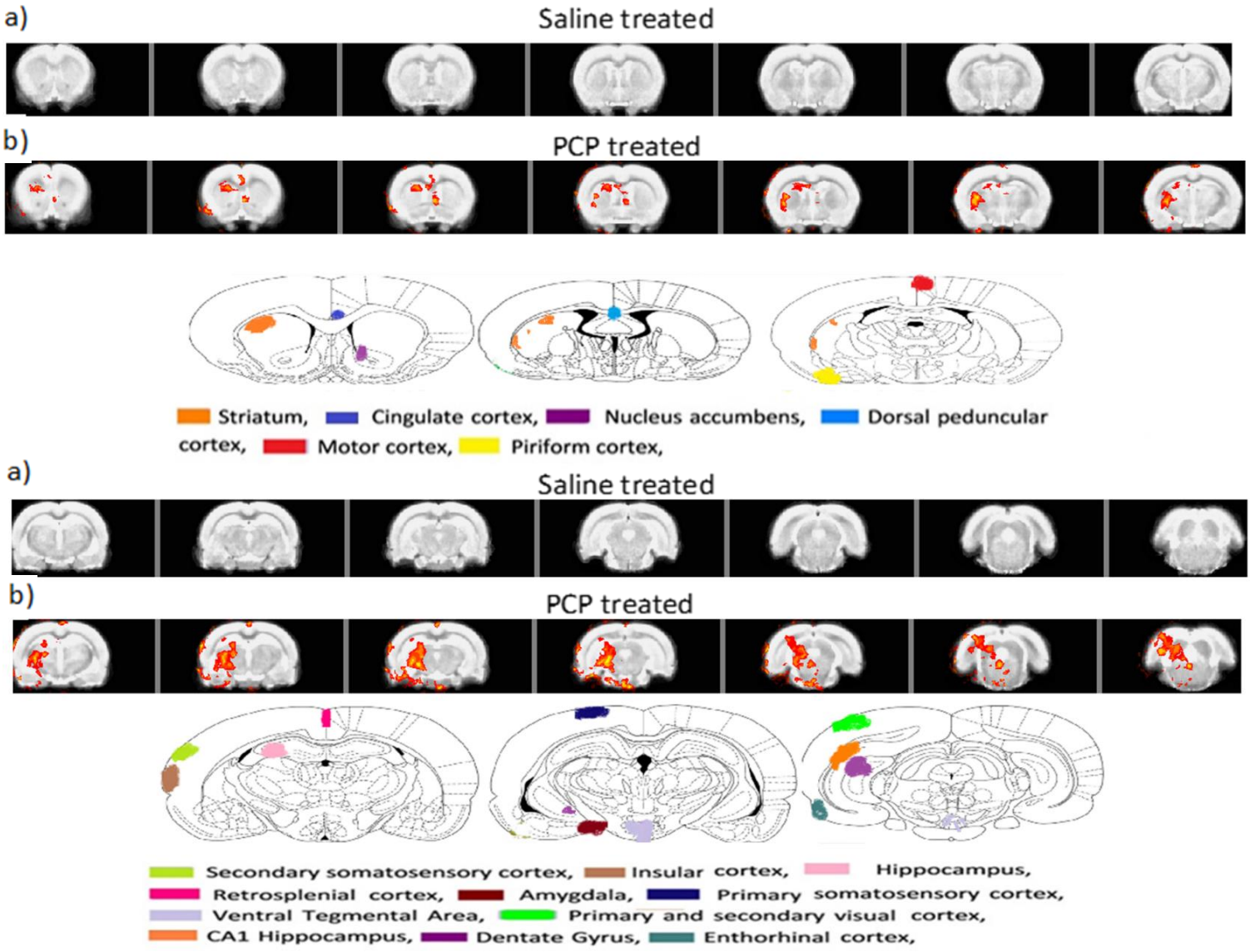


Figure 4.14 Result of group level analysis across all subjects. a) Regions showing for nicotine challenge following saline treatment. No changes in activity were seen for nicotine. b) Regions showing downregulated activation for nicotine challenge following PCP treatment.

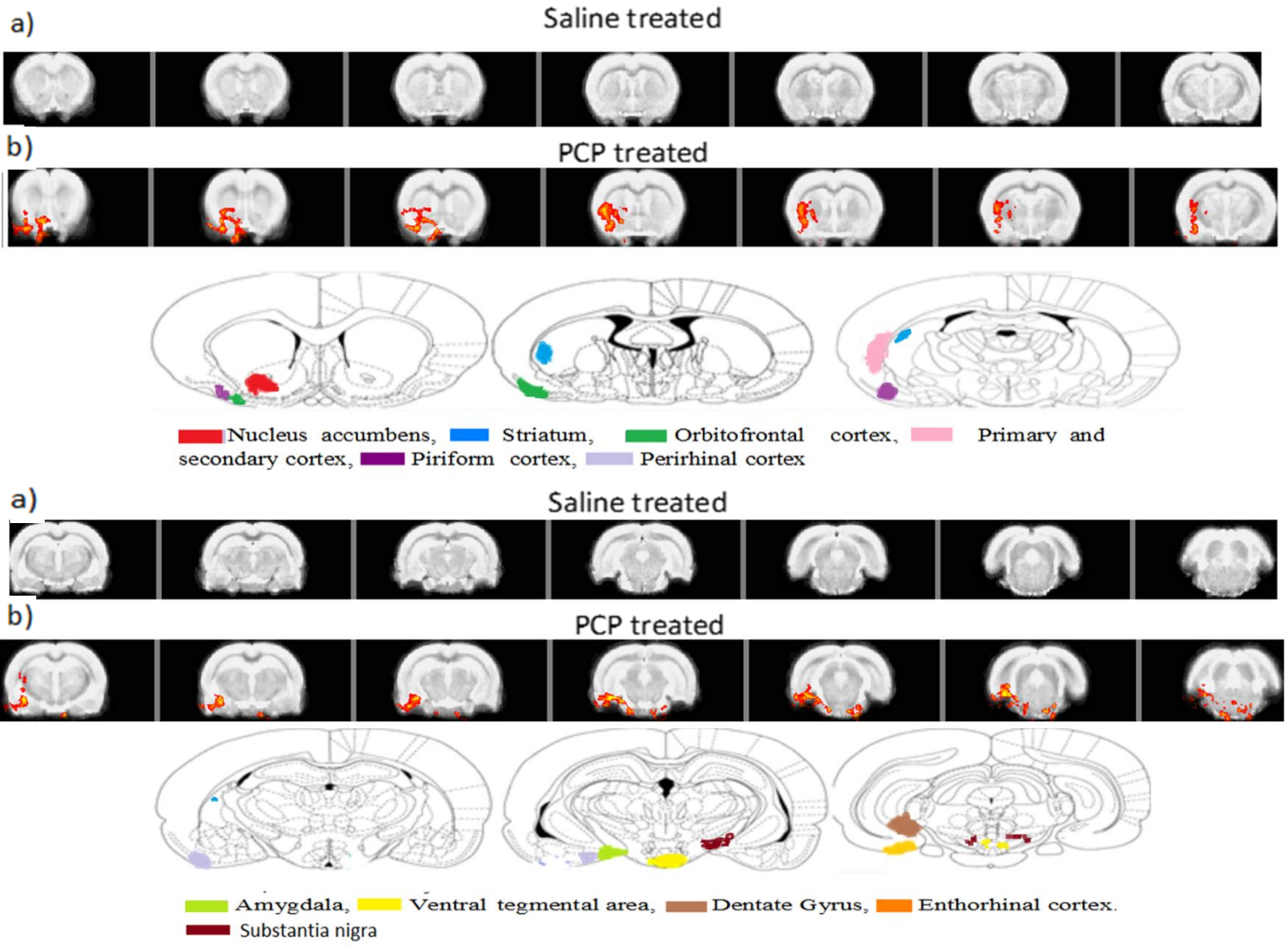


Figure 4.15 Result of group level analysis across all subjects. a) Regions showing for amphetamine challenge following saline treatment. No changes in activity were seen for amphetamine. b) Regions showing downregulated activation for amphetamine challenge following PCP treatment.

These data obtained from the group-level analysis show that subchronic PCP pretreatment changed brain activity patterns induced by amphetamine and nicotine. Specifically, PCP pretreatment attenuated both dopaminergic and cholinergic mechanisms for amphetamine and nicotine in many brain areas, but did not show any areas where activity was enhanced. Attenuation for amphetamine was mainly observed in the NAc, striatum, orbitofrontal cortex, primary and secondary cortex, piriform cortex, perirhinal cortex, amygdala, VTA, dentate gyrus, and enthorhinal cortex (regions identified by comparison of group level results with the rat brain atlas (Paxinos and Watson, 1998), whereas attenuation for nicotine was mainly observed in the dorsal striatum, motor cortex, NAc, dorsal peduncular cortex, piriform cortex, secondary somatosensory cortex, insular cortex, hippocampus, retrosplenial cortex, amygdala, primary somatosensory cortex, VTA, primary and secondary visual cortex, CA1 hippocampus, dentate gyrus, enthorhinal cortex.

These results indicated region-dependent changes in BOLD activation evoked by acute nicotine or amphetamine, after sub-chronic PCP treatment. Interestingly, these effects were found to be unilateral rather than bilateral, with attenuation of drug stimuli by PCP pretreatment only evident in the right hemisphere.

On the other hand, saline pretreatment did not show either enhancement or attenuation for amphetamine and nicotine challenges regions in where seen for PCP treated animals. In other words, dopaminergic and cholinergic mechanisms activated by amphetamine and nicotine respectively showed neither increase or decrease in BOLD signalling changes in saline treated rats. Thus the changes seen after PCP treatment can be attributed to the effects of the drug, rather than to changes over time, or due to repeated scanning sessions (figure 4.13).

4.5.3 Resting state MRI results

In addition to BOLD signalling changes brought by nicotine and amphetamine before and after PCP treatment, rsMRI was applied to investigate whether functional connectivity changes occurred after PCP treatment on same group animals. Resting state analysis of pre-amphetamine rest blocks was performed to compare pre- and post-PCP resting state networks. Results showed that neither PCP nor saline treatment caused significant resting-state changes, albeit signal that changes were obtained from the data. Since functional data aligned to structural template it somehow left obtained signal from cortical regions out of the template in figure 4.16. However, we consider that these activations in those regions still represent cortical functional connectivity changes brought on by subchronic PCP treatment. Artefact somehow occurred in data represent by blue colour in data.

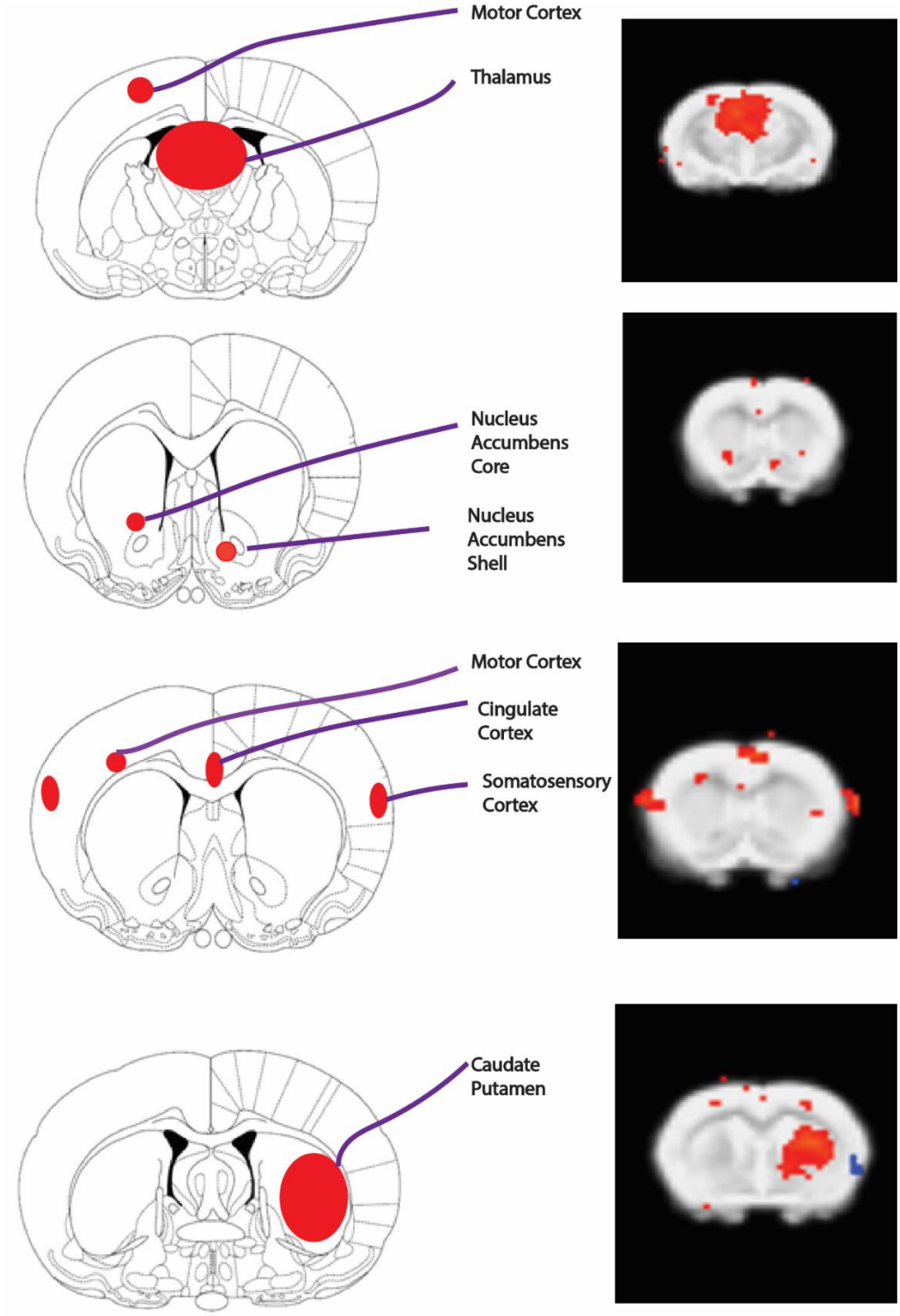


Figure 4.16 Exemplar for functional connectivity networks; pre-PCP and post-PCP. Functional connectivity changes observed in motor cortex, thalamus, nucleus accumbens, cingulate cortex, somatosensory cortex, caudate putamen. More changes seen in appendix.

PCP induced functional connectivity changes from voxels indicate that these regions were activated by PCP treatment. These results imply that rsMRI experiment were able to show an effect of PCP on resting brain activity, but PCP did not change functional connectivity networks in the same data set. In other words, molecular changes brought by PCP, but not neural resting state functional connectivity networks. Those networks represent spontaneous fluctuations in brain activity and changes in those spontaneous fluctuations is implicated in pathological conditions such as schizophrenia (Fox and Raichle 2007). The resting state networks in figure 4.14 show brain regions that are "active" in a correlated way while the brain is at rest. Gozzi's study showed that acute PCP changed rCBV in cortical brain regions, thalamus, cingulate gyrus, NAc (Gozzi, 2008). The main difference between the current study and Gozzi's study is that we were assessing whether functional connectivity changes were brought about by chronic PCP treatment. Although results in the present study were not significant changed brain regions data show that functional connectivity networks in resting state (see chapter 1). (More data form components in appendix).

There is not a significant change observed functional connectivity changes by PCP regarding strengthen or weakening of which connections, but effect of PCP was observed on changes of resting brain activity (see results and appendix).

4.6 Discussion

A wealth of evidence suggests that dopamine release is dysregulated in schizophrenia, yet the core deficit appears to lie in glutamate dysfunction (Javitt, 1999). A possible explanation is that core deficits in glutamate seen in schizophrenia lead to dopamine dysregulation through interactions between these mechanisms (Kretschmer, 1999). In addition to dopamine, cholinergic mechanisms are likely to be involved in dopamine modulation (Threlfell *et al.*, 2012), and may be disrupted by PCP. To address whether the cholinergic mechanism, which modulates dopamine release, is affected by subchronic PCP treatment we conducted a longitudinal phMRI experiment. These experimental results indicate that acetylcholine signalling is attenuated by PCP treatment. Dopamine signalling was attenuated in a regionally dependent manner. Animals were challenged by amphetamine and nicotine before and after subchronic PCP treatment. Both agents evoked BOLD activation in different brain regions before and after PCP pretreatment. High-level analysis showed that PCP treatment changed dopaminergic and cholinergic mechanisms. Attenuation for amphetamine was mainly observed in the NAc, striatum, orbitofrontal cortex, primary and secondary cortex, piriform cortex, perirhinal cortex, amygdala, VTA, dentate gyrus, and enthorhinal cortex, whereas attenuation for nicotine was mainly observed in the dorsal striatum, motor cortex, NAc, dorsal peduncular cortex, piriform cortex, secondary somatosensory cortex, insular cortex, hippocampus, retrosplenial cortex, amygdala, primary somatosensory cortex, VTA, primary and secondary visual cortex, CA1 hippocampus, dentate gyrus, enthorhinal cortex by PCP treatment. In other words, those regions were active for amphetamine and nictone, but inactivated by PCP treatment. Moreover, there is no enhancement either for amphetamine or nicotine challenge by PCP treatment. Saline-treated animals did not show either attenuation or enhancement with nicotine and amphetamine. In this experiment, animals

underwent longitudinal phMRI scanning; long-term experiments carried out using the same group of animals across multiple time points. It is possible that age-related changes somehow affected the results. It is known that there are age-dependent changes in receptor composition within the brain. For example, NMDA pre- and postsynaptic receptor structure shows changes in a maturing brain in different brain regions (Bouvier *et al.,* 2015). However, in our experiments, changes in BOLD signalling were unlikely to be mediated by age-dependent factors since the changes were restricted to the PCP treated group, and were not seen in saline-treated animals. Significant changes were obtained from PCP treated animals, but not saline-treated animals and both groups are similar ages.

Approximately 2% of the striatal neurons are cholinergic interneurons and these neurons modulate dopamine release by activation of nicotinic or muscarinic receptor activation on dopaminergic axon terminals in the nucleus accumbens. Glutamatergic afferents from cortical brain regions influence dopamine modulation via cholinergic interneurons in the striatum (Kosillo *et al.,* 2016). Therefore, dysfunctional glutamate release may change dopamine activation somewhat in those regions. Previous studies showed that PCP treatment changes the dopamine signalling in striatal brain regions. However, acetylcholine activation shows differences after PCP treatment as well. In the present study, PCP treatment inactivated acetylcholine activation in the dorsal striatum, but not ventral striatum. Striatal brain regions are important regarding the reward mechanism and motivation, which are interrupted in schizophrenia. In addition to cholinergic mechanisms dopaminergic mechanisms mainly in the NAc, SN and VTA are inactivated by PCP. These results show the utility of longitudinal phMRI to assess different brain regions for various agents in targeted brain regions. Psychoactive compounds that can potentially disrupt neurovascular coupling and can render results difficult to interpret as unknown drug effects on neurovascular coupling may mask true neuronal activity at

neurotransmitter receptors. For this reason, in some cases it has been reported that a target drug may not match the expected functional sites (Ogawa *et al.,* 1993).Therefore, interpretation of results regarding PCP effects on dopaminergic and cholinergic mechanism needs caution. Changes in vasculature representative of neuronal activity measured by BOLD signal caused by amphetamine and nicotine challenges provide a measurement of possible dopaminergic and cholinergic mechanisms in the brain. Further, these vasculature changes for amphetamine and nicotine in brain downregulated after subchronic PCP treatment, but not saline treatment.

4.6.1 Dopamine sensitization by amphetamine and nicotine successive injections

These results show significant signalling differences for amphetamine and nicotine; it should be kept in mind that successive administration of amphetamine may result in sensitization. Amphetamine is a sensitization agent, and its sensitization effects can be seen in behavioural experiments, whereas studies shows dopamine sensitization and locomotor sensitization for nicotine is unlikely occurs by insufficient number of nicotine injection (Clarke and Kumar, 1983) rather upregulation of nicotinic receptors and behavioural sensitization for nicotine usually occurs 5 to 15 days nicotine exposure (Collins *et al.,* 1988; Collins *et al.,* 1990; Mugnaini *et al.,* 2002). Motor behavioural changes can be seen at first-day amphetamine treatment, but rats that underwent amphetamine pretreatment showed sensitization after about 35 days withdrawal compared to the saline group (Febo and Pira, 2011). Moreover, amphetamine-sensitized rats show functional brain differences after the scent of 2,4,5-trimethylthiazoline (TMT), which evokes innate fear and activates neuronal signatures of stress in rodents' ventral hippocampus and midbrain areas (Febo and Pira, 2011). Therefore, these results show

that BOLD signal changes after amphetamine sensitization can be measured using the phMRI technique. It is possible that these changes reflected sensitization for amphetamine or cross-sesitization for amphetamine and nicotine administration in our experiments. It is unlikely to account for the effects of single injection amphetamine before and after PCP treatment as the interval times between the first (before PCP treatment), and the second (after PCP treatment) amphetamine administration should be enough time to avoid potential sensitization or cross-sensitization (nicotine and amphetamine). Since behavioural experiments were not included in this present study, future behavioural experiments are required to test whether sensitization occurs one-week following amphetamine injections. In addition to sensitization, these changes may be reflect desensitization for nicotine. However, different subunits of nAChRs show different desensitization properties For instance, glutamatergic terminals express α7 and glutamatergic synaptic transmission enhances by nicotine through nAChRs whereas, GABA neurons express α4 and β2, and nicotine depression of GABAergic transmission via desensitization of nAChRs on GABA neurons also affects the nicotine-mediated desensitization in VTA (Mansvelder, *et al.*, 2002).

Desensitization is mediated by the α4β2 subunit of nAChRs by acute nicotine treatment (Lopez-Hernandez and Sabchez-Padilla, 2004). It is not known whether the sensitisation that occurs at a cellular level following single nicotine injections is expressed as behavioural sensitisation. Further experiments to test whether nicotine with single injections with one-week interval leads to desensitization at cellular levels and in turn, the outcome of behavioural desensitization needs to be investigated by behavioural experiments. It is also possible that both challenges nicotine and amphetamine induced tolerance and caused BOLD signalling changes related to tolerance development Behavioural experiments indicated that tolerance development for amphetamine occurs

by chronic amphetamine and nicotine administration (Schuster *et al.,* 1966; Marks *et al.,* 1983). Apart from these challenges, PCP by itself may cause desensitization although rsMRI data did not show any functional changes for PCP treatment. However, we have seen different signalling changes in different brain regions for amphetamine and nicotine. The reason behind of failure of rsMRI may be that PCP does not change functional connectivity in circuits but instead rather changes molecular function of synapses. The other potential reason may be that the experiment was under-powered, with too few animals included in the analysis (n= 4 for PCP and 2 for saline). However, MRI experiments give reliable results with small number of subjects, therefore it is not clear whether rsMRI results can be changed by increasing the number of animals in further experiments.

4.6.2 Molecular changes measured by HPLC partially supported obtained results from phMRI

HPLC analysis was conducted following the MRI scan experiments. HPLC with electrochemical detection was used in present study (see appendex) to measure oxidable compounds such as dopamine and its metabolites (DOPAC and HVA), and serotonin and its metabolite (5-hydroxyindoleacetic acid: 5HIAA) applying electrochemical detectors in target brain areas. Compounds can be separated dependent on each compounds retention time on the analytical column and detected electrochemically at different time points. The standard run before and after mixed run as providing a reference to compounds coming off time points. Although time resolution is poor, with the measurements providing only a ‘snap-shot’ of the brain at the time of death, this technique enables measurement of different compounds from the mixed solution in same

run condition: thus dopamine, DOPAC, HVA, serotonin and 5HIAA were measured in each sample, allowing changes in these compounds caused by in vivo pharmacological manipulations to be measured.

The rationale for measuring these compounds in brain homogenates was to investigate whether chronic PCP treatment changed mainly dopamine and its metabolites in different brain regions such as dorsal striatum, NAc, FCx, and VTA. Although PCP is a non-competitive NMDAR antagonist (chapter 1) dopamine levels show difference in different species and different brain regions. After the end of the longitudinal imaging experiments, animals were culled and the brains dissected for regional post-mortem HPLC analysis to provide further support for molecular changes brought by PCP, measured by phMRI. However, one drawback of this technique is that following homogenisation, extracellular and intracellular neurotransmitter changes cannot be separated. Therefore, to what extend the changes in neurotransmitters brought by PCP occur at extracellular or intracellular level is not clear after homogenisation of the brain. On the other hand measurement of dopamine and its metabolites and serotonin and its metabolites in different brain regions would show whether any region dependent difference occurred after PCP treatment. Results obtained from HPLC analysis indicated that chronic PCP treatment may have decreased dopamine and HVA levels in dorsal striatum and NAc respectively. However, serotonin and its metabolite 5HIAA were unlikely to have changed by chronic PCP treatment in brain regions after chronic PCP treatment. Given the variability seen in the measurements of the neurotransmitters and their metabolites, and the relatively small numbers of brains tested in the present experiments, these data must be considered as preliminary, and therefeore are not presented in full: they are given in Appendix D. It was difficult to interpret PCP effects on dopamine and its metabolites. However, the data did give some indications of possible

changes, which should be investigated further in future experiments. For example, in the saline treated group, dopamine levels were highest in dorsal striatum compared to the other brain, although measurements in FCx and VTA were highly variable. In addition, the analysis gave some indication of which compounds changed after PCP treatment. DOPAC is the primary metabolites of dopamine, produced by enzymatic breakdown by monoamine oxidase, and has been implicated in dopamine related behavioural modulations in brain: dysfunctions of this metabolite may be implicated in psychiatric diseases. On the other hand HVA is catalysed from DOPAC via catechol-O-methyl transferase, and is the final product in the breakdown and clearance of dopamine. Although PCP treatment did not change DOPAC levels HVA levels were significantly attenuated in NAc by chronic PCP treatment. HVA activation is related to metabolic stress in schizophrenia, therefore it has an important role in schizophrenia research. On the other hand, serotonin and its metabolite 5HIAA did not show any significant differences after PCP treatment, although the high variability observed from the data makes interpretation difficult. As mentioned above, the high variability of the data likely relate to the low power of the analysis, due to the small sample size: normally, to obtain sufficient power for these kinds of measurements, n-values of 8 to 10 per group would be used. However, in these experiments we were limited by the number of animals undergoing fMRI. The preliminary data obtained nevertheless indicate that increasing the subject number may provide less variation and interpretable data to address how PCP changes monoamine transmitters and monoamine transmitter metabolites.

Attenuation in dopamine levels within dorsal striatum and HVA levels in the NAc may reflect the dopamine sensitization in dorsal striatum and NAc respectively by PCP treatment. However, absolute dopamine levels in PCP pretreated animals were lower than in saline pretreated animals, hence dopamine sensitization is unlikely to be induced by a

single injection of amphetamine. The animals were challenged by nicotine and amphetamine before and after PCP treatment, hence dopamine sensitization by amphetamine and dopamine desensitization by nicotine are potential confounds. However, single injection challenges at one week intervals between challenges are unlikely to cause either sensitization or desensitization. In the literature, dopamine sensitization is described as increased motivation, confidence, focus, and sense of well-being in humankind (Center for Substance Abuse Treatment 1999; Blum *et al.,* 2008). However, it is difficult to measure those aspects of dopamine sensitization in animals, locomotor activation and food consumption can be measured as an index of dopamine sensitization. Further behavioural experiments may shed light to understand whether dopamine sensitization occurred by either nicotine or amphetamine. On the other hand, dopamine desensitization or developing tolerance to drugs (most likely for amphetamine) might be an object to investigate. Attenuation of dopamine and metabolites in PCP treated animal cause to think about whether PCP treatment leads to desensitization of NMDARs, which, in turn, decreases dopamine release. The decreasing dopamine by PCP is unlikely to occur either through depletion of the releasable pool or cytotoxicity, but might be desensitization to PCP (Dwoskin *et al.,* 1992). Further experiments might be useful regarding dopamine receptor changes after PCP treatment which may shed light to understandwhether PCP treatment causes functional changes either directly or indirectly on dopaminergic axon terminals. In fact, any changes brought by PCP onto dopaminergic axon terminals may help to understand underlying factors of the condition of schizophrenia.

Therefore, the preliminary data are unlikely to represent sensitization, desensitization, or tolerance development, albeit behavioural experiments and increasing the number of

animals in conjunction with neurochemistry data would be needed to address these issues in future experiments.

4.6.3 Anaesthesia regime and injection route

It should be mentioned that the anaesthesia regime used may induce signalling changes. The animals were anaesthetised with isoflurane which interacts with excitatory and inhibitory mechanisms in the brain; hence nicotine and amphetamine before and after PCP treatment might interact with isoflurane and affect the BOLD signalling changes. Therefore, these experiments could be run under scan without anaesthesia to ascertain that results are not comprised by anaesthesia effect. The administration route of amphetamine and nicotine, which was i.p injection, was kept consistent between intervals during the experiment period. A different route of administration can lead to different physiological responses. For example, cocaine administration changed the cardiovascular, respiratory responses via intravenous (i.v.) injection, but not intracerebroventricular (i.c.v.) injection (Febo *et al.,* 2004). In our experiments, i.p. injection of amphetamine activated specific brain regions. However, amphetamine-evoked BOLD signalling is largely widened to areas of the brain by i.v. injection is observed (Dixon *et al.,* 2005). Therefore, results from the present study need cautious interpretation considering the number of the animals, anaesthesia regime, and administration route of drugs. All these factors may change the BOLD signalling by changing physiological mechanisms.

In order to qualify the data obtained from phMRI study, awake animals would be a good subject to assess dopaminergic and cholinergic mechanisms for PCP under scan. In fact, stimulation the target brain circuits and obtained the signalling changes by phMRI on

awake animals provide cutting through data such as avoiding anaesthesia effect and focusing target circuits which may resulted in more objective data. However, MRI in unanaesthetised rats is not widely used for both technical and ethical reasons.

4.7 Conclusion

Functional MRI is a novel technique to measure BOLD signalling changes in the brain and an intermediate technique between clinical and preclinical research. Longitudinal phMRI experiments indicated that subchronic PCP pretreatment changed the signalling on the dopaminergic and cholinergic mechanisms in a region-dependent manner in the rat brain. PCP treatment attenuated for amphetamine and nicotine altered brain activation but did not enhance it. Those changes were not observed in saline-treated animals. rsMRI showed PCP treatment did not change functional connections which comprise anatomically separated, but functionally connected, regions. Those regions are displaying a high level of correlated BOLD signal activity. Cholinergic systems, in addition to dopaminergic systems, need to be taken into consideration in schizophrenia. The recent human study showed that glutamate is dysfunctional onset of schizophrenia (Kim *et al.*, 2017). However, the glutamate system has complex interactions with other systems and acetylcholine malfunction may be important in changing dopamine levels or activation. These processes need to be further investigated to understand how glutamate signalling is involved in schizophrenia. We found that cholinergic and dopaminergic systems changed after PCP treatment, modelling schizophrenia, in rats. Molecular changes are not correlative to functional changes. In other words, PCP treatment did not cause functional connectivity changes. However, these preliminary data should be strengthened by increasing the number of animals to understand molecular and functional changes in

cholinergic and dopaminergic mechanisms brought by dysfunctional glutamate systems mainly mediated through NMDARs. Further studies need to conduct similar experiment design on awake animals to avoid anaesthesia in the event that isoflurane change inhibitory and excitatory mechanisms which may confound the BOLD response to target drugs.

5. DISCUSSION

Schizophrenia is a psychiatric disease which affects around 1% of the population around the world. There are different approaches used to explain the underlying factors of this condition, yet its full explanation remains elusive. Dopamine and glutamate theories are two of these approaches classified in environmental factors. The dopamine theory can explain positive symptoms, but it does not provide an explanation for the negative symptoms and cognitive dysfunction, whereas the glutamate theory is more promising in this regard as it can explain both negative and cognitive symptoms as well as positive symptoms (Javitt 1987). Administration of PCP induces the full spectrum of the symptoms seen in people with schizophrenia in all three domains; positive, negative and cognitive. It also causes behavioural changes in animals which mimic schizophrenia-like behaviours. In schizophrenia, studies of this condition indicate that striatal areas, more precisely the NAc, are dysfunctional regarding its structural and physiological condition. Animal studies also support the human studies. Therefore, the studies reported in this thesis have mainly focused on the NAc in rats. The NAc is important in the control of motivation-related processes, including reward, stress and arousal. It is separated into two anatomically and functionally distinct subregions, the core and the shell: the core region connects predominantly with basal ganglia motor structures, whereas the shell connects with more limbic-related subcortical structures (Sesack and Grace, 2009; Ito *et al.*, 2004). Functionally, the shell subregion is predominantly involved in the acquisition and expression of motivated behaviour, while the core region is more concerned with the motor components of such behaviour (Zahm and Brog, 1992; Deutch, 1993). Dopaminergic neurones comprising the mesolimbic pathway innervate both regions extensively and exercise modulatory control of information throughput (Sesack and

Grace, 2009). In the context of schizophrenia, the shell region appears to be particularly implicated, with its critical involvement in processes such as stimulus salience processing, attentional selection (Berridge and Robinson, 1998; Zahm, 2000) and cognitive flexibility (Ding, 2014), which are dysfunctional in people living with positively symptomatic schizophrenia: our studies therefore focussed on the shell region. These regions are part of the limbic system, and get dopaminergic afferents from the VTA. The dopaminergic axon terminals in NAc express a wide variety of receptors, such as GABAergic receptors (Xi and Stein, 1998), mGluRs, and ACh receptors. In NAc, glutamate and dopamine receptor activity can regulate ACh release (Zmarowski *et al*., 2005). However, acetylcholine activation also regulates dopamine signalling, which shows a differentiation between the dorsal striatum and the NAc (Parikh *et al.,* 2010; Nelson *et al.,* 2003). Tonic and phasic activation also modulate dopamine release differently through cholinergic systems (Kemel, 1992; Threlfell *et al.,* 2010). The phasic activation of dopamine in the NAc is mainly related to reward signalling, which is interrupted in schizophrenia. In fact, acetylcholine modulation of dopamine release is changed by PCP pretreatment (Del Arco *et al*., 2007), which indicates that this changes acetylcholine activation either via a direct effect of PCP on cholinergic receptors or indirectly via glutamatergic control of cholinergic activity. However, the extent to which PCP activation of the cholinergic system leads to psychotomimetic effects is not yet clear, despite the fact that cholinergic interneurons constitute ~2% of striatal neurons and their subunit population differentiates between the NAc and VTA. For example, β4 and α7 are found in VTA, but not on dopamine axon terminals in striatum. However, the β2 subunit of nAChRs is expressed on striatal axon terminals in rodents (Salminen *et al.,* 2004). . Receptor types of cholinergic systems also modulate dopamine release in the opposite

way. mAChRs decrease dopamine release (Kemel, 1992), whereas nAChRs enhance dopamine release (Threlfell *et al.,* 2010).

Summary of conclusion from experimental chapters

In chapter 2, modulation of potassium-stimulated dopamine release by NMDA was evaluated by using FSCV with carbon fibre electrodes. In this experiment, potassium was superfused through the tissue chamber before (stimulation K1) and after (stimulation K2) drug administration, and the effects of the drugs were assessed by comparing the dopamine release at K1 and K2, expressed as a K2:K1 ratio. Previous experiments in the lab showed consistent stimulations at K1 and K2 (i.e. K2:K1 ratio close to 1.0) in control (non-drug treated) slices, allowing the effects of drugs to be easily assessed (Gupta and Young, unpublished). However, the experiments reported here failed to show such consistency between K1 and K2 in non-treated slices, which made it difficult to interpret the changes seen after NMDA treatment. Thus, modulation of dopamine release by NMDA was ambiguous due to inconsistencies between the repeated potassium stimulation: that is between the K1 and K2 peaks. In terms of the effect of PCP pretreatment, the potassium-evoked dopamine release at K1 was increased in slices taken from subchronic PCP pretreated animals, compared to those taken from either saline pretreated animals or non-pretreated animals. The K2/K1 ratio was used to assess potassium-evoked dopamine release in slices taken from both non-pretreated animals and PCP or saline pretreated animals, whereas baseline changes (not the K2/K1 ratio) could be used to assess NMDA activation of dopamine release without potassium perfusion into the tissue bath for slices taken from both PCP or saline-pretreated animals and non-pretreated animals. Baseline dopamine release analysis showed significant dopamine

release by NMDA in slices form both non-pretreated and PCP or saline-pretreated animals. However, NMDA did not increase potassium-stimulated dopamine release for non-pretreated brain slices, whereas there was a significant effect of NMDA on potassium-stimulated dopamine release on PCP-pretreated brain slices. It is worth bearing in mind that the data obtained from the K2/K1 ratio analysis are ambiguous regarding this ratio equality. In our experiments, the K2/K1 ratio was not equal to 1.0, which means the results obtained from those experiments may not represent NMDA modulation of potassium-evoked dopamine release.

Before starting the experiments, several different concentrations of potassium were used to choose an appropriate concentration. A 100 mM concentration gave the most consistent and reliable dopamine release, and was employed for the main experiments. Using this high concentration of potassium also resulted in some problems which need to be taken into consideration. Potassium is a strong depolarising agent; hence the high concentration of potassium might result in widespread depolarisation across the whole slice, and so the activation is not very specific. Although the first stimulation gave a consistent dopamine release across different slices, the second stimulation was always lower than first, making it difficult to evaluate potassium-stimulated dopamine changes. As an alternative to the inconsistent K2/K1 ratio, the K3/K2 ratio was calculated. The K3/K2 ratio gave a more consistent ratio (close to 1.0). At the end of the experiments, the K2/K1 and the K3/K2 ratios were compared. Results showed that the K2/K1 ratio was lower than the K3/K2 ratio. Baseline changes caused by the potassium would have been evaluated only by considering the K3/K2 ratio; however, changes obtained from such experiments may not represent a reliable drug effect if three-time successive potassium simulation applied into tissue bath. Therefore, we moved on to use electrical stimulation to measure dopamine signalling changes.

In Chapter 3, electrical stimulation of dopamine release was assessed to try to overcome some of the problems encountered with potassium stimulation. Electrical stimulationin within the context of FSV is a good technique to measure electroactive species which are oxidizable catecholamines at the electrode surface in FSCV. Electrical stimulation produced a rapid dopamine release which returned to the baseline level within a few seconds after stimulation, was discrete, and enabled us to measure reproducible drug effects over time. Initial experiments developed parameters that were capable of measuring dopamine release in different experiments. Electrical stimulation provides a more discrete, local stimulation and dopamine release returns to baseline levels in only a few seconds, which makes this technique more precise than potassium stimulation. The reproducibility of the stimulated release enabled repeated stimulations, at 3 min intervals,during which drugs could be applied to assess their effects. The results show that NMDA caused a dose-dependent receptor-specific decrease in electrically stimulated dopamine release. It is possible that these changes reflected excitotoxic damage brought by NMDA perfusion. While we cannot rule this out for the highest concentration tested (100 µM), it is unlikely to account for the effects measured with a concentration of 30 µM, since (1) we saw at least a partial return to baseline levels, (2) the effects could be blocked by antagonists, and (3) staining of a representative sample of the slices with the mitochondrial function marker TTC after completion of recording showed strong staining, indicating a healthy slice (i.e. good mitochondrial function) (Yavas and Young, 2017). Therefore, NMDA modulation of dopamine release is unlikely to be due to excitotoxicity.

Dopaminergic axon terminals in the NAc express scarce NMDARs; therefore, an indirect route may be involved in those interactions in between NMDA and dopamine in the NAc. GABAergic receptor activation is one possible intermediate factor by giving feedback on

dopaminergic neurons in the striatum. Those receptors expressed on both MSN and dopaminergic axon terminals in NAc, but it is unlikely they modulate NMDA activation on dopamine signalling because the GABA-A antagonist, picrotoxin, neither reversed NMDA depression of dopamine release nor changed dopamine levels by its own activation. However, studies from our lab have shown that both the GABA-A agonist, muscimol, and the GABA-B agonist, baclofen, caused a significant dose-dependent attenuation of electrically stimulated dopamine release in NAc (Ferdinand, Peters & Young, unpublished data). Another possibility is that mGluRs may be involved, since these receptors are known to modulate dopamine release (Hu *et al.*, 1999). Although, for the most part, their action is reported to be excitatory (Rubio *et al.*, 2012), the mGluR2/3 subunit of metabotropic receptors has an inhibitory action (Shigemoto *et al.*, 1997), and may interact with NMDARs. The current study proposes that NMDA causes the release of an excessive spillover of glutamate from postsynaptic receptors, which binds to mGluR2/3 receptor terminals , which in turn inhibits endogenous dopamine release by triggering a protein kinase signalling cascade in NAcS. Although metabotropic receptors are expressed on dopaminergic axon terminals, they may also mediate dopamine activation from cortical areas as coronal slices have glutamatergic afferents from the cortex. High electrical stimulation may activate the terminals of these neurons, and they have a feedback onto accumbal regions.

In addition to metabotropic receptors, nAChRs also attenuated NMDA-depressed electrically stimulated dopamine release. The α4β2 subunit of the nAChRs antagonist-DHβE was perfused with NMDA and reversed NMDA dopamine attenuation. However, the drug alone also enhanced dopamine release by it is own activation without NMDA. This increase, as brought about by DHβE, possibly reflected the stimulation parameters

used in this study. Previous studies showed that high-frequency electrical stimulation enhanced dopamine release (Cragg, 2006).

DHβE gave a dose-dependent profile in slices obtained from both non-pretreated and saline-pretreated animals; however, slices obtained from PCP pretreated animals did not show a dose dependent dopamine release profile by DHβE. PCP pretreatment indicates a somewhat changed cholinergic mechanism in NAc, which resulted in an effect on dopamine activation in NAc. In addition to antagonism of nAChRs, the agonist, nicotine, was also used to evaluate nAChRs activation on dopamine release. The slices from both saline- and PCP-pretreated animals showed a significant increase on electrically stimulated dopamine release by nicotine administration. However, potentiation of these nAChRs, by activating their allosteric site, differentiated between saline- and PCP-pretreated slices. Both slices from saline-pretreated animals and PCP-pretreated animals showed a dose-dependent decrease via perfusion of nicotine concomitant with HEPES, a positive allosteric modulator for the β2 subunit of nAChRs in NAc. Therefore, potentiation via the activation of nicotonic allosteric receptors by nicotine concomitant with HEPES-enhanced ACh release turns on attenuation of electrically stimulated dopamine release in slices from both saline- and PCP-pretreated animals. Besides, the effect of nicotine on peak dopamine responses in slices for both saline- and PCP-pretreated animals, there may be changes in the reuptake of dopamine. Peak dopamine following nicotine does not return to baseline levels in either the saline or PCP groups. This may be due to changes in the activation of DAT. To assess possible changes in DAT, tau, a measure of the whole slope of decay, was calculated as a measure of dopamine reuptake from the FSCV responses. Some drugs, such as cocaine, block dopamine reuptake transporters which lead to dopamine accumulation in the synaptic cleft due to the fact that neurotransmitters is not being cleared. Tau calculation results show that

slices obtained from PCP-pretreated animals show a significant difference in the washout period in terms of tau activation, whereas there is no significant difference in stimulation period. On the other hand, slices from saline-pretreated animals did not show a significant difference in tau activation; however, slices from neither saline-pretreated animals nor PCP-pretreated slices showed any significant difference in terms of tau changes during the drug stimulation period. Therefore, nicotine does not activate dopamine transporters and does not change re-uptake process while the drug is on receptors. Those experiments, obtained from FSCV, show how phasic dopamine activation, as modulated by different systems, is changed by PCP pretreatment. Phasic dopamine activation is critical to our understanding of reward and reward-related mechanisms which are located in the NAc. The present study showed that PCP pretreatment disrupts phasic dopamine activation via the glutamatergic and cholinergic mechanisms. However, tonic activation of dopamine regulation using low stimulation parameters may also shed light on whether any other possibility can change dopamine activation by glutamatergic and cholinergic modulation.

Behavioural experiments, in addition to the neurochemistry studies in the current study, provided information as to whether subchronic PCP pretreatment is effective. NOR test served as a positive control for the neurochemistry experiments. Basically, attention, episodic memory and visual memory are evaluated by this test. During the testing stage, animals pretreated with saline showed the expected preference to explore the novel object over the familiar one. However, animals pretreated with PCP did not indicate this preference, but instead spent similar amounts of time exploring each object. The discrimination index (DI) is a measure of how much animals discriminate between the two objects, with a value of zero indicating no discrimination (Grayson *et al.,* 2007). These data indicate that the PCP pretreatment was effective, therefore the lack of an effect of PCP pretreatment on NMDA-mediated attenuation of dopamine release, cannot simply

be attributed to the pretreatment being ineffective. Those behavioural changes may be the outcome of neurochemical changes brought by PCP in a different brain region, or there may be no enduring direct effect of PCP in NAc. Interestingly, antagonism of nAChRs reversed the NMDA attenuation of electrically stimulated dopamine release in saline-treated rats, whereas no significant change was observed in slices for PCP pretreated animals in the NAc. It is likely that PCP pretreatment leads to conformational and morphological changes on nAChRs in the NAc, which then negatively modulates dopamine signalling. Behavioural experiments supported PCP pretreatment change was effective as well. However, FSCV experiments do not allow us to clarify the sort of analysis. Rather, FSCV experiments indicated that PCP pretreatment alters the cholinergic system, which, in turn, regulates dopamine signalling in the NAc region. To confirm whether nAchRs undergo receptor-related changes, further analysis could be helpful. For example, polymerase chain reaction (PCR) analysis can be applied for investigation as to whether the receptor density of those receptors is modified by PCP.

In the fourth chapter, further experiments using phMRI support that the cholinergic and dopaminergic mechanisms are altered in region-dependent brain areas by subchronic PCP treatment. The phMRI technique allows us to see global changes in brain activation by tracking down BOLD activation changes. Those changes enable the prediction of possible pathologies. Therefore, the effect of subchronic PCP treatment on dopaminergic and cholinergic activation measured by phMRI in the current study. Nicotine and amphetamine evoked global BOLD activation before and after PCP pretreatment. In previous studies nicotine and amphetamine effects on BOLD activation was measured by phMRI (Dixon *et al.*, 2005, Huang *et al.*, 2015). These results for nicotine and amphetamine-evoked BOLD activation were replicated in our study. However, those cholinergic and dopaminergic regions were downregulated by PCP pretreatment. In other

words, nicotine-evoked brain regions, mainly the dorsal striatum, VTA and SN were inactivated by PCP and amphetamine-evoked brain regions mainly in the cortical areas, NAc, ventrolateral striatum, and VTA are inactivated by subchronic PCP treatment. In saline-treated animals, areas do not show either upregulation or downregulation for amphetamine- and nicotine-evoked brain regions, which is a good indication that these changes were the result of specific subchronic PCP treatment, possibly mediated through the disruption of cholinergic and dopaminergic signalling, either indirectly via glutamatergic control of cholinergic and dopaminergic activity, or directly through the effect of PCP on cholinergic and dopaminergic receptors. The nicotine effect on BOLD activation in similar brain regions was observed by age, dose, and anaesthesia regime dependence in previous studies. However, previous studies, to our knowledge, did not observe subchronic PCP pretreatment on cholinergic mechanisms although acute PCP treatment imaging studies have been undertaken elsewhere else (Risterucci *et al.,* 2005; Gozzi *et al.,* 2008a; Gozzi *et al.,* 2008b; Gozzi *et al.,* 2008c; Broberg *et al.,* 2013). Acetylcholine, which is known to modulate dopamine release in the striatum (Threlfell *et al.,* 2012) also shows changes after PCP treatment (Del Arco *et al.,* 2007). However, it is not known how those changes are brought by PCP in different brain regions. The results obtained from longitudinal phMRI showed that the cholinergic system was inactivated by subchronic PCP treatment, which may shed light on the understanding of the underlying neuropathological mechanism of schizophrenia. It still remains to be seen whether a dysfunctional cholinergic system can lead to the psychotomimetic effects observed in schizophrenia.

In addition to cholinergic systems, dopaminergic systems were also altered by PCP. The amphetamine model is widely used in schizophrenia research to understand dysfunctional dopaminergic mechanisms. Imaging studies indicate that amphetamine causes global

BOLD activation in various brain areas (Dixon *et al.*, 2005). We chose amphetamine to challenge brain activation and to observe how these activations for amphetamine are changed by PCP. Dopaminergic mechanisms, mainly in the midbrain areas, were inactivated for amphetamine after subchronic PCP treatment.

On the other hand, rsMRI analysis did not show significant differences in functional connectivity networks by PCP pretreatment, but these data obtained from rsMRI showed where the resting brain activity for PCP is. Resting state analysis of pre-amphetamine rest blocks was performed to compare pre- and post-PCP resting state networks. The results showed that neither PCP nor saline treatment caused significant resting-state changes. These results imply that the rsMRI experiment showed an effect of PCP on resting brain activity, but PCP did not change functional connectivity networks. Therefore, PCP pretreatment does not change core functional connectivity in different brain regions. To our knowledge, this is the first imaging experiment that measures how PCP pretreatment changes BOLD signalling activation for dopaminergic and cholinergic mechanism and indeed whether these changes comprise functional connectivity network changes by PCP pretreatment.

Although BOLD signalling changes were obtained from these experiments, it is worth bearing in mind that psychoactive compounds can potentially disrupt neurovascular coupling, and this, in turn, can render results difficult to interpret as unknown drug effects on neurovascular coupling affect the BOLD signal and may mask true neuronal activity at neurotransmitter receptors. For this reason, it has been reported in some cases that a target drug may not match the expected functional sites (Ogawa *et al.*, 1993; Herkenham, 1987). To confirm whether BOLD signalling changes represent molecular changes, further experiments were performed by HPLC analysis. Dopamine level attenuation was only observed in the dorsal striatum, whereas HVA level attenuation was observed in the

NAc. Hernandez *et al*. (1988) showed that PCP perfusion through brain did not change HVA levels in the NAc. However, in this study, HVA levels were measured at extracellular levels, whereas in our experiments HVA levels were measured in homogenised brain samples, which comprises both intracellular and extracellular neurotransmitters. In fact, Hernandez's study (1988) focused on PCP infusion into the local brain area, whereas animals were undergoing subchronic PCP pretreatment in our study. On the other hand, PCP (i.v. injection) did not show a significant difference for extracellular dopamine levels in the striatum in awake primates, whereas dopamine levels increased by PCP (dialysis perfusion) on rats in microdialysis studies (Adams *et al.,* 2002; McCullough and Salamone, 1992). Those contrary findings indicate that the action of sub-chronic PCP on dopamine regulation in striatal areas is not clear. In fact, those results may reflect differences between species such as primates or rats and drug delivery such as drug perfusion through the brain or i.v. injection. In our experiments, intra- and extracellular dopamine release was significantly reduced in the dorsal striatum by subchronic PCP pretreatment. Beside, PCP inactivation for dopaminergic mechanisms was observed in VTA and SN by phMRI analysis, HPLC analysis failed to support PCP inactivated changes in the VTA. Although the dissection aimed to separate VTA from SN, because the two regions are very close together, we cannot definitely say that the samples contained only VTA Therefore, VTA and SN regions are homogenised together in HPLC. However, variation in data needs to be considered when comparing between phMRI and HPLC of brain homogenates in the present study.

In fact, those results may be counteracted by nicotine and amphetamine challenges which may cause cross-sensitization, although it is not an issue as the one-week intervals between nicotine and amphetamine injections provide enough time to avoid cross-sensitization (details in chapter 4 and 5 discussion sections)

To sum up, *in vitro*, FSCV and fMRI data show that PCP pretreatment changes cholinergic systems. phMRI studies supporting FSCV experiments for PCP changes on cholinergic mechanisms, and behavioural experiments supported those changes brought by PCP affecting memory and attention. However, it should be taken into consideration that the anaesthesia and injection route may also change phMRI data. Isoflurane causes hypotension, respiratory depression is a potent cerebral vasodilator (Hansen *et al*., 1988), but may change neurotransmitter activation by increasing dopamine release and decreasing acetylcholine release (Opacka *et al.*, 1991; Shichino *et al.*, 1997). However, isoflurane does not negate the BOLD signal, hence the results obtained from fMRI are still trustworthy. PCP pretreatment did not change functional connectivity data obtained from rsMRI, but molecular changes were confirmed by phMRI. Brain homogenised HPLC analysis followed by phMRI partially supported the proposal that PCP treatment attenuated dopamine release for the dorsal striatum. Although functional connectivity networks were intact in these regions, molecular changes were observed for both nicotine and amphetamine challenges.

Further experiments are required to confirm those modifications in the cholinergic system and their effect on dopamine signalling. The effect of PCP pretreatment on dopamine signalling involves cholinergic modulation of NMDARs. However, it remains elusive as to how PCP pretreatment changes mAChR activation and their modulation on dopamine signalling. However, little is known about how mAChR modulation of dopamine signalling alterations is brought by subchronic PCP pretreatment. In the present study, nAChRs indicated a pivotal role on dopamine changes brought by subchronic PCP treatment with *in vitro* and *in vivo* experiments (chapter 2 and chapter 4). Ongoing experiments in our lab aim to address this question: preliminary data suggest that the mAChR agonist, oxotremorine-M shows a differential effect on electrically stimulated

dopamine release, dependent on stimulation frequency (O'Conner, Yavas and Young, unpublished data).

In the present study (see appendix), microdialysis experiments were conducted to assess whether the effect of subchronic PCP pretreatment on dopamine release in the NAcS was mediated by presynaptic interaction with cholinergic receptors, by using DHbE in freely moving rats.

Bilateral probes were implanted into NAc and tissue was stimulated by infusion of high potassium aCSF through the probes. DHβE was infused through the probe unilaterally, so that one side showed the drug effect, while the other side acted as the control (no drug). To assess subchronic PCP pretreatment effect endogenous acetylcholine release via α4β2 subunit of nAChRs activation, which, , changes endogenous dopamine release in NAc. Animals underwent PCP or saline pretreatment (twice a day for 5 days) and were given 7 days for washout to make sure there was no drug on receptors. After the washout period animals underwent stereotaxic surgery and chronic probe implantation. Dopamine and its metabolites, DOPAC and HVA, and the serotonin metabolite 5HIIA were measured in the dialysates by HPLC. However, due to technical problems with the microdialysis procedure, only a very limited amount of data were collected, and therefore the experiments are not presented in full here: details, including discussion of the reasons behind the technical problems are in Appendix E.

The results obtained in the present study indicate that the cholinergic system plays a pivotal role in the interaction between glutamate and dopamine theories and thus our understanding of the underlying factors in schizophrenia. Current antipsychotic treatments mainly targets the dopamine receptors . However, dopamine is regulated by the α4β2 subunit of nAChRs, and subchronic PCP treatment disrupts this regulation in

cholinergic systems (chapter 4) as well as in dopaminergic systems (chapter 2 and chapter 4). Given the body of evidence, a significant proportion of patients with schizophrenia are known to be heavy smokers. However, it still remains as to whether smoking can reduce symptoms; further experiments are required to address this question. We suggestthat nicotine could enhance dopamine release, but potentiation of the β2 subunit of nAChRs by the PAM, HEPES, increases ACh release, which reduces increased dopamine release. Potentiated ACh either accelerates the desensitization effect of nicotine, which reduces potentiated dopamine release, or increased levels of ACh suppress dopamine release (Figure 5.1). Therefore, targeting acetylcholine, or more specifically the allosteric site of the β2 subunit of nAChRs, may represent a promising treatment for this condition in the future.

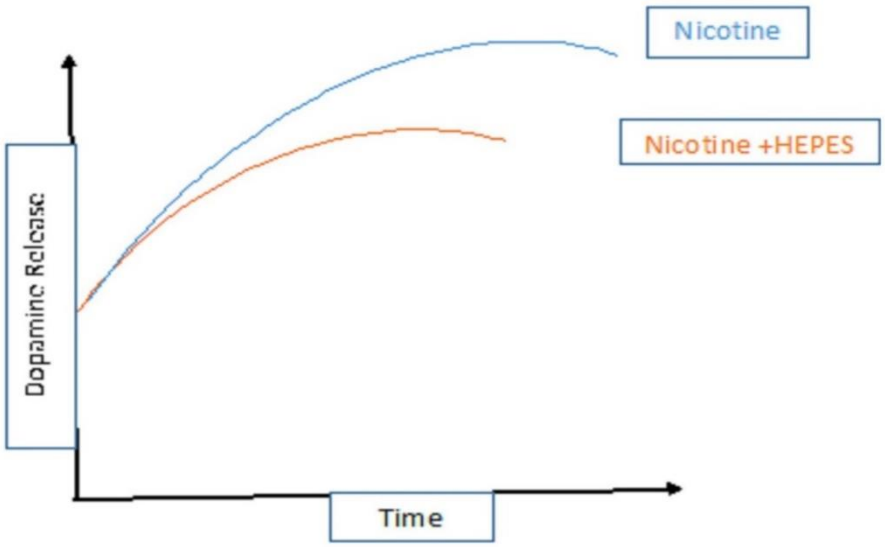


Figure 5.1 Dopamine release modulation by nicotine alone and nicotine concomitant with HEPES in NAc. Nicotine enhanced electrically stimulated dopamine release, but potentiation of β2 subunit of nAChRs enhanced acetylcholine release which, turn on, suppress evoked dopamine release.

6. APPENDIX

Appendix A : Silver – Silver Chloride Reference Electrode

(NPI Electronics, Tamm, Germany)

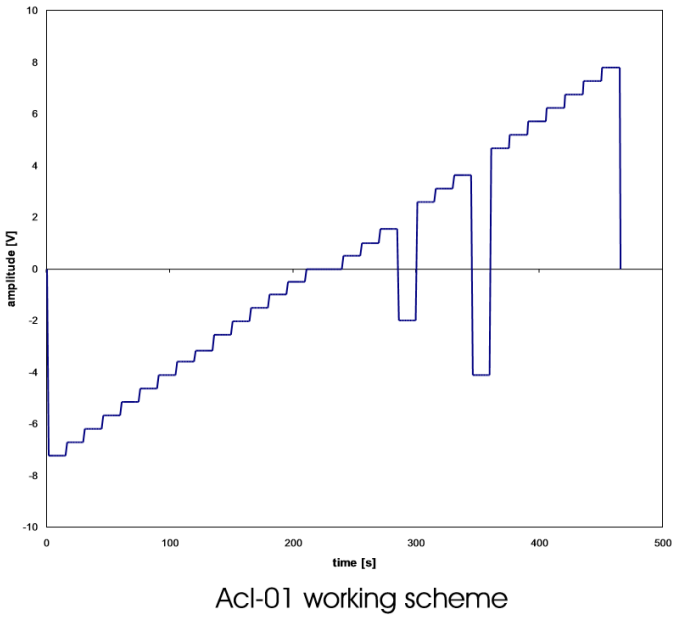


Figure 6.1 Graphical representation of the changes in applied voltage used by ACl-01 for electrode coating. (From NPI Electronics web site – see below)

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 Göttingen, 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 B : NOR scoring and checking reliability

Scoring and checking for reliability

- Both sets of scorer data were compared

Novel Object Recognition Score Sheet										
	Scorer	Andrew								
Animal ID Code	Acquisition				Test					
	Left		Right		Novel Side	Left		Right		
	Time	Visits	Time	Visits		Time	Visits	Time	Visits	
A1	19.856	17	18.407	14	R	19.932	12	18.784	10	
A2	4.129	8	5.616	5	R	5.576	3	4.358	4	
A3	23.908	14	25.875	12	L	35.141	14	9.966	6	
A4	24.038	11	18.639	8	L	15.526	6	11.876	5	
A5	21.235	14	17.277	16	R	10.865	10	23.44	17	
A6	23.293	9	16.544	11	R	7.976	5	21.531	11	
A7	19.68	14	20.413	17	R	13.649	8	26.043	16	
A8	19.599	10	22.927	15	R	8.572	4	21.939	9	
A9	34.047	18	28.677	20	L	22.216	15	19.369	12	
A10	26.656	14	29.589	15	L	30.773	12	26.3	8	
A11	23.626	9	40.344	13	L	12.346	8	17.892	8	
A12	29.287	13	22.176	14	L	24.959	12	25.329	17	
A13	14.006	10	25.35	17	L	16.436	10	7.584	9	
A14	17.346	11	20.556	12	L	31.55	13	22.939	12	
A15	18.713	15	18.716	16	L	11.591	12	4.993	7	
A16	25.479	13	17.122	12	L	10.906	12	8.156	8	
A17	30.098	14	37.663	17	R	34.036	15	34.989	17	
A18	33.402	18	28.401	12	R	20.745	7	32.058	11	
A19	10.67	8	20.747	9	R	9.211	8	11.346	10	
A20	7.185	5	8.08	8	R	13.122	8	12.999	8	
A21	16.898	10	42.037	18	R	9.194	9	15.163	13	
A22	32.276	17	27.387	21	R	19.144	11	22.796	12	
A23	13.802	8	43.067	21	R	12.504	9	27.677	18	
A24	18.952	5	17.971	12	R	3.448	3	6.321	4	
A25	19.881	12	18.518	13	R	1.217	2	12.09	5	
A26	25.314	15	25.279	17	R	16.36	6	23.995	11	
A27	18.056	13	28.86	17	R	9.307	7	26.238	12	
A28	11.377	12	20.489	19	R	7.626	10	10.329	13	
A29	14.859	13	12.302	13	L	9.616	10	8.13	11	
A30	24.681	6	25.257	8	L	30.765	11	13.641	8	
A31	14.389	7	16.538	10	L	31.442	9	34.245	11	
A32	29.113	18	23.783	13	R	8.355	9	39.698	12	

Novel Object Recognition Score Sheet										
	Scorer	Ersin (transposed L to R)								
Animal ID Code	Acquisition				Test					
	Left		Right		Novel Side	Left		Right		
	Time	Visits	Time	Visits		Time	Visits	Time	Visits	
A1	32.141	21	21.278	14	R	20.233	14	18.262	9	
A2					R					
A3	28.198	16	30.861	14	L	29.957	15	9.992	6	
A4	26.273	13	16.664	9	L	14.005	14	9.025	5	
A5	25.441	14	15.598	16	R	13.222	12	21.991	20	
A6	23.796	11	15.277	13	R	8.599	5	17.24	11	
A7	18.11	13	21.133	19	R	8.908	7	20.636	21	
A8	21.806	13	19.973	17	R	9.272	3	20.96	12	
A9	35.914	22	29.966	23	L	21.447	14	21.223	15	
A10	24.648	20	29.291	18	L	33.485	13	22.832	11	
A11	19.592	11	49.25	14	L	11.329	10	20.382	13	
A12	30.005	16	22.463	15	L	18.134	15	27.025	17	
A13	13.587	10	30.024	21	L	16.079	10	9.794	12	
A14	17.325	11	25.571	12	L	25.637	15	24.119	14	
A15	19.18	15	21.675	16	L	9.685	13	4.928	7	
A16	28.575	14	20.11	15	L	8.848	11	7.319	12	
A17	30.77	17	39.018	24	R	40.454	18	40.297	17	
A18	37.127	19	28.28	12	R	20.384	9	24.663	13	
A19	10.52	8	18.879	12	R	10.127	10	11.741	10	
A20	6.282	5	6.82	7	R	12.156	9	12.75	12	
A21	16.993	11	39.95	21	R	9.214	9	20.678	14	
A22	30.119	20	27.768	20	R	17.238	13	24.352	17	
A23	13.997	8	43.189	28	R	14.857	11	24.327	20	
A24	18.987	7	15.698	11	R	3.128	3	4.248	4	
A25	14.492	11	16.343	13	R	0.736	2	12.67	6	
A26	24.067	16	26.356	18	R	18.961	7	20.914	9	
A27	16.757	13	31.666	17	R	12.105	9	24.265	16	
A28	11.434	9	28.339	19	R	5.939	8	7.184	13	
A29	14.611	13	13.38	13	L	9.381	11	7.229	8	
A30	29.903	8	28.9	10	L	30.1	14	16.002	9	
A31	14.892	7	17.286	10	L	25.422	12	34.991	16	
A32	43.475	26	21.324	15	R	8.19	10	46.673	15	

- compared reliability of differences (rather than of each individual score. This takes account of the fact that our absolute values may be different, but the overall pattern may be the same. Took each of our Left:Right ratio for Acquisition and Test, and then compared across testers for each stage. I was looking for consistency values

Reproducibility test					
Acquisition			Test		
AY	EY	% Different	AY	EY	% Different
0.93	0.66	71	0.94	0.90	96
1.36			0.78		
1.08	1.09	101	3.53	3.00	85
0.78	0.63	82	1.31	1.55	119
0.81	0.61	75	2.16	1.66	77
0.71	0.64	90	2.70	2.00	74
1.04	1.17	113	1.91	2.32	121
1.17	0.92	78	2.56	2.26	88
0.84	0.83	99	1.15	1.01	88
1.11	1.19	107	1.17	1.47	125
1.71	2.51	147	0.69	0.56	81
0.76	0.75	99	0.99	0.67	68
1.81	2.21	122	2.17	1.64	76
1.19	1.48	125	1.38	1.06	77
1.00	1.13	113	2.32	1.97	85
0.67	0.70	105	1.34	1.21	90
1.25	1.27	101	1.03	1.00	97
0.85	0.76	90	1.55	1.21	78
1.94	1.79	92	1.23	1.16	94
1.12	1.09	97	0.99	1.05	106
2.49	2.35	95	1.65	2.24	136
0.85	0.92	109	1.19	1.41	119
3.12	3.09	99	2.21	1.64	74
0.95	0.83	87	1.83	1.36	74
0.93	1.13	121	9.93	17.21	173
1.00	1.10	110	1.47	1.10	75
1.60	1.89	118	2.82	2.00	71
1.80	2.48	138	1.35	1.21	89
0.83	0.92	111	1.18	1.30	110
1.02	0.97	94	2.26	1.88	83
1.15	1.16	101	0.92	0.73	79
0.82	0.49	60	4.75	5.70	120

Saline					
Acquisition		Test		DI	
Left	Right	Familiar	Novel		
26.00	19.84	20.08	18.52	-0.04	
26.05	28.37	9.98	32.55	0.53	
23.34	16.44	12.04	22.72	0.31	
23.54	15.91	8.29	19.39	0.40	
20.70	21.45	8.92	21.45	0.41	
25.65	29.44	24.57	32.13	0.13	
18.95	20.20	4.96	10.64	0.36	
27.03	18.62	7.74	9.88	0.12	
35.26	28.34	20.56	28.36	0.16	
10.60	19.81	9.67	11.54	0.09	
13.90	43.13	13.68	26.00	0.31	
18.97	16.83	3.29	5.28	0.23	
17.19	17.43	0.98	12.38	0.85	
17.41	30.26	10.71	25.25	0.40	
27.29	27.08	14.82	30.43	0.34	
36.29	22.55	8.27	43.19	0.68	

	Acquisition		Test		DI
Mean	23.01	23.48	11.16	21.86	0.33
SEM	1.74	1.80	1.59	2.56	0.06
n	16	16	16	16	16

PCP				
Acquisition		Test		DI
Left	Right	Familiar	Novel	
4.13	5.62	5.58	4.36	-0.12
25.16	17.65	10.45	14.77	0.17
18.90	20.77	11.28	23.34	0.35
34.98	29.32	20.30	21.83	0.04
21.61	44.80	19.14	11.84	-0.24
29.65	22.32	26.18	21.55	-0.10
13.80	27.69	8.69	16.26	0.30
17.34	23.06	23.53	28.59	0.10
30.43	38.34	37.25	37.64	0.01
6.73	7.45	12.64	12.87	0.01
16.95	40.99	9.20	17.92	0.32
31.20	27.58	18.19	23.57	0.13
24.69	25.82	17.66	22.45	0.12
11.41	24.41	6.78	8.76	0.13
14.74	12.84	7.68	9.50	0.11
14.64	16.91	34.62	28.43	-0.10

	Acquisition		Test		DI
Mean	19.77	24.10	16.82	18.98	0.08
SEM	2.25	2.76	2.42	2.16	0.04
n	16	16	16	16	16

between 75 and 125%, which most of them were, therefore showing good consistency.

- On this basis, simply took the mean of our two scores for the remainder of the calculations

Figure 6.2 NOR test scoring scoring and checking reliability

Appendix C : Examples of FSCV for potassium stimulated dopamine release

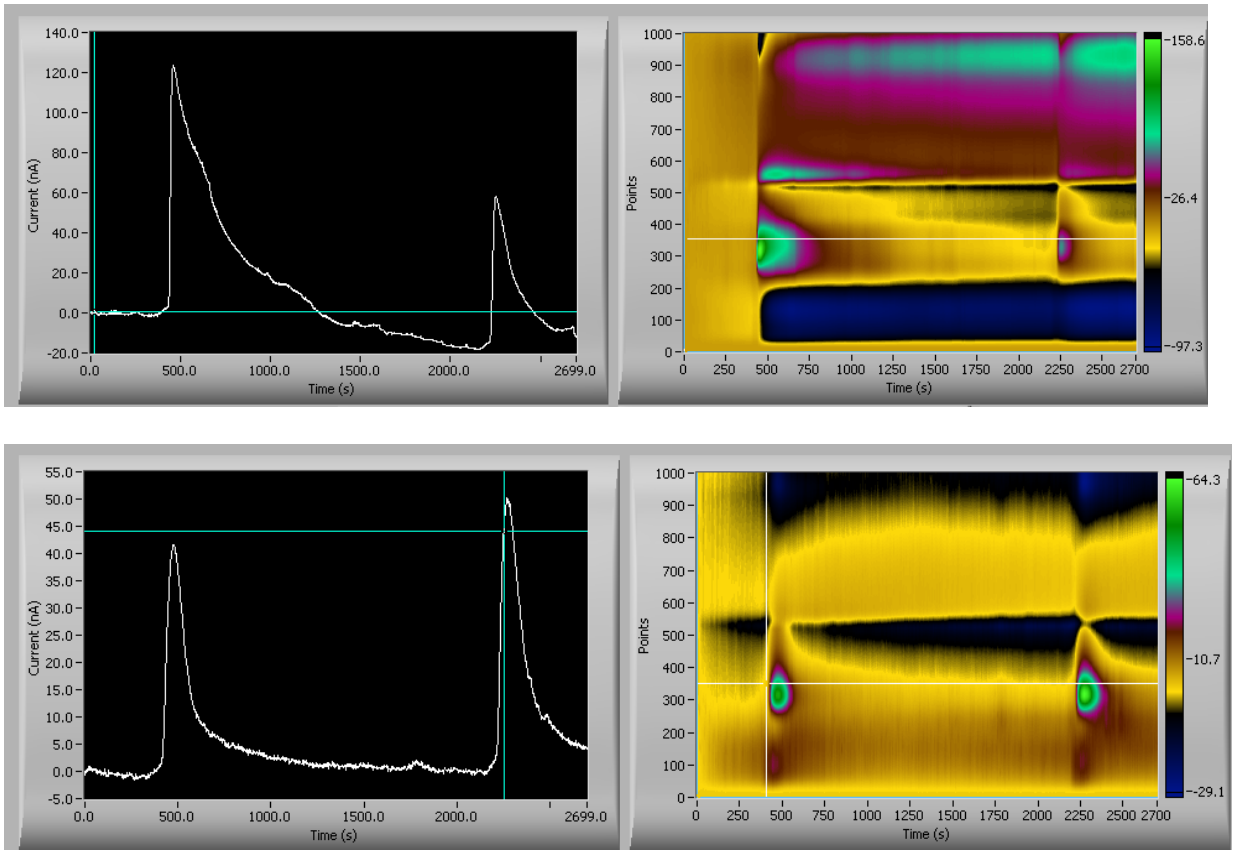
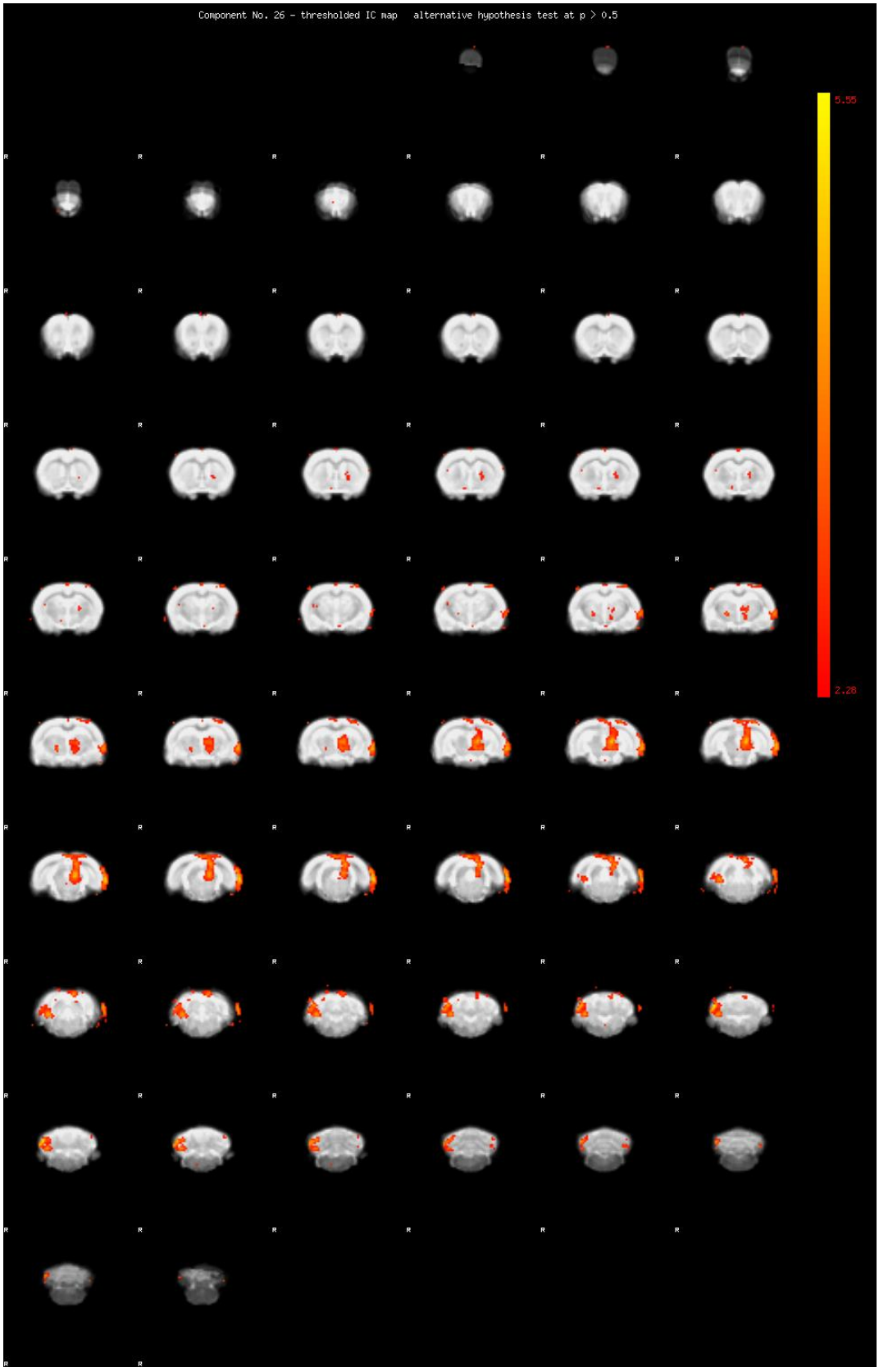
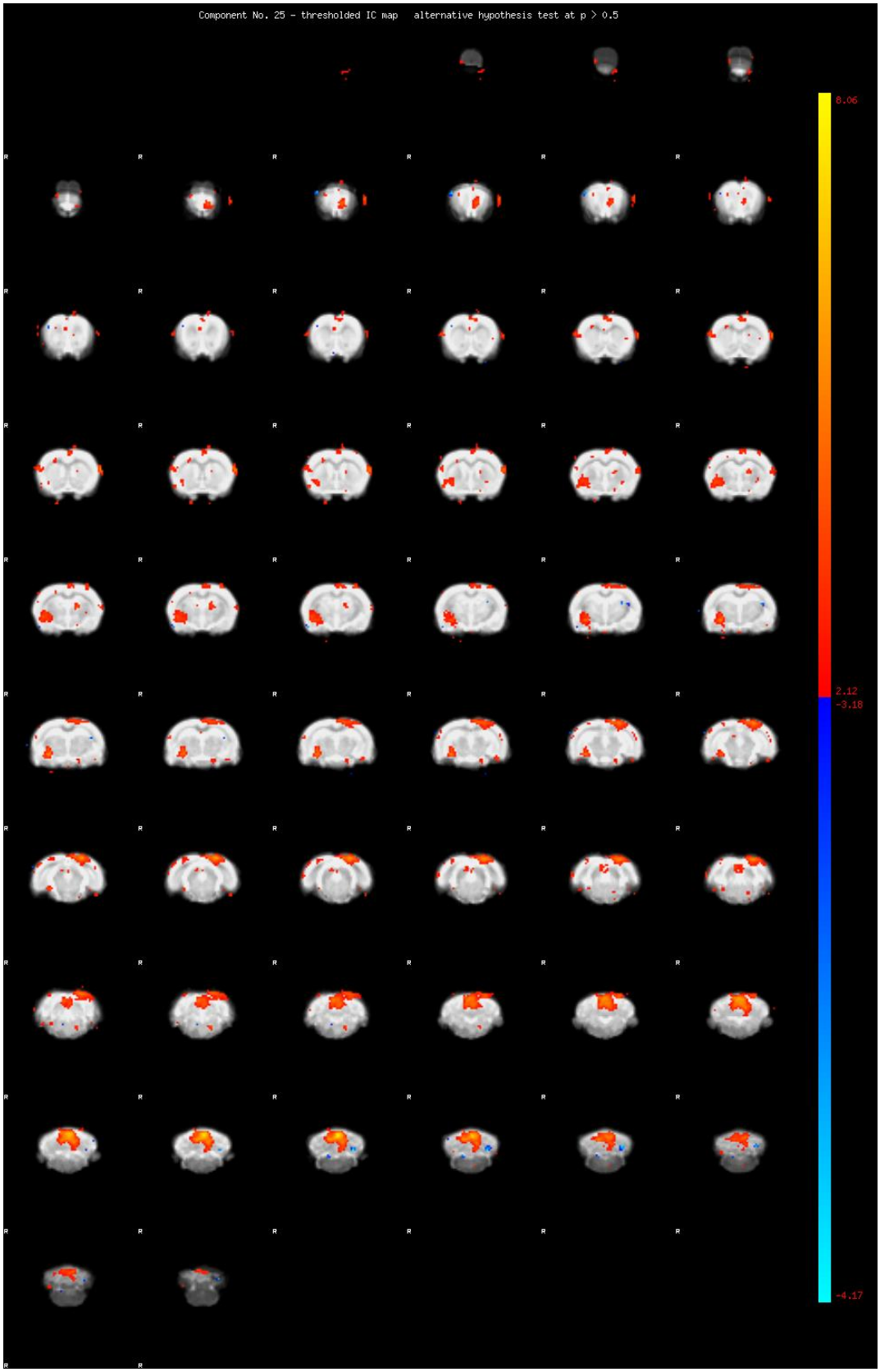
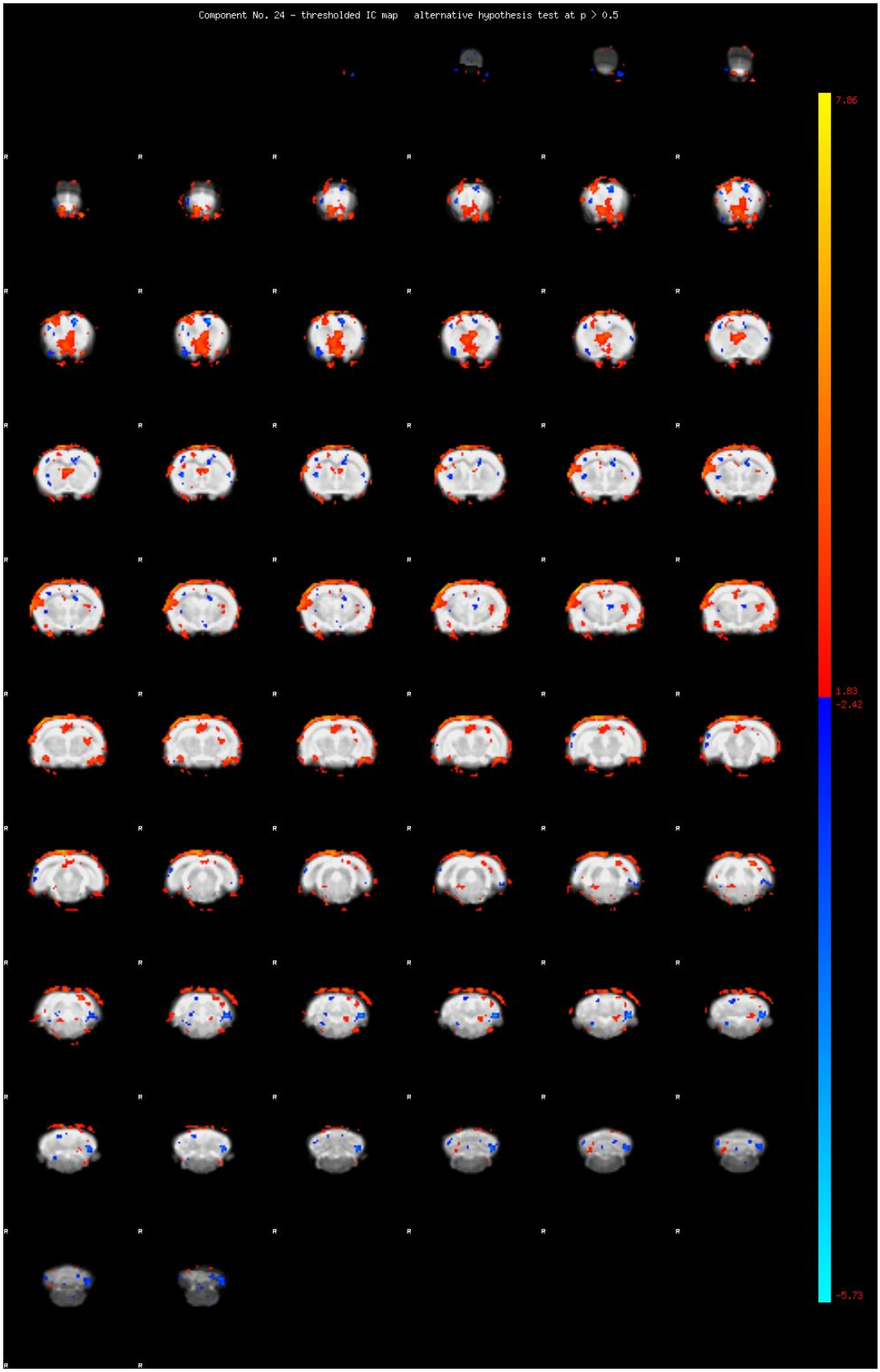


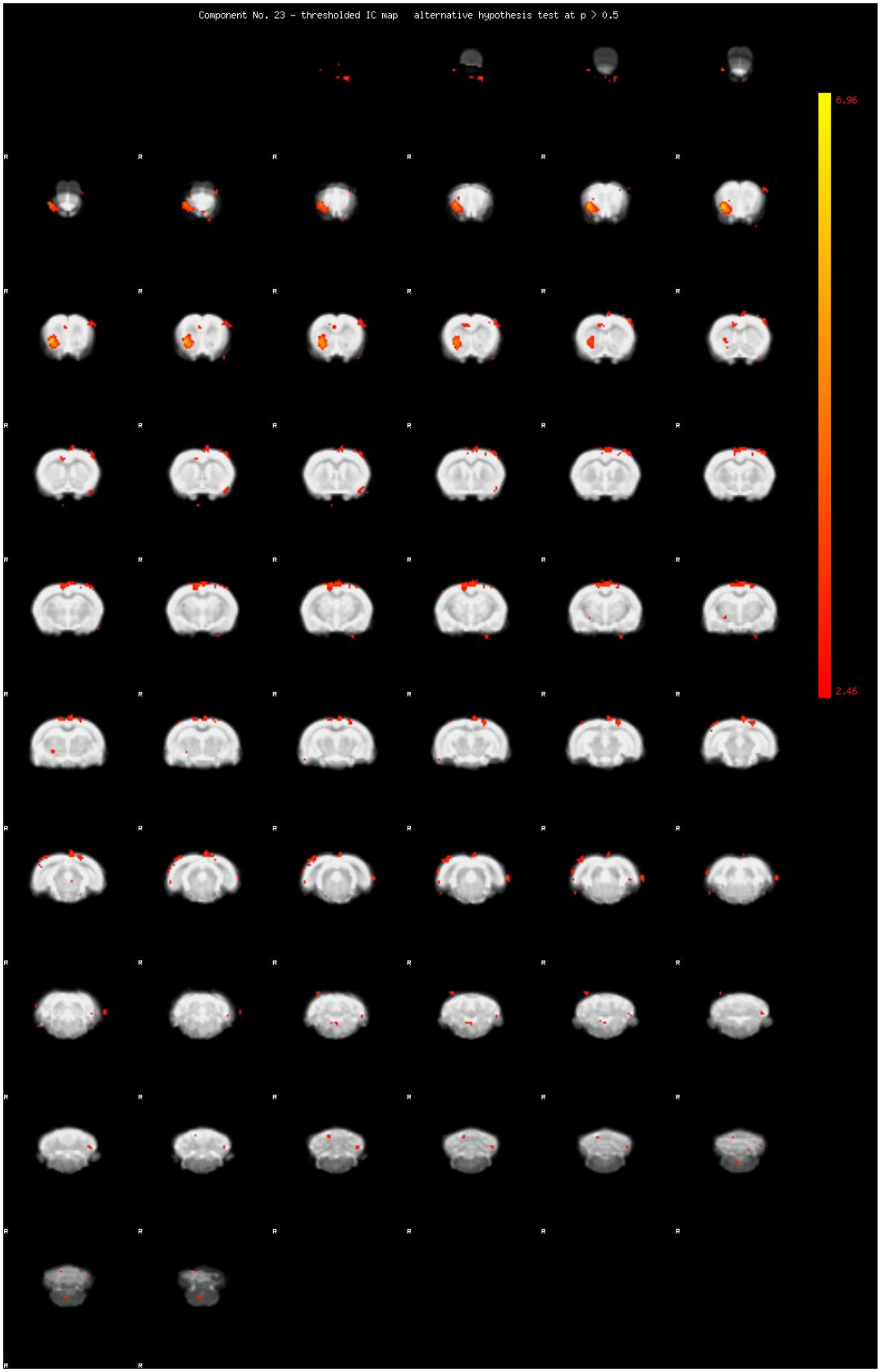
Figure 6.3: K1 and K2 potassium stimulated dopamine release. a) a bad K2/K1 ratio obtained from potassium stimulation. b) A good K2/K1 ratio obtained from potassium stimulation. (see section 2.4 and 2.7.4)

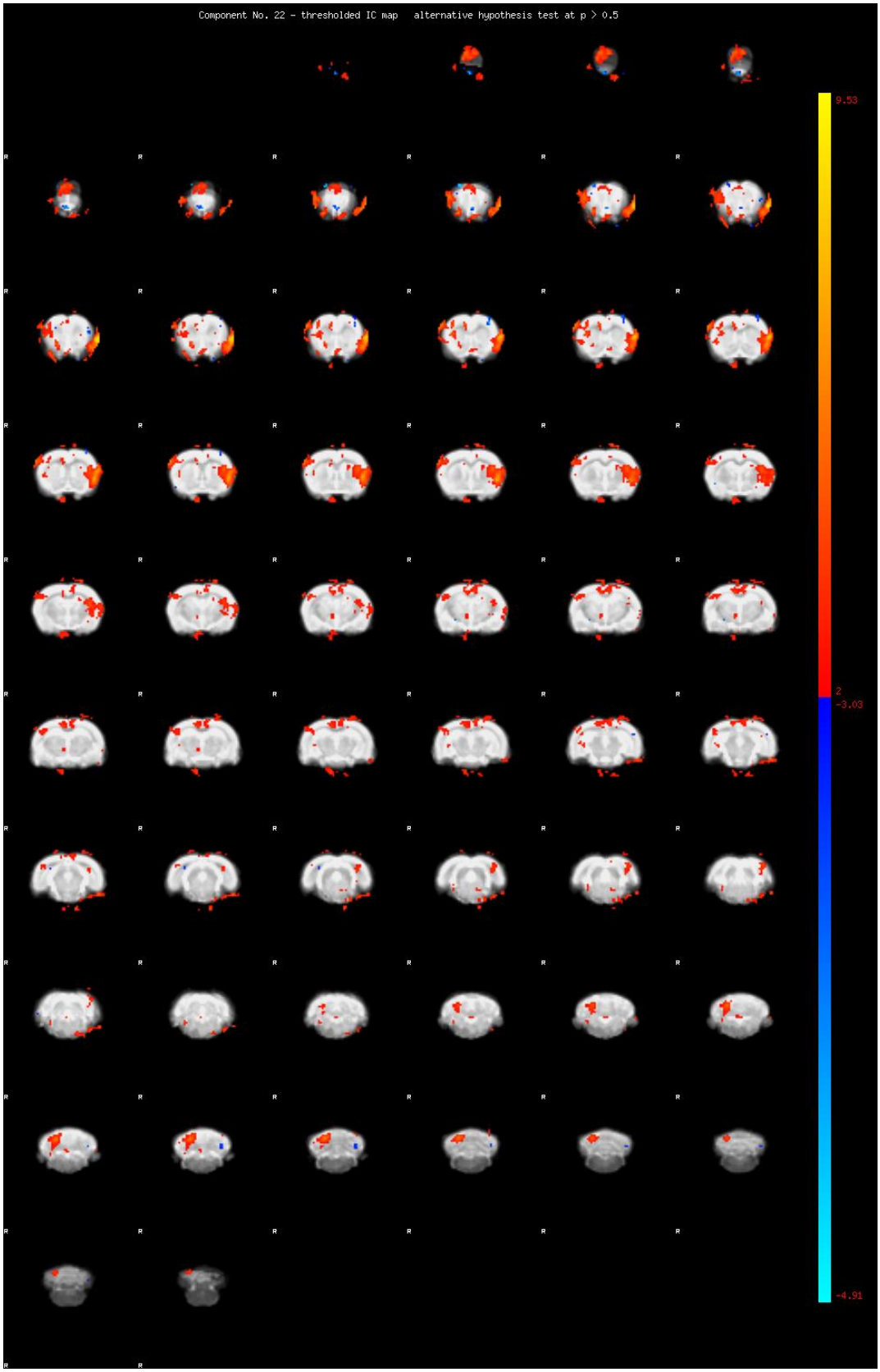
Appendix D :Resting State functional connectivity networks

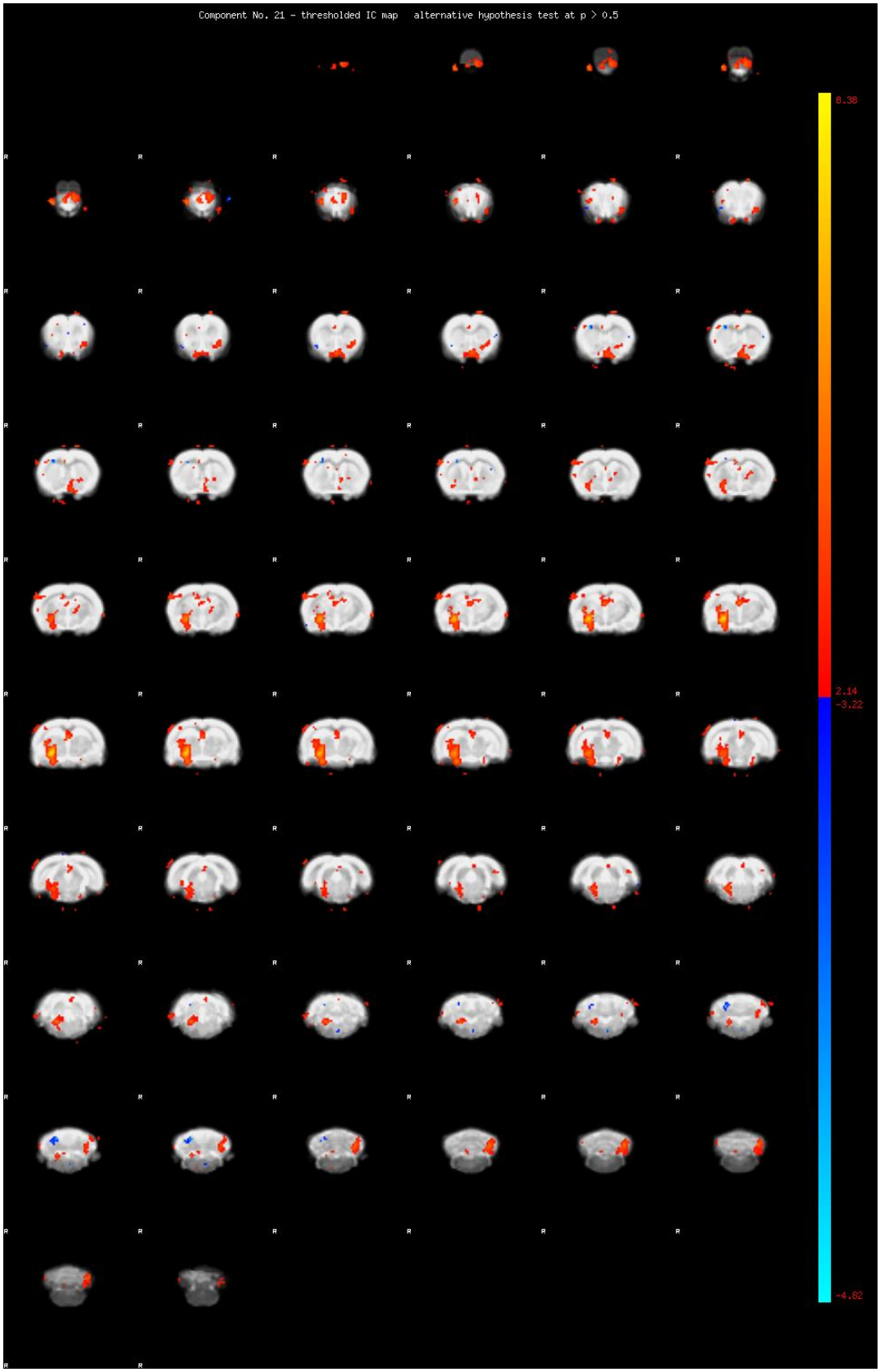


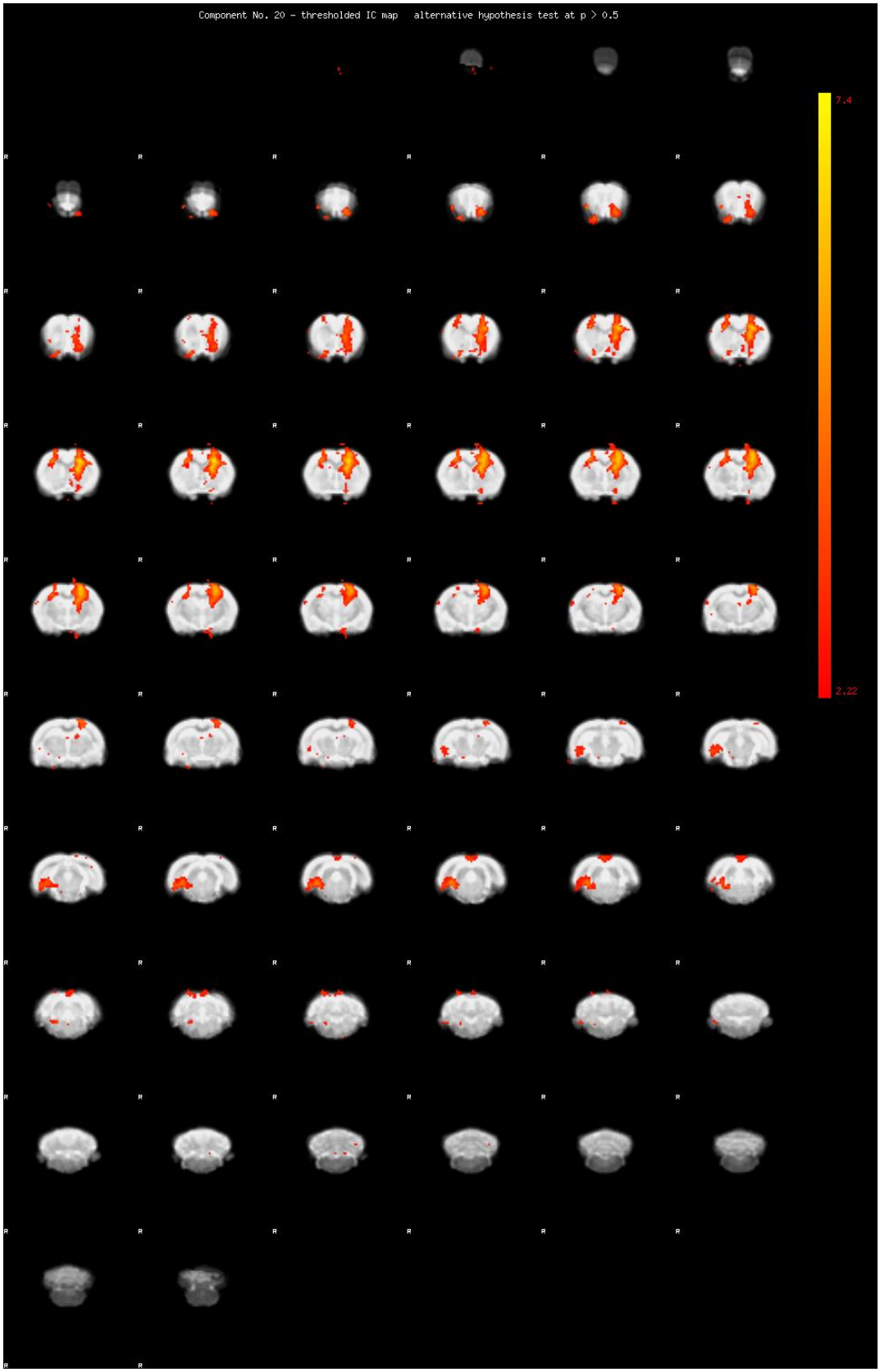

Component No. 25 - thresholded IC map alternative hypothesis test at p > 0.5
8.06
2.12
-3.18
-4.17

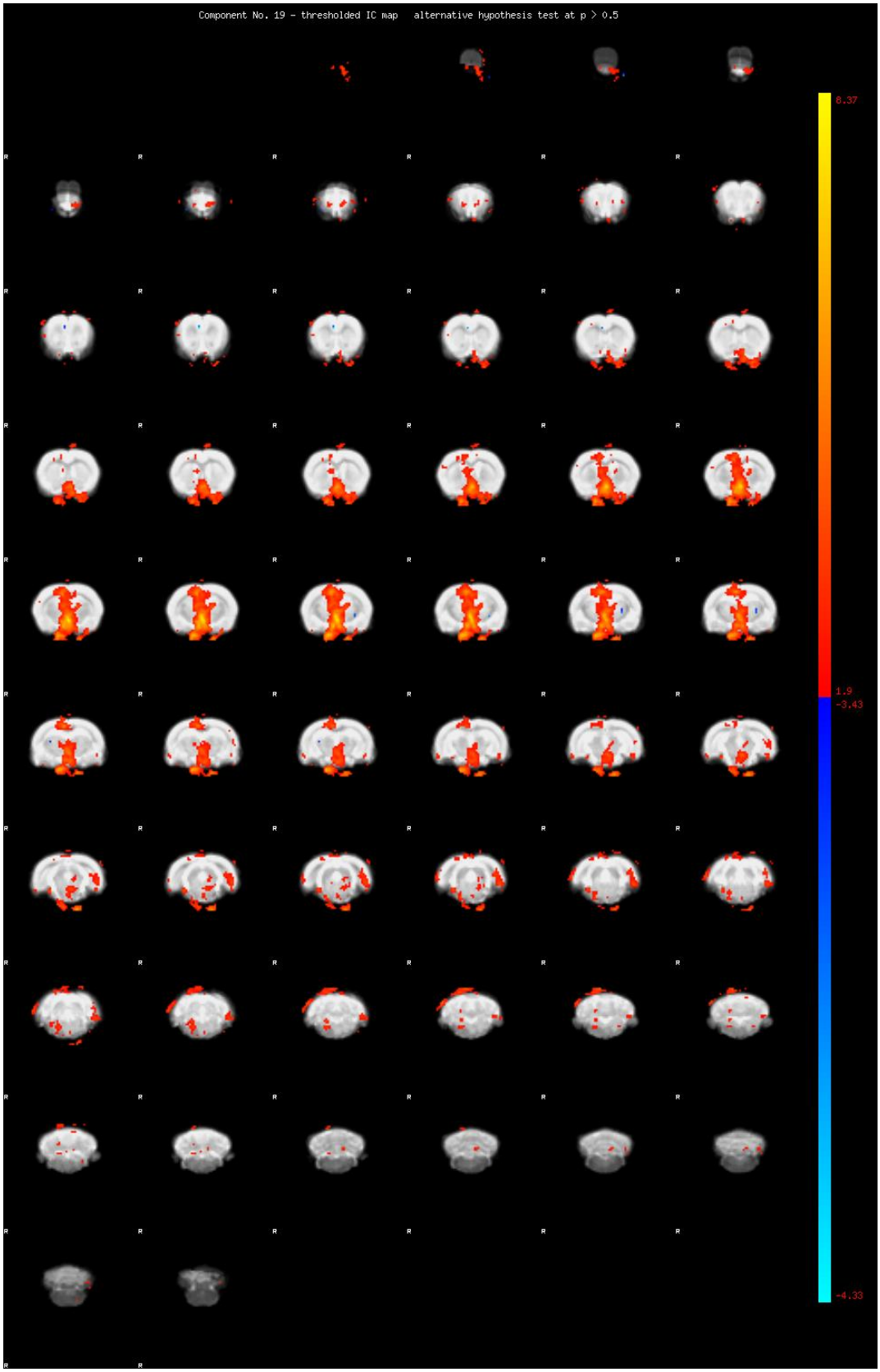

Component No. 24 - thresholded IC map alternative hypothesis test at p > 0.5
7.86
1.83
-2.42
-6.73

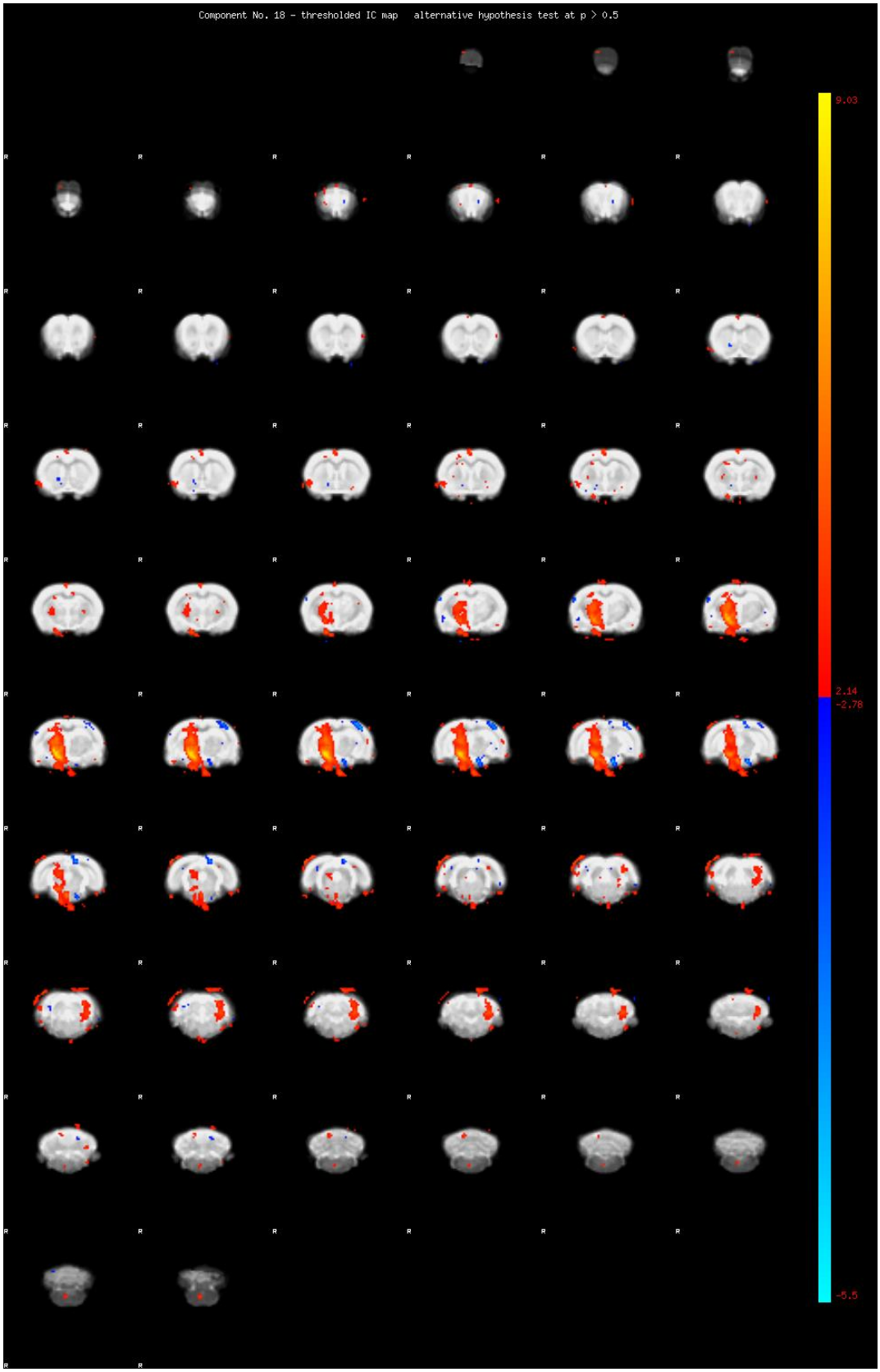

Component No. 23 - thresholded IC map alternative hypothesis test at p > 0.5
8.96
2.46

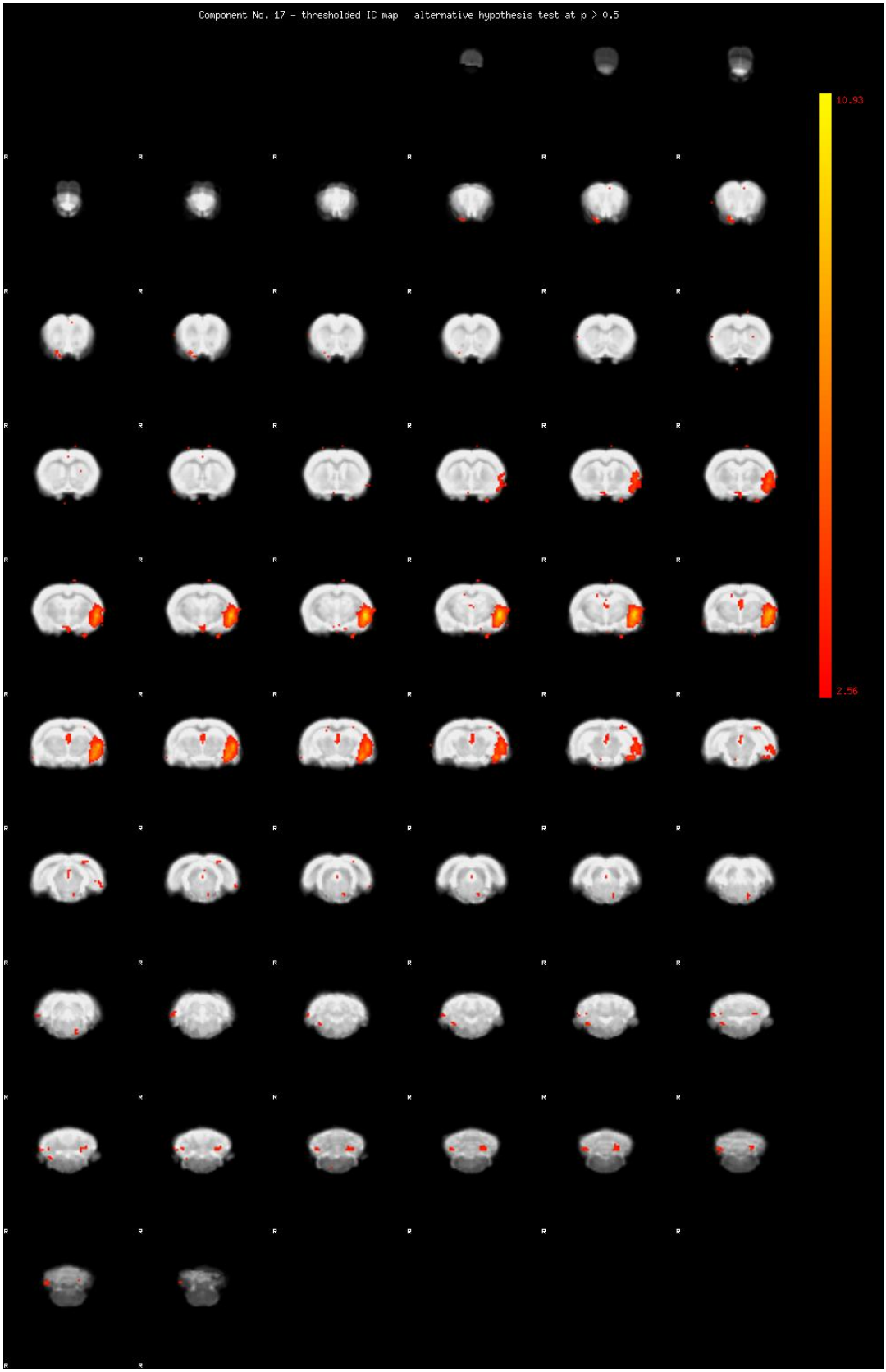

Component No. 22 - thresholded IC map alternative hypothesis test at p > 0.5
9.53
2
-3.03
-4.91

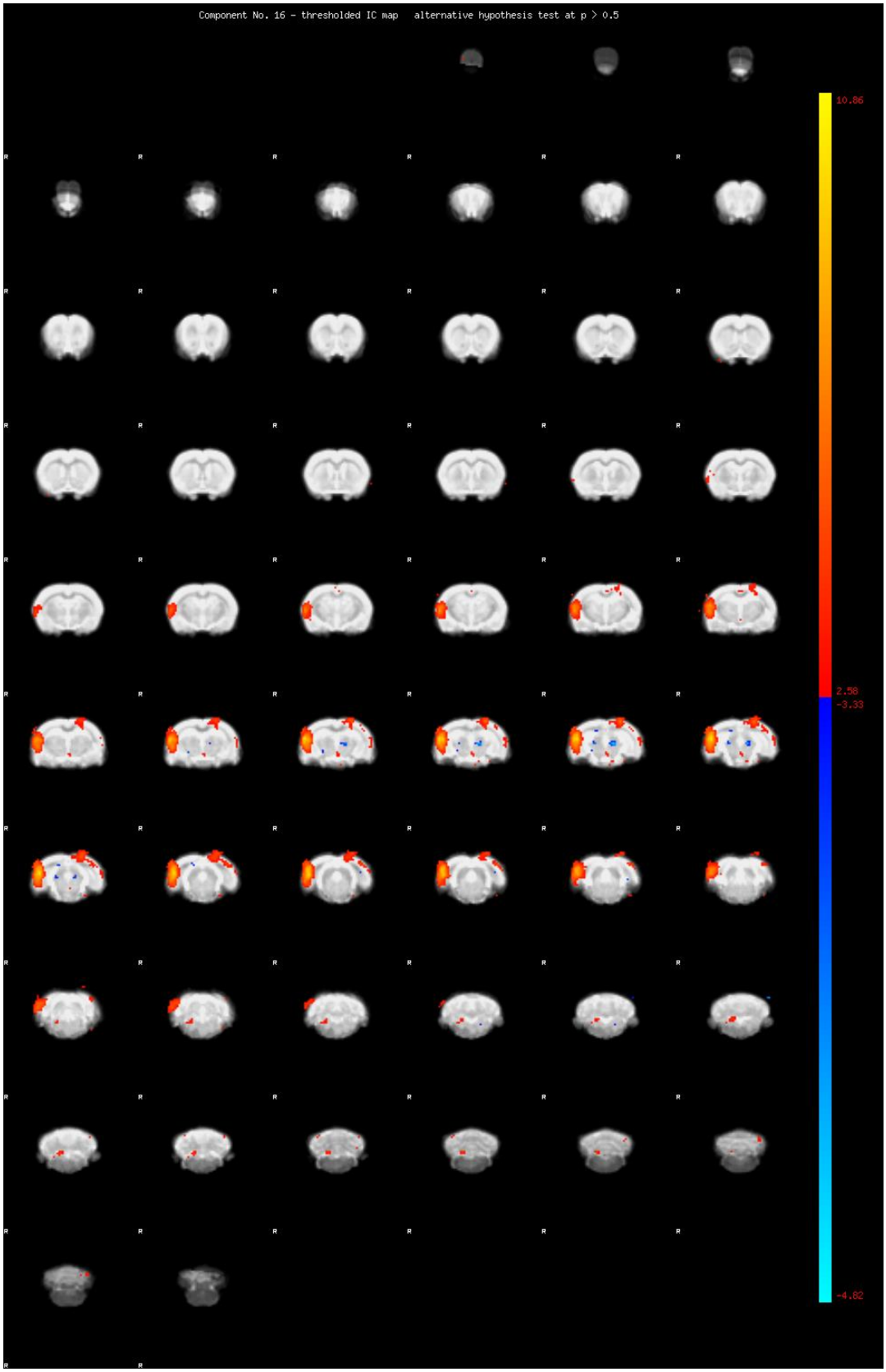

Component No. 21 - thresholded IC map alternative hypothesis test at p > 0.5
8.38
2.14
-3.22
-4.82

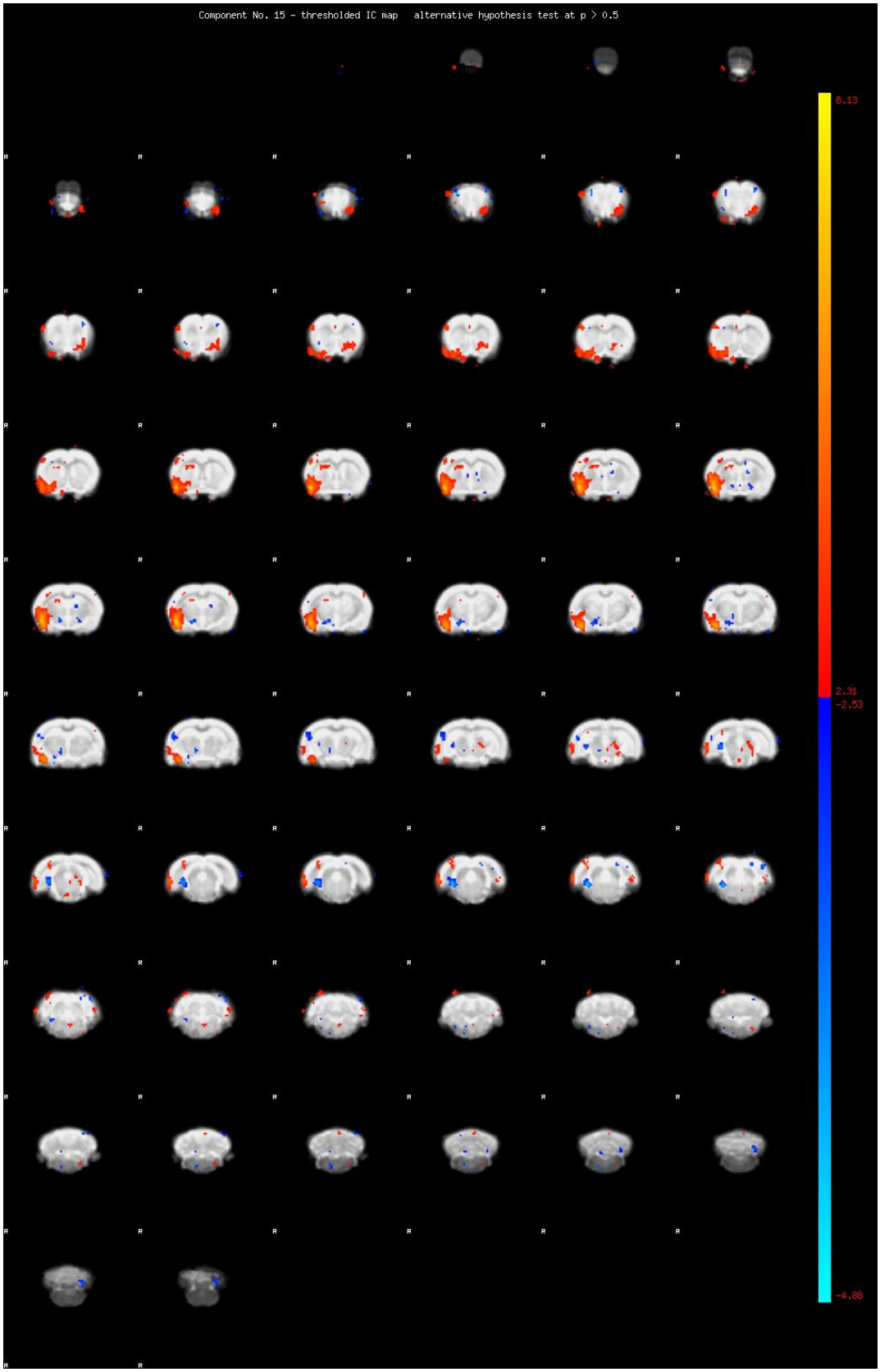

Component No. 20 - thresholded IC map alternative hypothesis test at p > 0.5
7.4
2.22

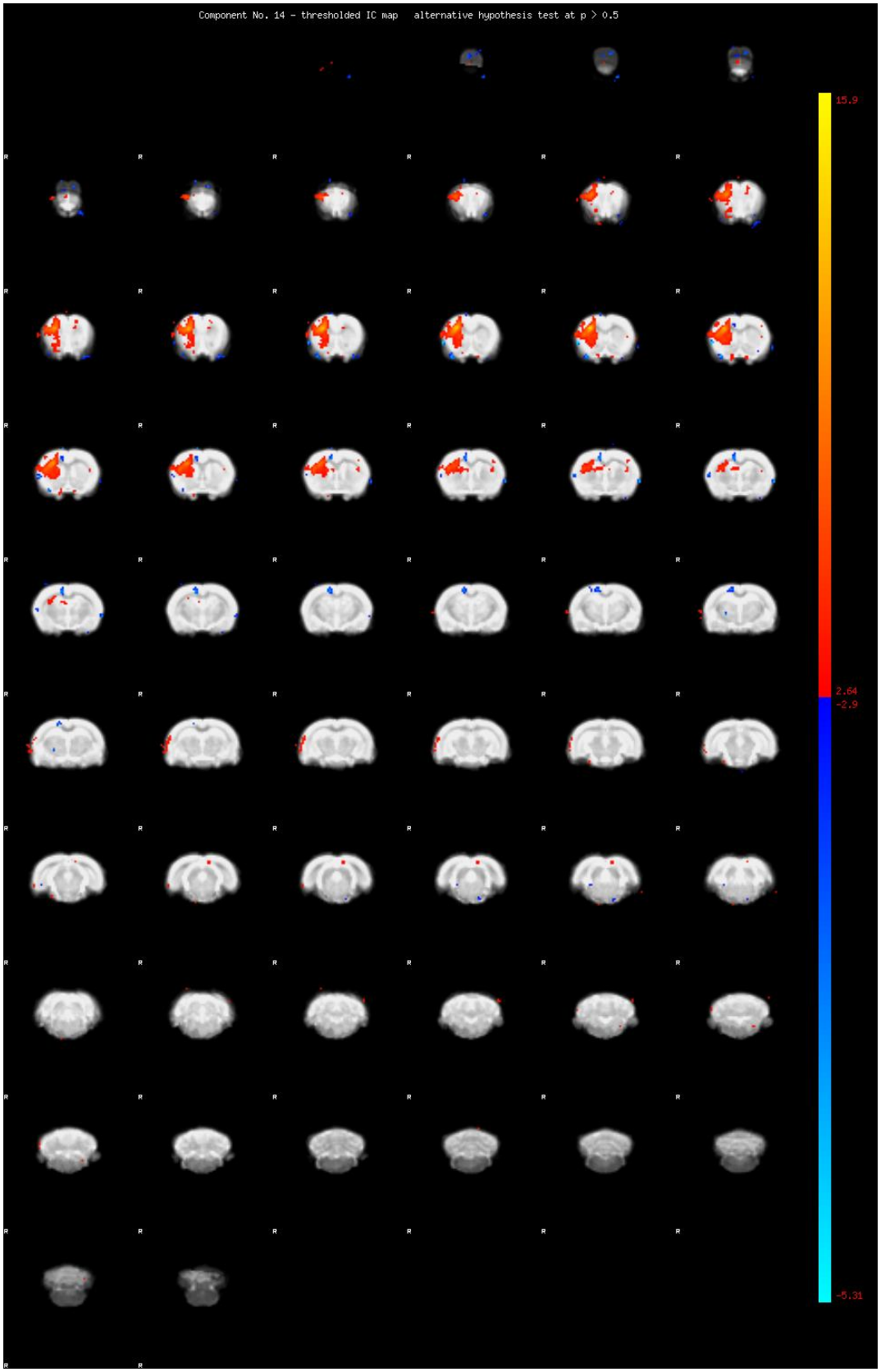

Component No. 19 - thresholded IC map alternative hypothesis test at p > 0.5
8.37
1.9
-3.43
-4.33

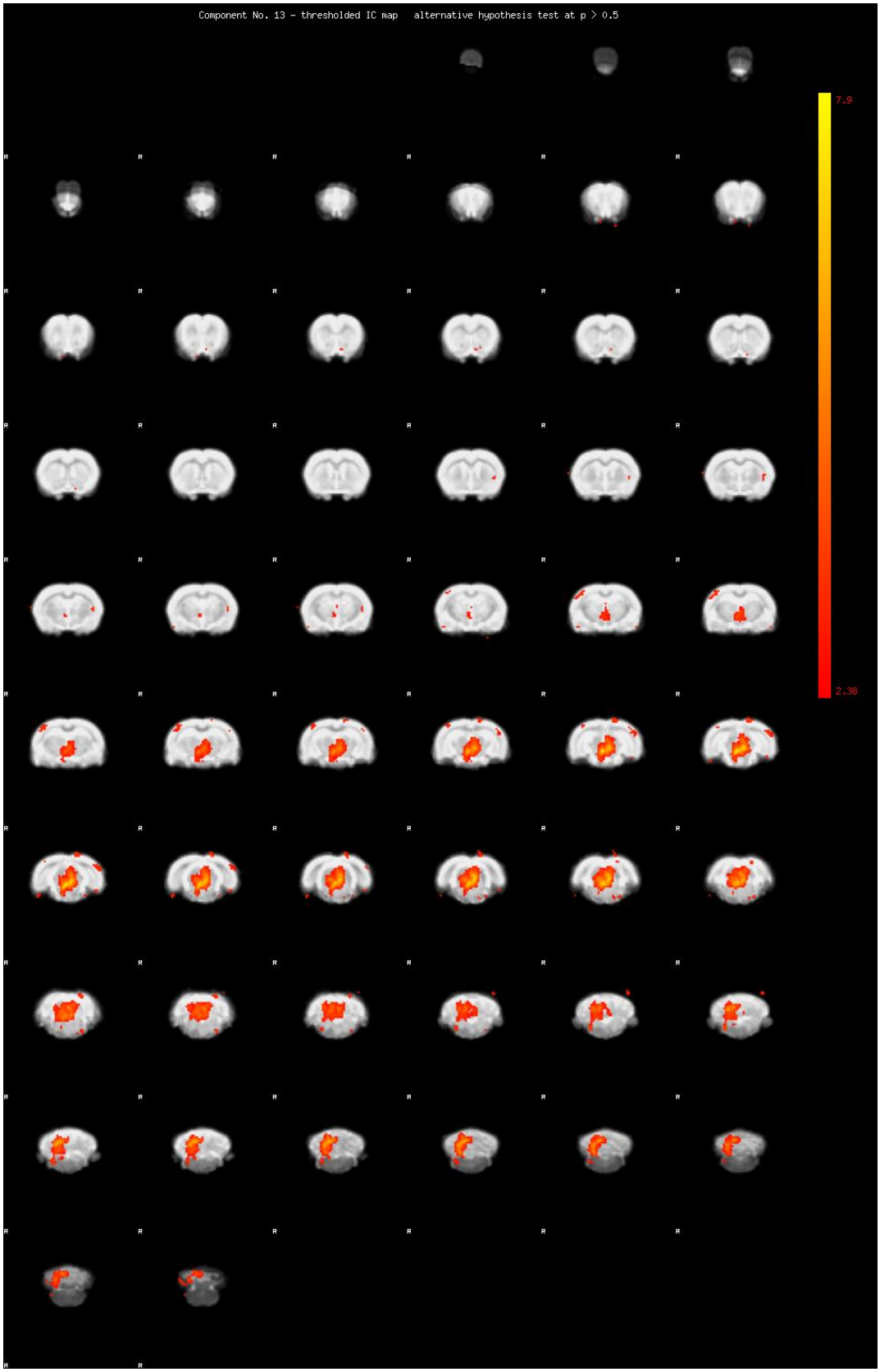

Component No. 18 - thresholded IC map alternative hypothesis test at p > 0.5
9.03
2.14
-2.78
-6.5

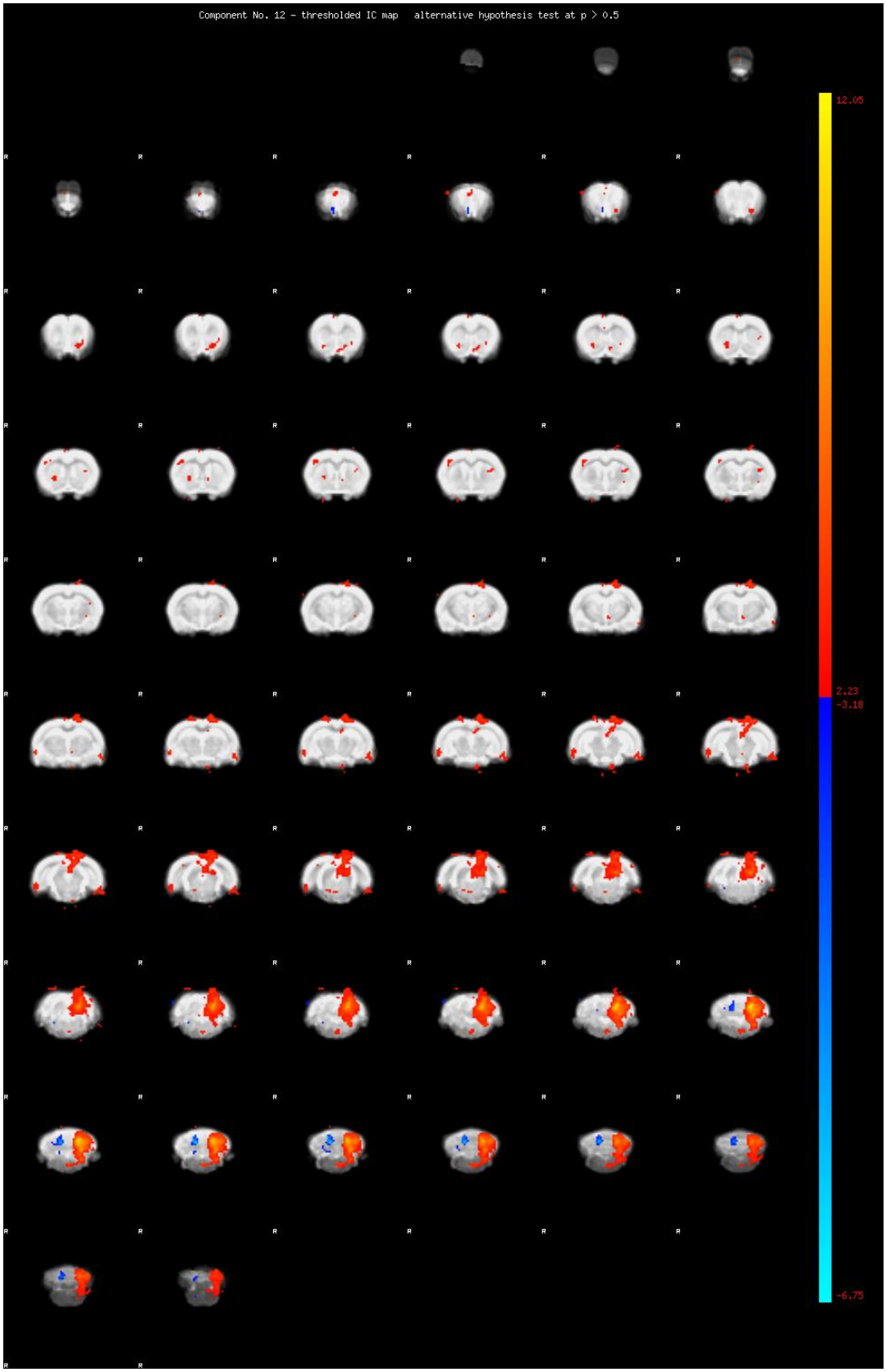

Component No. 17 - thresholded IC map alternative hypothesis test at p > 0.5
10.93
2.56

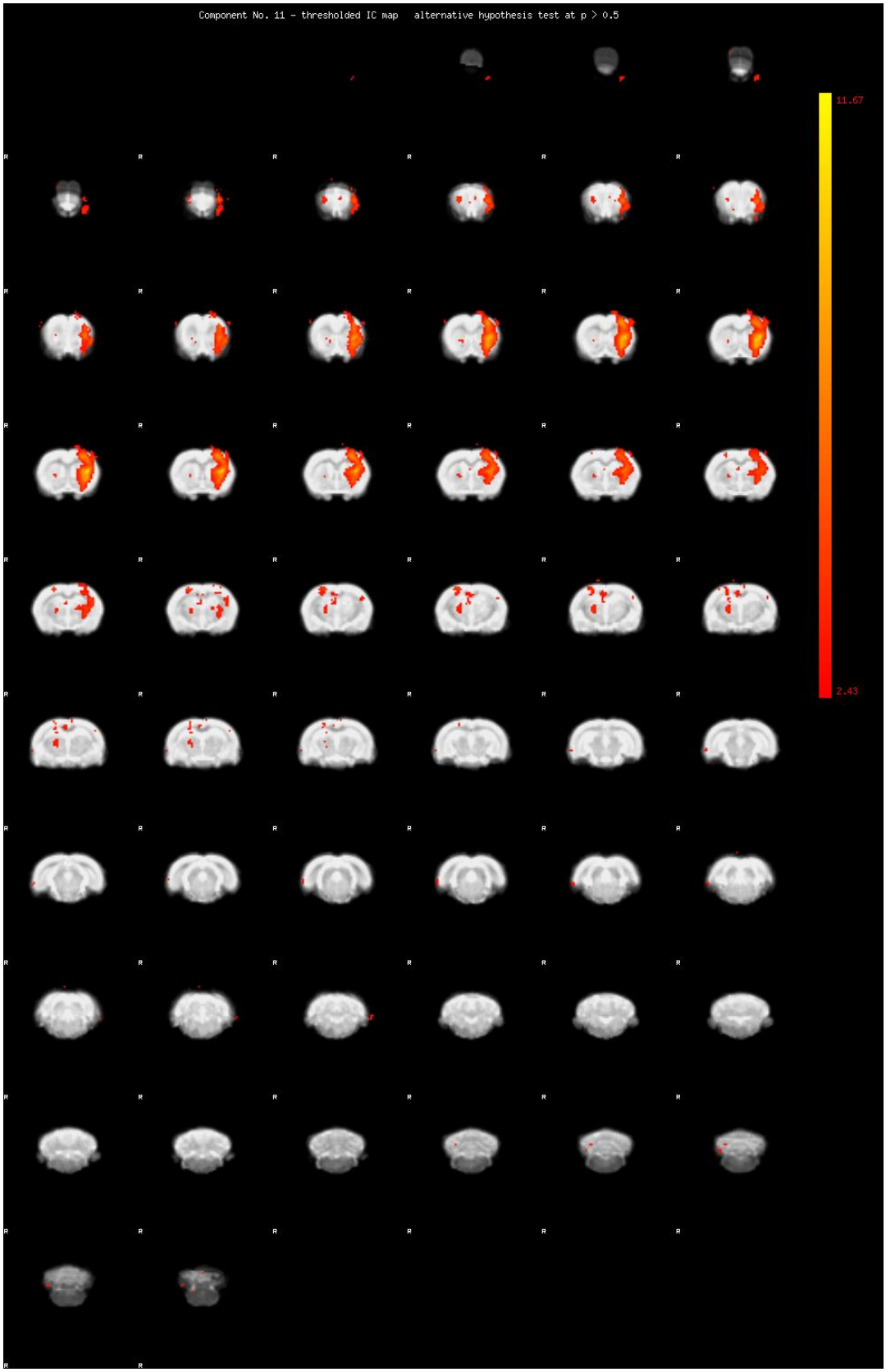

Component No. 16 - thresholded IC map alternative hypothesis test at p > 0.5
10.86
2.58
-3.33
-4.82

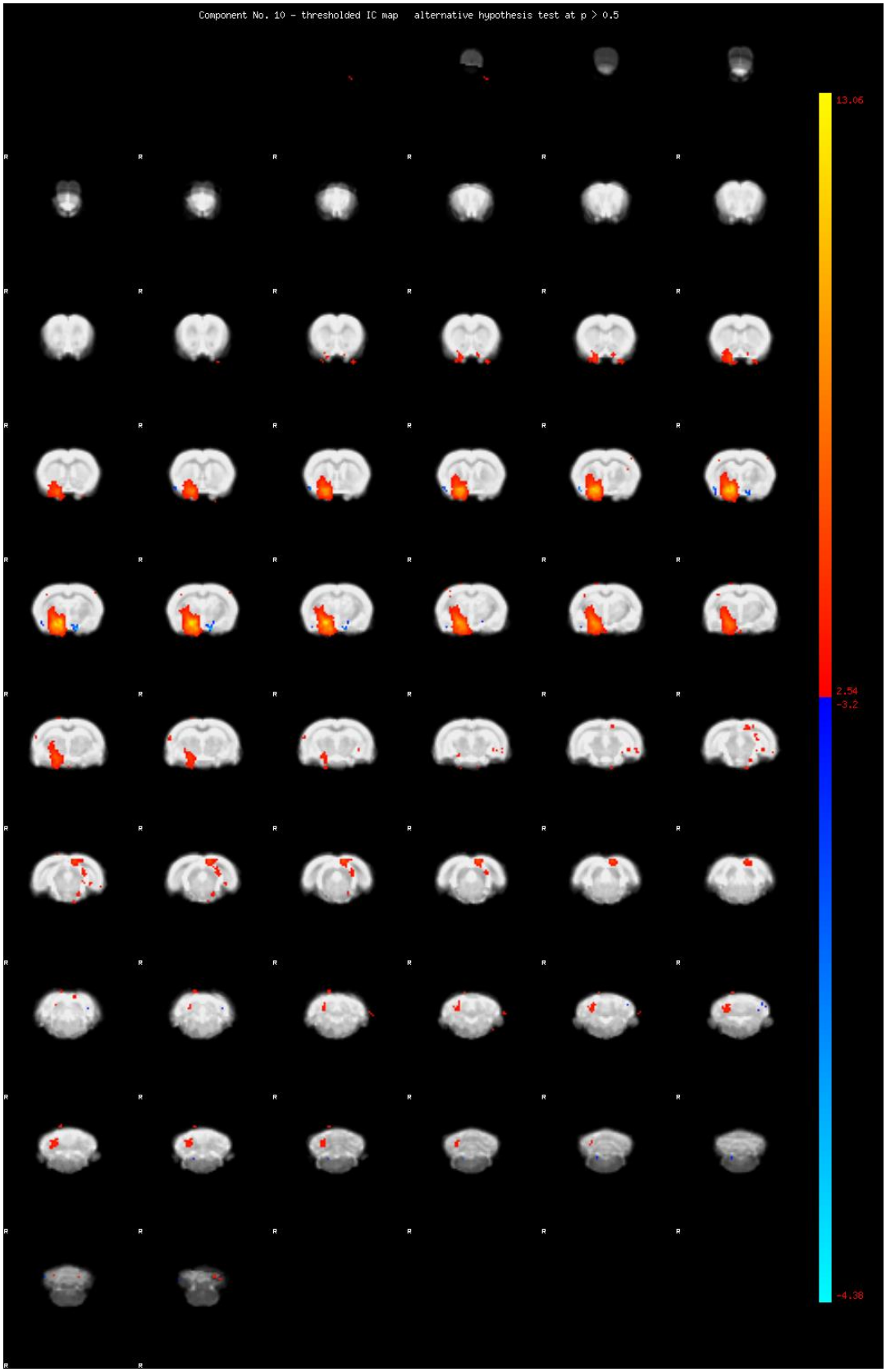

Component No. 15 - thresholded IC map alternative hypothesis test at p > 0.5
8.13
2.31
-2.53
-4.88

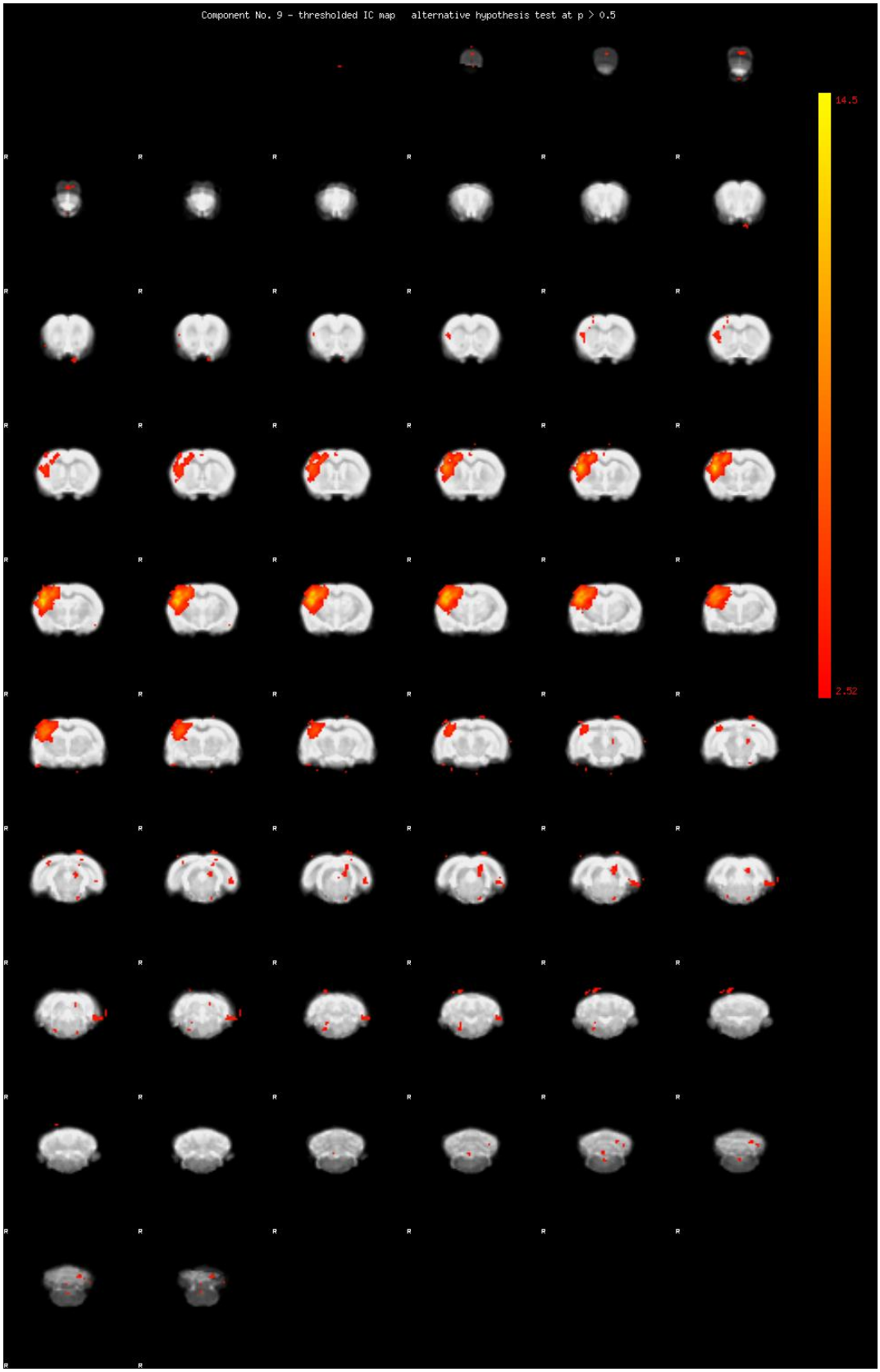

Component No. 14 - thresholded IC map alternative hypothesis test at p > 0.5
15.9
2.64
-2.9
-5.31

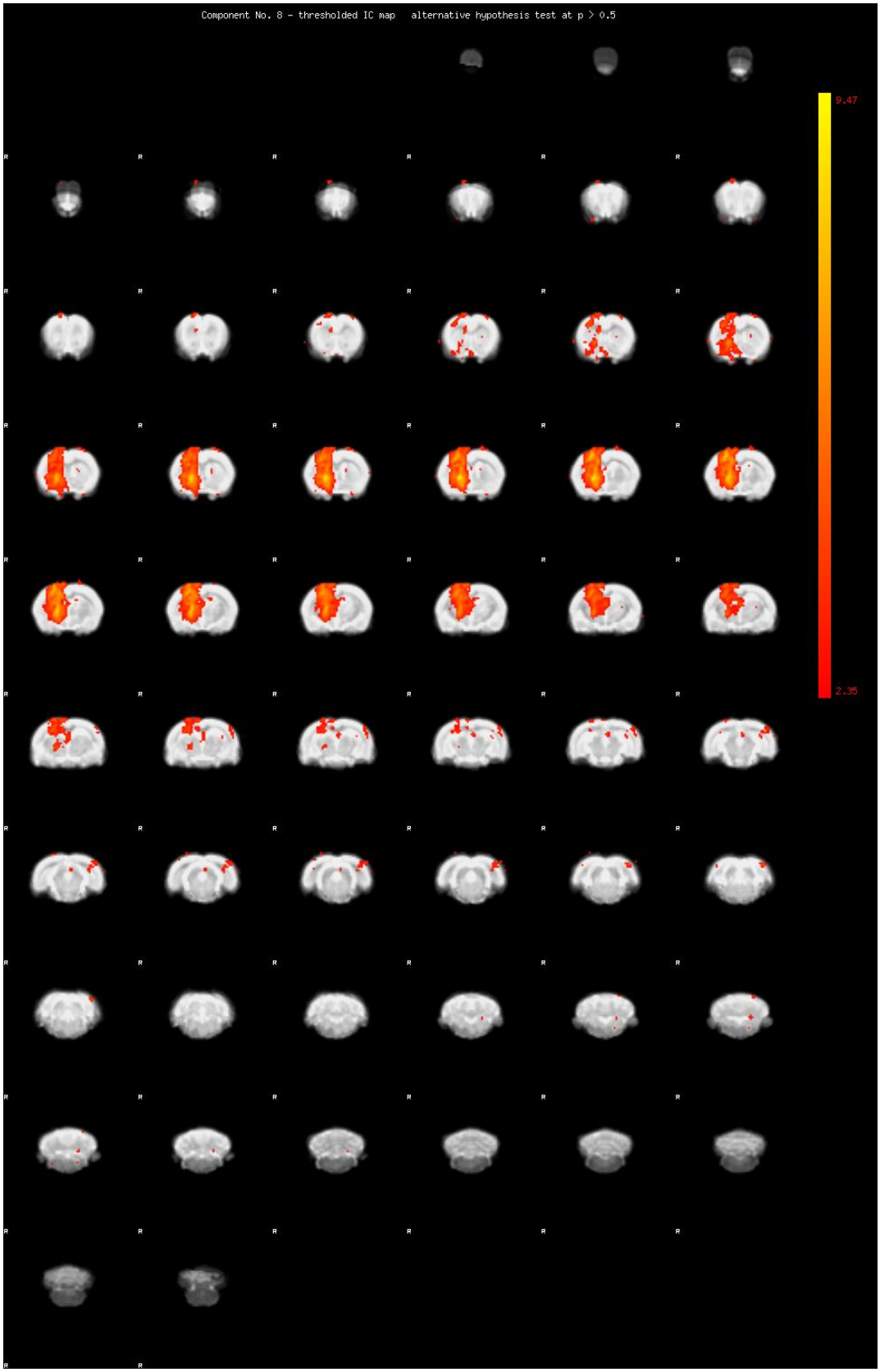

Component No. 13 - thresholded IC map alternative hypothesis test at p > 0.5
7.9
2.38

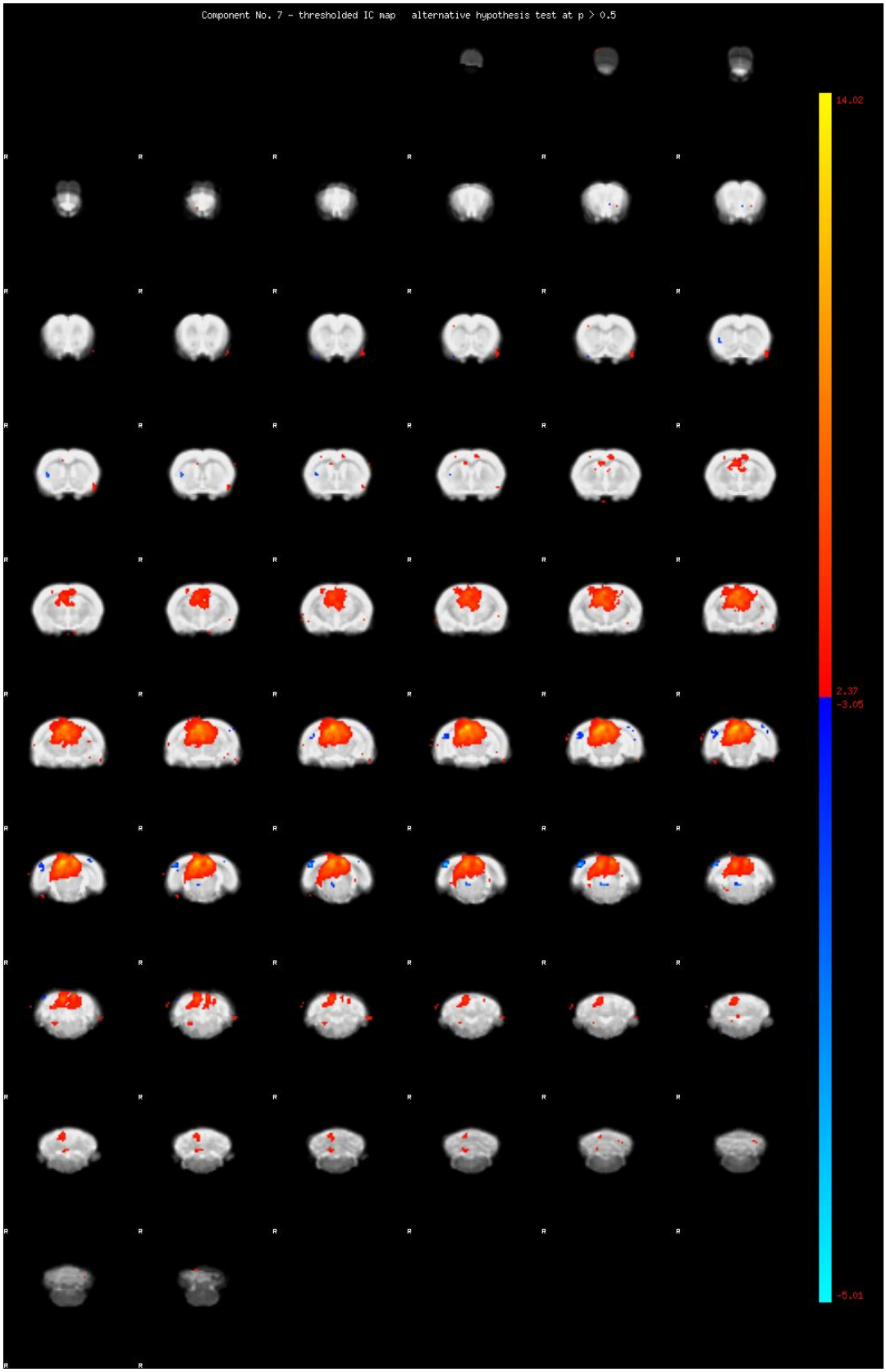

Component No. 12 - thresholded IC map alternative hypothesis test at p > 0.5
12.05
2.23
-3.18
-6.75

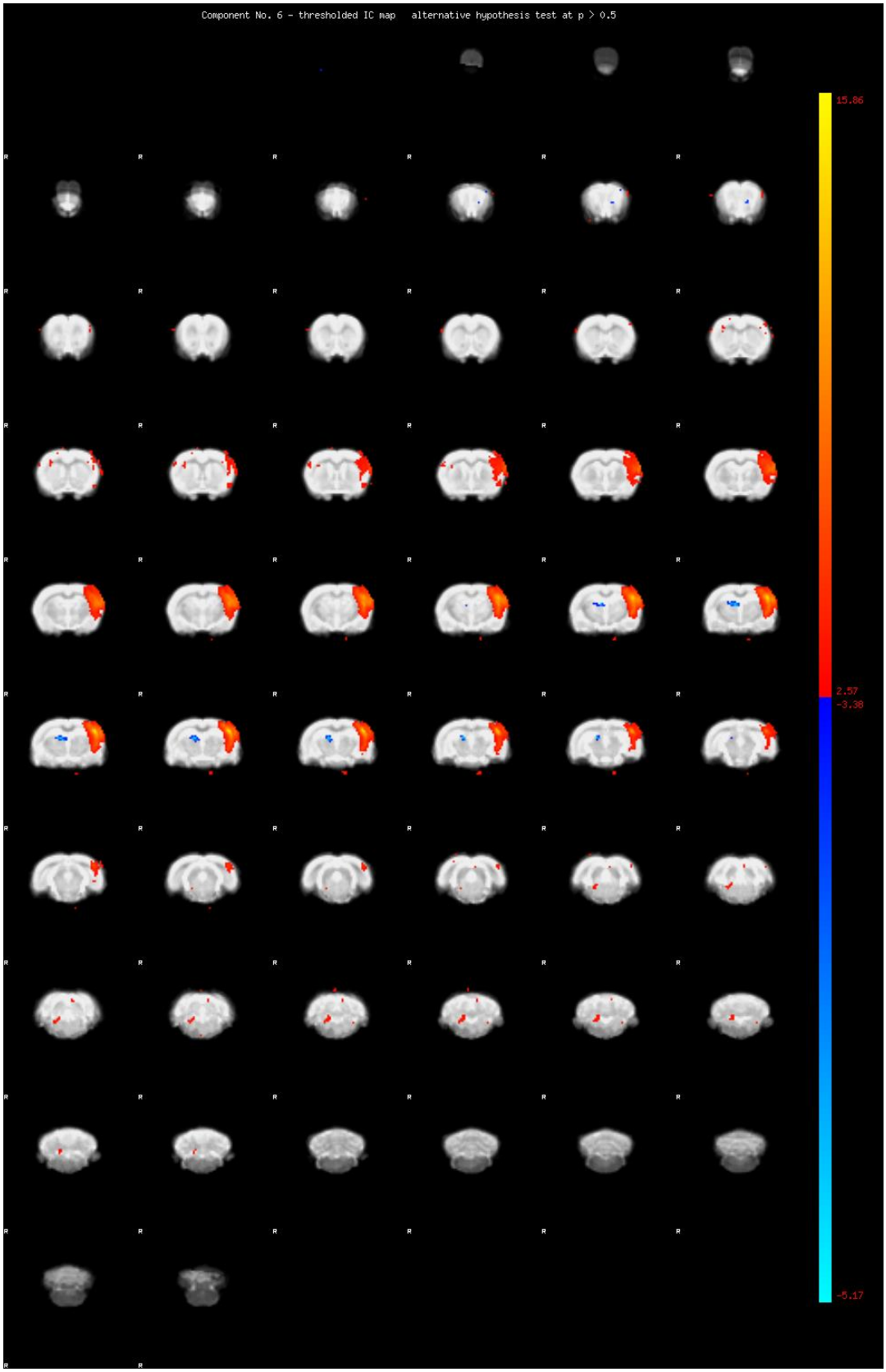

Component No. 11 - thresholded IC map alternative hypothesis test at p > 0.5
11.67
2.43

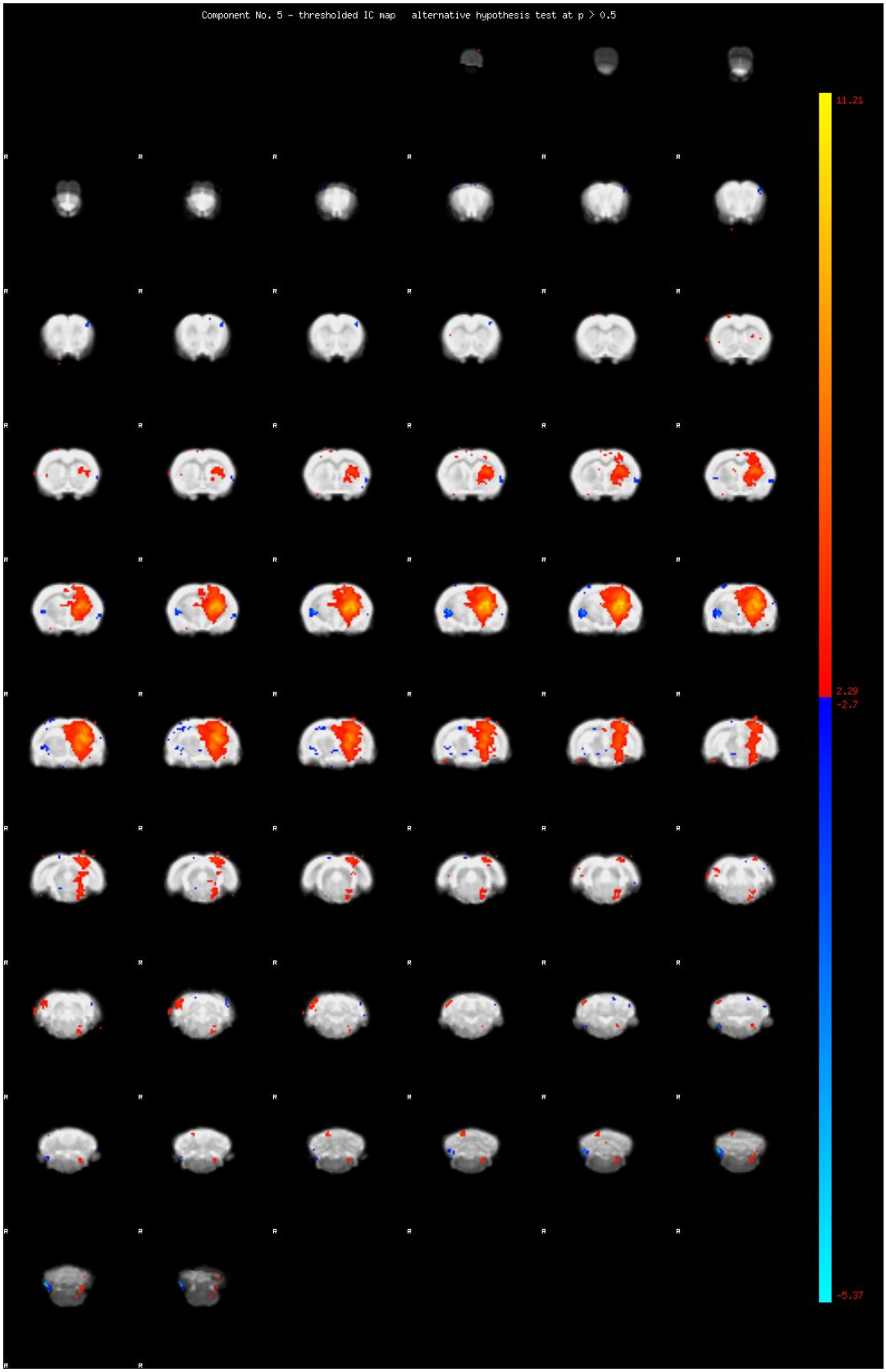

Component No. 10 - thresholded IC map alternative hypothesis test at p > 0.5
13.06
2.54
-3.2
-4.38

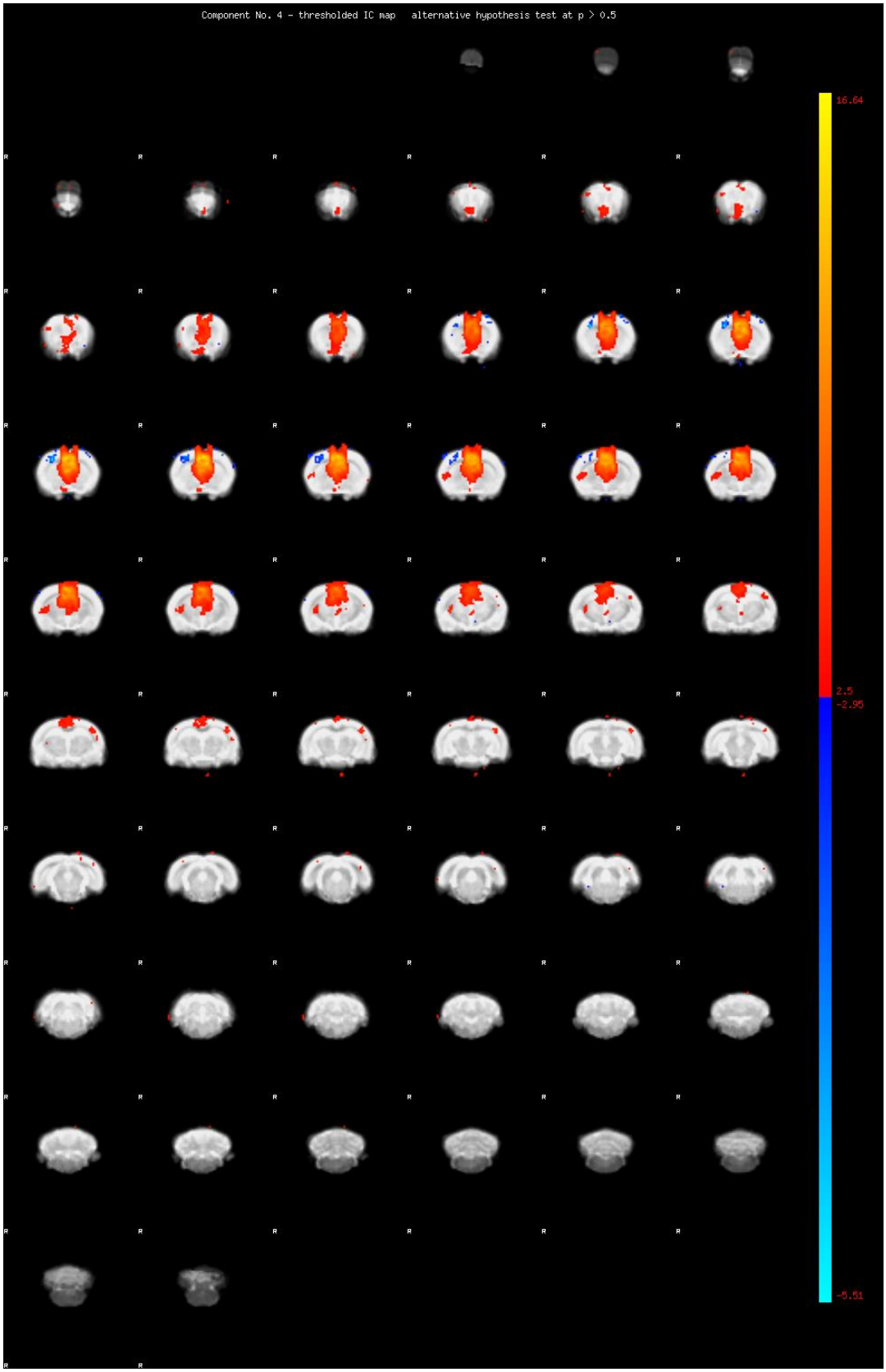

Component No. 9 - thresholded IC map alternative hypothesis test at p > 0.5
14.5
2.52

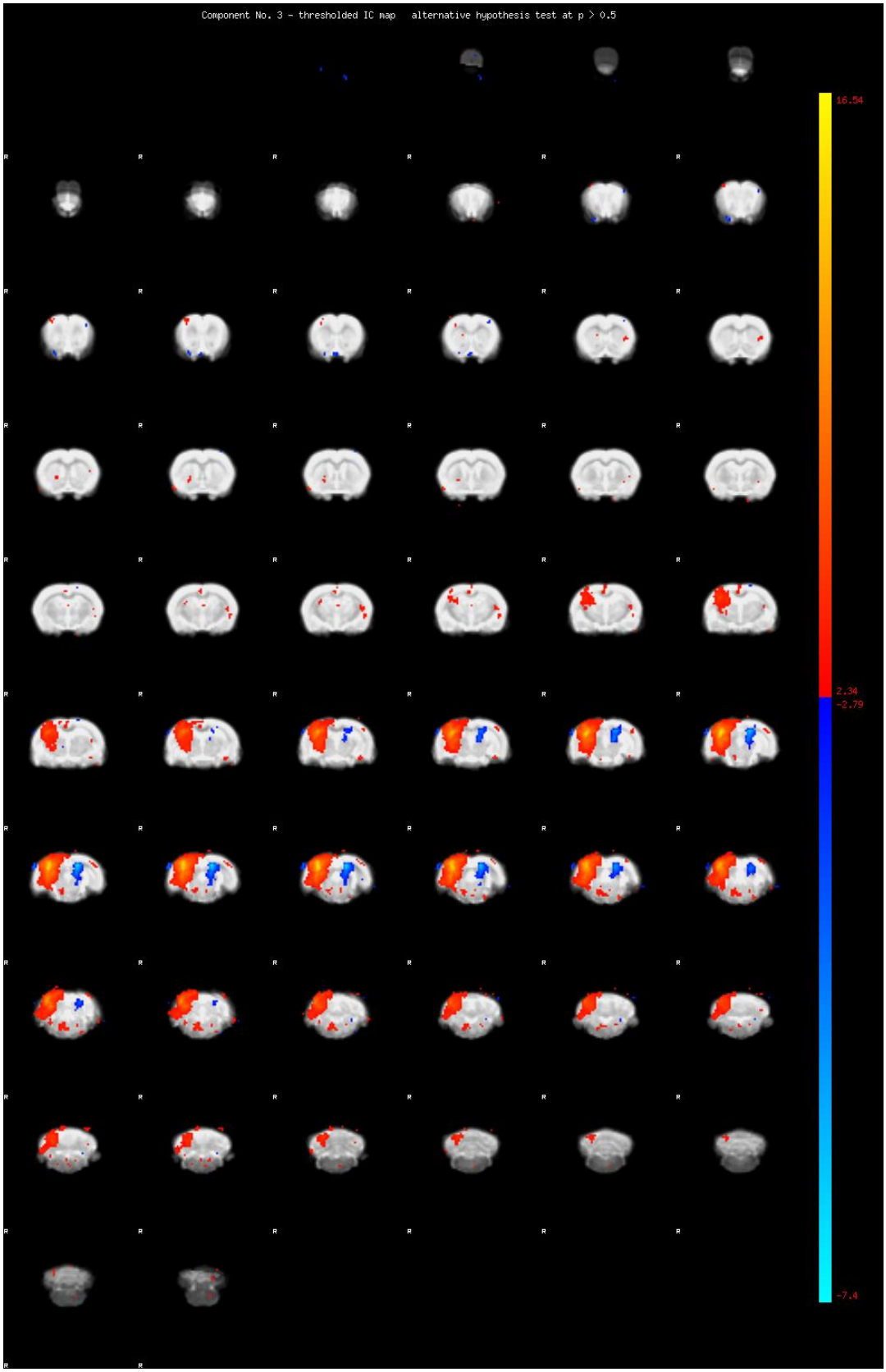

Component No. 8 - thresholded IC map alternative hypothesis test at p > 0.5
9.47
2.35

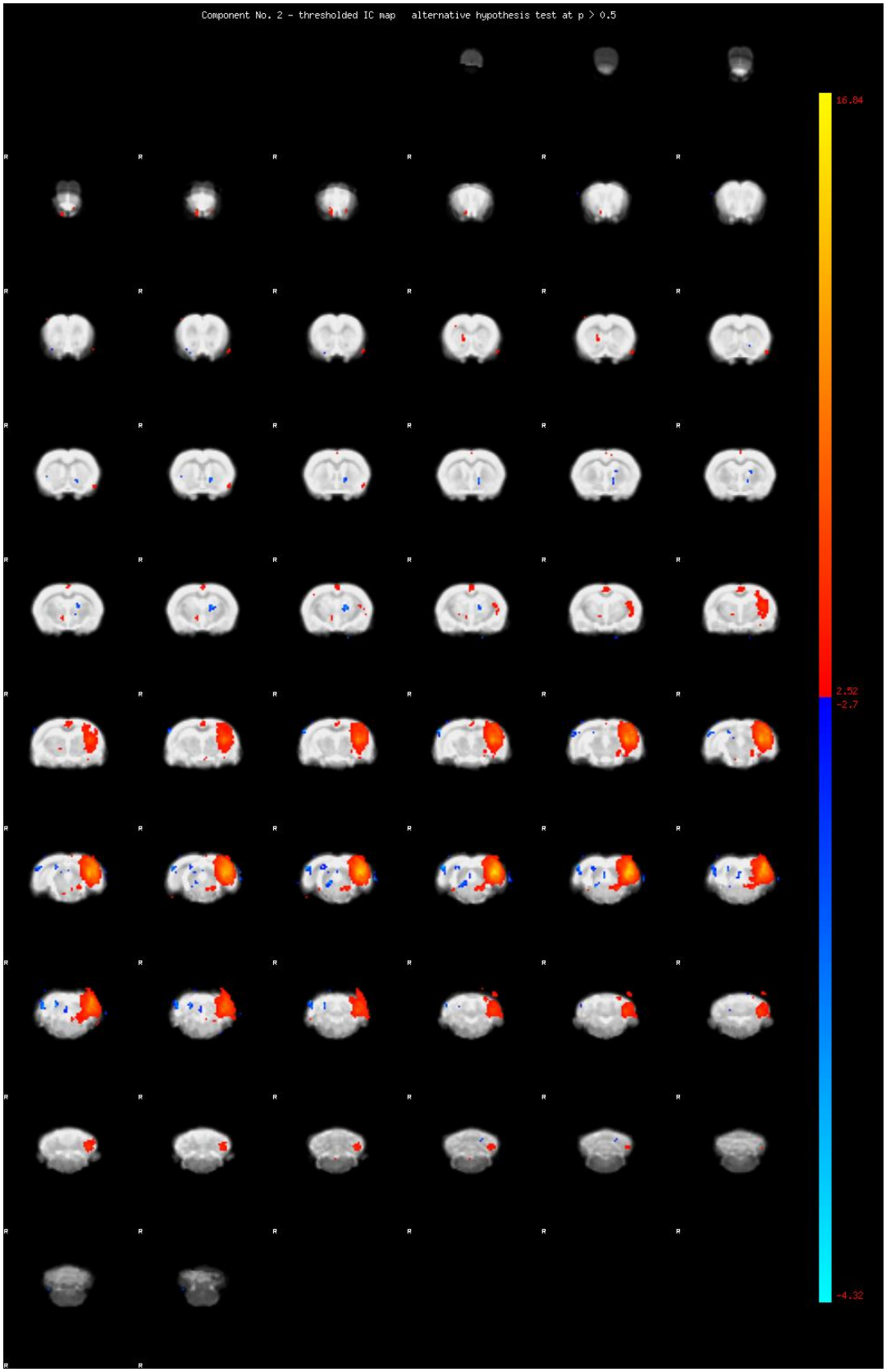

Component No. 7 - thresholded IC map alternative hypothesis test at p > 0.5
14.02
2.37
-3.05
-5.01

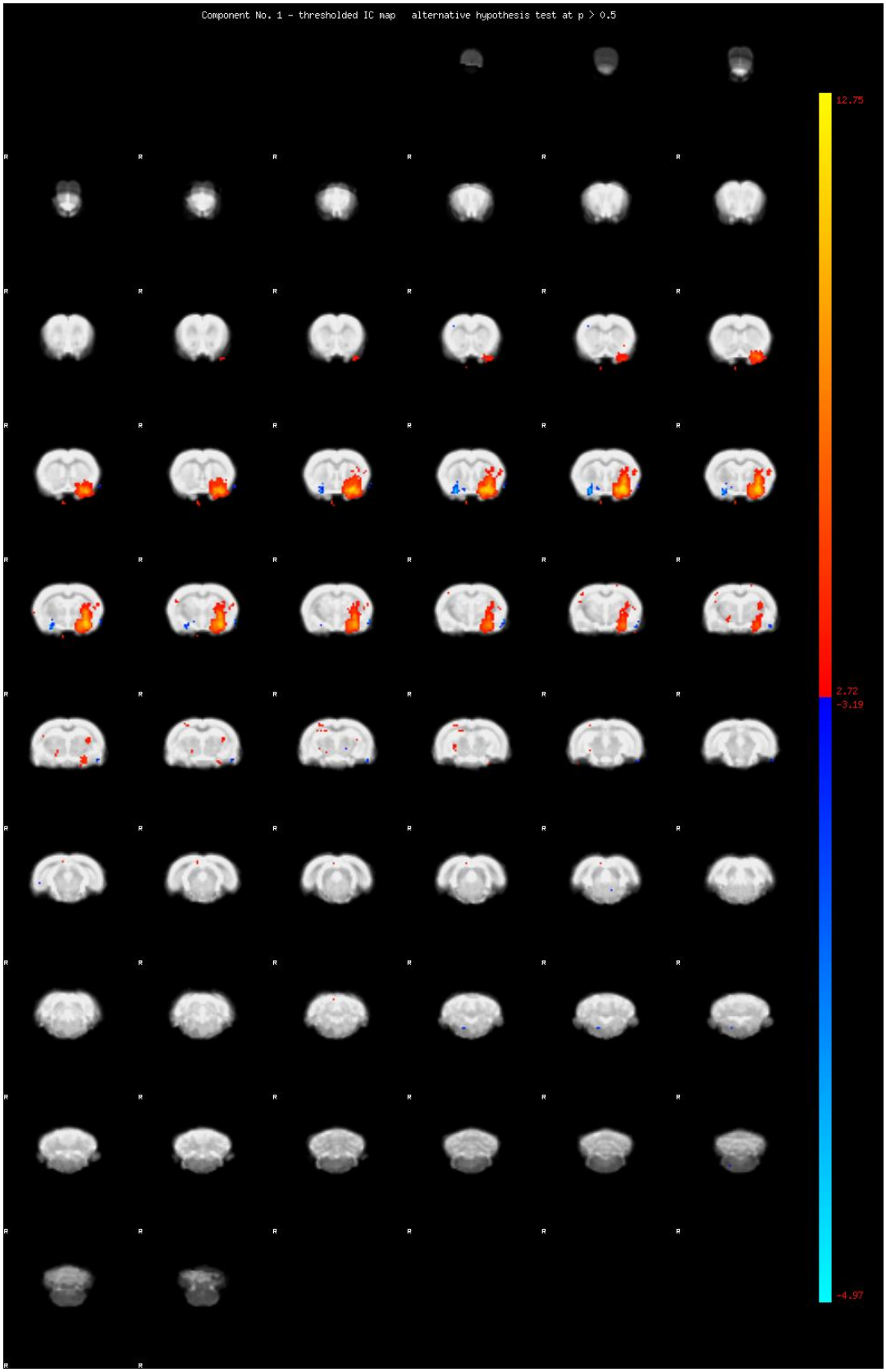

Component No. 6 - thresholded IC map alternative hypothesis test at p > 0.5
15.86
2.57
-3.38
-5.17

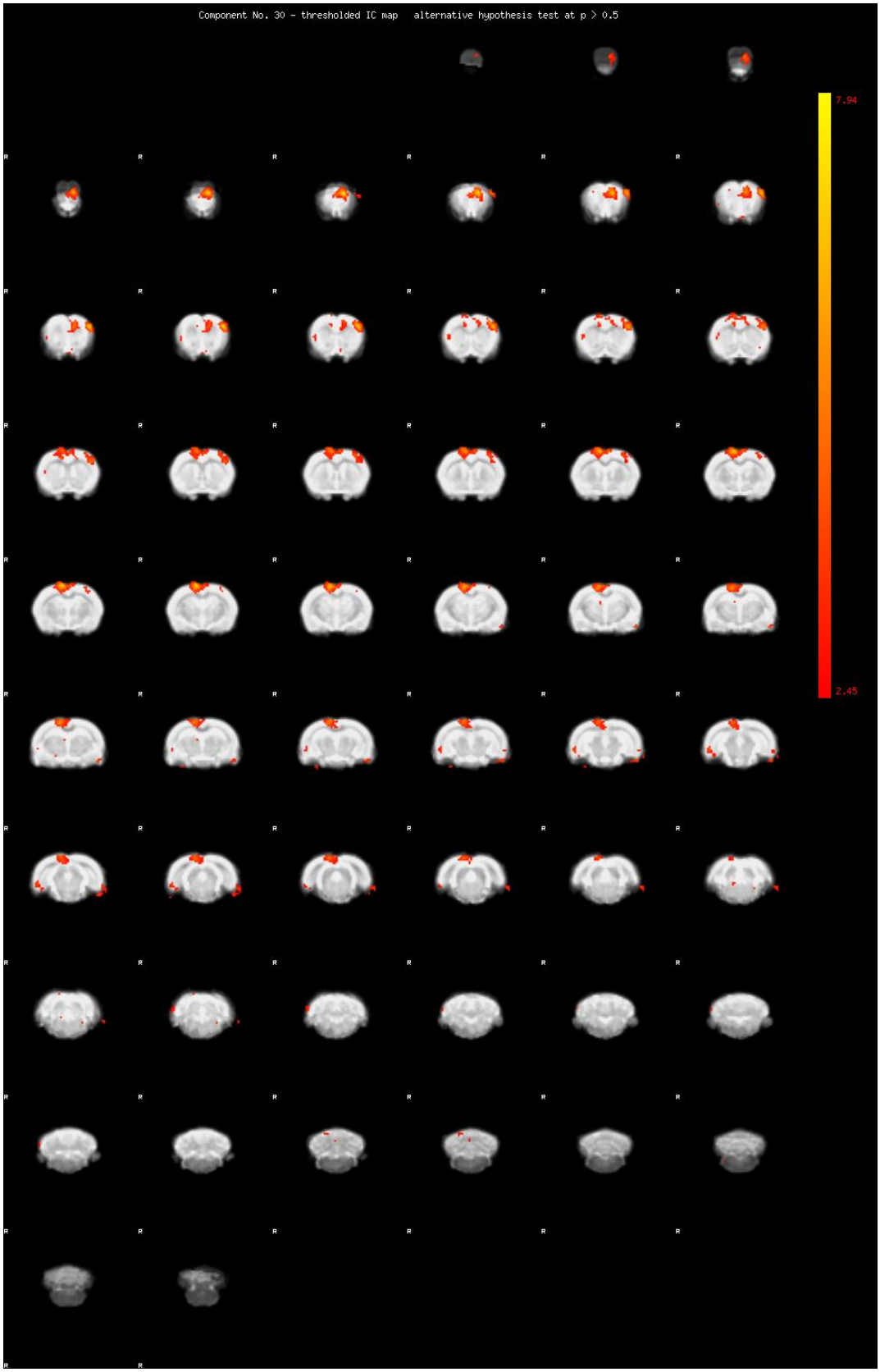

Component No. 5 - thresholded IC map alternative hypothesis test at p > 0.5
11.21
2.29
-2.7
-5.37

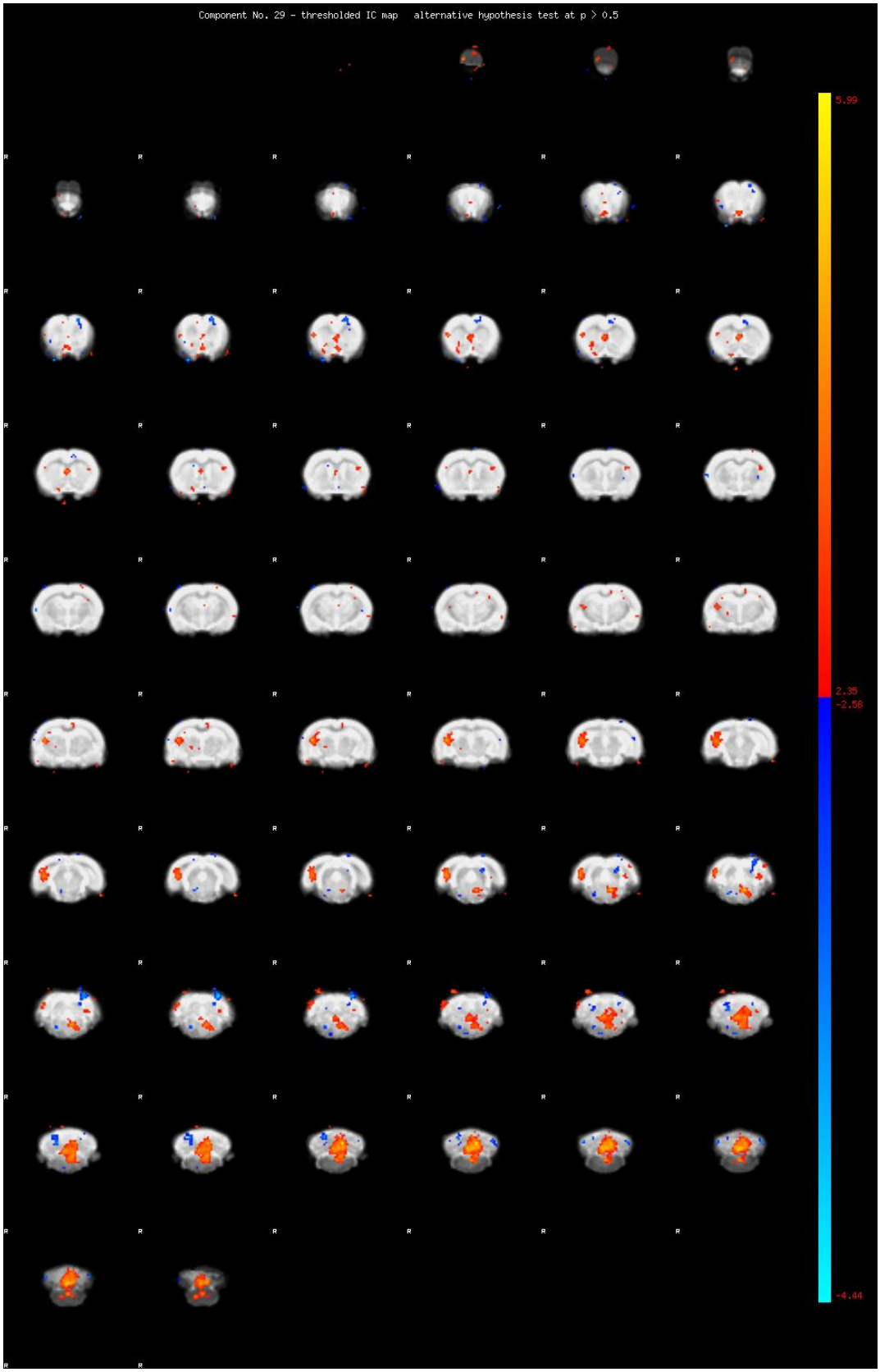

Component No. 4 - thresholded IC map alternative hypothesis test at p > 0.5
16.64
2.5
-2.95
-5.51

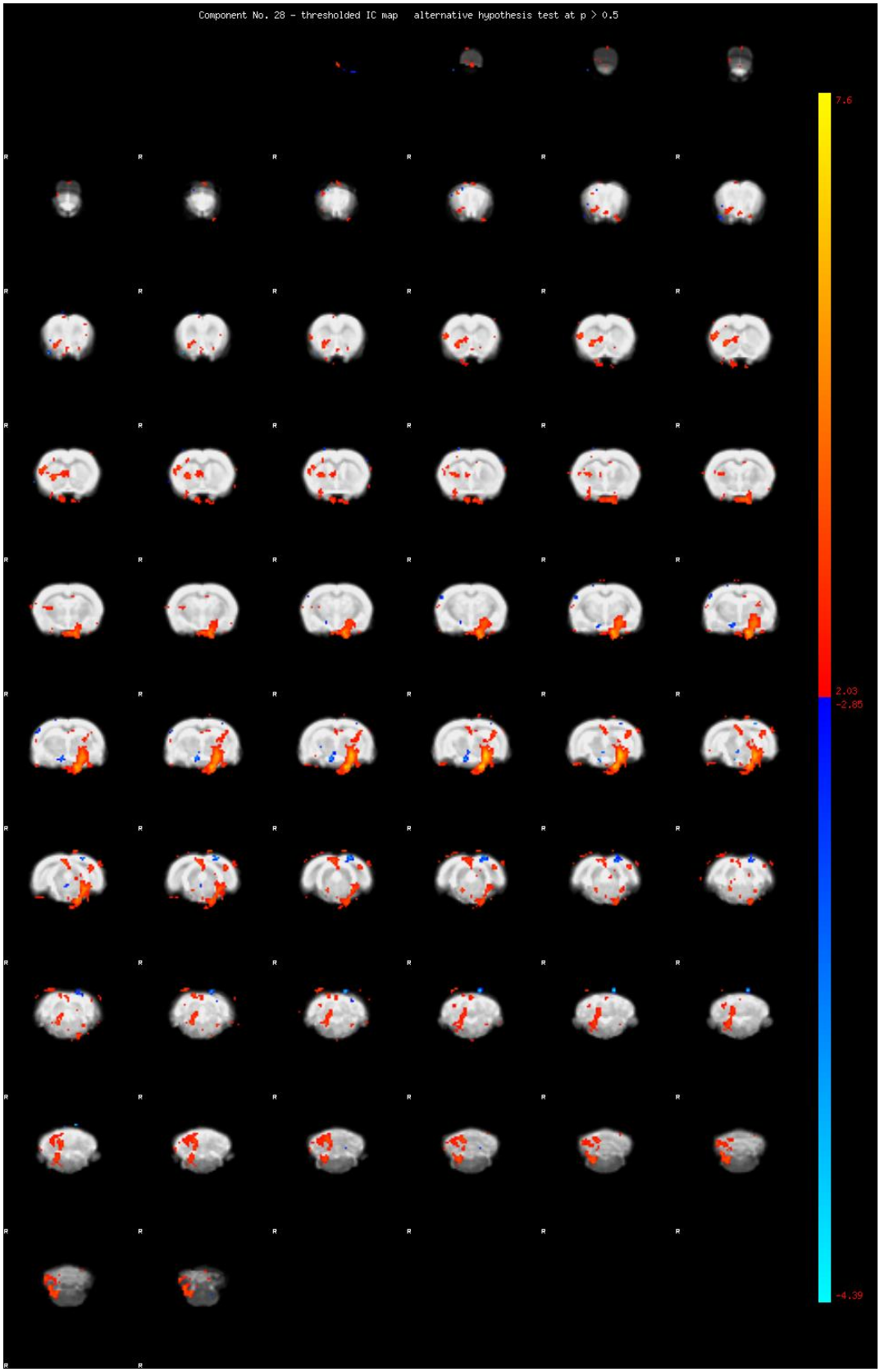

Component No. 3 - thresholded IC map alternative hypothesis test at p > 0.5
16.54
2.34
-2.79
-7.4


Component No. 2 - thresholded IC map alternative hypothesis test at p > 0.5
16.84
2.52
-2.7
-4.32


Component No. 1 - thresholded IC map alternative hypothesis test at p > 0.5
12.75
2.72
-3.19
-4.97


Component No. 30 - thresholded IC map alternative hypothesis test at p > 0.5
7.94
2.45


Component No. 29 - thresholded IC map alternative hypothesis test at p > 0.5
5.99
2.35
-2.58
-4.44


Component No. 28 - thresholded IC map alternative hypothesis test at p > 0.5
7.6
2.03
-2.85
-4.39

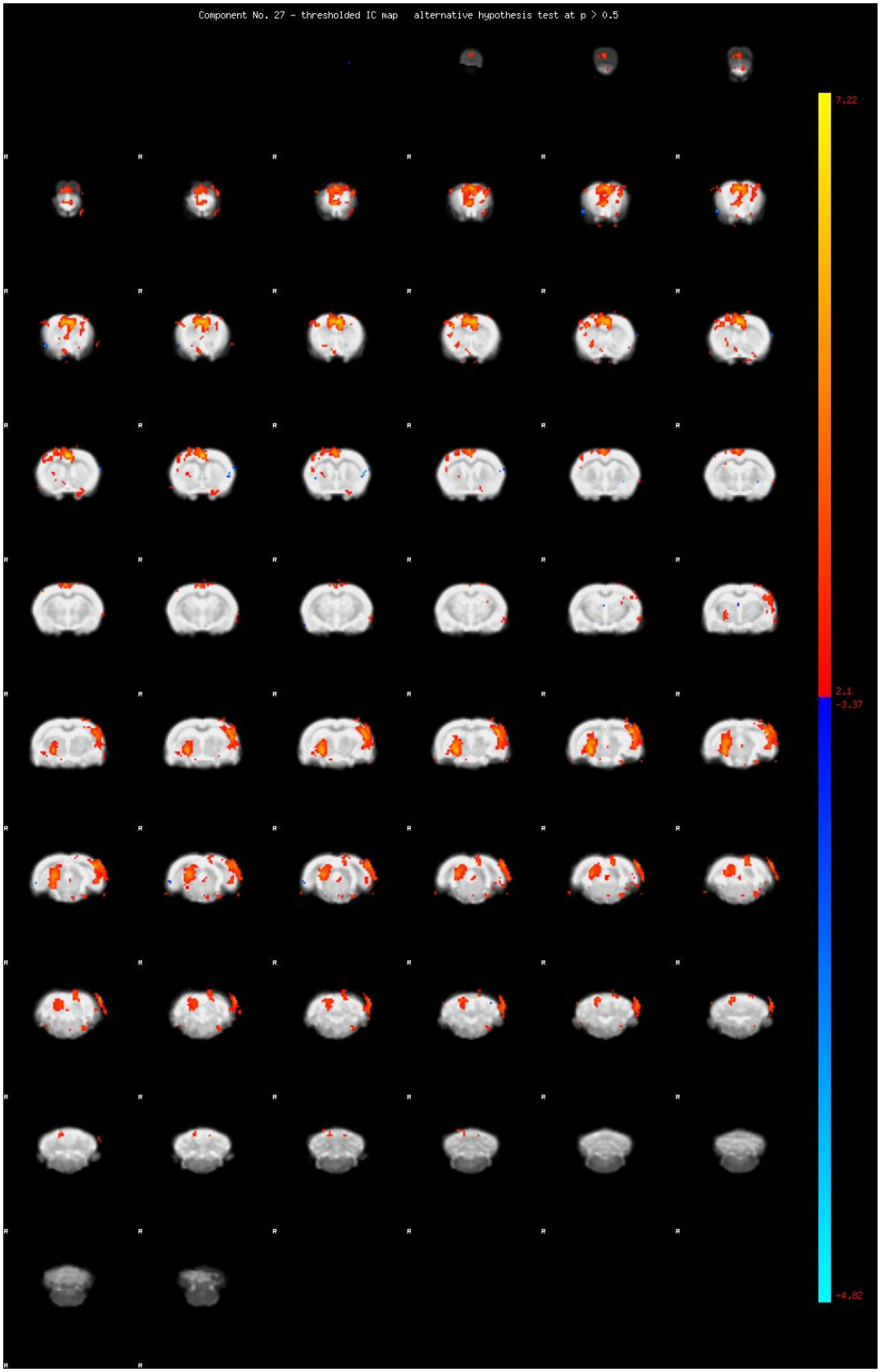


Figure 6.4. Resting state networks showing 30 independent components. Each of these components/ spatial maps show regions of the brain that are correlated in their resting state "activity". PCP did not induce any changes in any of these 30 networks.

Appendix E: HPLC analysis of dopamine, serotonin and their metabolites in brain region homogenates

Background

phMRI provides information of BOLD activation of brain regions after pharmacological manipulations. However, this does not provide a direct measure of neurochemical function. To help interpret the data from phMRI, post mortem analysis with HPLC was used to quantify regional dopamine levels in brain homogenates. HPLC is a widely used method in psychopharmacological experiments, for quantitative measurement of individual compounds in a mixed solution. In present study HPLC was used to measure dopamine and its metabolites, DOPAC and HVA and serotonin and its metabolite 5HIAA in four brain regions (dorsal striatum, NAc, FCx, and VTA) in brains taken from (see appendix)

Method and material

Brain dissection

At the end of imaging experiments (Chapter 4) the saline and PCP treated animals were humanely killed, and their brains were taken for HPLC analysis of the tissue content of dopamine, its metabolites DOPAC and HVA, and 5HT and its metabolite 5HIAA in four regions of interest, NAc, dorsal striatum, FCX and midbrain (containing VTA). Thus all animals had received two MRI scanning sessions a week apart during which either amphetamine or nicotine were administered, followed by a week of daily treatment with PCP (2 mg/kg, twice daily for five days) or saline (1 ml/kg twice daily for five days) and a week of drug-free washout. The two MRI scans were then repeated a week apart, with

amphetamine or nicotine administered again (see chapter 4 for full details). One week after the final MRI scan, animals were deeply anaesthetised with isoflurane and killed by cervical dislocation, and the brain was removed and placed in an ice cold stainless steel brain slicer matrix with 1.0 mm coronal section slices intervals (BSRAS001-1). Brain slices isolating the regions of interest were cut with razor blades, as follows: the first blade was inserted 2mm from the rostral pole of the cortex, and two further blades were inserted at 2 mm intervals, providing slices containing FCx, NAc and dorsal striatum respectively. Two more blades were inserted 3 and 5 mm caudal to these, providing a single slice containing VTA. The individual slices were then dissected to isolate the required regions, which were placed in Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C until analysis.

Homogenisation Methods

Each individual brain region sample was weighed and then homogenised in 0.1 M perchloric acid for approximately 2 min to ensure that the tissue was well dispersed. The homogenate solution was then transferred into 1.5 ml Eppendorf and centrifuged using a bench centrifuge for 45 min at 4°C. The supernatant was then carefully aspirated off using a Pasteur pipette ready for HPLC analysis, taking care not to disturb the pellet.

HPLC Methods

Chromatographic separation was achieved using a 2.00 x 150 mm C18, 5µm column (LUNA(2): Phenomenex, UK) perfused with a 75 mM phosphate running buffer (75 mM $NaH_2PO_{4)}$, 1 mM ethylenediaminetetra acetic acid (EDTA); 0.6 mM octane sulfonic acid, 10% methanol, pH 7.4) at a flow rate of 300 µl/min delivered by a high pressure

pump. Detection of electroactive compounds was achieved with an Antec electrochemical detector, incorporating a VT-03 low volume flow cell (Antec, Netherlands), with the working electrode set at 800 mV relative to a Ag/AgCl reference electrode. Samples supernatant from tissue homogenates were injected manually through a Rheodyne high pressure valve, incorporating a 20 µl sample loop. Supernatant samples were analysed in duplicate and levels of dopamine, DOPAC, HVA were measured.

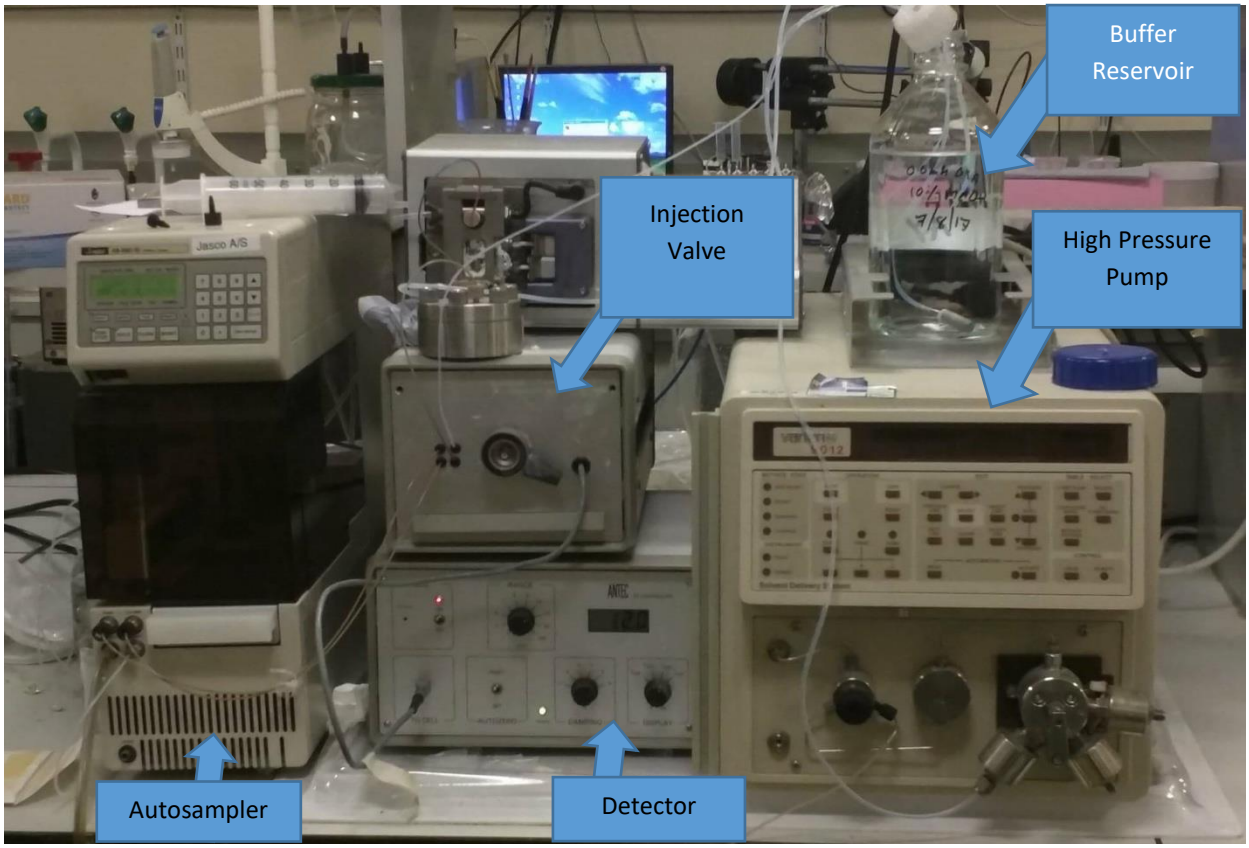


Figure 6.5 HPLC system. Comprising; Autosampler, injection valve, detector, buffer reservoir, high pressure pump.

Data Analysis

Peak areas for compounds of interest were determined using a PC-based integrator (Chrom Perfect) and concentrations were calculated for each with reference to peak areas in a standard solution (50 nM) made freshly each day. Mean concentrations were calculated for each tissue sample, by taking an average of the two duplicates, and then this value was corrected for the weight of tissue. Statistical analysis were performed two way ANOVA in GraphPad Prism 7.

Results

Brain homogenate levels of monoamine transmitters and monoamine transmitter metabolites, measured by HPLC with electrochemical detection

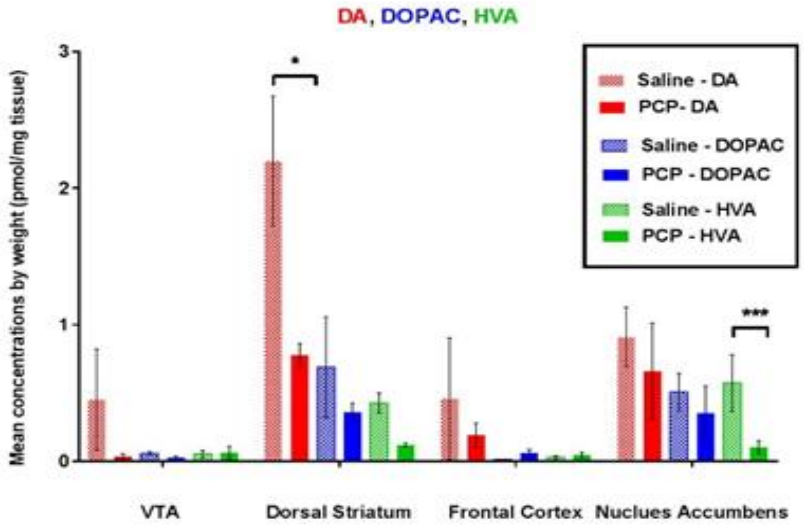


Figure 6.6 Effect of PCP treatment on HPLC analysed monoamine transmitters and monoamine metabolites. mean concentrations by weight of dopamine (DA), DOPAC, HVA. * $p < .05$; difference from control; *** 0.0002

Measurements of dopamine in NAc, midbrain and FCx showed no significant differences between saline and PCP pretreated animals. However, there was a significant main effect of brain area ($F[3,19] = 1.899$; $p = 0.1640$) and of drug ($F[1,19] = 8.18$; $p = 0.0100$), but no significant interaction ($F[3,19] = 1.899$; $p = 0.1640$). Dopamine levels in PCP treated animals were lower than in saline treated animals in dorsal striatum.

Monoamine transmitter metabolites DOPAC did not show significant main effect of region ($F [3, 19] = 5.627$ $P=0.0062$) drug effect ($F [3, 19] = 0.6$ $P=0.6228$), or interaction ($F [1, 19] = 1.141$ $P=0.2988$). However, for HVA levels there were significant main effects of region ($F [3, 18] = 14.55$ $P<0.0001$) , and drug ($F [1, 18] = 22.68$ $P=0.0002$), and a significant interaction ($F [3, 18] = 9.121$ $P=0.0007$). In NAc, HVA levels were significantly decreased after PCP pretreatment.

In present study, results indicated that chronic PCP treatment attenuated dopamine and HVA levels in dorsal striatum and NAc respectively. However, given the variability of monoamine neurotransmitters and their metabolites in figure 6.6 it is difficult to interpret PCP effects on dopamine and its metabolites. This is likely to be due to the relative lower power of the study, with only n = 3 to 4 per group. HPLC analysis is mainly conducted using the higher level of sample size (n = 8 to 10) to gain sufficient power. Therefore, increasing the subject number may provide less variation and interpretable data to address how PCP change monoamine transmitters and monoamine transmitter metabolites.

Discussion

Brain homogenisation in HPLC experiments showed some differences in levels between saline and PCP tissue, but indicate that, in general, the differences were not significant. However, the extreme variability and lack of power, mean that the results should be

interpreted cautiously and further similar experiments should be carried out to understand to what extent PCP alters dopamine function in different brain regions and its relationship with schizophrenia.

Appendix F : Microdialysis measurements of dopamine in freely moving rats

Background

The experiments described in this thesis demonstrate changes in modulation of dopamine release after PCP pretreatment, involving nAChR mechanisms both *in vitro* (FSCV) and *in vivo* (phMRI). A further aim of the project was to confirm and extend these findings in vivo using brain microdialysis. This technique allows sampling of the brain extracellular fluid collection in intact animals through a chronically implanted microdialysis probe, located in the brain area(s) of interest. This is valuable in neuropharmacology studies as it allows measurement of local neurotransmitter release and also allows for delivery of drugs into discrete brain regions which would not otherwise cross the blood-brain barrier. The probe is comprised of two pieces of capillary tubing that are covered in a semipermeable membrane, and neurotransmitters and metabolites in extracellular space in brain, diffuse across the semipermeable membrane. The dialysate obtained is then used for analysis of compounds of interest in the brain using HPLC. A constant artificial cerebrospinal fluid (aCSF) flow is maintained during the microdialysis process in order to keep the balance of the ionic environment the brain aCSF; circulation also creates a concentration gradient between the dialysate fluid and the extracellular space fluid.

Aims

- To investigate the effect of subchronic PCP pretreatment on dopamine release in the NAc in conscious, freely moving rats *in vivo*.
- On the basis of results obtained *in vitro*, to investigate whether the effect of subchronic PCP pretreatment on dopamine release in the NAcS is mediated through nAChR mechanisms.

Methods

Animals

Eight female Wistar rats (Division of Biomedical Services, University of Leicester) were used. Animals had accessed to food ad libitum and animal welfare was monitored daily. All procedures using animals were carried out with appropriate personal and project licence (project number; 60/4390 personal licence; I67DDB722) approval under the Animals (Scientific Procedures) Act, 1986, and with local ethical approval from the University of Leicester Animal Welfare and Ethical Review Body (AWERB).

Pretreatment

Animals were pretreated subchronically with PCP (2 mg/kg, i.p.) or vehicle (0.9% saline) (volume 1 ml/kg, i.p.) administered twice daily for five days, and the animals were then left for at least five days (drug free) before testing. Full details of the procedure was as previously described (Chapter 2 and3).

Implantation of guide cannulae and microdialysis probes

After the washout period animals underwent to stereotaxic surgery and guide cannulae (Microbiotech AB, Sweden) were implanted bilaterally into NAc. Animals were anaesthetised with isoflurane (2-3% isoflurane; flow rate 2 l/min), and the top of the head shaved and cleaned ready for surgery. Veterinary ophthalmic ointment (Lacrilube) was applied into each of the eyes to protect from drying and debris. The surface of the skull was exposed and cleaned and the position the medio-lateral and antero-posterior coordinates (AP, 1.4; ML ± 1.0, Paxinos and Watson, 1988) for implantation were marked, relative to bregma. Holes were drilled, using a hand drill, and the guide cannulae were inserted to a depth of 5.5 mm, and secured, with the aid of two 1 mm stainless steel anchor screws placed in the parietal bones, using dental acrylic. In addition a wire fixing post was cemented in posterior to the guide cannulae to aid attachment of the delivery tubes. The wound was closed with sutures, physiological saline (5 ml; s.c) was injected to prevent dehydration, and the animal was allowed to recover from surgery. The animal was giving peri- and post-operative analgesia (Carprieve, 0.1 ml/kg bodyweight per day) for 3 days and antibiotics (Baytril, 0.2 ml/kg bodyweight per day) for 5 days.

Microdialysis recording

Animals were given a week to recovery from the surgery. On the experiment day animals were lightly anaesthetised with isoflurane, and dialysis probes (MAB6, Microbiotech AB, Sweden) were inserted through the guide cannulae to lie in NAc. After recovery from anaesthesia, animals were put into plexiglass recording box and probes were connected to the delivery tubes and perfusion with aCSF (2.5 µl/min; delivered from an Instech low flow rate syringe pump) was started immediately. After one hour

equilibration perfusion, 10 min dialysate samples were collected into 2.5 µl of 1M H3PO4, and stored on ice. Potassium stimulation (100 mM) was delivered bilaterally via the dialysis probes to stimulate brain activation in sample 5 and again in sample 10. 10 min after the first potassium stimulation, the perfusate on one side was changed for aCSF containing DHβE (1uM). At the end of the experiment samples were kept in -80 to run for HPLC analysis with electrochemical detection. Following the completion of the recording, rats were humanely killed by pentobarbitone overdose, and the brain removed to confirm probe placement.

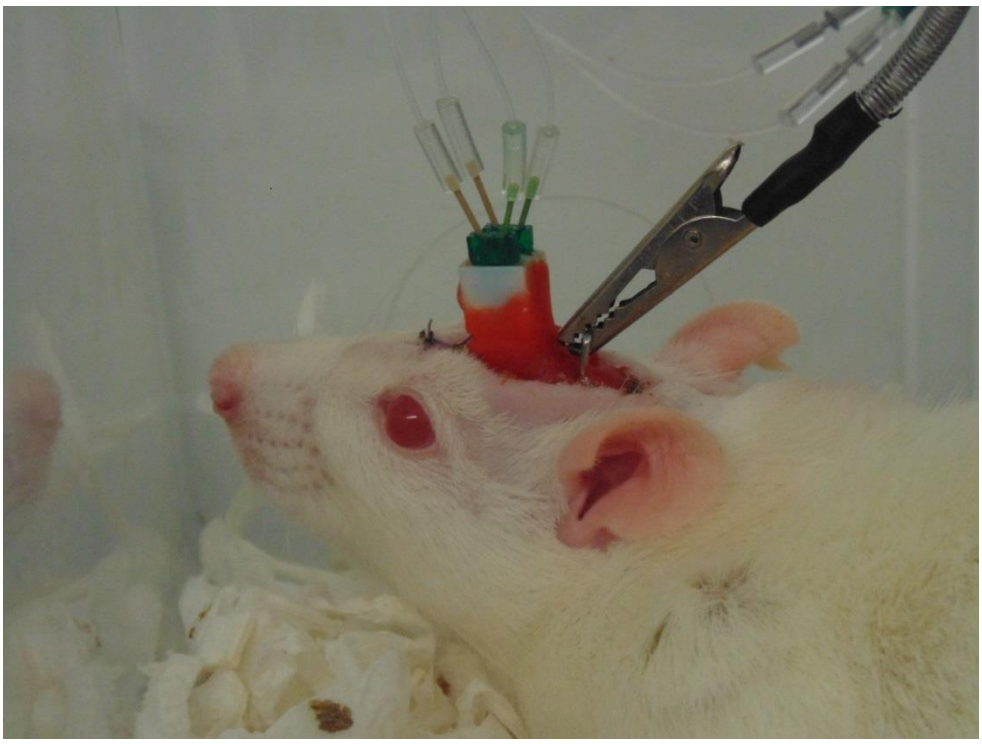


Figure 6.7 Photograph of an animal connected up for brain microdialysis. Guide cannulae (white) were implanted inder general anaesthesia, then, on the day of recording, dialysis probes (green) were inserted into the guides, and connected to the delivery tubing for dialysis perfusion. The crocodile clip (right) attached to a stainless steel wire to provide a tether for the delivery tubes, taking strain off the capillary tube connections.

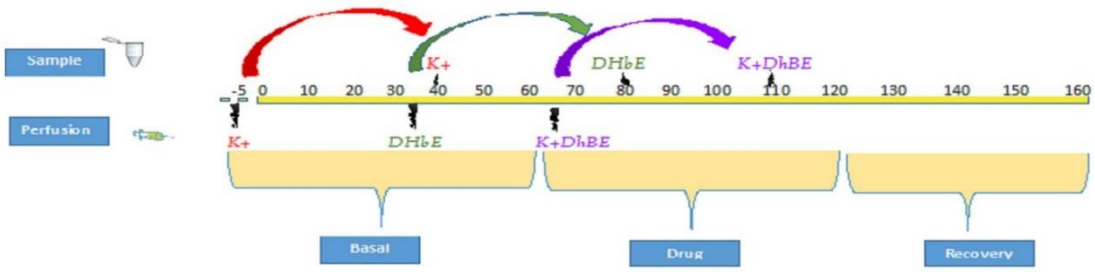


Figure 6.8 Sample collection time schedule. 100mMK^+ perfused through brain by tube 5 min before starting sample collection. Potassium stimulated brain sample was collected at 35 min after potassium perfused. 1µM DhβE perfused at 35 min and DhβE mediated brain sample was collected at 70 min. 100mM K^+ comcominat with 1µM DhβE perfused at 65 min and of which evoked sample collected at 100 min. first 60 min was baseline sample period, a second 60 min was drug period, and last 40 min was recovery period.

HPLC analysis

HPLC analysis was performed using an ANTEC Intro electrochemical detector, incorporating a VT-03 flow cell (ANTEC, Netherlands). Running buffer (75 mM NaH_2PO_4; 1 mM EDTA; 1.2 mM octanesulfonic acid; 10 % methanol; pH 3.7) was pumped at a flow rate of 0.11 ml.min (Jasco high pressure pump), and separation was achieved using a C18 reverse phase column (Phenomenex, LUNA(2) C18; 1.0 x 150 mm). Concentrations of dopamine, DOPAC, HVA and 5HIAA were assessed from the resulting chromatograms, with reference to a 10 nM standard, using ChromPerfect analytical software.

Results

Technical problems encountered with the dialysis procedure meant that the data collected were not reliable. Problems included damage to the probes during implantation and blocking of the probe tips. In addition, maintaining a constant dialysis flow during application of potassium and drug to the perfusate was problematic due to the liquid switches no working. Therefore, some experiments gave no data, while in others the levels were very variable: this may be a result of changes in the flow rates since the sample volumes were not consistent. This not only caused problems for the HPLC analysis, but also meant that the timings of potassium and drug applications were uncertain. Nevertheless, improvements in the procedure did allow reliable measurements

to be made in two animals, although no conclusions can be made about the effects of the drugs (Figure 6.X)

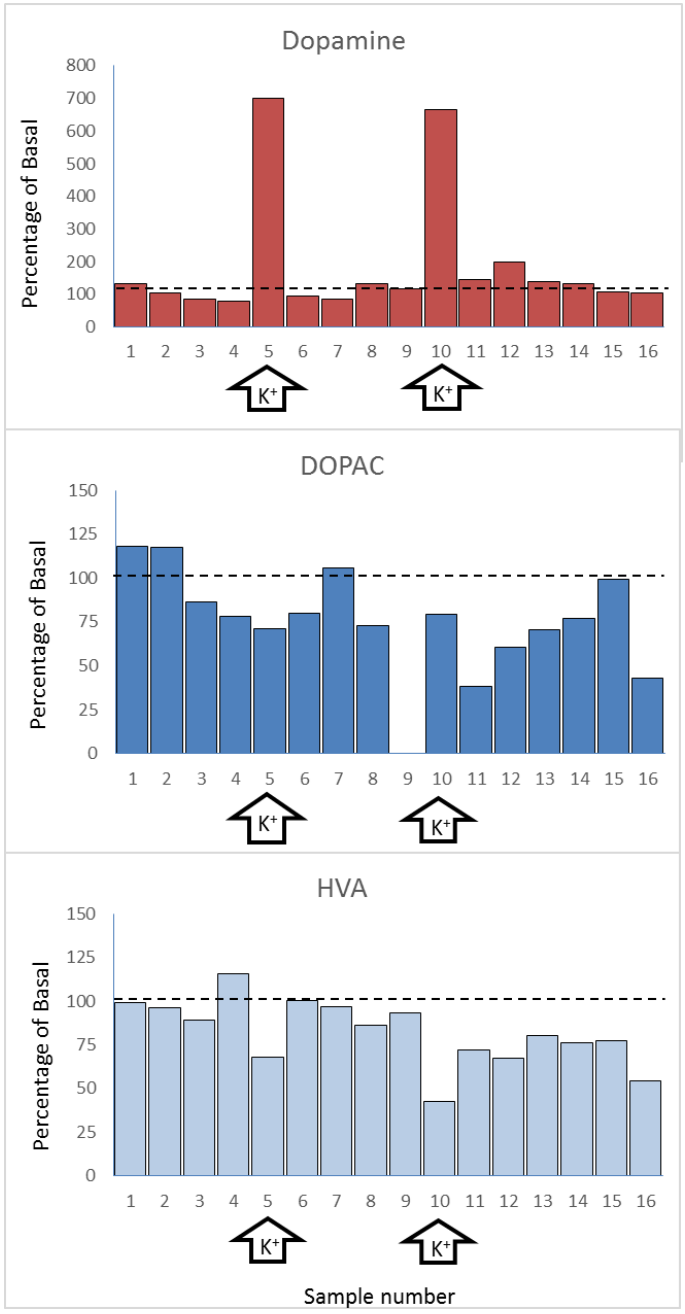


Figure 6.9: Example results from a single microdialysis experiment. Data are expressed as percentages of the basal levels measured during the first four samples, and show changes in dopamine, DOPAC and HVA in the non-drug treated side of a PCP pretreated animal. The dotted line shows 100% (i.e. no change from basal).

Discussion

Microdialysis is a sampling technique and widely used in neuropharmacology research. Small molecules diffuse across to semipermeable probe and collected sample run in HPLC. Therefore, we used microdialysis experiments to assess cholinergic modulation of extracellular dopamine release in NAc, and test whether antagonism of α4β2 subunit of nAChRs by DHβE changed the extracellular dopamine levels in freely moving rats. Microdialysis experiments are mainly used to assess the tonic dopamine activation and its changes by target ligands or drugs. Therefore, in these experiments we intended to assess tonic dopamine activation in NAc by DHβE, and its modulation of potassium stimulated release. Using bilateral probes implanted into NAc so that one side was used as a drug infusion, whereas the other side had no drug and acted as a control.

Thus, although this protocol is capable of measure acetylcholine modulation of extracellular dopamine release, technical problems encountered prevented reliable data being collected. Microdialysis has been a routine procedure in the lab for many years using probes constructed in the lab. However, in the present studies, commercial probes were used, and a number of problems were encountered, including blocking of the probe and damage to the tip during implantation, in part due to the rapid final movement of the probe 'clicking' into position in the guide cannula. In addition, difficulties were encountered with switching between drugs without interruption of the dialysis flow. Problems encountered with the liquid switches meant that the changeovers could not be made without interrupting the flow. Nevertheless, these problems were ultimately overcome, and some appropriate data were collected, all be it that they were not sufficient to address the scientific questions posed.

7. REFERENCES

Abeliovich, A., Schmitz, Y., Fariñas, I., Choi-Lundberg, D., Ho, W. -., Castillo, P. E., . . . Rosenthal, A. (2000). Mice lacking a-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron*, *25*(1), 239-252.

Adams, B. W., Bradberry, C. W., & Moghaddam, B. (2002). NMDA antagonist effects on striatal dopamine release: Microdialysis studies in awake monkeys. *Synapse*, *43*(1), 12-18.

Adrian, R. H. (1956). The effect of internal and external potassium concentration on the membrane potential of frog muscle. The Journal of Physiology, 133(3), 631-658.

Akiyama, G., Ikeda, H., Matsuzaki, S., Sato, M., Moribe, S., Koshikawa, N., & Cools, A. R. (2004). GABA A and GABA B receptors in the nucleus accumbens shell differentially modulate dopamine and acetylcholine receptor-mediated turning behaviour. *Neuropharmacology*, *46*(8), 1082-1088.

Albuquerque, E. X., Tsai, M. C., Aronstam, R. S., Witkop, B., Eldefrawi, A. T., & Eldefrawi, M. E. (1980). Phencyclidine interactions with the ionic channel of the acetylcholine receptor and electrogenic membrane. *Proceedings of the National Academy of Sciences of the United States of America*, 77(2), 1224-1228.

Alger, B. E., & Nicoll, R. A. (1980). Epileptiform burst afterhyperolarization: calcium-dependent potassium potential in hippocampal CA1 pyramidal cells. Science, 210(4474), 1122-1124.

American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders, (DSM-5®) American Psychiatric Pub.

Amitai, N., & Markou, A. (2010). Effects of metabotropic glutamate receptor 2/3 agonism and antagonism on schizophrenia-like cognitive deficits induced by phencyclidine in rats. *European Journal of Pharmacology, 639*(1-3), 67-80.

Andersen, P. H., Gingrich, J. A., Bates, M. D., Dearry, A., Falardeau, P., Senogles, S. E., & Caron, M. G. (1990). Dopamine receptor subtypes: Beyond the D1/D2 classification. *Trends in Pharmacological Sciences, 11*(6), 231-236.

Andreasen, N. C., Ehrhardt, J. C., Swayze II, V. W., Tyrell, G., Cohen, G., Ku, J. S., & Arndt, S. (1991). T1 and T2 relaxation times in schizophrenia as measured with magnetic resonance imaging. *Schizophrenia Research, 5*(3), 223-232. doi:10.1016/0920-9964(91)90080-B

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cognitive Processing, 13*(2), 93-110.

Aosaki, T., Tsubokawa, H., Ishida, A., Watanabe, K., Graybiel, A. M., & Kimura, M. (1994). Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 14*(6), 3969-3984.

Aosaki, T. O. S. H. I. H. I. K. O., Kimura, M. I. N. O. R. U., & Graybiel, A. M. (1995). Temporal and spatial characteristics of tonically active neurons of the primate's striatum. Journal of neurophysiology, 73(3), 1234-1252.

Aramakis, V. B., & Metherate, R. (1998). Nicotine selectively enhances NMDA receptor-mediated synaptic transmission during postnatal development in sensory neocortex. *Journal of Neuroscience, 18*(20), 8485-8495.

Arthurs, O. J., & Boniface, S. (2002). How well do we understand the neural origins of the fMRI BOLD signal*? Trends in Neurosciences, 25*(1), 27-31.

Asif-Malik, A., Dautan, D., Young, A. M., & Gerdjikov, T. V. (2017). Altered cortico-striatal crosstalk underlies object recognition memory deficits in the sub-chronic phencyclidine model of schizophrenia. *Brain Structure and Function,* 1-12.

Azam, L., Winzer-Serhan, U. H., Chen, Y., & Leslie, F. M. (2002). Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs within midbrain dopamine neurons. *Journal of Comparative Neurology, 444*(3), 260-274. doi:10.1002/cne.10138

Baldo, M. A., Daniele, S., & Mazzocchin, G. A. (1998). A study on the suitability of carbon disk microelectrodes for trace analysis of lead and copper by ASV. *Electroanalysis, 10*(6), 410-416.

Baldo, M. A., Daniele, S., Corbetta, M., & Mazzocchin, G. A. (1995). Performance of platinum-based spherical mercury microelectrodes in cyclic voltammetry and stripping analysis. *Electroanalysis, 7*(10), 980-986.

Balla, A., Hashim, A., Burch, S., Javitt, D. C., Lajtha, A., & Sershen, H. (2001). Phencyclidine-induced dysregulation of dopamine response to amphetamine in prefrontal cortex and striatum. *Neurochemical Research, 26*(8-9), 1001-1006.

Balla, A., Koneru, R., Smiley, J., Sershen, H., & Javitt, D. C. (2001). Continuous phencyclidine treatment induces schizophrenia-like hyperreactivity of striatal dopamine release. *Neuropsychopharmacology, 25*(2), 157-164.

Bard, A. J., Faulkner, L. R., Leddy, J., & Zoski, C. G. (1980). *Electrochemical methods: Fundamentals and applications* .Wiley: New York; Vol.2.

Beaulieu, J. -., & Gainetdinov, R. R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacological Reviews, 63*(1), 182-217. doi:10.1124/pr.110.002642

Bechtholt-Gompf, A. J., Walther, H. V., Adams, M. A., Carlezon, W. A., Öngür, D., & Cohen, B. M. (2010). Blockade of astrocytic glutamate uptake in rats induces signs of anhedonia and impaired spatial memory. *Neuropsychopharmacology, 35*(10), 2049-2059.

Bennett, D. A., Bernard, P. S., Amrick, C. L., Wilson, D. E., Liebman, J. M., & Hutchison, A. J. (1989). Behavioral pharmacological profile of CGS 19755, a competitive antagonist at N-methyl-D-aspartate receptors. *Journal of Pharmacology and Experimental Therapeutics, 250*(2), 454-460.

Bennett, S., & Gronier, B. (2005). Modulation of striatal dopamine release in vitro by agonists of the glycineB site of NMDA receptors; interaction with antipsychotics. *European Journal of Pharmacology, 527*(1–3), 52-59. doi:http://dx.doi.org/10.1016/j.ejphar.2005.10.015

Berendse, H. W., & Richfield, E. K. (1993). Heterogeneous distribution of dopamine D1 and D2 receptors in the human ventral striatum. Neuroscience letters, 150(1), 75-79.

Bernardi, G., Floris, V., Marciani, M. G., Morocutti, C., & Stanzione, P. (1976). The action of acetylcholine and L-glutamic acid on rat caudate neurons. *Brain Research, 114*(1), 134-138. doi:10.1016/0006-8993(76)91014-3

Berridge, K.C. and Robinson, T.E. (1998) What is the role of dopamine in reward: Hedonic impact, reward learning, or incentive salience? *Brain Research Reviews 28:*309-369.

Bloomfield, C., O'Donnell, P., French, S., & Totterdell, S. (2007). Cholinergic neurons of the adult rat striatum are immunoreactive for glutamatergic N-methyl-d-aspartate 2D but not N-methyl-d-aspartate 2C receptor subunits. *Neuroscience, 150*(3), 639-646.

Blum, K., Chen, A. L., Chen, T. J., Braverman, E. R., Reinking, J., Blum, S. H., ... & Prihoda, T. J. (2008). Activation instead of blocking mesolimbic dopaminergic reward circuitry is a preferred modality in the long term treatment of reward deficiency syndrome (RDS): a commentary. Theoretical Biology and Medical Modelling, 5(1), 24.

Bouvier, G., Bidoret, C., Casado, M., & Paoletti, P. (2015). Presynaptic NMDA receptors: Roles and rules. *Neuroscience,* 322-340. doi:https://doi.org/10.1016/j.neuroscience.2015.10.033

Breiter, H. C., Gollub, R. L., Weisskoff, R. M., Kennedy, D. N., Makris, N., Berke, J. D., . . . Hyman, S. E. (1997). Acute effects of cocaine on human brain activity and emotion. *Neuron*, 591-611.doi:https://doi.org/10.1016/S0896-6273(00)80374-8

Broberg, B. V., Madsen, K. H., Plath, N., Olsen, C. K., Glenthøj, B. Y., Paulson, O. B., . . . Søgaard, L. V. (2013). A schizophrenia rat model induced by early postnatal phencyclidine treatment and characterized by magnetic resonance imaging. *Behavioural Brain Research, 250*, 1-8.

Broxterman, H., & Mos, J. (1980). Dopamine hypoactivity measured by in vivo voltammetry. European *Journal of Pharmacology*, *68*(3), 389-391.

Brozoski, T. J., Brown, R. M., Rosvold, H. E., & Goldman, P. S. (1979). Cognitive deficit caused by regional depletion of dopamine in prefrontal cortex of rhesus monkey. Science, 205(4409), 929-932.

Bruijnzeel, A. W., Alexander, J. C., Perez, P. D., Bauzo-Rodriguez, R., Hall, G., Klausner, R., . . . Febo, M. (2014). Acute nicotine administration increases BOLD fMRI signal in brain regions involved in reward signaling and compulsive drug intake in rats. International Journal of *Neuropsychopharmacology, 18*(2), pyu011.

Caballero-Gaudes, C., & Reynolds, R. C. (2017). Methods for cleaning the BOLD fMRI signal. *Neuroimage, 154,* 128-149.

Calabresi, P., Centonze, D., Gubellini, P., Pisani, A., & Bernardi, G. (2000). Acetylcholine-mediated modulation of striatal function. *Trends in Neurosciences, 23*(3), 120-126. doi:10.1016/S0166-2236(99)01501-5

Calderan, L., Chiamulera, C., Marzola, P., Fabene, P. F., Fumagalli, G. F., & Sbarbati, A. (2005). Sub-chronic nicotine-induced changes in regional cerebral blood volume and transversal relaxation time patterns in the rat: A magnetic resonance study.

Neuroscience Letters, 377(3), 195-199. doi:http://dx.doi.org/10.1016/j.neulet.2004.12.001

Callado, L. F., Hopwood, S. E., Hancock, P. J., & Stamford, J. A. (2000). Effects of dizocilpine (MK 801) on noradrenaline, serotonin and dopamine release and uptake. *Neuroreport, 11*(1), 173-176.

Campagna, J. A., Miller, K. W., & Forman, S. A. (2003). Mechanisms of actions of inhaled anesthetics. *New England Journal of Medicine, 348*(21), 2110-2124.

Cao, J., Nesil, T., Wang, S., Chang, S. L., & Li, M. D. (2016). Expression profile of nicotinic acetylcholine receptor subunits in the brain of HIV-1 transgenic rats given chronic nicotine treatment. *Journal of Neurovirology,* , 1-8. doi:10.1007/s13365-016-0438-3

Carlsson, A. (1988). The current status of the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology,* 1(3), 179-186.

Carter, C. S., Braver, T. S., Barch, D. M., Botvinick, M. M., Noll, D., & Cohen, J. D. (1998). Anterior cingulate cortex, error detection, and the online monitoring of performance. *Science (New York, N.Y.), 280*(5364), 747-749.

Castañé, A., Santana, N., & Artigas, F. (2015). PCP-based mice models of schizophrenia: Differential behavioral, neurochemical and cellular effects of acute and subchronic treatments. *Psychopharmacology,* doi:10.1007/s00213-015-3946-6

Cellard, C., Tremblay, S., Lehoux, C., & Roy, M. A. (2007). Processing spatial–temporal information in recent-onset schizophrenia: the study of short-term memory and its susceptibility to distraction. Brain and cognition, 64(3), 201-207.

Center for Substance Abuse Treatment. (1999). —How Stimulants Affect the Brain and Behavior.

Cepeda, C., Buchwald, N. A., & Levine, M. S. (1993). Neuromodulatory actions of dopamine in the neostriatum are dependent upon the excitatory amino acid receptor subtypes activated. *Proceedings of the National Academy of Sciences of the United States of America, 90*(20), 9576-9580.

Cespuglio, R., Faradji, H., Hahn, Z., & Jouvet, M. (1984). Voltammetric detection of brain 5-hydroxyindoleamines by means of electrochemically treated carbon fibre electrodes: Chronic recordings for up to one month with movable cerebral electrodes in the sleeping or waking rat. Measurement of Neurotransmitter Release in Vivo. (ed. C.A. Marsden), J.Wiley&Sons, 173-191.

Chase, T., Katz, R., & Kopin, I. (1969). Release of [3h] serotonin from brain slices1. *Journal of Neurochemistry*, *16*(4), 607-615.

Cheer, J. F., Wassum, K. M., & Wightman, R. M. (2006). Cannabinoid modulation of electrically evoked pH and oxygen transients in the nucleus accumbens of awake rats. *Journal of Neurochemistry, 97*(4), 1145-1154.

Chen, Y. I., Choi, J., Andersen, S. L., Rosen, B. R., & Jenkins, B. G. (2005)(a). Mapping dopamine D2/D3 receptor function using pharmacological magnetic resonance imaging. *Psychopharmacology*, *180*(4), 705-715.

Chen, Z., Silva, A. C., Yang, J., & Shen, J. (2005)(b). Elevated endogenous GABA level correlates with decreased fMRI signals in the rat brain during acute inhibition of GABA transaminase. *Journal of Neuroscience Research, 79*(3), 383-391.

Choi, J. -., Mandeville, J. B., Iris Chen, Y., Kim, Y. R., & Jenkins, B. G. (2006). High resolution spatial mapping of nicotine action using pharmacologic magnetic resonance imaging. *Synapse, 60*(2), 152-157. doi:10.1002/syn.20284

Clark, J. J., Sandberg, S. G., Wanat, M. J., Gan, J. O., Horne, E. A., Hart, A. S., . . . Phillips, P. E. M. (2010). Chronic microsensors for longitudinal, subsecond dopamine detection in behaving animals. *Nature Methods, 7*(2), 126-129.

Clarke, P., Hommer, D., Pert, A., & Skirboll, L. (1987). Innervation of substantia nigra neurons by cholinergic afferents from pedunculopontine nucleus in the rat: Neuroanatomical and electrophysiological evidence. *Neuroscience, 23*(3), 1011-1019.

Clarke, P. B. S., & Kumar, R. (1983). Characterization of the locomotor stimulant action of nicotine in tolerant rats. British journal of pharmacology, 80(3), 587-594.

Collins, A. C., Romm, E., & Wehner, J. M. (1988). Nicotine tolerance: an analysis of the time course of its development and loss in the rat. Psychopharmacology, 96(1), 7-14.

Collins, A. C., Romm, E., & Wehner, J. M. (1990). Dissociation of the apparent relationship between nicotine tolerance and up-regulation of nicotinic receptors. Brain research bulletin, 25(3), 373-379.

Conn, P. J., & Pin, J. -. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annual Review of Phamacology and Toxicology*. 205-237.

Cortese, B. M., & Phan, K. L. (2005). The role of glutamate in anxiety and related disorders. *CNS Spectrums, 10*(10), 820-830.

Cotman, C. W., & Monaghan, D. T. (1988). Excitatory amino acid neurotransmission: NMDA receptors and hebb-type synaptic plasticity. *Annual Review of Neuroscience, 11*(1), 61-80.

Couturier, S., Bertrand, D., Matter, J., Hernandez, M., Bertrand, S., Millar, N., . . . Ballivet, M. (1990). A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron, 5*(6), 847-856. doi:http://dx.doi.org/10.1016/0896-6273(90)90344-F

Coyle, J. T. (2006). Glutamate and schizophrenia: Beyond the dopamine hypothesis. *Cellular and Molecular Neurobiology, 26*(4-6), 365-384.

Cragg, S. J. (2006). Meaningful silences: How dopamine listens to the ACh pause. *Trends in Neurosciences, 29*(3), 125-131. doi:10.1016/j.tins.2006.01.003

Cragg, S. J., & Rice, M. E. (2004). DAncing past the DAT at a DA synapse. *TRENDS in Neuroscience,* 270-277. doi:https://doi.org/10.1016/j.tins.2004.03.011

Cull-Candy, S. G., & Leszkiewicz, D. N. (2004). Role of distinct NMDA receptor subtypes at central synapses. Science's STKE : *Signal Transduction Knowledge Environment, 2004*(255), re16. doi:stke.2552004re16 [pii]

Dani JA, Bertrand. D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Review of Pharmacology and Toxicology, 47:699-729.*, 699-729.

Del Arco, A., Mora, F., Mohammed, A., & Fuxe, K. (2007). Stimulation of D2 receptors in the prefrontal cortex reduces PCP-induced hyperactivity, acetylcholine release

and dopamine metabolism in the nucleus accumbens. *Journal of Neural Transmission, 114*(2), 185-193.

Deuker, L., Bullmore, E. T., Smith, M., Christensen, S., Nathan, P. J., Rockstroh, B., & Bassett, D. S. (2009). Reproducibility of graph metrics of human brain functional networks. *Neuroimage, 47*(4), 1460-1468.

Deutch, A. Y., Tam, S. Y., Freeman, A. S., Bowers Jr, M. B., & Roth, R. H. (1987). Mesolimbic and mesocortical dopamine activation induced by phencyclidine: contrasting pattern to striatal response. European journal of pharmacology, 134(3), 257-264.

Deutch, A. Y. (1993). Prefrontal cortical dopamine systems and the elaboration of functional corticostriatal circuits: Implications for schizophrenia and parkinson's disease. *Journal of Neural Transmission - General Section, 91*(2-3), 197-221.

Dettmers, C., Hartmann, A., Rommel, T., Krämer, S., Pappata, S., Young, A., ... & Baron, J. C. (1994). Immersion and perfusion staining with 2, 3, 5-triphenyltetrazolium chloride (TTC) compared to mitochondrial enzymes 6 hours after MCA-occlusion in primates. Neurological research, 16(3), 205-208.

Dickinson, J. A., Kew, J. N., & Wonnacott, S. (2008). Presynaptic alpha 7- and beta 2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. *Molecular Pharmacology, 74*(2), 348-359. doi:10.1124/mol.108.046623 [doi]

Ding, X., Qiao, Y., Piao, C., Zheng, X., Liu, Z., & Liang, J. (2014). N -methyl-D-aspartate receptor-mediated glutamate transmission in nucleus accumbens plays a

more important role than that in dorsal striatum in cognitive flexibility. *Frontiers in Behavioral Neuroscience, 8*(SEP)

Dixon, A., Prior, M., Morris, P., Shah, Y., Joseph, M., & Young, A. (2005). Dopamine antagonist modulation of amphetamine response as detected using pharmacological MRI. *Neuropharmacology, 48*(2), 236-245.

Dodick, D. W., & Gargus, J. J. (2008). Why migraines strike. *Scientific American, 299(2),* 56-63.

Doherty, J. D., Simonovic, M., So, R., & Meltzer, H. Y. (1980). The effect of phencyclidine on dopamine synthesis and metabolism in rat striatum. *European Journal of Pharmacology, 65*(2-3), 139-149.

du Sert, N. P., Bamsey, I., Bate, S. T., Berdoy, M., Clark, R. A., Cuthill, I., . . . Moon, L. (2017). The experimental design assistant. *PLoS Biology, 15*(9), e2003779.

Dwoskin, L., Jewell, A., Buxton, S., & Carney, J. (1992). Phencyclidine-induced desensitization of striatal dopamine release. *Neuropharmacology*, 31(10), 1033-1039.

Eckenstein, F., & Thoenen, H. (1983). Cholinergic neurons in the rat cerebral cortex demonstrated by immunohistochemical localization of choline acetyltransferase. *Neuroscience Letters, 36*(3), 211-215.

Ellenbroek, B. A., van den Kroonenberg, P. T., & Cools, A. R. (1998). The effects of an early stressful life event on sensorimotor gating in adult rats. Schizophrenia research, 30(3), 251-260.

Elsworth, J. D., Groman, S. M., Jentsch, J. D., Leranth, C., Redmond, D. E., Kim, J. D., ... & Roth, R. H. (2015). Primate phencyclidine model of schizophrenia: sex-specific effects on cognition, brain derived neurotrophic factor, spine synapses, and dopamine turnover in prefrontal cortex. International Journal of Neuropsychopharmacology, 18(6).

Ennaceur, A., & Delacour, J. (1988). A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behavioural brain research, 31(1), 47-59.

Escobar, A. P., Cornejo, F. A., Olivares-Costa, M., González, M., Fuentealba, J. A., Gysling, K., . . . Andrés, M. E. (2015). Reduced dopamine and glutamate neurotransmission in the nucleus accumbens of quinpirole-sensitized rats hints at inhibitory D2 autoreceptor function. *Journal of Neurochemistry*, *134*(6), 1081-1090. doi:10.1111/jnc.13209

Exley, R., & Cragg, S. (2008). Presynaptic nicotinic receptors: A dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. *British Journal of Pharmacology*, *153*(S283-297).

Exley, R., Clements, M. A., Hartung, H., McIntosh, J. M., & Cragg, S. J. (2008). a6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. *Neuropsychopharmacology, 33*(9), 2158-2166. doi:10.1038/sj.npp.1301617

Febo, M., & Pira, A. S. (2011). Increased BOLD activation to predator stressor in subiculum and midbrain of amphetamine-sensitized maternal rats. *Brain Research, 1382*, 118-127. doi:http://dx.doi.org/10.1016/j.brainres.2010.11.092

Ferrari, R., Le Novère, N., Picciotto, M. R., Changeux, J. P., & Zoli, M. (2002). Acute and long-term changes in the mesolimbic dopamine pathway after systemic or local single nicotine injections. *European Journal of Neuroscience, 15*(11), 1810-1818. doi:10.1046/j.1460-9568.2001.02009.x

Ferris, M. J., Milenkovic, M., Liu, S., Mielnik, C. A., Beerepoot, P., John, C. E., . . . Ramsey, A. J. (2014). Sustained N-methyl-d-aspartate receptor hypofunction remodels the dopamine system and impairs phasic signaling. *European Journal of Neuroscience, 40*(1), 2255-2263.

Flecknell, P. A. (1987). Laboratory animal anaesthesia. an introduction for research workers and technicians. Academic Press.

Fox, M. D., & Raichle, M. E. (2007). Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. Nature reviews neuroscience, 8(9), 700.

Friston, K. J., Harrison, L., & Penny, W. (2003). Dynamic causal modelling. *Neuroimage, 19*(4), 1273-1302.

Fuxe, K., Borroto-Escuela, D. O., Tarakanov, A., Fernandez, W. R., Manger, P., Rivera, A., . . . Filip, M. (2013). Understanding the balance and integration of volume and synaptic transmission. relevance for psychiatry. *Neurology, Psychiatry and Brain Research, 19*(4), 141-158.

Garcia-Munoz, M., Lopez-Huerta, V. G., Carrillo-Reid, L., & Arbuthnott, G. W. (2014). Extrasynaptic glutamate NMDA receptors: Key players in striatal function. *Neuropharmacology, 89*, 54-63.

Goulart, B. K., De Lima, M. N. M., De Farias, C. B., Reolon, G. K., Almeida, V. R., Quevedo, J., ... & Roesler, R. (2010). Ketamine impairs recognition memory consolidation and prevents learning-induced increase in hippocampal brain-derived neurotrophic factor levels. Neuroscience, 167(4), 969-973.

Ge, S., & Dani, J. A. (2005). Nicotinic acetylcholine receptors at glutamate synapses facilitate long-term depression or potentiation. *Journal of Neuroscience, 25*(26), 6084-6091. doi:10.1523/JNEUROSCI.0542-05.2005

Gibbons, A. S., Thomas, E. A., & Dean, B. (2009). Regional and duration of illness differences in the alteration of NCAM-180 mRNA expression within the cortex of subjects with schizophrenia. Schizophrenia research, 112(1-3), 65-71.

Goldlust, E. J., Paczynski, R. P., He, Y. Y., Hsu, C. Y., & Goldberg, M. P. (1996). Automated measurement of infarct size with scanned images of triphenyltetrazolium chloride-stained rat brains. Stroke, 27(9), 1657-1662.

Gotti, C., Clementi, F., Fornari, A., Gaimarri, A., Guiducci, S., Manfredi, I., . . . Zoli, M. (2009). Structural and functional diversity of native brain neuronal nicotinic receptors. Biochemical Pharmacology, 703-711.doi:https://doi.org/10.1016/j.bcp.2009.05.024

Goutier, W., Lowry, J., McCreary, A., & O'Connor, J. (2016). Frequency-dependent modulation of dopamine release by nicotine and dopamine D1 receptor ligands: An in vitro fast cyclic voltammetry study in rat striatum. *Neurochemical Research, 41*(5), 945-950.

Gozzi, A., Herdon, H., Schwarz, A., Bertani, S., Crestan, V., Turrini, G., & Bifone, A. (2008). Pharmacological stimulation of NMDA receptors via co-agonist site suppresses fMRI response to phencyclidine in the rat. *Psychopharmacology, 201*(2), 273-284.

Gozzi, A., Large, C. H., Schwarz, A., Bertani, S., Crestan, V., & Bifone, A. (2008). Differential effects of antipsychotic and glutamatergic agents on the phMRI response to phencyclidine. *Neuropsychopharmacology, 33*(7), 1690-1703.

Gozzi, A., Schwarz, A., Crestan, V., & Bifone, A. (2008). Drug–anaesthetic interaction in phMRI: The case of the psychotomimetic agent phencyclidine. *Magnetic Resonance Imaging, 26*(7), 999-1006.

Grace, A. A. (1991). Phasic versus tonic dopamine release and the modulation of dopamine system responsivity: A hypothesis for the etiology of schizophrenia. *Neuroscience,* 1-24. doi:https://doi.org/10.1016/0306-4522(91)90196-U

Grace, A. A. (2000). The tonic/phasic model of dopamine system regulation and its implications for understanding alcohol and psychostimulant craving. *Addiction, 95*(8s2), 119-128.

Grace, A. A., & Onn, S. P. (1989). Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded in vitro. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 9*(10), 3463-3481.

Grace, A., & Bunney, B. (1983). Intracellular and extracellular electrophysiology of nigral dopaminergic neurons—3. evidence for electrotonic coupling. *Neuroscience, 10*(2), 333-348.

Gracy, K. N., & Pickel, V. M. (1996). Ultrastructural immunocytochemical localization of the N-methyl-D-aspartate receptor and tyrosine hydroxylase in the shell of the rat nucleus accumbens. *Brain Research, 739*(1), 169-181.

Graybiel, A. M. (1990). Neurotransmitters and neuromodulators in the basal ganglia. *Trends in Neurosciences, 13*(7), 244-254. doi:http://dx.doi.org/10.1016/0166-2236(90)90104-I

Grayson, B., Adamson, L., Harte, M., Leger, M., Marsh, S., Piercy, C., & Neill, J. C. (2014). The involvement of distraction in memory deficits induced by NMDAR antagonism: Relevance to cognitive deficits in schizophrenia. *Behavioural Brain Research, 266*, 188-192.

Greengard, P. (2001). The neurobiology of slow synaptic transmission. *Science, 294*(5544), 1024-1030. doi:10.1126/science.294.5544.1024

Gross, N. B., & Marshall, J. F. (2009). Striatal dopamine and glutamate receptors modulate methamphetamine-induced cortical fos expression. *Neuroscience, 161*(4), 1114-1125.

Gross, N. B., & Marshall, J. F. (2009). Striatal dopamine and glutamate receptors modulate methamphetamine-induced cortical fos expression. *Neuroscience, 161*(4), 1114-1125.

Grupe, M., Paolone, G., Jensen, A. A., Sandager-Nielsen, K., Sarter, M., & Grunnet, M. (2013). Selective potentiation of (α4)3(β2)2 nicotinic acetylcholine receptors augments amplitudes of prefrontal acetylcholine- and nicotine-evoked glutamatergic transients in rats. *Biochemical Pharmacology*, 86(10), 1487-1496. doi:https://doi.org/10.1016/j.bcp.2013.09.005

Guo, J. -., Tredway, T. L., & Chiappinelli, V. A. (1998). Glutamate and GABA release are enhanced by different subtypes of presynaptic nicotinic receptors in the lateral geniculate nucleus. *Journal of Neuroscience, 18*(6), 1963-1969.

Haber, S. N., Fudge, J. L., & McFarland, N. R. (2000). Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum. *Journal of Neuroscience*, *20*(6), 2369-2382.

Hahn, B., Sharples, C. G. V., Wonnacott, S., Shoaib, M., & Stolerman, I. P. (2003). Attentional effects of nicotinic agonists in rats. *Neuropharmacology*, *44*(8), 1054-1067. doi:http://dx.doi.org/10.1016/S0028-3908(03)00099-6

Hammond, R. S., Tull, L. E., & Stackman, R. W. (2004). On the delay-dependent involvement of the hippocampus in object recognition memory. Neurobiology of learning and memory, 82(1), 26-34.

Hansen, T. D., Warner, D. S., Todd, M. M., Vust, L. J., & Trawick, D. C. (1988). Distribution of cerebral blood flow during halothane versus isoflurane anesthesia in rats. *Anesthesiology, 69*(3), 332-337.

Hardingham, G. E., Fukunaga, Y., & Bading, H. (2002). Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nature Neuroscience, 5*(5), 405-414.

Heimer, L. (1975). The subcortical projections of allocortex: similarities in the neuronal associations of the hippocampus, the piriform cortex and the neocortex. In *Golgi centennial symposium proceedings* (pp. 173-193). Raven.

Herkenham, M. (1987). Mismatches between neurotransmitter and receptor localizations in brain: Observations and implications. *Neuroscience, 23*(1), 1-38.

Hernandez, L., Auerbach, S., & Hoebel, B. G. (1988). Phencyclidine (PCP) injected in the nucleus accumbens increases extracellular dopamine and serotonin as measured by microdialysis. *Life Science, 42(18), 1713-1723.* doi:http://dx.doi.org/10.1016/0024-3205(88)90037-9

Hillman, H., & McIlwain, H. (1961). Membrane potentials in mammalian cerebral tissues in vitro: Dependence on ionic environment. *The Journal of Physiology, 157*(2), 263-278.

Hillman, H., Campbell, W., & McIlwain, H. (1963). Membrane potentials in isolated and electrically stimulated mammalian cerebral cortex. effects of chlorpromazine, cocaine, phenobarbitone and protamine on the tissue's electrical and chemical responses to stimulation. *Journal of Neurochemistry, 10*(5), 325-339.

Hitzemann, R. J., Loh, H. H., & Domino, E. F. (1973). Effect of phencyclidine on the accumulation of 14 C-catecholamines formed from 14 C-tyrosine. *Archives Internationales De Pharmacodynamie Et De Therapie, 202*(2), 252-258.

Hluštík, P., Noll, D. C., & Small, S. L. (1998). Suppression of vascular artifacts in functional magnetic resonance images using MR angiograms. *NeuroImage*, 7(3), 224-231. doi:https://doi.org/10.1006/nimg.1998.0320

Hodgkin, A. L., & Huxley, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. The Journal of physiology, 117(4), 500-544.

Hoebel, B. G., Avena, N. M., & Rada, P. (2007). Accumbens dopamine-acetylcholine balance in approach and avoidance. *Current Opinion in Pharmacology*, 7 (6), 617-627. doi:https://doi.org/10.1016/j.coph.2007.10.014

Hondo, H., Yonezawa, Y., Nakahara, T., Nakamura, K., Hirano, M., Uchimura, H., & Tashiro, N. (1994). Effect of phenycyclidine on dopamine release in the rat prefrontal cortex; an in vivo microdialysis study. *Brain Research, 633*(1-2), 337-342. *doi:10.1016/0006-8993(94)91558-X*

Howard, E., Schier, C., Wetzel, J., Duvauchelle, C., & Gonzales, R. (2008). The shell of the nucleus accumbens has a higher dopamine response compared with the core after non-contingent intravenous ethanol administration. *Neuroscience, 154*(3), 1042-1053.

Hu, G., Duffy, P., Swanson, C., Ghasemzadeh, M. B., & Kalivas, P. W. (1999). The regulation of dopamine transmission by metabotropic glutamate receptors. *The Journal of Pharmacology and Experimental Therapeutics, 289*(1), 412-416.

Huang, W., Tam, K., Fernando, J., Heffernan, M., King, J., & DiFranza, J. R. (2015). Nicotine and resting-state functional connectivity: Effects of intermittent doses. *Nicotine & Tobacco Research, 17*(11), 1311-1317.c

Huang, Y. H., Ishikawa, M., Lee, B. R., Nakanishi, N., Schlüter, O. M., & Dong, Y. (2011). Searching for presynaptic NMDA receptors in the nucleus accumbens. Journal of Neuroscience, 31(50), 18453-18463.

Hutchison, R. M., Leung, L. S., Mirsattari, S. M., Gati, J. S., Menon, R. S., & Everling, S. (2011). Resting-state networks in the macaque at 7T. *NeuroImage*, 56 (3), 1546-1555. doi:https://doi.org/10.1016/j.neuroimage.2011.02.063

Iannetti, G., & Wise, R. G. (2007). BOLD functional MRI in disease and pharmacological studies: Room for improvement? *Magnetic Resonance Imaging, 25*(6), 978-988.

Iraji, A., Calhoun, V. D., Wiseman, N. M., Davoodi-Bojd, E., Avanaki, M. R. N., Haacke, E. M., & Kou, Z. (2016). The connectivity domain: Analyzing resting state fMRI data using feature-based data-driven and model-based methods. *NeuroImage*, 494-507. doi:https://doi.org/10.1016/j.neuroimage.2016.04.006

Iravani, M. M., & Kruk, Z. L. (1996). Real-time effects of N-methyl-D-aspartic acid on dopamine release in slices of rat caudate putamen: A study using fast cyclic voltammetry. *Journal of Neurochemistry, 66*(3), 1076-1085.

Ito, R., Robbins, T. W., & Everitt, B. J. (2004). Differential control over cocaine-seeking behavior by nucleus accumbens core and shell. *Nature Neuroscience, 7*(4), 389-397. doi:10.1038/nn1217

Javitt, D. C. (1987). Negative chronic symptomotology and the PCP (Phencyclidine) model of schizophrenia. *The hill side journal of clinical psychology, 9* (1), 12-35.

Javitt, D. C. (2007). Glutamate and schizophrenia: Phencyclidine, N-Methyl-d-Aspartate receptors, and Dopamine–Glutamate interactions. *International Review of Neurobiology*, *78*, 69-108. doi:http://dx.doi.org/10.1016/S0074-7742(06)78003-5

Javitt, D. C., & Zukin, S. R. (1990). The role of excitatory amino acids in neuropsychiatric illness. *Journal of Neuropsychiatry and Clinical Neurosciences, 2*(1), 44-52.

Javitt, D. C., Balla, A., Burch, S., Suckow, R., Xie, S., & Sershen, H. (2004). Reversal of phencyclidine-induced dopaminergic dysregulation by N-methyl-D-aspartate receptor/glycine-site agonists. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *29*(2), 300-307. doi:10.1038/sj.npp.1300313 [doi]

Javitt, D. C., Balla, A., Sershen, H., & Lajtha, A. (1999). Reversal of phencyclidine-induced effects by glycine and glycine transport inhibitors. *Biological Psychiatry*, *45*(6), 668-679. doi:http://dx.doi.org/10.1016/S0006-3223(98)00237-6

Jenkins, B. G. (2012). Pharmacologic magnetic resonance imaging (phMRI): Imaging drug action in the brain. *Neuroimage*, *62*(2), 1072-1085. doi:http://dx.doi.org/10.1016/j.neuroimage.2012.03.075

Jenkinson, M. and Smith, S.M.. A global optimisation method for robust affine registration of brain images. *Medical Image Analysis*, 5(2):143-156, 2001.

Jenkinson, M., Beckmann, C.F., Behrens, T.E., Woolrich, M.W., Smith, S.M. FSL. *NeuroImage,* 62:782-90, 2012

Jentsch, J. D., Elsworth, J. D., Redmond Jr, D. E., & Roth, R. H. (1997). Phencyclidine increases forebrain monoamine metabolism in rats and monkeys: modulation by the isomers of HA966. Journal of Neuroscience, 17(5), 1769-1775.

Jentsch, J. D., & Roth, R. H. (1999). The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology, 20(3), 201-225.

Jones, L. J., McCutcheon, J. E., Young, A. M., & Norton, W. H. (2015). Neurochemical measurements in the zebrafish brain. *Frontiers in Behavioral Neuroscience, 9,* 246. doi:10.3389/fnbeh.2015.00246 [doi]

Jones, S. M., Snell, L. D., & Johnson, K. M. (1987). Inhibition by phencyclidine of excitatory amino acid-stimulated release of neurotransmitter in the nucleus accumbens. *Neuropharmacology, 26*(2–3), 173-179. doi:http://dx.doi.org/10.1016/0028-3908(87)90206-1

Jones, S. M., Snell, L. D., & Johnson, K. M. (1987). Phencyclidine selectively inhibits N-methyl-D-aspartate-induced hippocampal [3H]norepinephrine release. *The Journal of Pharmacology and Experimental Therapeutics, 240*(2), 492-497.

Jones, G. H., Hernandez, T. D., Kendall, D. A., Marsden, C. A., & Robbins, T. W. (1992). Dopaminergic and serotonergic function following isolation rearing in rats: study of behavioural responses and postmortem and in vivo neurochemistry. Pharmacology Biochemistry and Behavior, 43(1), 17-35.

Kantrowitz, J. T., & Javitt, D. C. (2010). N-methyl-d-aspartate (NMDA) receptor dysfunction or dysregulation: The final common pathway on the road to schizophrenia? *Brain Research Bulletin, 83*(3), 108-121.

Karasawa, J. -., Yoshimizu, T., & Chaki, S. (2006). A metabotropic glutamate 2/3 receptor antagonist, MGS0039, increases extracellular dopamine levels in the nucleus accumbens shell. *Neuroscience Letters, 393*(2-3), 127-130.

Karch, S., Leicht, G., Giegling, I., Lutz, J., Kunz, J., Buselmeier, M., . . . Meindl, T. (2009). Inefficient neural activity in patients with schizophrenia and nonpsychotic relatives of schizophrenic patients: Evidence from a working memory task. *Journal of Psychiatric Research, 43*(15), 1185-1194.

Karlsson, R., Tanaka, K., Saksida, L. M., Bussey, T. J., Heilig, M., & Holmes, A. (2008). Assessment of glutamate transporter GLAST (EAAT1)-deficient mice for phenotypes relevant to the negative and Executive/Cognitive symptoms of schizophrenia. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology, 34*(6), 1578-1589.

Keefe, K. A., Zigmond, M. J., & Abercrombie, E. D. (1992). Extracellular dopamine in striatum: Influence of nerve impulse activity in medial forebrain bundle and local glutamatergic input. *Neuroscience, 47*(2), 325-332.

Keithley, R. B., Takmakov, P., Bucher, E. S., Belle, A. M., Owesson-White, C. A., Park, J., & Wightman, R. M. (2011). Higher sensitivity dopamine measurements with faster-scan cyclic voltammetry. *Analytical Chemistry, 83*(9), 3563-3571.

Kemel, M. L., Gauchy, C., Desban, M., Krebs, M. O., & Glowinski, J. (1992). Control of dopamine release by acetylcholine and dynorphin in the striosomal and matrix compartments of the cat caudate nucleus. *Neurochemistry International, 20*(SUPPL.), 111-114. doi:10.1016/0197-0186(92)90221-C

Kim, S., Kaufman, M. J., Cohen, B. M., Jensen, J. E., Coyle, J. T., Du, F., & Öngür, D. (2017). In Vivo brain glycine and glutamate concentrations in patients with first-episode psychosis measured by echo Time–Averaged proton magnetic resonance spectroscopy at 4T. *Biological Psychiatry, In press, Corrected Proof.* doi:https://doi.org/10.1016/j.biopsych.2017.08.022

Kissinger, P. T., & Heineman, W. R. (1983). Cyclic voltammetry. *Journal of Chemical Education, 60*(9), 702-706.

Klink, R., De Kerchove D'Exaerde, A., Zoli, M., & Changeux, J. -. (2001). Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *Journal of Neuroscience, 21*(5), 1452-1463.

Kohl, R. R., Katner, J. S., Chernet, E., & McBride, W. J. (1998). Ethanol and negative feedback regulation of mesolimbic dopamine release in rats. Psychopharmacology, 139(1-2), 79-85.

Kosillo, P., Zhang, Y., Threlfell, S., & Cragg, S. J. (2016). Cortical control of striatal dopamine transmission via striatal cholinergic interneurons. *Cerebral Cortex, 26*(11), 4160-4169.

Kotecha, S. A., Oak, J. N., Jackson, M. F., Perez, Y., Orser, B. A., Van Tol, H. H. M., & MacDonald, J. F. (2002). A D2 class dopamine receptor transactivates a receptor

tyrosine kinase to inhibit NMDA receptor transmission. *Neuron, 35*(6), 1111-1122. doi:10.1016/S0896-6273(02)00859-0

Kretschmer, B. D. (1999). Modulation of the mesolimbic dopamine system by glutamate: Role of NMDA receptors. *Journal of Neurochemistry, 73*(2), 839-848. doi:10.1046/j.1471-4159.1999.0730839.x

Krimer, L. S., Muly, E. C., Williams, G. V., & Goldman-Rakic, P. S. (1998). Dopaminergic regulation of cerebral cortical microcirculation. *Nature Neuroscience, 1*(4), 286-289.

Krnjevic, K., & Phillis, J. W. (1963). Iontophoretic studies of neurones in the mammalian cerebral cortex. *The Journal of Physiology, 165*, 274-304.

Kulagina, N. V., Zigmond, M. J., & Michael, A. C. (2001). Glutamate regulates the spontaneous and evoked release of dopamine in the rat striatum. *Neuroscience, 102*(1), 121-128.

Lacourse, W and Mark P . Olson. (2004). Chapter 17. voltammetry. In Jack Cazes (Ed.), Analytical instrumentation handbook, (Third Edition ed., pp. 529-544). USA: CRC Press. doi:10.1201/9780849390395.ch17

Lacy, R. T., Brown, R. W., Morgan, A. J., Mactutus, C. F., & Harrod, S. B. (2016). Intravenous prenatal nicotine exposure alters METH-induced hyperactivity, conditioned hyperactivity, and BDNF in adult rat offspring. *Developmental Neuroscience,* doi:10.1159/000446563

Lapper, S., & Bolam, J. (1992). Input from the frontal cortex and the parafascicular nucleus to cholinergic interneurons in the dorsal striatum of the rat. *Neuroscience, 51*(3), 533-545.

Lehre, K. P., & Danbolt, N. C. (1998). The number of glutamate transporter subtype molecules at glutamatergic synapses: Chemical and stereological quantification in young adult rat brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 18*(21), 8751-8757.

Li, S. -., & Ju, J. -. (2012). Functional roles of synaptic and extrasynaptic NMDA receptors in physiological and pathological neuronal activities. *Current Drug Targets, 13*(2), 207-221. doi:10.2174/138945012799201630

Li, F., Irie, K., Anwer, M. S., & Fisher, M. (1997). Delayed triphenyltetrazolium chloride staining remains useful for evaluating cerebral infarct volume in a rat stroke model. Journal of Cerebral Blood Flow & Metabolism, 17(10), 1132-1135.

Liang, X., Zou, Q., He, Y., & Yang, Y. (2013). Coupling of functional connectivity and regional cerebral blood flow reveals a physiological basis for network hubs of the human brain. *Proceedings of the National Academy of Sciences of the United States of America, 110*(5), 1929-1934. doi:10.1073/pnas.1214900110 [doi]

Liljequist, S. (1991). NMDA receptor antagonists inhibit ethanol-produced locomotor stimulation in NMRI mice. *Alcohol,* 8(4), 309-312. doi:https://doi.org/10.1016/0741-8329(91)90449-7

Linden, D. J., Dickinson, M. H., Smeyne, M., & Connor, J. A. (1991). A long-term depression of AMPA currents in cultured cerebellar purkinje neurons. *Neuron, 7*(1), 81-89.

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., ... & Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science, 277(5332), 1659-1662.

Lipska, B. K., Jaskiw, G. E., & Weinberger, D. R. (1993). Postpubertal emergence of hyperresponsiveness to stress and to amphetamine after neonatal excitotoxic hippocampal damage: a potential animal model of schizophrenia. Neuropsychopharmacology, 9(1), 67.

Lopez-Hernandez, G. Y., Sanchez-Padilla, J., Ortiz-Acevedo, A., Lizardi-Ortiz, J., Salas-Vincenty, J., Rojas, L. V., & Lasalde-Dominicci, J. A. (2004). Nicotine-induced up-regulation and desensitization of alpha4beta2 neuronal nicotinic receptors depend on subunit ratio. *The Journal of Biological Chemistry, 279*(36), 38007-38015. doi:10.1074/jbc.M403537200 [doi]

Maex, R., Grinevich, V. P., Grinevich, V., Budygin, E., Bencherif, M., & Gutkin, B. (2014). Understanding the role α7 nicotinic receptors play in dopamine efflux in nucleus accumbens. *ACS Chemical Neuroscience, 5*(10), 1032-1040.

Mandeville, J. B. (2012). IRON fMRI measurements of CBV and implications for BOLD signal. *Neuroimage, 62*(2), 1000-1008.

Mansvelder, H. D., & McGehee, D. S. (2000). Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron, 27*(2), 349-357.

Mansvelder, H. D., Keath, J. R., & McGehee, D. S. (2002). Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron, 33*(6), 905-919. doi:http://dx.doi.org/10.1016/S0896-6273(02)00625-6

Marcott, P. F., Gong, S., Donthamsetti, P., Grinnell, S. G., Nelson, M. N., Newman, A. H., ... & Ford, C. P. (2018). Regional heterogeneity of D2-receptor signaling in the dorsal striatum and nucleus accumbens. Neuron, 98(3), 575-587.

Marcus, M. M., Mathé, J. M., Nomikos, G. G., & Svensson, T. H. (2001). Effects of competitive and non-competitive NMDA receptor antagonists on dopamine output in the shell and core subdivisions of the nucleus accumbens. *Neuropharmacology, 40*(4), 482-490.

Marks, M. J., Burch, J. B., & Collins, A. C. (1983). Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. *The Journal of Pharmacology and Experimental Therapeutics, 226*(3), 817-825.

Mathie, A., Colquhoun, D., & Cull-Candy, S. G. (1990). Rectification of currents activated by nicotinic acetylcholine receptors in rat sympathetic ganglion neurones. The Journal of Physiology, 427(1), 625-655.

McCullough, L. D., & Salamone, J. D. (1992). Increases in extracellular dopamine levels and locomotor activity after direct infusion of phencyclidine into the nucleus accumbens. *Brain Research, 577*(1), 1-9.F

McIlwain, H. (1955). In Neurochemistry, ed. Elliott, KAC, Page, JH & Quastel, JHCC Illinois: Thomas, Springfield.

Mcllwain, H., & Snyder, S. H. (1970). Stimulation of piriform and neocortical tissues in an in vitro flow-system: Metabolic properties and relase of putative neurotransmitters. *Journal of Neurochemistry*, 17, 521-530.

Mela, F., Marti, M., Fiorentini, C., Missale, C., & Morari, M. (2006). Group-II metabotropic glutamate receptors negatively modulate NMDA transmission at striatal cholinergic terminals: Role of P/Q-type high voltage activated ca++ channels and endogenous dopamine. *Molecular and Cellular Neuroscience, 31*(2), 284-292. doi:http://dx.doi.org/10.1016/j.mcn.2005.09.016

Melander, T., Staines, W. A., Hökfelt, T., Rökaeus, Å., Eckenstein, F., Salvaterra, P., & Wainer, B. (1985). Galanin-like immunoreactivity in cholinergic neurons of the septum-basal forebrain complex projecting to the hippocampus of the rat. *Brain Research, 360*(1), 130-138.

Melis, M., Pillolla, G., Luchicchi, A., Muntoni, A. L., Yasar, S., Goldberg, S. R., & Pistis, M. (2008). Endogenous fatty acid ethanolamides suppress nicotine-induced activation of mesolimbic dopamine neurons through nuclear receptors. Journal of Neuroscience, 28(51), 13985-13994.

Meredith, G., Blank, B., & Groenewegen, H. (1989). The distribution and compartmental organization of the cholinergic neurons in nucleus accumbens of the rat. *Neuroscience, 31*(2), 327-345.

Michael, A. C., Borland, L. M., John, C. E., & Jones, S. R. (2007). *Fast scan cyclic voltammetry of dopamine and serotonin in mouse brain slices.* Electrochemical Methods for Neuroscience (e,d. Michael and Borland). Boca Raton (FL): CRC Press/Taylor & Francis. Chapter 4.

Miller, D. W., & Abercrombie, E. D. (1996). Effects of MK-801 on spontaneous and amphetamine-stimulated dopamine release in striatum measured with in vivo microdialysis in awake rats. *Brain Research Bulletin, 40*(1), 57-62.

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., & Caron, M. G. (1998). Dopamine receptors: From structure to function. *Physiological Reviews, 78*(1), 189-225.

Mitrano, D. A., Pare, J. F., & Smith, Y. (2010). Ultrastructural relationships between cortical, thalamic, and amygdala glutamatergic inputs and group I metabotropic glutamate receptors in the rat accumbens. Journal of Comparative Neurology, 518(8), 1315-1329.

Miyauchi, M., Neugebauer, N. M., Oyamada, Y., & Meltzer, H. Y. (2016?). Nicotinic receptors and lurasidone-mediated reversal of phencyclidine-induced deficit in novel object recognition. *Behavioural Brain Research, 301*, 204-212. doi:http://dx.doi.org/10.1016/j.bbr.2015.10.044

Mouri, A., Noda, Y., Enomoto, T., & Nabeshima, T. (2007). Phencyclidine animal models of schizophrenia: approaches from abnormality of glutamatergic neurotransmission and neurodevelopment. Neurochemistry international, 51(2-4), 173-184.

Moffett, J., Tieman, S. B., Weinberger, D. R., Coyle, J. T., & Namboodiri, A. M. (2006). *N-acetylaspartate: A unique neuronal molecule in the central nervous system* Springer Science & Business Media.

Mogenson, G. J., Jones, D. L., & Yim, C. Y. (1980). From motivation to action: Functional interface between the limbic system and the motor system. *Progress in Neurobiology, 14*(2-3), 69-97.

Momiyama, A., Feldmeyer, D., & Cull-Candy, S. G. (1996). Identification of a native low-conductance NMDA channel with reduced sensitivity to Mg2+ in rat central neurones. *The Journal of Physiology, 494 (Pt 2)*(Pt 2), 479-492.

Morris, G., Arkadir, D., Nevet, A., Vaadia, E., & Bergman, H. (2004). Coincident but distinct messages of midbrain dopamine and striatal tonically active neurons. *Neuron, 43*(1), 133-143. doi:10.1016/j.neuron.2004.06.012

Mos, J., Broxterman, H. J., & Van Bennekom, W. P. (1981). In vivo voltammetric investigations into the action of HA-966 on central dopaminergic neurons. *Brain Research, 207*(2), 465-470.

Mosharov, E. V., & Sulzer, D. (2005). Analysis of exocytotic events recorded by amperometry. *Nature Methods, 2*(9)

Mugnaini, M., Tessari, M., Tarter, G., Pich, E. M., Chiamulera, C., & Bunnemann, B. (2002). Upregulation of [3H] methyllycaconitine binding sites following continuous infusion of nicotine, without changes of α7 or α6 subunit mRNA: an autoradiography and in situ hybridization study in rat brain. European Journal of Neuroscience, 16(9), 1633-1646.

Neill, J. C., Barnes, S., Cook, S., Grayson, B., Idris, N. F., McLean, S. L., . . . Harte, M. K. (2010). Animal models of cognitive dysfunction and negative symptoms of

schizophrenia: Focus on NMDA receptor antagonism. *Pharmacology & Therapeutics*, 128(3),419-432doi:https://doi.org/10.1016/j.pharmthera.2010.07.004

Nelson, M. E., Kuryatov, A., Choi, C. H., Zhou, Y., & Lindstrom, J. (2003). Alternate stoichiometries of a4ß2 nicotinic acetylcholine receptors. *Molecular Pharmacology, 63*(2), 332-341. doi:10.1124/mol.63.2.332

Neuhoff, H., Neu, A., Liss, B., & Roeper, J. (2002). I(h) channels contribute to the different functional properties of identified dopaminergic subpopulations in the midbrain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 22*(4), 1290-1302. doi:22/4/1290 [pii]

Nicola, S. M., Surmeier, D. J., & Malenka, R. C. (2000). Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. *Annual Review of Neuroscience, 23*(1), 185-215.

Nomura, T., Oyamada, Y., Fernandes, H. B., Remmers, C. L., Xu, J., Meltzer, H. Y., & Contractor, A. (2016). Subchronic phencyclidine treatment in adult mice increases GABAergic transmission and LTP threshold in the hippocampus. Neuropharmacology, 100, 90-97.

O'Neill, R. D., Chang, S., Lowry, J. P., & McNeil, C. J. (2004). Comparisons of platinum, gold, palladium and glassy carbon as electrode materials in the design of biosensors for glutamate. *Biosensors & Bioelectronics*, 19 (2004), 1521-1528.doi:https://doi.org/10.1016/j.bios.2003.12.004

Oakman, S. A., Faris, P. L., Kerr, P. E., Cozzari, C., & Hartman, B. K. (1995). Distribution of pontomesencephalic cholinergic neurons projecting to substantia

nigra differs significantly from those projecting to ventral tegmental area. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 15*(9), 5859-5869.

O'Donnell, P., & Grace, A. A. (1995). Synaptic interactions among excitatory afferents to nucleus accumbens neurons: Hippocampal gating of prefrontal cortical input. *Journal of Neuroscience, 15*(5 I), 3622-3639.

Ogawa, S., Menon, R., Tank, D., Kim, S., Merkle, H., Ellermann, J., & Ugurbil, K. (1993). Functional brain mapping by blood oxygenation level-dependent contrast magnetic resonance imaging. A comparison of signal characteristics with a biophysical model. *Biophysical Journal, 64*(3), 803-812.

Olson, C., & Adams, R. N. (1960). Carbon paste electrodes application to anodic voltammetry. *Analytica Chimica Acta*, *22*, 582-589.

O'Neill, R. D., & Lowry, J. P. (1995). On the significance of brain extracellular uric acid detected with in-vivo monitoring techniques: A review. *Behavioural Brain Research, 71*(1), 33-49.

Opacka-Juffry, J., Ahier, R. G., & Cremer, J. E. (1991). Nomifensine-induced increased in extracellular striatal dopamine is enhanced by isoflurane anaesthesia. *Synapse, 7*(2), 169-171.

Oswald, M. J., Schulz, J. M., Kelsch, W., Oorschot, D. E., & Reynolds, J. N. (2015). Potentiation of NMDA receptor-mediated transmission in striatal cholinergic interneurons. Frontiers in Cellular *Neuroscience, 9,* 116. doi:10.3389/fncel.2015.00116 [doi]

Paasonen, J., Salo, R. A., Shatillo, A., Forsberg, M. M., Närväinen, J., Huttunen, J. K., & Gröhn, O. (2016). Comparison of seven different anesthesia protocols for nicotine pharmacologic magnetic resonance imaging in rat. *European Neuropsychopharmacology, 26*(3), 518-531.

Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease. *Nature Reviews Neuroscience, 14*(6), 383-400.

Papouin, T., & Oliet, S. H. (2017). Synaptic and extra-synaptic NMDA receptors in the CNS. The NMDA receptors (pp. 19-49) Springer.

Parikh, V., Ji, J., Decker, M. W., & Sarter, M. (2010). Prefrontal ß2 subunit-containing and a7 nicotinic acetylcholine receptors differentially control glutamatergic and cholinergic signaling. *Journal of Neuroscience, 30*(9), 3518-3530. doi:10.1523/JNEUROSCI.5712-09.2010

Park, C. K., Mendelow, A. D., Graham, D. I., McCulloch, J., & Teasdale, G. M. (1988). Correlation of triphenyltetrazolium chloride perfusion staining with conventional neurohistology in the detection of early brain ischaemia. Neuropathology and applied neurobiology, 14(4), 289-298.

Paxinos, G., & Watson, C. (1998). The rat brain in stereotaxic coordinates. *Academic Press*.

Pidoplichko, V. I., DeBiasi, M., Williams, J. T., & Dani, J. A. (1997). Nicotine activates and desensitizes midbrain dopamine neurons. *Nature, 390*(6658), 401.

Rahman, S., & McBride, W. J. (2002). Involvement of GABA and cholinergic receptors in the nucleus accumbens on feedback control of somatodendritic dopamine release in the ventral tegmental area. Journal of neurochemistry, 80(4), 646-654.

Rahman, S., Zhang, J., & Corrigall, W. A. (2003). Effects of acute and chronic nicotine on somatodendritic dopamine release of the rat ventral tegmental area: In vivo microdialysis study. *Neuroscience Letters*, *348*(2), 61-64. doi:http://dx.doi.org/10.1016/S0304-3940(03)00723-7

Rehncrona, S., Mela, L., & Siesjö, B. K. (1979). Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. Stroke, 10(4), 437-446.

Reynolds, L. M., Cochran, S. M., Morris, B. J., Pratt, J. A., & Reynolds, G. P. (2005). Chronic phencyclidine administration induces schizophrenia-like changes in N-acetylaspartate and N-acetylaspartylglutamate in rat brain. *Schizophrenia Research, 73*(2–3), 147-152. doi:http://dx.doi.org/10.1016/j.schres.2004.02.003

Rezvani, A. H. (2006). Involvement of the NMDA system in learning and memory. *Animal Models of Cognitive Impairment, ,* 37-48.

Rice, M. E., & Cragg, S. J. (2004). Nicotine amplifies reward-related dopamine signals in striatum. *Nature Neuroscience, 7*(6), 583-584. doi:10.1038/nn1244

Richards, C. (1998). What the actions of anaesthetics on fast synaptic transmission reveal about the molecular mechanism of anaesthesia. *Toxicology Letters, 100,* 41-50.

Richter, F., Bauer, R., Lehmenkühler, A., & Schaible, H. (2008). Spreading depression in the brainstem of the adult rat: Electrophysiological parameters and influences on

regional brainstem blood flow. *Journal of Cerebral Blood Flow & Metabolism, 28*(5), 984-994.

Risterucci, C., Jeanneau, K., Schöppenthau, S., Bielser, T., Künnecke, B., von Kienlin, M., & Moreau, J. L. (2005). Functional magnetic resonance imaging reveals similar brain activity changes in two different animal models of schizophrenia. *Psychopharmacology,* 180(4), 724-734.

Robinson, D. L., Phillips, P. E., Budygin, E. A., Trafton, B. J., Garris, P. A., & Wightman, R. M. (2001). Sub-second changes in accumbal dopamine during sexual behavior in male rats. *Neuroreport, 12*(11), 2549-2552.

Robinson, D. L., Venton, B. J., Heien, M. L. A. V., & Wightman, R. M. (2003). Detecting subsecond dopamine release with fast-scan cyclic voltammetry in vivo. *Clinical Chemistry, 49*(10), 1763-1773.

Robinson, E. C., Jbabdi, S., Andersson, J., Smith, S., Glasser, M. F., Van Essen, D. C., . . . Jenkinson, M. (2013). Multimodal surface matching: Fast and generalisable cortical registration using discrete optimisation. *International Conference on Information Processing in Medical Imaging,* 475-486.

Rodríguez, G., Neugebauer, N. M., Yao, K. L., Meltzer, H. Y., Csernansky, J. G., & Dong, H. (2017). Δ9-tetrahydrocannabinol (Δ9-THC) administration after neonatal exposure to phencyclidine potentiates schizophrenia-related behavioral phenotypes in mice. Pharmacology Biochemistry and Behavior, 159, 6-11.

Rohrer, S. P., Jacobson, E. B., Hayes, E. C., Birzin, E. T., & Schaeffer, J. M. (1994). Immunoaffinity purification of avermectin-binding proteins from the free-living

nematode caenorhabditis elegans and the fruitfly drosophila melanogaster. *The Biochemical Journal, 302 (Pt 2)*(Pt 2), 339-345.

Rondou, P., Haegeman, G., & Van Craenenbroeck, K. (2010). The dopamine D4 receptor: Biochemical and signalling properties. *Cellular and Molecular Life Sciences, 67*(12), 1971-1986.

Ross, C. A., Margolis, R. L., Reading, S. A. J., Pletnikov, M., & Coyle, J. T. (2006). Neurobiology of schizophrenia. *Neuron, 52*(1), 139-153. doi:http://dx.doi.org/10.1016/j.neuron.2006.09.015

Rowland, L., Bustillo, J. R., & Lauriello, J. (2001). Proton magnetic resonance spectroscopy (H-MRS) studies of schizophrenia. *Seminars in Clinical Neuropsychiatry, 6*(2), 121-130.

Rubio, M. D., Drummond, J. B., & Meador-Woodruff, J. H. (2012). Glutamate receptor abnormalities in schizophrenia: Implications for innovative treatments. *Biomolecules and Therapeutics, 20*(1), 1-18.

Runnels, P. L., Joseph, J. D., Logman, M. J., & Wightman, R. M. (1999). Effect of pH and surface functionalities on the cyclic voltammetric responses of carbon-fiber microelectrodes. Analytical *Chemistry, 71*(14), 2782-2789.

Salamone, A., Zappettini, S., Grilli, M., Olivero, G., Agostinho, P., Tomé, A. R., . . . Marchi, M. (2014). Prolonged nicotine exposure down-regulates presynaptic NMDA receptors in dopaminergic terminals of the rat nucleus accumbens. *Neuropharmacology, 79*, 488-497. doi:http://dx.doi.org/10.1016/j.neuropharm.2013.12.014

Salminen, O., Murphy, K. L., McIntosh, J. M., Drago, J., Marks, M. J., Collins, A. C., & Grady, S. R. (2004). Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Molecular Pharmacology, 65*(6), 1526-1535. doi:10.1124/mol.65.6.1526

Sams-Dodd, F., Lipska, B. K., & Weinberger, D. R. (1997). Neonatal lesions of the rat ventral hippocampus result in hyperlocomotion and deficits in social behaviour in adulthood. Psychopharmacology, 132(3), 303-310.

Sands, S. B., & Barish, M. E. (1992). Neuronal nicotinic acetylcholine receptor currents in phaeochromocytoma (PC12) cells: dual mechanisms of rectification. The Journal of Physiology, 447(1), 467-487.

Sarter, M., Parikh, V., & Howe, W. M. (2009). Phasic acetylcholine release and the volume transmission hypothesis: Time to move on. *Nature Reviews Neuroscience, 10*(5), 383-390. doi:10.1038/nrn2635

Savchenko, A., Braun, G. B., & Molokanova, E. (2016). Nanostructured antagonist of extrasynaptic NMDA receptors. *Nano letters,* 16(9), 5495-5502.

Schilström, B., Rawal, N., Mameli-Engvall, M., Nomikos, G. G., & Svensson, T. H. (2003). Dual effects of nicotine on dopamine neurons mediated by different nicotinic receptor subtypes. *International Journal of Neuropsychopharmacology, 6*(1), 1-11. doi:10.1017/S1461145702003188

Schoffelmeer, A. N. M., Vanderschuren, L. J. M. J., De Vries, T. J., Hogenboom, F., Wardeh, G., & Mulder, A. H. (2000). Synergistically interacting dopamine D1 and

NMDA receptors mediate nonvesicular transporter-dependent GABA release from rat striatal medium spiny neurons. *Journal of Neuroscience, 20*(9), 3496-3503.

Schulte, M. K., Khatri, S., Huang, Y., DeCristofano, L., & LeBlan, G. G. (2016). Allosteric modulation and potential therapeutic applications of heteromeric nicotinic acetylcholine receptors. *Neurotransmitter, 3*

Schultz, W. (2002). Getting formal with dopamine and reward. *Neuron, 36*(2), 241-263. doi:10.1016/S0896-6273(02)00967-4

Schuster, C., Dockens, W., & Woods, J. (1966). Behavioral variables affecting the development of amphetamine tolerance. *Psychopharmacology, 9*(2), 170-182.

Schwartz, T. L., Sachdeva, S., & Stahl, S. M. (2012). Glutamate neurocircuitry: Theoretical underpinnings in: Schizophrenia. *Frontiers in Pharmacology, 3 NOV* doi:10.3389/fphar.2012.00195

Schwarz, A. J., Gozzi, A., Chessa, A., & Bifone, A. (2012). Voxel scale complex networks of functional connectivity in the rat brain: Neurochemical state dependence of global and local topological properties. *Computational and Mathematical Methods in Medicine,* 1-15.

Segovia, G., & Mora, F. (2001). Involvement of NMDA and AMPA/kainate receptors in the effects of endogenous glutamate on extracellular concentrations of dopamine and GABA in the nucleus accumbens of the awake rat. *Brain Research Bulletin, 54*(2), 153-157. doi:http://dx.doi.org/10.1016/S0361-9230(00)00432-9

Sershen, H., Balla, A., Aspromonte, J. M., Xie, S., Cooper, T. B., & Javitt, D. C. (2008). Characterization of interactions between phencyclidine and amphetamine in rodent

prefrontal cortex and striatum: Implications in NMDA/glycine-site-mediated dopaminergic dysregulation and dopamine transporter function. *Neurochemistry International, 52*(1–2), 119-129. doi:http://dx.doi.org/10.1016/j.neuint.2007.07.011

Sesack, S.R., and Grace, A.A. (2009) Cortico-basal ganglia reward network: microcircuitry. *Neuropsychopharmacology 35,* 27-47.

Sforazzini, F., Schwarz, A. J., Galbusera, A., Bifone, A., & Gozzi, A. (2014). Distributed BOLD and CBV-weighted resting-state networks in the mouse brain. *NeuroImage,* 15(87), 403-415. doi:https://doi.org/10.1016/j.neuroimage.2013.09.050

Shatillo, A., Salo, R. A., Giniatullin, R., & Gröhn, O. H. (2015). Involvement of NMDA receptor subtypes in cortical spreading depression in rats assessed by fMRI. *Neuropharmacology, 93,* 164-170.

Shichino, T., Murakawa, M., Adachi, T., Nakao, S., Shinomura, T., Kurata, J., & Mori, K. (1997). Effects of isoflurane on in vivo release of acetylcholine in the rat cerebral cortex and striatum. *Acta Anaesthesiologica Scandinavica, 41*(10), 1335-1340.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., . . . Mizuno, N. (1997). Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 17*(19), 7503-7522.

Shin, J. H., Adrover, M. F., Wess, J., & Alvarez, V. A. (2015). Muscarinic regulation of dopamine and glutamate transmission in the nucleus accumbens. *Proceedings of the National Academy of Sciences of the United States of America, 112*(26), 8124-8129. doi:10.1073/pnas.1508846112 [doi]

Sicard, K., Shen, Q., Brevard, M. E., Sullivan, R., Ferris, C. F., King, J. A., & Duong, T. Q. (2003). Regional cerebral blood flow and BOLD responses in conscious and anesthetized rats under basal and hypercapnic conditions: Implications for functional MRI studies. Journal of Cerebral Blood *Flow & Metabolism, 23*(4), 472-481.

Silvers, J. M., Harrod, S. B., Mactutus, C. F., & Booze, R. M. (2007). Automation of the novel object recognition task for use in adolescent rats. Journal of neuroscience methods, 166(1), 99-103.

Smith, S.M., Jenkinson, M., Woolrich, M.W., Beckmann, C.F., Behrens, T.E.J., Johansen-Berg, H. , Bannister, P.R. , De Luca, M., Drobnjak, I., Flitney, D.E., Niazy, R., Saunders, J., Vickers, J., Zhang, Y., De Stefano, N., Brady, J.M., and Matthews, P.M. Advances in functional and structural MR image analysis and implementation as FSL. *NeuroImage*, 23(S1):208-19, 2004

Snigdha, S., Neill, J. C., McLean, S. L., Shemar, G. K., Cruise, L., Shahid, M., & Henry, B. (2011). Phencyclidine (PCP)-induced disruption in cognitive performance is gender-specific and associated with a reduction in brain-derived neurotrophic factor (BDNF) in specific regions of the female rat brain. Journal of molecular neuroscience, 43(3), 337-345.

Snyder, G. L., Fienberg, A. A., Huganir, R. L., & Greengard, P. (1998). A dopamine/D1 receptor/protein kinase A/dopamine- and cAMP-regulated phosphoprotein (mr 32 kDa)/protein phosphatase-1 pathway regulates dephosphorylation of the NMDA receptor. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 18*(24), 10297-10303.

Snyder, S. H. (2006). Dopamine receptor excess and mouse madness. *Neuron, 49*(4), 484-485. doi:http://dx.doi.org/10.1016/j.neuron.2006.02.002

Sofroniew, M., Priestley, J., Consolazione, A., Eckenstein, F., & Cuello, A. (1985). Cholinergic projections from the midbrain and pons to the thalamus in the rat, identified by combined retrograde tracing and choline acetyltransferase immunohistochemistry. *Brain Research, 329*(1), 213-223.

Sood, P., Idris, N. F., Cole, S., Grayson, B., Neill, J. C., & Young, A. M. (2011). PD168077, a D(4) receptor agonist, reverses object recognition deficits in rats: Potential role for D(4) receptor mechanisms in improving cognitive dysfunction in schizophrenia. *Journal of Psychopharmacology (Oxford, England), 25*(6), 792-800. doi:10.1177/0269881110387840 [doi]

Spano, P. F., Govoni, S., & Trabucchi, M. (1978). Studies on the pharmacological properties of dopamine receptors in various areas of the central nervous system. *Advances in Biochemical Psychopharmacology, 19*, 155-165.

Stamford, J. A., Kruk, Z. L., & Millar, J. (1985). Ascorbic acid does not modulate stimulated dopamine release: In vivo voltammetric data in the rat. *Neuroscience Letters,* 60(3), 357-362. doi:https://doi.org/10.1016/0304-3940(85)90603-2

Stamford, J. A., Kruk, Z. L., & Millar, J. (1986). Sub-second striatal dopamine release measured by in vivo voltammetry. *Brain Research*, 381(2), 351-355. doi:https://doi.org/10.1016/0006-8993(86)90087-9.

Stamford, J. A., Palij, P., Davidson, C., Jorm, C. M., & Phillips, P. E. (1995). Fast cyclic voltammetry in brain slices. Voltammetric Methods in Brain Systems, , 81-116.

Stamford, J., Crespi, F., & Marsden, C. (1992). In vivo voltammetric methods for monitoring monoamine release and metabolism. *Monitoring Neuronal Activity,* , 113-145.

Steinpreis, R. E., & Salamone, J. D. (1993). The role of nucleus accumbens dopamine in the neurochemical and behavioral effects of phencyclidine: A microdialysis and behavioral study. *Brain Research, 612*(1–2), 263-270. doi:http://dx.doi.org/10.1016/0006-8993(93)91671-E

Steward, C., Prior, M., Chapman, V., Morris, P., & Marsden, C. (2004). Mapping functional changes in rat brain in response to altered serotonergic function using BOLD fMRI. *Proceedings (12th) of the International Society of Magnetic Resonance in Medicine,* 1169.

Stokes, C., Treinin, M., & Papke, R. L. (2015). Looking below the surface of nicotinic acetylcholine receptors. Trends in pharmacological sciences, 36(8), 514-523.

Supprian, T., Hofmann, E., Warmuth-Metz, M., Franzek, E., & Becker, T. (1997). MRI T2 relaxation times of brain regions in schizophrenic patients and control subjects. *Psychiatry Research: Neuroimaging, 75*(3), 173-182. doi:http://dx.doi.org/10.1016/S0925-4927(97)00052-8

Svensson, A., Carlsson, A., & Carlsson, M. (1992). Differential locomotor interactions between dopamine D1/D2 receptor agonists and the NMDA antagonist dizocilpine in monoamine-depleted mice. *Journal of Neural Transmission, 90*(3), 199-217.

Takasu, M. A., Dalva, M. B., Zigmond, R. E., & Greenberg, M. E. (2002). Modulation of NMDA receptor-dependent calcium influx and gene expression through EphB

receptors. *Science (New York, N.Y.), 295*(5554), 491-495. doi:10.1126/science.1065983 [doi]

Tanii, Y., Nishikawa, T., Umino, A., & Takahashi, K. (1990). Phencyclidine increases extracellular dopamine metabolites in rat medial frontal cortex as measured by in vivo dialysis. *Neuroscience Letters, 112*(2), 318-323. doi:http://dx.doi.org/10.1016/0304-3940(90)90223-V

Threlfell, S., Clements, M. A., Khodai, T., Pienaar, I. S., Exley, R., Wess, J., & Cragg, S. J. (2010). Striatal muscarinic receptors promote activity dependence of dopamine transmission via distinct receptor subtypes on cholinergic interneurons in ventral versus dorsal striatum. *Journal of Neuroscience, 30*(9), 3398-3408. doi:10.1523/JNEUROSCI.5620-09.2010

Threlfell, S., Lalic, T., Platt, N. J., Jennings, K. A., Deisseroth, K., & Cragg, S. J. (2012). Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. *Neuron, 75*(1), 58-64.

Tiberi, M., Jarvie, K. R., Silvia, C., Falardeau, P., Gingrich, J. A., Godinot, N., . . . Caron, M. G. (1991). Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D1 dopamine receptor subtype: Differential expression pattern in rat brain compared with the D1A receptor. *Proceedings of the National Academy of Sciences of the United States of America, 88*(17), 7491-7495.

Timofeeva, O. A., & Levin, E. D. (2011). Glutamate and nicotinic receptor interactions in working memory: Importance for the cognitive impairment of schizophrenia. *Neuroscience, 195*, 21-36. doi:10.1016/j.neuroscience.2011.08.038

Toner, C. C., & Stamford, J. A. (1996). ‘Real time’ measurement of dopamine release in an in vitro model of neostriatal ischaemia. *Journal of Neuroscience Methods*, 67(2), 133-140. doi:https://doi.org/10.1016/0165-0270(96)00030-1

Toner, C. C., & Stamford, J. A. (1997). Characteristics of the NMDA receptor modulating hypoxia/hypoglycaemia-induced rat striatal dopamine release in vitro. *European Journal of Pharmacology, 340*(2–3), 133-143. doi:http://dx.doi.org/10.1016/S0014-2999(97)01422-2

Tseng, K. Y., & O'Donnell, P. (2004). Dopamine-glutamate interactions controlling prefrontal cortical pyramidal cell excitability involve multiple signaling mechanisms. *Journal of Neuroscience, 24*(22), 5131-5139.

Tyszkiewicz, J. P., Gu, Z., Wang, X., Cai, X., & Yan, Z. (2004). Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-dependent mechanism in pyramidal neurones of rat prefrontal cortex. *The Journal of Physiology, 554*(3), 765-777.

Uchida, S., & Hotta, H. (2009). Cerebral cortical vasodilatation mediated by nicotinic cholinergic receptors: Effects of old age and of chronic nicotine exposure. *Biological and Pharmaceutical Bulletin, 32*(3), 341-344.

Upadhyay, J., Anderson, J., Schwarz, A. J., Coimbra, A., Baumgartner, R., Pendse, G., . . . Bishop, J. (2011). Imaging drugs with and without clinical analgesic efficacy. *Neuropsychopharmacology, 36*(13), 2659-2673.

Van Essen, D. C., Smith, S. M., Barch, D. M., Behrens, T. E., Yacoub, E., Ugurbil, K., & Wu-Minn HCP Consortium. (2013). The WU-minn human connectome project: An overview. *Neuroimage, 80*, 62-79.

Verberne, A. J., & OWENS, N. C. (1998). Cortical modulation of the cardiovascular system. *Progress in Neurobiology, 54*(2), 149-168.

Vincent, J. L., Patel, G. H., Fox, M. D., Snyder, A. Z., Baker, J. T., Van Essen, D. C., . . . Raichle, M. E. (2007). Intrinsic functional architecture in the anaesthetized monkey brain. *Nature, 447*(7140), 83-86.

Wanat, M., Hopf, F., Stuber, G., Phillips, P., & Bonci, A. (2008). Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of ih. *The Journal of Physiology, 586*(8), 2157-2170.

Wang, L., Saalmann, Y. B., Pinsk, M. A., Arcaro, M. J., & Kastner, S. (2012). Electrophysiological low-frequency coherence and cross-frequency coupling contribute to BOLD connectivity. *Neuron, 76*(5), 1010-1020.

Weissleder, R., Elizondo, G., Wittenberg, J., Rabito, C. A., Bengele, H. H., & Josephson, L. (1990). Ultrasmall superparamagnetic iron oxide: Characterization of a new class of contrast agents for MR imaging. *Radiology, 175*(2), 489-493. doi:10.1148/radiology.175.2.2326474 [doi]

Weltzin, M. M., & Schulte, M. K. (2011). Interaction of HEPES analogues with high and low affinity alpha4beta2 nicotinic acetylcholine receptors. *The FASEB Journal, 25*(1 Supplement), 798.1-798.1.

Weltzin, M. M., Huang, Y., & Schulte, M. K. (2014). Allosteric modulation of alpha4beta2 nicotinic acetylcholine receptors by HEPES. *European Journal of Pharmacology, 732*, 159-168.

Williamson, P., Pelz, D., Merskey, H., Morrison, S., Karlik, S., Drost, D., . . . Conlon, P. (1992). Frontal, temporal, and striatal proton relaxation times in schizophrenic patients and normal comparison subjects. *American Journal of Psychiatry*, *149*(4), 549-551.

Wilson, C. (1998). In the synaptic organization of the brain. *Basal ganglia* (pp. pp. 279–316.) Oxford, UK: Oxford Univ. Press.

Wolf, M. E., & Roth, R. H. (1990). Autoreceptor regulation of dopamine synthesis. *Annals of the New York Academy of Sciences, 604*(1), 323-343.

Wood, P. L. (1982). Actions of GABAergic agents on dopamine metabolism in the nigrostriatal pathway of the rat. *The Journal of Pharmacology and Experimental Therapeutics, 222*(3), 674-679.

Wood, T. C., Lythgoe, D. J., & Williams, S. C. (2013). rBET: Making BET work for rodent brains. *PLoS ONE,* 1-23.

Woolf, N. J. (1991). Cholinergic systems in mammalian brain and spinal cord. *Progress in Neurobiology, 37*(6), 475-524. doi:10.1016/0301-0082(91)90006-M

Woolrich, M.W., Jbabdi, S., Patenaude, B., Chappell, M., Makni, S., Behrens, T., Beckmann, C., Jenkinson, M., Smith, S.M. Bayesian analysis of neuroimaging data in FSL. *NeuroImage*, 45:S173-86, 2009

World Health Organization. (1992). The ICD-10 classification of mental and behavioural disorders: Clinical descriptions and diagnostic guidelines Geneva: World Health Organization.

Wroge, C. M., Hogins, J., Eisenman, L., & Mennerick, S. (2012). Synaptic NMDA receptors mediate hypoxic excitotoxic death. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 32*(19), 6732-6742. doi:10.1523/JNEUROSCI.6371-11.2012 [doi]

Wu, Y., Pearl, S. M., Zigmond, M. J., & Michael, A. C. (2000). Inhibitory glutamatergic regulation of evoked dopamine release in striatum. *Neuroscience, 96*(1), 65-72. doi:http://dx.doi.org/10.1016/S0306-4522(99)00539-4

Wu, Y. N., & Johnson, S. W. (2015). Memantine selectively blocks extrasynaptic NMDA receptors in rat substantia nigra dopamine neurons. *Brain research*, 1603, 1-7.

Xi, Z.X. Stein, E.A. (1998). Nucleus Accumbens Dopamine Release Modulation by Mesolimbic GABAa Receptors- an In Vivo electrochemical Study. *Brain Research, 798*,156-65.

Xu, J., Zhu, Y., & Heinemann, S. F. (2006). Identification of sequence motifs that target neuronal nicotinic receptors to dendrites and axons. Journal of Neuroscience, 26(38), 9780-9793.

Yan, H., Ishihara, K., Serikawa, T., & Sasa, M. (2003). Activation by N-Acetyl-l-Aspartate of acutely dissociated hippocampal neurons in rats via metabotropic glutamate receptors. *Epilepsia, 44*(9), 1153-1159.

Yan, Z., Song, W. J., & Surmeier, D. J. (1997). D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-insensitive pathway. Journal of neurophysiology, 77(2), 1003-1015.

Yavas, E., & Young, A. M. (2017). N-methyl-D-aspartate modulation of nucleus accumbens dopamine release by metabotropic glutamate receptors: Fast cyclic voltammetry studies in rat brain slices in vitro. ACS Chemical Neuroscience, 8(2), 320-328.

Yorgason, J. T., España, R. A., & Jones, S. R. (2011). Demon voltammetry and analysis software: Analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *Journal of Neuroscience Methods, 202*(2), 158-164

Yun, I. A., Nicola, S. M., & Fields, H.L. (2004). Contrasting effects of dopamine and glutamate receptor antagonist injection in the nucleus accumbens suggest a neuronal mechanism underlying cue-evoked goal-directed behavior. *European Journal of Neuroscience, 20*, 249-263.

Zahm, D. S., & Brog, J. S. (1992). On the significance of subterritories in the "accumbens" part of the rat ventral striatum. *Neuroscience, 50*(4), 751-767. doi:http://dx.doi.org/10.1016/0306-4522(92)90202-D

Zahm, D.S. (2000) An integrative neuroanatomical perspective on some subcortical substrates of adaptive responding with emphasis on the nucleus accumbens. *Neuroscience and Biobehavioral Reviews 24*:85-105.

Zappettini, S., Grilli, M., Olivero, G., Chen, J., Padolecchia, C., Pittaluga, A., . . . Marchi, M. (2014). Nicotinic alpha7 receptor activation selectively potentiates the function of NMDA receptors in glutamatergic terminals of the nucleus accumbens. *Frontiers in Cellular Neuroscience, 8,* 332. doi:10.3389/fncel.2014.00332 [doi]

Zhang, H., & Sulzer, D. (2012). Regulation of striatal dopamine release by presynaptic auto-and heteroreceptors. *Basal Ganglia, 2*(1), 5-13.

Zhang, W., Yamada, M., Gomeza, J., Basile, A. S., & Wess, J. (2002). Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1–M5 muscarinic receptor knock-out mice. Journal of Neuroscience, 22(15), 6347-6352.

Zhou, F., Liang, Y., & Dani, J. A. (2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. *Nature Neuroscience, 4*(12), 1224-1229.

Zittel, H., & Miller, F. (1965). A glassy-carbon electrode for voltammetry. *Analytical Chemistry, 37*(2), 200-203.

Zmarowski, A., Sarter, M., & Bruno, J. P. (2005). NMDA and dopamine interactions in the nucleus accumbens modulate cortical acetylcholine release. *European Journal of Neuroscience, 22*(7), 1731-1740. doi:10.1111/j.1460-9568.2005.04333.x

Quik, M., Vailati, S., Bordia, T., Kulak, J. M., Fan, H., McIntosh, J. M., ... & Gotti, C. (2004). Subunit composition of nicotinic receptors in monkey striatum; effect of MPTP and L-dopa treatments. Molecular pharmacology.