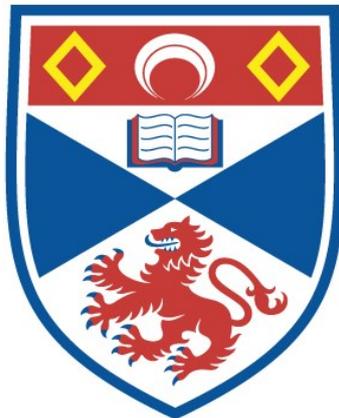


**ATTENTION REGULATION AND
BEHAVIOURAL FLEXIBILITY IN RATS WITH
RELEVANCE TO SCHIZOPHRENIA**

Alonzo J. Whyte

**A Thesis Submitted for the Degree of PhD
at the
University of St Andrews**



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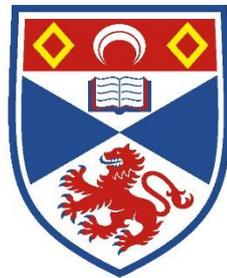
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RELEVANCE TO SCHIZOPHRENIA

Alonzo J. Whyte



University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of
PhD

at the University of St Andrews

University of St Andrews

September 2016

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Acknowledgements

First and foremost, I must express my thankfulness to Professor Verity Brown, for her superb mentorship. When I selected her as mentor all those years ago, I did not expect to end up doing a PhD in Scotland. However, through a series of fortunate events, that is what occurred and I am ever improved thanks to her expertise and guidance in the field of behavioural neuroscience. Professor Brown has become a person whom I recognise as being of the utmost quality of character, and whose advice I will gladly accept as I advance past the PhD. My gratitude also goes to Dr Eric Bowman, and Dr David Tait. Dr Bowman for the statistical advisement; and Dr Tait, for the practical implementation of all the set-shifting studies, as well as always being available for an impromptu discussion on the science (or differences in American and British English!). I would also like to thank Drs James Ainge, and Gareth Miles, for their advice throughout the course of the degree. As well as the rest of the Brown lab, Animal care unit, and the Psychology and Neuroscience staff. Also thanks to my B.S. and M.S. educators for training me to succeed at the next level.

This thesis is dedicated to my mother, Joy, who while studying for her own degree in psychology, exposed me to this interesting and exciting field, and started my thirst for knowledge about the brain and behaviour. Also, thanks to my mother, father, sister, extended family, and friends back in America, for the positive messages and support. Special thank you to Andrea (the best running buddy) for all the packages!

A deep thanks to my all my new friends and colleagues. The dungeon that is the Lilac room would have been less bearable if not for the humorous and exciting experiences I have been able to share with Andy, Sonny, and others. And of course to Monica for being there day after day, as someone to share life outside of lab with. These people have all made sure I have enjoyed my time in St Andrews in addition to the abundance of learning.

Financial support for the work in this thesis was provided by the University of St Andrews 600th Anniversary Scholarship.

Abstract

Schizophrenia is a neuropsychological disorder in which the neural systems which regulate attention allocation, primarily the dorsolateral prefrontal cortex, are dysfunctional, resulting in deficient gating of attention to irrelevant inputs from the environment. This sensory processing dysfunction hinders goal-directed behaviour to the extent that the subsequent cognitive deficits of schizophrenia prevent many chronic patients from leading normal lives. It is the onus of neuroscience to understand the nature of deficits induced by the disorder, thus providing target mechanisms for remediation of those deficits in patients. To accomplish this, manipulations in rats with relevance to schizophrenia are examined in assays with translation to human neurobiology and behaviour. In this thesis, three manipulations with relevance to schizophrenia, were examined for attentional regulation in the attentional set-shifting task, and similar assays, to determine how different forms of schizophrenia-related pathology influence attentional regulation and behavioural flexibility.

The foremost findings of the experiments herein were that manipulations inducing schizophrenia-related neurobiology, resulted in impaired performance in extradimensional set-shifting and reversal learning. These deficits were found following: acute inhibition of the mPFC in adult rats, in adult rats who had been exposed to a glutamate receptor antagonist during the neonatal period of development, and/or in adult rats who had gestational disruption of neuron proliferation. Across all three manipulations, a clear behavioural pattern of deficient sensory gating, evidenced by responding to irrelevant stimuli during the set-shifting task was found.

These findings suggest that at the core of the cognitive deficits in schizophrenia is the 'loosening of associations' such that patients suffer the inability to regulate attention, and limit sensory processing to relevant information. The subsequent aberrant learning about irrelevant information then impairs performance during goal-directed behaviours.

Chapter 1. Introduction to Attentional Regulation and Schizophrenia

Attentional regulation is a key process in navigating changing environments. Under most conditions it is best to limit attention (i.e. sensory processing) to certain features of the environment. However, there are certain conditions, when not having biased attentional processing, may lead to gathering more beneficial information about the environment. Unfortunately, the latter appears to be the default state of persons suffering from schizophrenia, such that they experience 'aberrant salience' which subsequently impairs their cognition. The neural substrates for attentional regulation are known to coalesce at the dorsolateral prefrontal cortex, whereby a process known as 'working memory' maintains task relevant information and biases attention. In this chapter I review how the pathology of schizophrenia, affects the brain and causes deficits in tasks dependent on the functioning of the dorsolateral prefrontal cortex (primarily attentional set-shifting). The findings in humans are then explored in rats with the aim of developing translational tools with potential for exploring and treating the cognitive symptoms of schizophrenia.

1.1 Overview

I was recently required to complete the matriculation process to begin the 4th year of my PhD. As an international student, an in-person visa check was mandated, requiring me to navigate the busy Gateway building. As it was the beginning of a new semester, the building was filled with undergraduates (also matriculating), whom I tried to ignore as much as possible, lest I truly felt old and out of place. The process took roughly 30 mins of navigating ill-placed A4-sized signs directing me to the correct queue, however, by selectively attending to the relevant environmental cues, I was successful in completing the process. Indeed, although I was vaguely aware of the teenagers chatting to each other, the details of those conversations eluded me as my focus of attention was on reaching the next queue and help desk. However, as I was leaving the Gateway building, this time fully focused on following the exit signs, I heard my name called. I turned to see some fellow doctoral students who were working some tables off to the side, at which point we then had a brief chat.

The brief narrative above actually depicts two key phenomena within attentional process. Specifically, the process by which I selectively attended to visual stimuli (the directions from the A4 signs), and used those cues to achieve my goal, while ignoring the auditory stimuli (chatter of the undergrads) which provided no information as to which queue I should be in, is the basis for 'attentional set' (Sutherland and Mackintosh, 1971; but see: Brown and Tait, 2016). That is to say, while the stimuli were changing, new relevant signs on each floor, as well as new irrelevant background stimuli, my ability to complete the matriculation process was enhanced by ignoring the irrelevant stimuli within the environment. Attentional set then, is a goal-directed process, wherein endogenous attentional regulation guides sensory processing. On the other hand, my colleague calling my name elicited the stimulus-driven (exogenous) attention process known known as the 'cocktail party effect' (Cherry, 1953; but see: Conway et al., 2001). The high importance of recognising my name being called, despite my attention being focused on visual stimuli, suggests that sensory systems are

tuned such that the salience of certain stimuli remains high under many circumstances. Indeed, shifting my sensory processing bias (attention) from gating auditory stimuli initially, to selectively attending to the conversation with my peers (while still gating irrelevant chatter in the background), is also an endogenous goal-directed process, known as ‘attentional set-shifting’ (Berg, 1948; but see: Roberts et al., 1988). These attentional processes depend on the functional integrity of several neural circuits, many of which are found to be dysfunctional in neuropsychiatric disorders. One disorder in which both processes are dysfunctional is schizophrenia. As described within this thesis introduction and empirical chapters, patients with schizophrenia, and animals with manipulations with relevance to schizophrenia, suffer from deficits in both exogenous attention (in the form of ‘aberrant salience’), as well as endogenous allocation of attention in goal-directed behavioural task. To further understand the psychological and neurobiological mechanisms underlying this pathology, this thesis focuses on impaired attentional regulation within the context of ‘schizophrenia-related’ manipulations in rats.

1.2 Schizophrenia genetics and neurochemistry

Schizophrenia is a complex neurodevelopmental disorder characterised by positive symptoms (e.g., hallucinations and delusions), negative symptoms (e.g., flat affect and social withdrawal), and cognitive symptoms (e.g., impairments in working memory, and attention; National Institute of Mental Health). Recent reviews of schizophrenia genetics have found that, although the genetic heritability of the disorder has been well evidenced (Owen et al., 2004), the disease has polygenetic risk factors (Sun et al., 2010; Farrell et al., 2015), and highly influenced by gene-environment interactions (Tsuang, 2000). Specifically, a review of the published genetic association studies through 2008 implicated over 10 genes including: DRD1, COMT, GRIN2B, and GABRB2 (Allen et al., 2008). However, while mutations in these genes increased the risk for development of schizophrenia, no single mutation determines whether a person will eventually

develop schizophrenia. Further studies have revealed that in combination with genetic risk factors for developing schizophrenia, environmental stressors during sensitive periods such as gestation (Hultman et al., 1999; Van et al., 2016), and throughout the lifespan (Howes et al., 2017), as well as the epigenetic modifications of genes (Zhang et al., 2010) can greatly exacerbate the risk.

The heterogeneity of the aetiology of schizophrenia has necessitated a focus on the common schizophrenia phenotypes resultant from any number of aetiologies. Of these common phenotypes, the positive symptoms can be considered the most severe of the core symptoms, and are of utmost importance for immediate treatment. Fortunately, early antipsychotic treatments targeting the dopaminergic system have proven efficacious for remediation of the positive symptoms (Seeman et al., 1975; for review see: Howes and Kapur, 2009). However, research into treatment of the negative and cognitive symptoms is ongoing, as the current antipsychotics do not remediate those for many patients (Lieberman et al., 2005a; Walters and Agius, 2014). Although many neurotransmitter systems show abnormal signalling in schizophrenia, in this section I review the most prominent (dopamine, glutamate, and gamma-aminobutyric acid; GABA), in the context of disease pathology and potential for

symptom alleviation. Figure 1.1 presents a representative diagram of the neurochemical findings discussed.

1.2.1 Dopamine

Currently, antipsychotic treatments for schizophrenia mainly target the dysfunctional dopaminergic neurotransmission. Dopaminergic neurotransmission can occur through several subtypes of dopamine receptors; D1-like receptors (D1Rs; D1 and D5) and D2-like receptors (D2Rs; D2, D3, and D5). Within these two major subdivisions, it was found that early efficacious antipsychotics targeted the D2Rs (Seeman et al., 1975). Activation of D2Rs typically initiates inhibitory intracellular signalling cascades. These early antagonists of D2Rs likely inhibited

	<u>Dopamine</u>	<u>Glutamate</u>	<u>GABA</u>
Positive symptoms:	D2R antagonism is effective for most patients but with severe side effects	NMDAR antagonism exacerbates positive symptoms in patients	Enhancing GABA signalling reduced symptom progression
Negative symptoms:	Not effective for many patients	Clinical trials show enhancement of NMDAR signalling reduces negative symptomatology	Enhancing GABA signalling reduced symptom progression
Cognitive symptoms:	Not effective for many patients	No evidence	Enhancing GABA _A R- α 2/3 signalling has mixed results for improving cognition in patients

Figure 1.1) Diagram of relevant findings for the role of dopamine, glutamate, and GABA in schizophrenia, from genetic and pharmacological studies.

this function of dopaminergic neurotransmission, resulting in enhanced stimulatory (D1R-mediated) signalling in cortical areas, while reducing hyperdopaminergic signalling in subcortical areas. While this approach has been largely effective at treating the positive symptoms associated with schizophrenia, the cognitive symptoms are not rescued by the currently available D2R pharmacotherapies (reviewed in: Conn et al., 2009).

In a recent review of the 'classical' dopamine hypothesis of schizophrenia, Howes and Kapur (2009) described how the dopamine hypothesis has undergone several revisions. In version I, the efficacy of D2R antagonists for treating the disorder led clinicians and researchers (in the 1970s and '80s) to consider the dopamine receptor dysfunctions as the aetiological beginning of the disorder. This 'dopamine receptor hypothesis' quickly came under criticism as new data indicated differential expression and functions of dopamine receptors based on neuroanatomical localisation, in addition to brain region-specific alterations in dopaminergic signalling. Version II, was based largely on the review published by Davis et al. (1991) which identified hypodopaminergic signalling in the prefrontal cortex (PFC) as a large contributing factor to the negative (and potentially the cognitive) schizophrenia symptoms, in addition to the hyperdopaminergic signalling in subcortical brain regions as the main cause the positive symptoms. Yet still, the second version was limited. Howes and Kapur postulate Version III, which incorporates disparate genetic findings by including a 'multiple hits', gene x environment, interaction into the aetiology of the disorder, as opposed to looking at D2Rs alone. Version III includes the well characterised presynaptic dopamine dysregulation, i.e. the driving hypo- or hyperdopaminergic neurotransmission in each brain region. Also, since D2R antagonists do not alleviate the negative or the cognitive symptoms, in Version III, dopamine dysregulation is limited to the aetiology of the positive symptoms. Indeed, Howes and Kapur, surmise that dopamine dysregulation alters the "appraisal of stimuli perhaps through a process of aberrant salience". This refinement was a step in the right direction, however it was still limited to dopaminergic neurotransmission. Many studies had also found that healthy patients treated with glutamate receptor antagonists had symptoms akin to those seen in schizophrenia patients (reviewed by: Javitt and Zukin, 1991). These and other findings from post-mortem, preclinical, and experimental studies, suggest that there is more to the neurochemical dysfunction than just dopamine. Furthermore, D2R antagonism also has unavoidable side effects which further motivated the discovery of new targets for treatment of symptoms (Marder,

1999). The next sections focus on the disruptions to, and the role of, glutamatergic and GABAergic signalling in schizophrenia. Since D2R antagonists have proven largely successful at alleviating the positive symptoms associated with the disorder, glutamatergic and GABAergic manipulations are examined primarily in the context of the negative and cognitive symptoms.

1.2.3 Glutamate

Key mediators of learning and memory, N-methyl-D-aspartate receptors (NMDARs) are highly implicated in the glutamatergic pathology of schizophrenia. Activation of NMDARs is multi-fold, requiring both voltage-dependent removal of the Mg^{2+} block, and binding of glutamate and a co-agonist (glycine or d-serine) to their respective sites on the receptor (Dingledine et al., 1999). Although they are only one of many types of glutamate receptors, antagonism of solely NMDARs is known to generate and exacerbate schizophrenia symptoms in both healthy controls and patients with schizophrenia respectively (Javitt and Zukin, 1991; Umbricht et al., 2000; Aalto et al., 2005; Krystal et al., 2005; Stone et al., 2007; Heekeren et al., 2008; Moore et al., 2011; Moghaddam and Javitt, 2012). The positive symptoms induced by NMDAR antagonists are likely due to increases in striatal dopaminergic signalling (Miller and O'Callaghan, 1995; Yan et al., 1997; Kegeles et al., 2000). Combined with alterations of genes, and proteins, associated with glutamatergic neurotransmission in schizophrenia patients (Akbarian et al., 1996; Mueller and Meador-Woodruff, 2004; Woo et al., 2004), these lines of evidence led to the 'NMDAR hypofunction' theory of schizophrenia. In 1994 and 1996, clinical trials with the NMDAR co-agonist glycine revealed that the addition of glycine, adjunctive to a patient's antipsychotic treatment, provided significant reduction in negative symptoms associated with the disorder (Javitt et al., 1994; Leiderman et al., 1996). Following this research, the same group found that pharmaceutically targeting the glycine binding site with the NMDAR agonist D-cycloserine also reduced negative symptoms in patients with schizophrenia (Heresco-Levy et al., 1998). Such early findings opened the door to treatment of

the previously untouchable negative symptoms associated with the disorder. Since glycine circulates above saturating levels in the brain (Ferraro and Hare, 1985), clinical trials researching the glutamatergic-mediated pathology shifted from targeting a receptor site which was likely already optimally functional, to enhancing NMDAR neurotransmission (or reducing presynaptic glutamate release) via other receptors or an NMDAR allosteric binding site.

Metabotropic glutamate receptors (mGluRs), like NMDARs, are activated by glutamate. However, unlike the ionotropic NMDARs, mGluRs are G-protein coupled receptors, meaning they signal through intracellular biochemical cascades (e.g., second messenger systems; Conn and Pin, 1997). Although there are a total of eight mGluR subtypes divided across three groups (Nakanishi, 1992), of interest to regulation of NMDAR signalling are mGluRs 2 & 3 (group II) and mGluR 5 (group I). Located presynaptically, mGluR 2/3 activity reduces glutamate release (Battaglia et al., 1997). Krystal et al. (2005), reported that administration of mGluR 2/3 agonist, LY354740, reduced ketamine-induced schizophrenia-related working memory deficits (as measured by the Hopkins Verbal Learning Test) in healthy human participants. A later generation mGluR 2/3 agonist (LY404039) reduced both positive and negative symptoms in human patients with schizophrenia (Patil et al., 2007). Unfortunately, these mGluR 2/3 agonists target the receptor's glutamate binding site, thus downregulation of the receptors, and desensitisation, may occur over time, limiting the efficacy of these drugs (Conn et al., 2009; Moghaddam and Javitt, 2012). On the postsynaptic terminal, activation of mGluR 5 enhances NMDAR neurotransmission via a stimulatory G-protein signalling cascade (Conn et al., 2009). Albeit positive thus far, studies with mGluR 5 have not however, progressed past preclinical findings in rodents (for review see: Matosin and Newell, 2013).

Until this point I have neglected to discuss NMDARs located on GABAergic interneurons. It is possible that the hyperactive hippocampus in patients with schizophrenia (described further in Chapter 1.3 Schizophrenia Neuroanatomy) is due specifically to NMDAR hypofunction on the GABAergic interneurons, which

are known to be more sensitive to NMDAR antagonists (Grunze et al., 1996), and have reduced expression in post-mortem brain samples from schizophrenia patients (Lewis et al., 2004; Lewis, 2013). Indeed, all of the NMDAR-directed pharmacotherapies discussed above could be efficacious through enhancement of GABAergic inhibition in the hippocampus. In the next section I briefly discuss GABAergic neurotransmission in relation to schizophrenia.

1.2.4 GABA

Findings in patients with schizophrenia of altered hippocampal anatomy (Weiss et al., 2005; Jaaro-Peled et al., 2010; Ledoux et al., 2014), and hyperactive hippocampus-related theta oscillations (Siekmeier and Stufflebeam, 2010; Kirihara et al., 2012), make it possible that altered hippocampal activity drives the dopaminergic dysfunctions evidenced in schizophrenia patients. In addition, the fact that several of the glutamatergic system alterations are densely localised to GABAergic interneurons (for review see: Nakazawa et al., 2012), suggests that perhaps hypofunction of hippocampal inhibitory interneurons may play a critical role in the overall dysregulation of the system.

GABA receptors come in two classes: ionotropic GABA-A receptors (GABA_AR), and metabotropic GABA-B receptors (Tao et al., 2013). The fast inhibitory actions of GABA act through GABA_AR-mediated Cl⁻ influx (Fritschy and Panzanelli, 2014), making it the ideal target for enhancement of GABAergic neurotransmission. GABA_ARs are unusually diverse, as their subunits can be composed from over 19 genes. Of these options, α 5-subunit-containing GABA_ARs are known to be upregulated in patients with schizophrenia (Guidotti et al., 2005). It is also known that upregulation of GABA_ARs is inverse to levels of GABA. That is to say that the increased expression of GABA_ARs in schizophrenia may be a compensatory mechanism by the system to normalise GABAergic neurotransmission which is reduced due to deficits in GABA synthesis (Guidotti et al.). Therefore, a method to increasing GABA levels is to increase the activity of existing GABA_ARs. This has proven of potential benefit in patients with

schizophrenia as the GABA_AR positive allosteric modulator (PAM) diazepam has been found to reduce positive and negative symptom progression (Carpenter et al., 1999). However, these compounds have yet to be shown as procognitive in preclinical or clinical trials. Further studies have examined the potential for enhancing GABA_AR activity through partial agonism of the $\alpha 2/\alpha 3$ subunit, and have provided positive, albeit limited, results for enhancing cognition in clinical trials (Lewis et al., 2008; Buchanan et al., 2011).

1.3 Schizophrenia neuroanatomy

The pathology of schizophrenia severely affects select brain regions. One of the most robust neuroanatomical markers obtained from human imaging studies is the enlargement of the lateral and third ventricles in schizophrenia patients compared to healthy controls (for review see: Jaaro-Peled et al., 2010). It is hypothesised that ventricular enlargement is progressive, as it is evident in first episode schizophrenia patients (Steen et al., 2006), and may continue to increase for several decades following the onset of symptoms (Jaaro-Peled et al.). This increase in ventricle size is likely related to the MRI findings of reductions in grey matter which are also widely reported (Wright et al., 2000; Ellison-Wright et al., 2008; Jaaro-Peled et al.), and is also purported as being progressive with time from the first episode (Borgwardt et al., 2008; Jaaro-Peled et al.). While whole brain volume is believed to be reduced (Wright et al., 2000), specific regions of reduction include: the temporal lobe (hippocampus), amygdala, thalamus, caudate putamen, and frontal cortex (Chakirova et al., 2010; Jaaro-Peled et al.; Takayanagi et al., 2010). Animal studies have revealed regulation of striatal dopamine activity by the hippocampus (for review see: Grace, 2012), and findings in schizophrenia patients of altered hippocampal anatomy (Weiss et al., 2005; Jaaro-Peled et al.; Ledoux et al., 2014), hyperactive hippocampus-related electroencephalography, and magnetoencephalographic measured oscillatory activity (Siekmeier and Stufflebeam, 2010; Kirihara et al., 2012), which are often positively correlated with positive symptom severity (Siekmeier and Stufflebeam, 2010), make it possible

that disrupted hippocampal activity drives the dopaminergic dysfunctions evidenced in schizophrenia patients (Grace). Thus, the altered dopaminergic dysfunctions are likely secondary to a hippocampal pathology. Furthermore, the hippocampal dysregulation is also linked to, and possibly caused by, the dysfunction of the PFC (Meyer-Lindenberg et al., 2005; Grace, 2012). Indeed, the brain region of origin for the pathology remains under considerable debate. One hypothesis focuses on the frontal lobe (frontal cortex) dysfunction, positing that a disruption of cognitive abilities renders the rest of the brain susceptible to further insult, whereas a competing theory posits a pathology arising from disrupted temporal lobe (hippocampus) connectivity (reviewed in: Ragland et al., 2007). The hippocampus *is* critical to the pathology of schizophrenia, and thus also occasionally revisited throughout the thesis. However, as the frontal cortex is the likely mediator of the cognitive deficits explored further within this thesis, the focus herein is primarily placed on the frontal lobe dysregulation.

The grey matter reductions in the frontal lobes (specifically, the dorsolateral PFC; dlPFC) are linked with severity of symptoms and chronicity (van Haren et al., 2007; Bonilha et al., 2008), and is often the area with the most evident reduction (Selemon et al., 1995; Selemon et al., 2003; van Haren et al.). Interestingly, although clinically available antipsychotic treatments do not effectively treat the cognitive symptoms for all patients, there is evidence that treatment with atypical antipsychotics, olanzapine or clozapine, can abate the grey matter loss. This is likely through their high affinity for serotonin receptors, whereas the high affinity D2R antagonist, haloperidol, does not hinder grey matter loss (Lieberman et al., 2005b; van Haren et al.). That cell-receptor level interactions can have such a profound effect on gross tissue level phenomena, necessitates the examination of cellular neuroanatomy associated with schizophrenia.

Despite the reduction in grey matter, the total number of neurons within post-mortem PFC tissue of schizophrenia patients, is not reduced (reviewed by: Garey, 2010); thus there is a higher density of neurons within the tissue (Selemon

et al., 1995; Rajkowska et al., 1998; Selemon et al., 1998). Further research has shown reductions in the number dendritic spines of PFC glutamatergic neurons, indicative of reduced information processing power through excitatory cell-cell communication (Garey, 2010). However, the neuronal density and dendritic arborisation disarray is likely reflective of disrupted neural development related to abnormal regulation of proteins involved in neuron localisation (reelin or Disrupted-in-Schizophrenia-1; DISC1), and cell-surface functions such as neurotransmitter (neuregulin 1) and synaptic (postsynaptic density protein 95; PSD95) signalling. Implicated in the PFC-hypofunction pathology, is enhanced NRG1 signalling which has been found to interact with PSD95, resulting in reduced NMDAR activity within the PFC (Hahn et al., 2006). Also reported are PFC reductions in reelin protein expression in ~50% of patients with schizophrenia (Impagnatiello et al., 1998). While reelin is implicated in developmental processes, that it is preferentially expressed within GABAergic interneurons of the PFC, suggests additional perturbations to the inhibitory neurons within the PFC.

The protein parvalbumin (PV) is a marker of interneurons which has proved useful in studying dysfunction outside of PFC pyramidal cells. While total number of PV-expressing neurons is not altered in schizophrenia patients (for review see: Lewis, 2013), there is evidence for dlPFC layer III and IV specific reductions in PV-mRNA (Hashimoto et al., 2003), as well as reduced mRNA expression of PV-expressing neuron potassium channel Kv9.3, which is involved in activation of PV-expressing interneurons. Furthermore, a subset of PV-expressing neurons also express GABA synthesising enzyme glutamate decarboxylase (isoform 67; GAD67). As consistent reductions in GAD67 mRNA are reported in post-mortem brains from schizophrenia patients (Lewis et al., 2005), the loss of inhibitory function of these PV-expressing neurons may contribute critically to the dysfunction of the PFC. Indeed, expression of GABA transporter subtypes (1 & 3) within the dlPFC is abnormal in tissue from schizophrenia patients, further suggestive of dysregulated GABA signalling (Schleimer et al., 2004).

While this brief review cannot nearly begin to highlight *all* the neurochemical (Figure 1.1) and anatomical pathology (Figure 1.2) induced by schizophrenia, it provides a basic framework for understanding many of the significant impacts of the disease on PFC circuitry. Thus, the following review of the behavioural consequences of these neurobiological pathologies can be understood in context. Furthermore, within the empirical chapters following the introduction, many of these neurobiological pathologies are re-explored in relation to observed (or un-observed) behavioural phenotypes, with a primary focus on deficits in behavioural flexibility and attentional regulation.

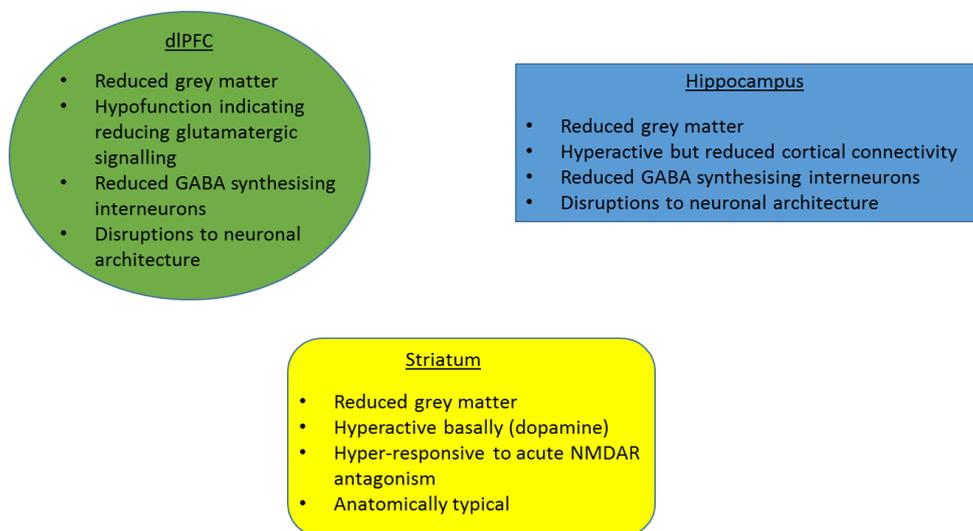


Figure 1.2) Diagram of schizophrenia-related anatomical alterations to the dlPFC, striatum, and hippocampus.

1.4 Schizophrenia behavioural flexibility and attentional regulation

When exposed to a weak auditory prestimulus, healthy persons have reduced blinking (startle reflex) in response to a subsequent (~120-150 ms later) additional pulse (Graham, 1975). Due to the biological abnormalities described in the previous section, patients with schizophrenia do not exhibit this effect to the same degree as controls (Braff et al., 1978). While the initial measurements have

technologically advanced to examine the amplitude of cortical electroencephalogram (EEG) detected potentials (e.g. the P50) in response to the auditory cue, the prepulse inhibition (PPI) paradigm has proven useful as a reliable index of deficient sensory gating in schizophrenia, and a window into the pathology of delusions and hallucinations (Javitt and Freedman, 2015). Interestingly, experiments have implicated the PFC in PPI response in both healthy controls and patients with schizophrenia (Tregellas et al., 2007; Mayer et al., 2013). Indeed, studies from lesion patients further evidence the role of the dlPFC in sensory gating (Knight et al., 1989; Yamaguchi and Knight, 1990; Knight et al., 1999). Critically, these sensorimotor gating deficits in schizophrenia, in addition to the hyperactive dopaminergic system are crucial to the higher-order, dlPFC-dependent, behavioural flexibility and attentional regulation deficits associated with the disorder. Indeed, deficits in sensory gating are evidenced not only during non-goal directed EEG recordings, but also during tasks which require attentional regulation such as dichotic listening (Rosburg et al., 2008). In the next sections, I focus on paradigms which test these functions during goal-directed behaviour.

1.4.1 Schizophrenia and the Wisconsin Card Sorting Test

While patients with schizophrenia are challenged with real world effects of deficits in attentional regulation, clinically measuring those deficits requires operationalising of the dysfunctions in conjunction with identifying the relevant neural substrates for the individual cognitive processes. An early tool for measuring attentional regulation is the Wisconsin Card Sorting Test (WCST; Berg, 1948). The task (Figure 1.3), as described by Berg, originally used a deck of 60 response cards. Each card contained information from each of three perceptual dimensions; colour (red, yellow, blue, green), shape (stars, crosses, triangles, circles), and number of stimuli on a card (ranging from one to four stimuli per card). The experimenter would present four 'stimulus cards' (such that each of the four stimuli within each of the three categories were presented once across all four cards) and inform the subject to sort the response cards based on which

stimulus card they thought it corresponded to. Following each placement, the experimenter would provide feedback on whether the subject's choice was correct or incorrect. This continued until the subject had sorted five cards consecutively using the correct category (e.g. number of stimuli), then the category changed to sorting based on a different perceptual dimension (e.g. colour or shape). This process continued for nine category shifts. Berg found that participants could be divided into three groups, A) those who were "successfully" aware of the category shifts, B) those who were "vaguely" aware of the shifts, and C) those who remained unaware of the shifts.

While group A showed an increase in errors for the first two category shifts, they performed near floor (sorting in five cards) for the remainder of the nine categories. Group B had a pattern of more errors through the entirety of the task than group A, and group C showed drastically worse performance than both A and B. Task performance critically reflects the ability to not only maintain, and respond based on, the relevant category (i.e. short-term and working memory

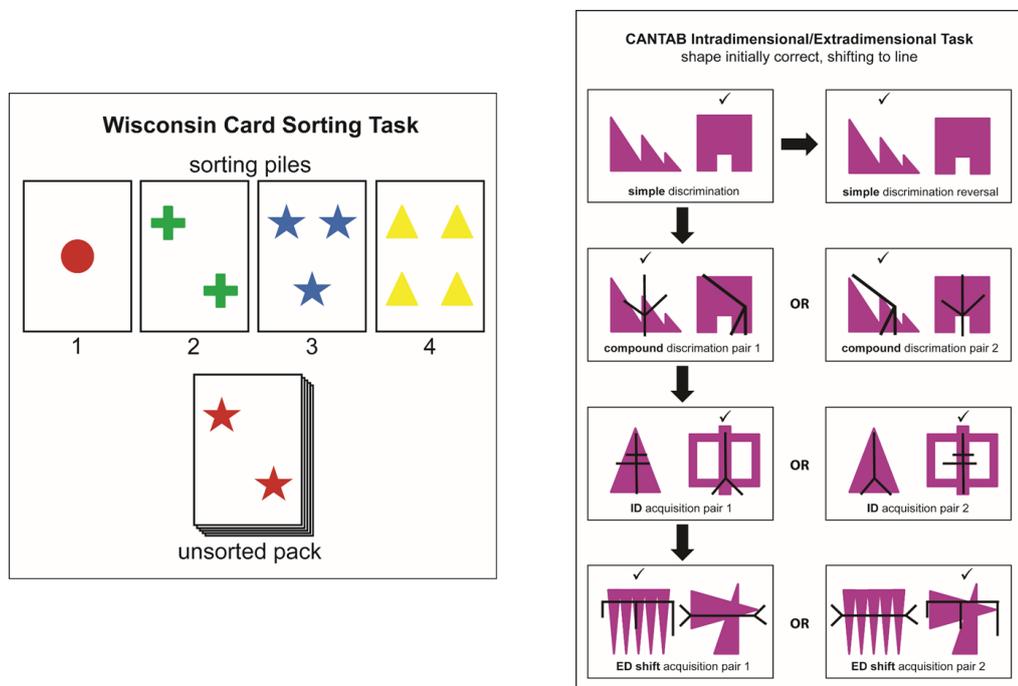


Figure 1.3) Diagrams of the Wisconsin Card Sorting Test (*left*), and the CANTAB ID/ED ASST (*right*; both images from Brown and Tait, 2016).

mechanisms), but to do so in the presence of distractors (i.e. attentional mechanisms). Thus, participants who reported the ability to ascertain and attend to the relevant sorting category had the best performance in the task. As described in the neuroanatomy section, patients with schizophrenia often exhibit a combination of neurochemical and neuroanatomical abnormalities within the PFC. Predictably, as tasks which involve attentional regulation rely on the integrity of the PFC for optimal performance, patients with schizophrenia exhibit deficits in the WCST. Indeed, WCST performance in schizophrenia patients is positively correlated with their PFC integrity and subsequent hypofunction (Berman et al., 1986; Weinberger et al., 1986; Deicken et al., 1995; Bonilha et al., 2008), thus the task is very popular as a measure of PFC function in schizophrenia patients.

A meta-analysis in 2004 reported over 300 studies in the PubMed library using the WCST in patients with schizophrenia (Li, 2004). The majority of these studies focus/report on a single metric derived from performance in the WCST: the number of perseverative errors (errors where the participant has used the same category for their choice as the previous choice, in spite of negative feedback). This type of error generally occurs following the completion of a category, when the subject is unaware a shift is required. Control subjects typically only make one error per switch, whereas schizophrenia patients are consistently reported to make significantly more perseverative errors (Li, 2004). Patients with frontal lobe lesions also make more perseverative errors in the WCST (Milner, 1963; Goldstein et al., 2004), thus researchers hypothesised that the perseverative errors made by schizophrenia patients were induced by PFC-hypofunction (reduced glutamatergic signalling), as opposed to the other neurochemical and neuroanatomical pathologies associated with the disorder. Albeit a step forward, the classification of errors as 'perseverative' does not indicate which of two possible mechanisms underlie the errors. The first possible mechanism for elevated perseverative errors is an inability to reallocate attention from the previously relevant rule/dimension. The second would be enhanced 'learned irrelevance' regarding the irrelevant dimension stimuli, thus impairment based on

reduced allocation of attention to the irrelevant dimension beyond the levels in controls. However, the nature of the WCST prevents analysis of these mechanisms.

Since the WCST uses card stimuli in which the exemplars within all dimensions are partially reinforced across the sorting categories (i.e. specific exemplars are repeated across stages, but require sorting into a different category), it is described as a 'partial change' design, which can confound processes such as reversal learning and set-shifting (Slamecka, 1968). A modification of the WCST, therefore, involves using novel stimuli, and 'stages' that don't require a category shift. This modification has been termed a 'total change' design and, with two-choice discriminations, allows for parsing the processes of stimulus-response contingencies (allowing for reversals within stimuli of the same perceptual dimension), as well as evidence of 'attentional set-formation', the phenomena by which attending to stimuli within one perceptual dimension enhances learning for stimuli within that dimension (within compound discriminations). For example, learning to attend to shape, across multiple stages, would speed discrimination of the correct stimulus, despite each stage presenting with novel shape, colour, and number exemplars. An early example of this total change attentional set-shifting task, for primates is the intradimensional/extradimensional (ID/ED) attentional set-shifting task (ASST) developed by Roberts et al. (1988).

1.4.2 Schizophrenia and the Attentional Set-Shifting Task

Generally, ASSTs measure reversal learning, and attentional set-shifting, additionally, set-formation can be inferred through the latter. In the commonly used touchscreen CANTAB ID/ED ASST for humans (Figure 1.3), shapes, lines, and solidity are the three perceptual dimensions used, with two unique, not readily verbally categorised, exemplars within each dimension (Sahakian and Owen, 1992). In a two-choice discrimination, the participant must select the appropriate stimulus to a criterion of beyond chance selection (normally six consecutive

correct responses). Upon completion of each discrimination between novel stimuli, the stimulus-reinforcement contingency is reversed (REV; i.e. the previously incorrect stimulus becomes the correct stimulus). The task begins with a simple discrimination (SD; e.g. between two differing line stimuli), then an SD reversal. Next the task progresses to compound discriminations in which the relevant exemplars (both correct and incorrect) presented in compound with novel exemplars in an irrelevant dimension (e.g. shape). In the first compound discrimination, the stimuli are presented side by side (C_D). Once participants perform that stage to criterion they advance to a CD in which the stimuli are presented overlapping. Participant must also complete that stage to criterion, and afterwards all stimuli are present as overlapping compounds. The next stage is a CD reversal (CDR), following which, a 'total change' is implemented for the ID acquisition. In the ID, the previously relevant dimension (shape or line) remains relevant, however new exemplars are used across all dimensions (i.e. new line and shape exemplars). In the final stage novel exemplars are again presented in both dimensions, however the correct exemplar is now from within the previously irrelevant dimension. This is an ED shift acquisition, and should induce a shift-cost (greater trials to criterion or errors to criterion) relative to the ID if an attentional set was formed (Eimas, 1966). Due to this structuring of stages researchers can parse trials to criterion (TTC) or errors to criterion for individual learning and attentional processes. Specifically, aside from the reversal stages, where reversing stimulus-reward contingencies is intended, the use of novel stimuli eliminates possible interference from stimulus-reward contingencies from previous stages, and the ED stage can be manipulated to examine perseveration to the previously relevant dimension compared to learned irrelevance of the previously irrelevant dimension (Owen et al., 1993).

While control participants are typically able to reach criterion for each stage within 50 trials (the cut-off for progression to the next stage), initial findings revealed that only ~50 - 60% of schizophrenia patients could complete the task (reaching criterion for each stage within 50 trials; Elliott et al., 1995), similar to

findings of reduced number of total shifts completed for schizophrenia patients in the WCST (Everett et al., 2001). Those patients who completed the task committed significantly more errors at the SD reversal and the CD. Additionally, the researchers used a manipulation at the ED to examine perseveration versus learned irrelevance. In the perseveration condition, the previously relevant dimension (e.g. line) became the irrelevant dimension, and the previously irrelevant dimension (e.g. shape) was replaced with a novel dimension (e.g. solidity) which the correct exemplar was derived from: thus, the participant was only be required to shift attention *away* from the previously relevant dimension. In the learned irrelevance condition, the previously relevant dimension (e.g. line) was replaced by a novel and irrelevant dimension (e.g. solidity), and novel exemplars were presented in the previously irrelevant dimension (e.g. shape), which became relevant: thus, the participant could only be require to shift *to* the previously irrelevant dimension. While schizophrenia patients showed no deficit at the ID or the learned irrelevance ED, they performed worse than controls during the perseveration ED, suggestive of an impairment in reallocating attention *away* from the previously relevant dimension. However, a subsequent study by the same research group found a different pattern of results (Pantelis et al., 1999), and is reviewed further in the next section.

Pantelis et al. (1999) tested schizophrenia patients in the ID/ED ASST, this time using a standard ED stage (where the relevant dimension became irrelevant, and vice versa). Critically, this differs from the modified task (Elliott et al., 1995), as the shift to the previously irrelevant dimension, could be still be due to learned irrelevance or perseveration, as a novel dimension was not introduced at the ED. This difference notwithstanding, they still found an impairment at the ED (similar to Elliot et al.). However, unexpectedly the researchers also found a significant impairment at the ID, in addition to the ID reversal. This apparently contradictory finding was very informative, as further examination of patient populations revealed that differences in the age of patients, and length of hospitalisation, may account for the discrepant findings. The patients in the Pantelis et al. experiment

were ~10 years older, and hospitalised for an average of 18 years, whereas the majority of the schizophrenia patients in the Elliot et al study were outpatients. Thus, the behavioural manifestation and nature of deficits can possibly change and/or exacerbate over the duration of the illness, with chronicity impairing set-formation (ID acquisition).

The possibility that the disease causes progressive deterioration, with time from onset correlating with impairments in attentional regulation, is actually supported by many additional behavioural studies. Using the same ID/ED ASST, (Hutton et al. (1998)) found that patients classified as first experiencing schizophrenia symptoms (first-episode), while failing to reach criterion at the ED, exhibited no other significant deficits at any other stages. Further, the percent of first-episode schizophrenia patients failing to reach criterion was less than as reported previously for chronic patients (Pantelis et al., 1999 1995). This finding was later replicated in a direct comparison of first-episode schizophrenia patients and chronic schizophrenia patients (Pantelis et al., 2009). Additional studies comparing amongst patients past the first-episode lend more support to the argument that the duration of illness influences the impairments (Pantelis et al., 2004; Hilti et al., 2010). However, a longitudinal study examining patients from their first-episode through follow up testing after 1, 3, and 6 years found no significant reduction in performance, as patients exhibited consistent significant reversal and ED shifting impairments, that did not differ by length of disease onset (Leeson et al., 2009). It possible that differences in patient selection methods can again explain these discordant results, as the Pantelis et al. (2009) study was a between-subjects design (no repeat ID/ED testing), whereas the Leeson et al., study was a within-subjects design with participants completing the task a total of four times. As patients in both studies were taking neuroleptic medication, these effects cannot be considered a consequence of reduced treatment. Overall, the progressive cortical atrophy (primarily PFC) reported in neuroanatomical findings would support the findings of the deficits in the ASST also being progressive.

As described in the neurochemistry section, the positive symptoms of the disorder are commonly tractable to treatment with D2R antagonists, while the cognitive symptoms are resistant to dopaminergic treatment (Conn et al., 2009). This is critical, as the degree of cognitive dysfunction is a strong indicator of long-term outcome in patients (Ventura et al., 2009; Meyer et al., 2014; Torgalsboen et al., 2014). Therefore, there is a desperate need for therapies which can enhance cognition in patients with schizophrenia. To this end, a large movement in preclinical rodent experiments has focused on developing: 1) rodent manipulations with relevance to schizophrenia, 2) tasks with translational validity in which to assay cognition in rats, 3) putative pharmacotherapies which can enhance cognition of the rats performing those tasks, following the schizophrenia related manipulation. While many of the visual discrimination based tasks (e.g. the CANTAB ID/ED) developed for human use are readily performed by non-human primates (Dias et al., 1996a, b), the lengthy developmental maturation period, and expense of performing tests with non-human primates, prevents high throughput testing. Thus, many preclinical researchers focus on experiments with manipulations in rats which eliminates many of those concerns, while providing access to many of the evolutionarily conserved neural structures and functions which are being targeted for investigation. In the next section I introduce some of the behavioural flexibility tasks that have been developed within this framework.

1.5 Tasks used to measure behavioural flexibility in rodents

One of the earliest perceptual set-shifting tasks in rats attempted to use visual stimuli (shapes and stripes) in a two-choice discrimination paradigm (Shepp and Eimas, 1964). Training required several days for rats to reach criterion with the discriminations (simple then compound), however once attained the following day the rats were tested in either an ID (e.g. continue responding to shape), or an ED (e.g. respond to stripe). This was the first instance of a total change design (i.e., the stimuli were novel) to assess set-shifting (in any mammal), and as expected the rats tested in the ID condition committed fewer errors than the rats tested in

the ED condition. Testing of behavioural flexibility in rats continued with this stage progression (acquisition phases followed by either ID, ED, or REV) for several decades, however (Birrell and Brown, 2000) reported on an improved perceptual set-shifting task for rodents, the rodent ASST (Figure 1.4).

Based on the ID/ED ASST developed for primates (marmoset monkeys and humans) by T.W. Robbins (Roberts et al., 1988; Sahakian and Owen, 1992), the rodent ASST relies on digging bowls containing olfactory and somatosensory stimuli, as opposed to visual discriminations which rats do not readily perform (Bussey et al., 1997). Indeed, by taking advantage of rodents' innate foraging behaviour, and discrimination based on odours and media, the training phase for the task can typically be done in the course of an hour, with complete testing proceeding the following day. Following closely to the CANTAB ID/ED ASST, the rodent ASST contains 7 stages (SD, CD, REV 1, ID, REV 2, ED, and REV 3). As a measure of construct and concurrent validity, the Birrell and Brown (2000) study examined if rats with medial PFC (mPFC) lesions exhibited similar performance as human patients with frontal lobe damage and monkeys with lateral PFC (IPFC) lesions. The rodent ASST was validated by this standard, as ibotenic acid-induced mPFC-lesioned rats had a deficit at the ED, consistent with the findings in human and non-human primates (Owen et al., 1991; Owen et al., 1993; Dias et al., 1996b). This task is the most widely used perceptual ID/ED set-shifting task, although other tasks also exist which measure 'switching' behavioural flexibility. These tasks are, however, typically more closely related to the WCST, as the stimuli remain constant throughout testing phases/stages.

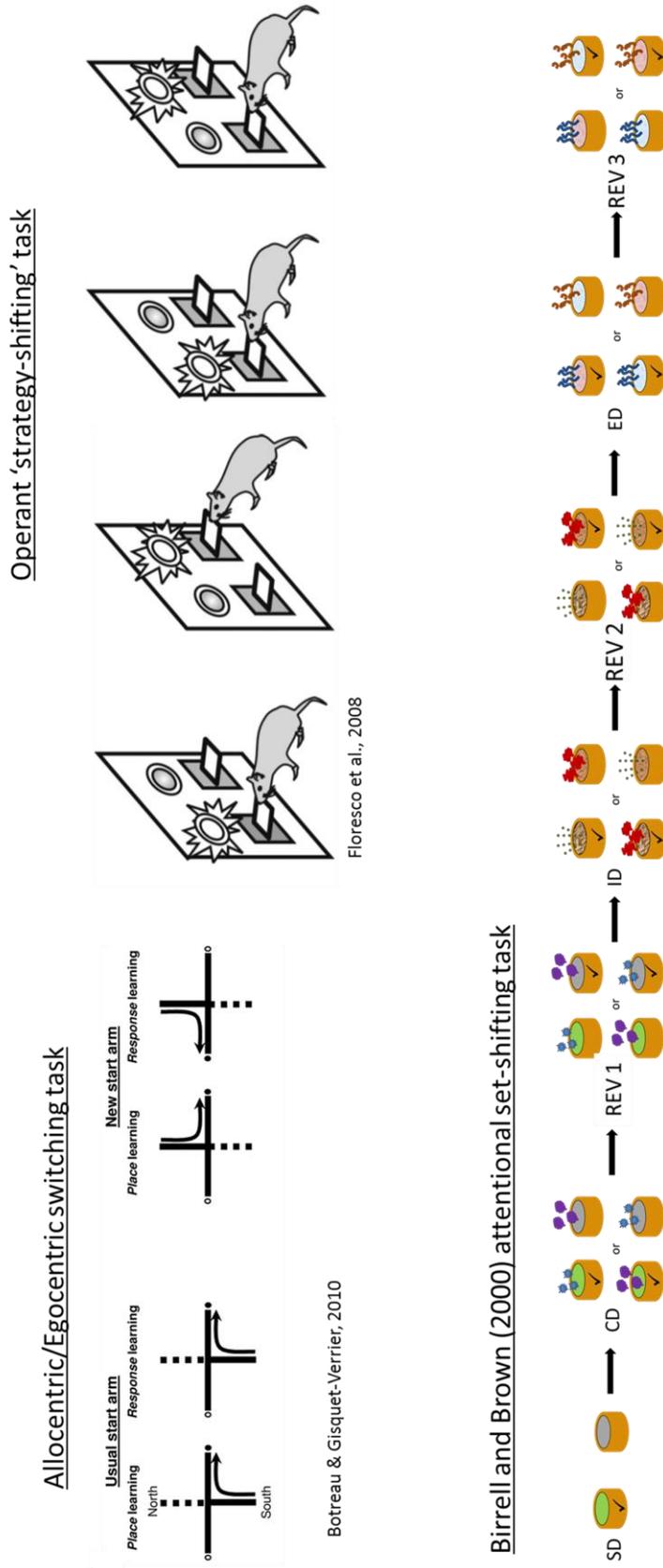


Figure 1.4) Diagrams representing rodent WCST-like tasks (top) and the CANTAB ID/ED based perceptual ASST (bottom).

In 1999, Ragozzino et al., tested mPFC-inhibited rats in a plus maze task (Thompson et al., 1980), in which either allocentric spatial location (each arm corresponding to north, south, east, or west), or an egocentric cue (always turn right regardless of starting location), served as rules to guide the animal to the baited arm. Rats were initially tested in the allocentric or egocentric discrimination conditions and then required to switch to responding to the opposite cue during the next phase. Rats with inactivation of the mPFC (pre- and infralimbic cortex) were impaired at the “cross-modal shifts” (e.g. switching from allocentric to egocentric responding), but not acquisition of either response strategy or “intra-modal” reversal of either strategy. The researchers concluded that mPFC is important for these cross-modal shifts.

Floresco et al. (2008) reported the use of a new automated procedure to measure behavioural flexibility in rats. In an operant chamber with a lever to the right and left of the reward pellet dispenser, and a light-emitting diode above each lever, rats were trained to respond either based on the lever location or the light location. Following simple discrimination learning, where either only the light cues or the lever cues were presented, there followed compound discrimination, wherein light and lever cues were presented in compound. During this stage the rat had to continue to respond to the cue from the SD (either the light cue or the egocentric lever cue). The ‘compound’ nature of the light/lever stimuli was considered an advancement over the plus maze rule-switching test, and removed the need for allocentric-based responding (Ragozzino et al., 1999). Inactivation of the mPFC in this task impaired the strategy-shift either from visual cue to egocentric response cue or vice versa (Floresco et al.). However, this task was not without limitations, which along with those of the plus maze task (Ragozzino et al.) are discussed in the next section.

In the operant chamber task (Floresco et al., 2008), clear differences in the difficulty of acquisition of, and shifting to, the egocentric lever cue strategy were evident in both mPFC-inactivated and control rats. Furthermore both tasks, plus maze and operant chamber, require days to weeks of habituation and training

prior to testing. The subsequent testing phase can also require days prior to acquisition by experimental and control groups. While these tasks are similar to the WCST in that they require a rule switch or strategy-shift, they cannot address questions of attentional set as the ID/ED tasks do, as they lack discriminations based on multiple stimuli within a perceptual dimension. The only possible manipulations within the tasks (Ragozzino et al., 1999; Floresco et al.) are location based, whereas the ID/ED ASST with multiple stimuli based within a dimension (odour or medium) can present those stimuli, in compound, in any spatial location, without confounding. Additionally, whereas the light stimulus is either on or off (Floresco et al.), ID/ED ASST dimensional stimuli can change while maintaining the higher order 'response rule', for example respond to an odour regardless of medium. Thus, the 'total change' nature of the perceptual rodent ID/ED ASST developed by Birrell and Brown (2000) provides the same advantages over the spatial-based behavioural flexibility tasks, as the ID/ED ASST over the WCST. Given the advantages of the Birrell and Brown ASST, all studies within this thesis adhere to that protocol, or are slightly modified from that protocol to address different learning and attentional processes.

1.6 Thesis rationale

The primary aim of using the ID/ED ASST in the following experiments is to study schizophrenia-related learning and attentional processes. To this end, three targeted manipulations in rats with relevance to schizophrenia were examined. The manipulations induce different schizophrenia-related phenotypes (both behavioural and biological), targeted at three different developmental time points corresponding to adulthood, juvenile, and gestational periods in humans. In Chapter 3, the manipulation examined is based on acute inhibition of mPFC glutamatergic neuronal signalling in adult rats. In Chapter 4, the effects of transient blockade of NMDARs during the neonatal period on adult behaviours is tested. Finally, in Chapter 5, another developmental manipulation is examined, as I report experiments in adult rats following inhibition of DNA translation within

neurons during critical period in gestation for cortical development. Figure 1.5 presents how each manipulation targets a known sensitive period for development of schizophrenia. The focus of these studies to test the hypotheses that: 1) schizophrenia-like ASST behaviour in rats can arise from targeted manipulation of the brain at several timepoints; 2) these targeted manipulations can allow investigation into the mechanisms underlying the ASST deficits.

Chapter 3: Inhibition of mPFC
glutamatergic signalling

- Adult PFC hypofunction induced by NMDAR antagonism or acute/chronic stress

Chapter 4: Neonatal-PCP treatment

- Late gestational/early childhood disruption of cortical glutamatergic signalling

Chapter 5: Gestational MAM-treatment

- Early gestational genetic disruption which alters cortical layering and neuronal density

Figure 1.5) Diagram of sensitive period targeted by each manipulation.

Chapter 2. General Methods

All experiments in this thesis were conducted in accordance with the regulations laid down in the United Kingdom Animals (Scientific Procedures) Act 1986, and performed with the authority of a Project License (PPL 60 / 4459) approved by the UK Home Office and the University of St Andrews Animal Welfare and Ethics Committee (personal licence number: IEB1CF8FE). Animals were kept under standard housing conditions (two-three per cage, 0700–1900 hr light phase, controlled temperature and humidity, ad libitum water).

2.1 ASST protocol

2.1.1 Apparatus

Training and testing took place in a modified plastic housing-cage (69.5 x 40.5 x 18.5 cm). The testing arena has two individually dividable chambers in which ceramic bowls containing digging stimuli and reward are placed.

2.1.2 Training

At least 12 hrs prior to training, each rat was given a bowl filled with home-cage sawdust and six pieces of food reward (half a Honey Loop cereal piece; Kellogg, UK) in the home-cage. Following exposure to the reward, rats were trained to dig in bowls filled with sawdust to obtain a food reward within the testing arena. To shape this response, the reward was placed on top of the sawdust on the initial trial. After the rat completed the trial by retrieving the reward, it was placed slightly deeper on the subsequent trial. This continued until the reward was completely buried at the bottom of the bowl and the rat was reliably retrieving it on each trial. Both chambers were baited and the rat was required to retrieve the reward from each side to prevent formation of a side bias. This 'digging' training regimen was typically completed in six trials. Next, two-choice simple discrimination training was performed. All rats were trained on the same exemplars for both odour (mint and oregano) and medium (shredded paper and polystyrene) discriminations. These exemplars were not used again. For the first four trials of a discrimination, rats were permitted to obtain the food reward from the correct bowl after an incorrect dig; however, after these four trials, an incorrect dig led to access to the correct bowl being blocked. Completion of a stage required rats to reach a criterion performance of consecutive correct responses which was greater than that predicted by chance 6-in-a-row ($p = 0.016$). Correct responses within the first four trials were included in this measure. The first test commenced the day following training. Subsequent tests were completed without the need for simple discrimination training.

2.1.3 Standard 7-stage ASST testing

The seven discrimination stages proceeded as follows:

- 1) a simple discrimination (SD) in which the rat either responds to an odour in sawdust, or an unscented digging medium
- 2) a compound discrimination (CD) in which the correct stimulus from the previous stage is paired with irrelevant stimuli from a different dimension
- 3) the first reversal (REV 1) in which the previously incorrect stimulus from the SD and CD becomes the correct stimulus
- 4) an intradimensional discrimination (ID) where new compound stimuli are presented however the correct stimulus is still within the same dimension as the preceding stages
- 5) a second reversal (REV 2) where the incorrect stimulus from the ID stage becomes the correct stimulus
- 6) an extradimensional shift (ED) in which all new compound stimuli pairs are presented and the correct stimulus is now one of the stimuli in the previously irrelevant dimension
- 7) lastly a third reversal (REV 3) in which the incorrect stimulus from the ED (within the same dimension) becomes the correct stimulus)

All exemplars used were counterbalanced for presentation order, and ED shift direction (i.e. odour to medium, or medium to odour). Table 2.1 depicts the exemplar pairs used.

Dimension	Training Pairs	Pair 1	Pair 2	Pair 3
Odours	Mint / Oregano	Cinnamon / Ginger	Sage / Paprika	Turmeric / Cloves
Media	Polystyrene/ Shredded Paper	Coarse Tea / Fine Tea	Sand / Grit	Coarse Shavings / Fine Shavings

Table 2.1) Exemplars used for standard ASST testing. Exemplars were presented in these pairs. Presentation order during testing was counterbalanced (e.g. 1 → 2 → 3 or 2 → 3 → 1).

2.2 Histology

Following behavioural testing rats were anaesthetised with 0.8 ml pentobarbital (ip; Pharmasol, Ltd, UK), then intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were stored in 20% sucrose for 24 hours at 4°C, then the brainstem and cerebellum were removed prior to weighing. Following weighing, brains were washed in distilled water, dried, placed in wells, covered in egg yolk, and placed in a formaldehyde bath for 72 hours. Afterwards, brains were sectioned at 50 µm on a freezing stage microtome (Jung Histoslide 2000, Reichert-Jung, Cambridge Instruments). Sections were stored in glycerol solution at -20°C.

For parvalbumin immunoreactivity detection, every 8th section of the frontal cortex was collected, and every 8th section of the hippocampus. Sections were placed in 9-hole netwells and petri dishes and washed four times for 5 min each in phosphate buffered saline (PBS: 158 M NaCl in 50% distilled H₂O and 50% 0.2 M phosphate buffer) on an automated rotator. Next the netwells were placed in blocking solution (1:5 normal goat serum, 1:100 10% Triton, in PBS) and rotated for an hour at room temperature. Sections were then washed in PBS three times

for 5 min each. Sections were placed in histology pots before being incubated with 5 ml of anti-parvalbumin antibodies (mouse anti-parvalbumin 1:8000, Sigma; in antibody diluting solution; ADS; 1:100 normal goat serum, 1:100 10% triton, in PBS) overnight at room temperature. The following day sections were washed in 9-hole netwells in PBS three times for 5 min each. Sections were then switched back into histology pots and incubated in 5 ml anti-mouse antibodies (goat anti-mouse, 1:200, Vectastain, in ADS) for one hour at room temperature. After incubation, sections were washed three times for 5 min in PBS, then placed in histology pots containing 5 ml of the biotinylation solution (Vectastain ABC KIT) and incubated for one hour. Sections were then washed three times for 5 min in PBS and parvalbumin immunoreactivity was detected by staining with 3,3'-Diaminobenzidine (DAB; one tablet per 20 ml, Sigma, in distilled H₂O). The sections were determined to be stained when landmark anatomical structures were clearly identifiable or until they had been incubated for 20 min. Following DAB staining sections were washed three times for 5 min in PBS, and stored in 9-hole netwells at 4°C until (up to 72 hours) they were mounted to treated glass slides and coverslipped with DPX.

All sections were imaged on a Zeiss Axio Imager M2 with ZEN software. Immunostaining was quantified using ImageJ automated cell counter. Images were grayscale transformed so that immunostaining appeared black. For the PFC 7-10 sections per brain were counted. For the hippocampus 3-4 sections per brain were counted. Regions of interest were outlined manually per the Paxinos and Watson atlas (1998). The settings for image J automated counting were (width:7; minimum distance between cells of 2.5; and opacity threshold of 2.5). Size measurements were also gathered from outlined regions of interest, with distance defined using the image scale bar for accuracy of measurements. An example of the analysis is provided in Figure 4.9 where it is first used.

2.3 Statistical analysis

2.3.1 Frequentist approach: Null hypothesis testing

Multi-factorial repeated measures ANOVA were performed with the relevant variables as the repeated factors (e.g. stage or test). Data were analysed in SPSS v21. When the repeated measures ANOVA revealed significant interactions, restricted comparisons were performed using corrected F values, calculated by dividing the mean square effect from the multivariate ANOVA, by the mean square error from the relevant interaction in the omnibus repeated measures ANOVA, and using the degrees of freedom associated with the error. This method of calculation is known as a corrected-omnibus analysis (Winer, 1971), which is a better estimate of the population error. To ensure statistical robustness, Huynh-Feldt corrected values were used for all F- and error values obtained from repeated measures ANOVAs. The Huynh-Feldt correction increases the error value to account for violations of sphericity/non-homogeneity of variance. For all figures *, indicates significantly different from controls, and #, indicates ID significantly different than ED, both $p < 0.05$.

2.3.2 Probability approach: Bayesian analysis

➤ *Issues with frequentist criterion standards for the ASST*

Confirmation of stage completion, within the ASST used for primates and rats, uses a frequency criterion level of performance, which is typically 6-correct-in-a-row ($p = 0.016$). This approach is a traditional 'null vs alternative hypothesis' framework, with 'random-guess' implied as the null hypothesis. However, there are three issues with the standard frequentist approach:

1. Although the p -value of 6-correct-in-a-row = 0.016, this value is only valid for independent instantiations of 6 trials. In a cumulative distribution, such as TTC measurements, the probability of attaining 6-correct-in-a-row rises as the number of trials rises;

2. Frequentist approaches are prone to under- and over-training, as they are insensitive to the subject's behavioural pattern outwith the window they measure (e.g. six trials);
3. There are other possible strategies that a subject may be using aside from the responding to the desired stimulus which need to be rejected. In the next sections I discuss some limitations of frequentist criterion and describe how a Bayesian analysis may alleviate these concerns.

➤ *Frequentist criterions are susceptible to false positives with increasing trials*

As mentioned above frequentist criterions are used based on the probability of getting k correct-out-of- N trials. The CANTAB version of the ASST uses the 6-in-a-row criterion with a limit of 50 trials (Sahakian and Owen, 1992). The rat task adopted the human ASST frequentist criterion (Birrell and Brown, 2000). However, some researchers using the task have a stricter criterion: mice typically require more trials and so for mouse ASST an 8-in-a-row criterion has been used (Bissonette et al., 2008), although, an automated set-shifting test for mice has used a less strict criterion of 8-out-of-10 criterion (Scheggia et al., 2014). Marmosets have been tested with even more strict criterion of 90% correct-out-of-32 trials on two consecutive sessions (Dias et al., 1997), and more recently 90% correct-out-of-40 within a single session (Clarke et al., 2005). The first issue with many of these frequentist criteria, is that the p -value only remains significant within N_k trials. This is evidenced by Monte Carlo simulations (simulations of trial by trial random choice behaviour during a two-choice discrimination) to determine the familywise error rate (mathematical false positive) for each of the above criteria (Figure 2.1). The simulated data represent the mathematical false positive rate given random responding in a two-choice discrimination. Specifically, for the rodent criteria, after 10 trials, the potential for a mathematical false positive rose above $p < 0.05$, revealing that the risk of a Type I error was no longer statistically desirable.

➤ *Frequentist criterions are susceptible to over- and undertraining subjects*

The second issue is the insensitivity to the entirety of the subject's decisions in the task. All of the frequentist criteria are limited to the k-out-of-N trials, however it is possible that 'learning' has occurred prior to this, or not occurred despite criterion being reached. Undertraining, reflects a circumstance where a subject progresses to the next stage after reaching the frequentist criterion, however, the subject is not actually making decisions based on the correct rule. This could occur by chance as seen in Figure 2.1 for the 6-in-a-row criterion. Indeed, the greater the TTC for a stage the higher the probability that a percentage of subjects are attaining the 6-in-a-row criterion by chance. This could be critical for stages which often require upwards of ten trials such as reversals or the ED and even more so in impaired subjects. A control measure for this would be raising the criterion standard to reduce the potential for undertraining. However, this approach is susceptible to unintended overtraining. The stringent criterion of the marmoset ASST, while reducing the occurrence of mathematical false positives to null (Figure 2.1), fails to actually detect when the subject has attained the learning rule. Thus, some subjects will receive additional trials beyond learning of the rule. In the case of the consecutive correct criterions, the progression correct resets after errors, thus repeated near attainments (e.g. 5-out-of-6 correct) are not recognised as a metric of the subjects' learning. If this pattern repeats again the subject could be unintentionally overtrained. This overtraining could potentially bias later decision making, such as the phenomenon of the overtraining reversal effect (Richman et al., 1972).

➤ *Frequentist criterions are insensitive*

If the aim of the criterion is to progress the task when the subject has evidenced 'learning' and applied the correct 'strategy', then all of these criteria are insensitive to the 'strategy' the subject is using. A Bayesian approach would allow for testing amongst a multitude of hypotheses including the intended strategy (responding based on the correct perceptual dimension), thus increasing

the likelihood of passing the subject to the next stage for the correct reason. Furthermore, when the subject is making decisions that reflect an incorrect strategy, such as choosing a location repeatedly instead of responding to the relevant stimulus dimension, a Bayesian approach can test that hypothesis and quantify the probability for that strategy. Recent papers have tried to 'predict' an animal's decision process by fitting behavioural data to Bayesian Inference models (Costa et al., 2015; Rygula et al., 2015). In the next section, I show how I have adapted Bayes' theorem for a similar purpose. To avoid confusion with the term 'strategy-shifting' used by others (Floresco et al., 2008), and to emphasise that the analysis within this thesis does not quantify what the rat is 'thinking', the term employed henceforth is 'behavioural pattern'. Thus, a rat's behavioural pattern

may reflect responding using a spatial pattern (e.g. place-match), or to a perceptual stimulus (e.g. cinnamon).

Frequentist Criteria

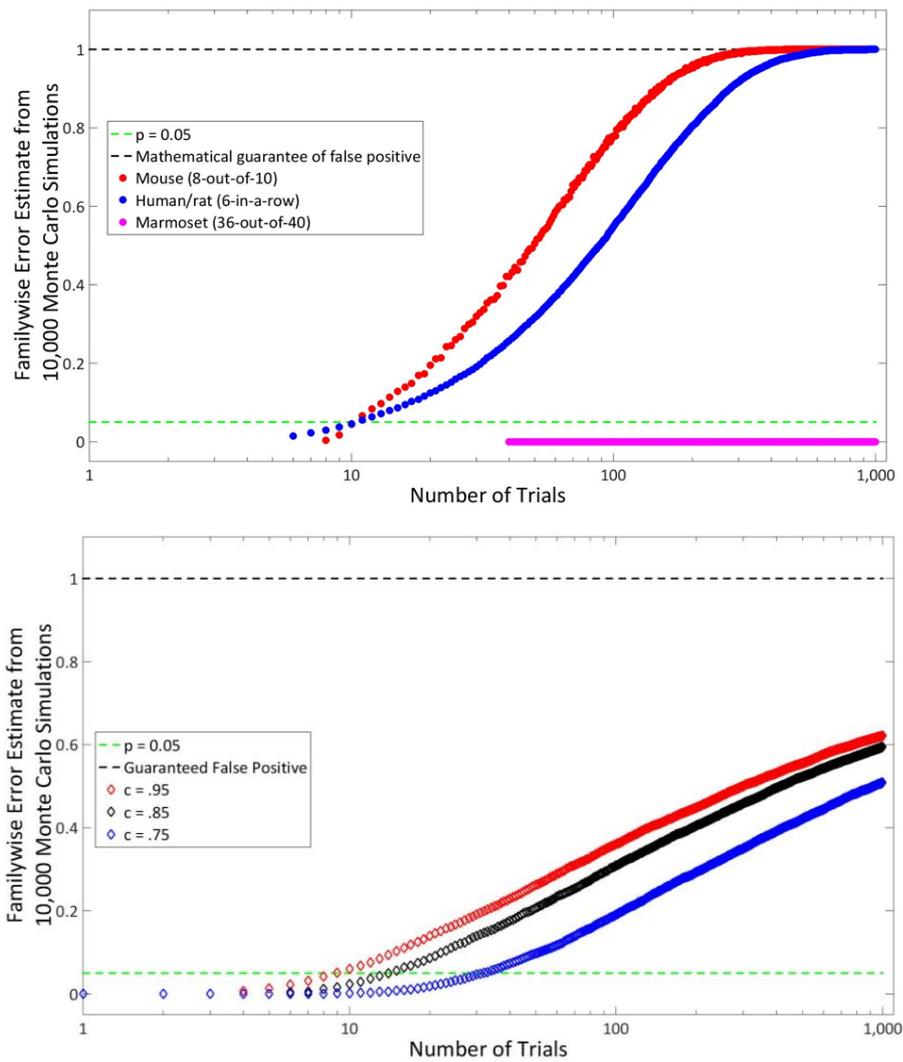


Figure 2.1) Simulated data for two-choice discriminations over cumulative trials. *Top.* Currently, used rodent criteria remain within the acceptable p -value range (< 0.05) up until the number of trials required for the stage exceeds ~ 10 . *Bottom.* While Bayesian criteria are also susceptible to generating false positives, this is at a greatly reduced rate.

➤ *Bayes Theorem Application*

Given a history of prior choices, and a specified number of hypotheses (behavioural patterns), Bayes theorem (1763) allows for calculation of the probability that each choice supports each of the hypotheses. This can be applied to the ASST to ascertain which of the behavioural patterns a rat's choice on a trial is evidencing, essentially a probability of 'what' the rat is doing. Mathematically, in the ASST the probability that a decision made by a rat on trial_(n) matches the desired correct response can be estimated given the rat's current decision and its decision on trials (1 through (n-1)). The formula to perform this calculation is in Figure 2.2.

From this formula provided by Bayes, it is possible to derive an algorithm that updates the probability for defined hypotheses (i.e. behavioural patterns) on a trial by trial basis. Thus, I have numerated how many 'logical' behavioural patterns a rat might provide evidence for during the task. To this end, the lab has determined that there are eight probable behavioural patterns. The performance data may show a spatial-based behavioural pattern (place-match: choosing the same side on consecutive trials; alternation; win-stay; or win-shift), or a perceptual-based behavioural pattern (to any one of the four stimuli from the two perceptual dimensions), as depicted in Figure 2.2. Behavioural patterns are simply what is seen in the data. It is not possible to know that the animal is necessarily selecting a response on the basis of that pattern. Nevertheless, the probability of each pattern can be tested against each other during each trial using Bayes theorem (see formulae in Figure 2.2). While each pattern starts with an equal probability ($p = 0.125$), representing equal evidence amongst them, following the rat's first choice, the probabilities update to increase for those supported by the choice and decrease for those patterns not supported. Using these criteria, the mathematical false positive rate during a discrimination is greatly reduced (Figure 2.1), while remaining sensitive to the entirety of the subject's choice behaviour. Aside from the number of behavioural patterns tested, the only free parameter within the algorithm is the likelihood of the evidence, that is to say how accurate

the choice is actually made based on the pattern(s) it supports. The likelihood parameter ‘c’ should ideally be 1. However, the analysis allows for the appearance of non-systematic choice behaviour, such as ‘explore-exploit’ (Berry and Fristedt, 1985). Therefore, I tested a range of values for ‘c’, with a Bayesian posterior

Possible patterns	Hypothesis	Behavioural pattern
Spatial _{Chamber}	H ₁₋₂	Side ₁ or Alternation ₂
Spatial _{Reward}	H ₃₋₄	Win-Stay ₃ or Win Shift ₄
Perceptual _{Medium(M)}	H ₅₋₆	Mn ₅ or Mn+1 ₆
Perceptual _{Odour(O)}	H ₇₋₈	On ₇ or On+1 ₈

Bayesian analysis formula and implementation

Bayes formula: Where H is hypothesis (behaviour pattern), E is the **new evidence**, and E* is the **past evidence**:

$$P(H1 | \{E, E^*\}) = \frac{P(E | H1, E^*) \cdot P(H1 | E^*)}{P(E | E^*)}$$

$$P(H2 | \{E, E^*\}) = \frac{P(E | H2, E^*) \cdot P(H2 | E^*)}{P(E | E^*)}$$

$$\dots$$

$$P(H8 | \{E, E^*\}) = \frac{P(E | H8, E^*) \cdot P(H8 | E^*)}{P(E | E^*)}$$

$$P(E | H_n, E^*) = \begin{cases} c & \text{consistent between } E \text{ and } H \\ 1-c & \text{otherwise} \end{cases}$$

Figure 2.2) *Top*. Table of possible behavioural patterns. *Bottom*. Formula for Bayesian calculation of the probability for each pattern.

probability threshold of 95% to compare to the p -value measure of frequentist criterions (Figure 2.1). There is deviation, with the lower value of $c = 0.75$ reducing the rate of mathematical false positives, and $c = 0.95$ slightly increasing mathematical false positive rates. As yet there is no empirical evidence for the actual value of 'c', which may vary across trials, and across individual rats. The value of $c = 0.85$, used in the analyses here is therefore based purely on assumption necessitated by an inability to determine what a rat is 'thinking' when it makes a decision. As the analysis here is merely used to guide experiments and reveal potential differences, additional manipulation of 'c' is not needed, however, future experiments can determine how varying 'c', changes the results presented here.

Due to the differences in TTC for individual rats, the group mean data presented here are presented in quartiles. For example, for a rat which completes the SD within 12 trials, the probabilities at trials, 3, 6, 9, and 12, were utilised. For irregular TTC the corresponding trial number was rounded, such that if criterion was reached in 6 trials, the probabilities at trials 2, 3, 5, and 6, were utilised. The resultant quartile probabilities were then averaged within a group. Additionally, for simplicity of graphs, the maximum evidence for either irrelevant dimension stimulus, or any of the four-possible spatial-based patterns is pooled into a single metric, irrelevant dimension or spatial, respectively.

Although the raw mean probabilities for each pattern can be used for comparison (with chance performance = 0.125), a more informative measure compares the Bayesian-derived posterior probability to chance evidence for the pattern. This measure is termed a Bayes factor. When the value yielded by the calculation is greater than 3 (reflecting 3 times greater than chance evidence for that pattern), the evidence is considered strong, as is consistent with standards used to reject the null hypothesis in frequentist measures (Jeffreys, 1939; Dienes, 2014). Indeed, within this thesis, in which the 6-in-a-row criterion was used for data collection, the subsequent behavioural pattern analysis revealed that across all stages for control rats the Bayes factor for the correct behavioural pattern was

greater than 3 at completion of the stage. The data here are presented as heatmaps with colour corresponding to the mean group Bayes factor. Also employed are bar graphs and tables for summary analysis.

For illustration of the method, Figure 2.3 shows an example of the 6-in-a-row criterion p -value changing trial by trial (a binomial calculation based on $N, k, q = 0.5$), compared to the Bayesian derived posterior probability reflecting responding based to the correct stimulus. In this example, the rat’s behavioural pattern indicates responding to the correct stimulus 3 trials prior to the frequentist criterion. Calculating the Bayes factor reveals that the rat is almost 8 times more likely to be responding based on the correct stimulus than any of the other possible patterns.

Example of Bayesian implementation

Trial	Left medium	Left odour	Right medium	Right odour	Rat's side choice	Correct choice?	Frequentist p -value <i>correct choice</i>	Posterior probability of <i>correct stimulus</i>
1	M13	O13	M14	O14	R	0	0	0.075
2	M14	O14	M13	O13	R	1	0.5	0.250
3	M13	O14	M14	O13	R	0	0	0.075
4	M14	O13	M13	O14	R	1	0.5	0.250
5	M14	O14	M13	O13	R	1	0.25	0.425
6	M14	O13	M13	O14	R	1	0.125	0.722
7	M13	O13	M14	O14	L	1	0.063	0.824
8	M14	O14	M13	O13	R	1	0.031	0.845
9	M13	O14	M14	O13	R	0	0	0.497
10	M14	O13	M13	O14	R	1	0.5	0.845
11	M13	O13	M14	O14	L	1	0.25	0.849
12	M13	O14	M14	O13	L	1	0.125	0.969
13	M14	O14	M13	O13	R	1	0.0625	0.970
14	M13	O13	M14	O14	L	1	0.0313	0.970
15	M13	O14	M14	O13	L	1	0.0156	0.995

Bayes factor calculation:

$$.995 \text{ (posterior)} \div .125 \text{ (chance)} = 7.96$$

Figure 2.3) Example table of the posterior probability changing based on the rat’s choice compared to the frequentist based p -value.

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

Experiments in this chapter focused on 'schizophrenia-related' performance in the attentional set-shifting task in rats with acute inhibition of the mPFC (mPFC_{in}). The aim was to model the PFC-hypofunction component of schizophrenia. The first experiment validated the methodology and replicated the finding of an extradimensional set-shifting deficit with mPFC_{in}. Analysis of the behavioural patterns implicated dysfunctional sensory gating in the deficit. Two further experiments employed modifications of the ASST. In both experiments, the hypothesis was that mPFC_{in} rats would learn about the redundant stimulus, whereas control rats would not. The experiments confirmed that mPFC_{in} induces a deficit in attention regulation wherein redundant information obtains associative strength and can guide behaviour.

3.1 Introduction to acute manipulations to induce 'schizophrenia-related' behaviour in rodents

Schizophrenia is typically characterised as a neurodevelopmental disorder, however 'schizophrenia-related' symptoms can be acute onset and induced via several 'acute' mechanisms including NMDAR antagonism (Krystal et al., 2005), amphetamine and cannabis abuse (Srisurapanont et al., 2003; Arendt et al., 2005), and extreme stress in vulnerable persons (Walker and Diforio, 1997; Walker et al., 2008). Back translation of these risk factors for acute onset schizophrenia-related symptomology has resulted in the development of animal manipulations based on: acute and sub-chronic drug treatments, chronic stress, and loss of function in select brain regions (i.e. lesion and temporary inactivation manipulations). As a measure of validity, sensory gating performance has been found to rely critically on the integrity of the mPFC in rats (Koch and Bubser, 1994; Sharpe and Killcross, 2014), as evidenced for the dlPFC in humans (see Chapter 1: Introduction), supporting the PFC-hypofrontality hypothesis of schizophrenia and the potential for translation between rodent and human studies. In this chapter, I review the literature on the acute manipulations in rodents which generate schizophrenia-related behavioural flexibility deficits, as well describe my own experiments with relevance for schizophrenia induced by acute mPFC-hypofunction in rats.

3.1.1 Adult sub-chronic/acute NMDA receptor antagonist manipulations

Disruption of glutamatergic signalling by NMDAR antagonists induces 'schizophrenia-related' symptomology in healthy volunteers (Umbricht et al., 2000; Krystal et al., 2005; see Chapter 1: Introduction for review). As a preclinical tool, researchers have extensively studied the effects of acute and sub-chronic NMDAR antagonists on behaviour and cognition, and the underlying neural networks, in adult rats and mice. Early experiments found that sub-chronic administration of phencyclidine (PCP; ~1 - 2 mg/kg daily for five days) induced metabolic hypofunction and reduction in parvalbumin mRNA and protein

expression in the PFC of rats (Cochran et al., 2003; Amitai et al., 2012). The physiological changes in response to sub-chronic PCP are reportedly severe enough to induce deficits in ASST performance, as many researchers have shown elevated trials-to-criterion (TTC) and/or errors at reversal stages and the ED stage (Rodefer et al., 2008; Broberg et al., 2009; Goetghebeur and Dias, 2009; Goetghebeur et al., 2010; Saland and Rodefer, 2011; Dawson et al., 2012; McLean et al., 2012). Behavioural flexibility effects are also evident in rats treated sub-chronically with ketamine (Nikiforuk and Popik, 2012), however, the ketamine treatment is only reported to cause a deficit at the ED stage, suggesting a slightly different mechanism of action than sub-chronic PCP-treatment.

The effects of NMDAR blockade on mPFC physiology are evident following a single exposure to a NMDAR antagonist. Reductions in GABAergic interneuron markers (PV and GAD67), and the neuronal activity marker, zif 268 mRNA, are evident in the thalamic reticular nucleus and mPFC of rats following treatment with 2 - 2.5 mg/kg PCP (Egerton et al., 2005; Amitai et al., 2012). Further supporting the role of NMDARs in 'schizophrenia-related' cognitive symptomology, acute blockade of NMDARs in rats also generates ASST deficits. First reported by Egerton et al. (2005), acute treatment with PCP (2.5 mg/kg), 24 hours prior to ASST testing, resulted in elevated TTC at the ED stage. Recently, Gastambide et al. (2013) reported that treatment with PCP (2.5 mg/kg) two hours prior to ASST induced deficits at the reversal and the ED stage. Treatment with ketamine (10 mg/kg) one hour prior to testing also diminished ASST performance however more selectively, as the rats only showed a significant deficit at the ED stage (Gastambide et al., 2013).

Ultimately the aim of this line of research is translational utility to understanding schizophrenia symptoms in human patients. The sub-chronic NMDAR antagonism manipulations (PCP and ketamine) show responsiveness to treatment with antipsychotics (particularly sertindole) already in use in human patients (Rodefer et al., 2008; Broberg et al., 2009; Goetghebeur and Dias, 2009; Nikiforuk and Popik, 2012), and the cognitive 'enhancing' drug modafinil

(Goetghebeur and Dias, 2009; Goetghebeur et al., 2010; Dawson et al., 2012). Although there are no known and validated treatments for the cognitive symptoms in human patients, the reported efficacy of antipsychotics in sub-chronic NMDAR antagonism manipulations supports the potential usefulness of acute manipulations to mimic schizophrenia symptomology in rats.

3.1.2 Stress-induced perturbations

The diathesis stress model of schizophrenia proposes that at-risk individuals are 'tipped over' the edge via chronic stress exposure (Walker and Diforio, 1997). Early research suggests that the 'tipping' and subsequent manifestation of symptoms was likely due to the impact of stress on the mPFC (Weinberger, 1987). Following this hypothesis, researchers began studying how stress alters the mPFC resulting in increased susceptibility to cognitive dysfunction.

A well validated method for increasing the stress response hormone glucocorticoid in rats is chronic and repeated stress (restraint, tail-pinch, cold-water swimming, etc.). Rats which undergo this treatment (typically, for 14 - 21 days) exhibit significantly elevated glucocorticoid levels, similar to the elevations in glucocorticoid levels measured in humans who experience chronic psychosocial stress such as 'work overload' (Kunz-Ebrecht et al., 2004; Schlotz et al., 2004). Using this technique to explore stress-induced alterations in the executive function network has yielded some interesting results with implications for cognitive deficits in schizophrenia.

Stress-induced alterations in rat mPFC morphology results in significant reductions in dendritic arborisation in mPFC, however no significant change in the orbitofrontal cortex (OFC; Liston et al., 2006). Consistent with this selective disruption of the mPFC, significant set-shifting deficits are found in chronically stressed rats, while reversal learning is spared (Liston et al., 2006; Nikiforuk and Popik, 2011; Naegeli et al., 2013; Nikiforuk and Popik, 2014). Of note, one paper reported deficits at the first reversal stage (REV 1) in chronically stressed rats (Jett

et al., 2015). The deficit at REV 1 may be due to the slightly different stress-induction method as the researcher used chronic unpredictable stress protocol while all but one (Naegeli et al., 2013) of the early studies utilised restraint stress alone. Indeed, it appears that physical stressors affect the OFC more so than the 'psychogenic' restraint stress procedure (Lapiz-Bluhm et al., 2009; Donegan et al., 2014). Chronic intermittent cold stress induces a select deficit at REV 1, but not at any other stages of the ASST (Lapiz-Bluhm et al.; Donegan et al.). This REV 1 deficit co-occurs with disruption of serotonin signalling in the OFC, (also known to be dysregulated in schizophrenia; Bleich et al., 1988), as well as altering levels of the interleukin-6 cytokine, a popular target for immune and inflammatory based schizophrenia research (Naudin et al., 1996). These data support the diathesis-stress model of combining the mPFC stress-induced effects with underlying neurochemical disruptions resulting in 'schizophrenia-related' cognitive and behavioural deficits. However, the basic of how the affected brain regions function to facilitate ASST performance remains to be explored fully. While the animal manipulations provide some insight, direct manipulation of an individual target region also provides beneficial information on underlying mechanisms in both typical and dysfunctional brains.

3.1.3 Lesion experiments

As described in the introduction (Chapter 1), several key brain regions are known to regulate performance in the ASST in humans (Rogers et al., 2000) and to be dysfunctional in patients with schizophrenia. Although schizophrenia affects multiple regions, comparison of select dysfunction of a single region grants insight into the potential underpinnings of individual symptoms. For example, the ED shift deficit in the ASST reported in patients with schizophrenia (Pantelis et al., 1999; Turner et al., 2004) may be largely due to dysfunction of the dlPFC in these patients. In the early '90s two papers highlighted the requirement of a functional dlPFC in normal performance of set-shifting (Owen et al., 1991; Owen et al., 1993). In a direct comparison between patients with frontal lobe damage and

schizophrenia patients, both groups exhibited deficits in set-shifting compared to controls (Pantelis et al.). It should be noted that this deficit was greater in the schizophrenia group, and that the schizophrenia group also had additional deficits in reversal learning which were not present in the frontal lobe patients, suggesting the additional neurobiological pathologies (e.g. OFC, hippocampal and striatal dysfunction) exacerbate the cognitive deficits.

The previous two sections highlighted how indirect manipulations (drug or stress-induced) affect the mPFC. In this section I will briefly, review data from direct manipulation (selective lesion) of areas implicated in schizophrenia, and how these findings in animal manipulations support findings in humans, and allow dissociation of behavioural phenotypes based on localisation, despite lacking direct aetiological fidelity to schizophrenia.

In rats, lesions to the OFC have been found to induce reversal learning impairments and loss of attentional set-formation (McAlonan and Brown, 2003; Chase et al., 2012), however TTC for the ED shift is spared (although it is questionable whether attentional set is formed following OFC-lesions). In mPFC-lesioned animals however, reversal learning stages are not reliably impaired but the ED stage requires more TTC compared to control animals (Birrell and Brown, 2000; Tait et al., 2009). Similar findings have been reported in mice (Bissonette et al., 2008) and marmosets (Dias et al., 1996a ,1997). The findings in lesioned animals therefore are consistent not only with the drug and stress manipulations, but also with the human findings (Owen et al., 1991; Owen et al., 1993). An advantage of animal experiments is that 'blue skies' research can be performed. For example, Chase et al., were able to replicate the rat OFC reversal learning deficit, and then follow up with a 4-ID task to examine the effect of removal of reversal stages on set-formation in rats with OFC lesions. In a surprising result, the removal of the reversal stages, and addition of three supplementary ID stages, revealed that OFC-lesioned rats *can* form an attentional set when provided ample opportunity, and that once an attentional set has been formed, they then manifest an ED shift deficit apparently similar to the deficit induced by mPFC lesions.

Although the direct translational impact of this research is perhaps, less clear than the sub-chronic NMDAR antagonist or chronically stressed rats, there is an obvious benefit from the challenge the results provide to the role of the OFC in 'solely' reversal learning. The knowledge gained by furthering understanding of the basic functionality and psychological mechanisms at work within the OFC may eventually provide additional treatment targets for persons suffering from OFC dysfunction. In the spirit of 'blue skies' research, I sought to examine the psychological mechanism underlying the ED deficit in rats with mPFC-disruptions. In the next section I briefly review the approach that was used to inhibit the mPFC.

3.1.4 Inactivation of the mPFC via designer receptors exclusively activated by designer drugs (DREADDs)

The technology available to the neuroscience field has advanced greatly since the 20th century. Many of these advancements allow for refinements in procedures, reduced animal usage, and replacement of certain procedures altogether. Designer receptors exclusively activated by designer drugs (DREADDs), is one such technology that provides all the mentioned advancements. DREADDs are engineered human muscarinic (hM) receptors which can be packaged into a virus and expressed in target cell populations (controlled by manipulation of the viral DNA promotor sequence) and specific brain regions (via microinjection surgery). Once the desired target is infected, DREADDs can be activated by the ligand clozapine *N*-oxide (CNO). Binding of CNO to the DREADD activates an intracellular G-protein signalling cascade, which can be either excitatory or inhibitory (Gq- or Gi-subunit coupled DREADDs respectively; Urban and Roth, 2015). The effectiveness of this approach is evident by numerous publications reporting robust physiological and behavioural effects following activation of the receptor (for review see: Roth, 2016; Smith et al., 2016). Of particular interest to this project is the inhibitory hM4Di-DREADD, which has been used effectively in the frontal cortex of monkeys (Eldridge et al., 2016), mice (Perova et al., 2015), and rats (Kerstetter et al., 2016). Inhibitory hM4Di receptor signalling can last up to 5 hours despite CNO only having a predicted half-life of 3 hours *in vivo* (den

Hartog et al., 2016). Further supporting the utility of targeted inactivation via DREADDs, the effect of temporary inactivation is reportedly similar to that of lesions. Ethanol-dependent mice with lesions to the lateral OFC exhibit elevated preference for ethanol compared to non-lesioned animals, and this preference is recapitulated in mice with inactivation of the lateral OFC via hM4Di-DREADDs activation (den Hartog et al., 2016).

As researchers, it is our duty to develop with the field and improve the methods we use to meet the highest standard of animal care and proper usage, and in this effort, I sought to utilise temporary DREADD-mediated inactivation of the mPFC as opposed to the ibotenic acid lesions which our lab has performed in the past (Birrell and Brown, 2000). Four key factors justified this position. The first benefit of employing this method was reducing the number of animals by half. With repeat testing and an AB design (treatment with CNO or vehicle) each animal was its own control thus our studies were within-subjects as opposed to between. A second benefit was the reduction of risk to the animal. Unlike with inhibitory ligands tetrodotoxin and muscimol, which would require placement of a guide cannula increasing risk for infection in the animal, once the DREADD receptor was delivered via microsurgery, the animal was no longer subjected to invasive procedures, apart from intraperitoneal (i.p.) injections of CNO. Third, the fluorescent tag incorporated in the DREADD receptor, allowed for visualisation of the extent of viral infection, a benefit not available with most other inactivation methods or PCP-administration. Last, the oral bioavailability of CNO (Lin et al., 1996; Chang et al., 1998), made activation of the DREADDs via a palatable gelatine tablet possible, further reducing the need for potentially stress inducing i.p. injections.

3.2 Rationale

In this chapter, I explore if temporary inactivation of the mPFC (via inhibitory DREADDs activation) would replicate the ED deficit induced by mPFC lesions (Birrell and Brown, 2000; Tait et al., 2009). Following replication of the ED

deficit, I sought to reconcile two seemingly opposing symptoms in schizophrenia, one being the inability to regulate attention and diminished sensory gating, and the other being perseveration and 'cognitive inflexibility'. The ED stage is prime for studying the role of attention to sensory stimuli, as normal rats effectively disregard the previously irrelevant dimension stimuli during the initial trials of the stage (a consequence of attentional set). This is despite the novelty of the stimuli, and results in increased TTC compared to the ID. However, in rats with mPFC-hypofunction, if they lack the ability to filter irrelevant stimuli and to behave based on optimal stimulus-reward contingences is 'cognitive inflexibility' in the form of reduced ability to shift attentional set actually the cause of the subsequent ED deficit?

3.3 Methods and materials

3.3.1 Animals

Adult male (n = 12) Lister Hooded rats (Charles River, UK) were pair-housed in standard housing conditions. Seven days prior to surgery rats were placed on mild food-control of 15-20 g per day. Water continued to be available ad libitum. Prior to surgery, all rats weighed 396 to 426 g. At the completion of all experiments, all rats showed evidence of weight gain (end weights 448 – 518 g).

3.3.2 Pharmacogenetic manipulation

After two weeks of acclimation to the new habitat, the rats were anaesthetised with a gaseous mix of isoflurane (5% at induction, then reduced to 1.5 - 2% for maintenance) and oxygen and given 0.05 ml of Carprieve (Carprofen: Pfizer, Kent, UK), via subcutaneous injection to reduce any potential surgery-related pain during recovery. Once the rats were unresponsive, their heads were shaved and cleaned with alcohol. Next, they were placed into a stereotaxic frame (Kopf, CA, USA), and secured into location with atraumatic ear and tooth bars (set and -3.3 mm to achieve level skull). All rats underwent bilateral microinjections of

1 μ l AAV5-CamKII-hM4Di-mCherry. Four injection sites were targeted to encompass the entirety of the mPFC. The Paxinos and Watson (Paxinos and Watson, 1998) coordinate system was used to calculate the location of all injection sites. Anterior/posterior measurements were calculated from Bregma at skull surface, while medial/lateral, and dorsal/ventral coordinates were calculated from the dura. The following coordinates were used: 1) anterior/posterior + 3.9, medial/lateral \pm 0.5, dorsal/ventral -3.1; 2) anterior/posterior + 2.9, medial/lateral \pm 0.5, dorsal/ventral -3.1. The skull was removed from the injections site with a drill, and a Hamilton syringe with a 30-gauge needle attached was used to deliver the virus over a 5-min period, followed by a 5 min post-injection *in situ* wait period for virus diffusion. Once all sites were injected the surgery site was closed with alcohol disinfected surgical staples. Following surgery, rats were single housed for 24 hours, and weighed daily for the next 48 hours to ensure no weight loss due to disrupted feeding behaviour.

For i.p. administration rats were habituated to the injection procedure with an i.p. injection of vehicle, for two days prior to test day. On test days, they received either CNO (10 mg/mg/ml, suspended in .09% saline) or the corresponding volume of vehicle. Following injections rats were isolated into a holding cage. For oral administration, 10 mg/kg CNO was suspended in a 'jelly' mix (60 ml sugar free blackcurrant flavoured Robinsons (Britvic, UK); 6 g, Dr Oetker Gelatine Sachet (12 g/sachet); stirred for 15 min). For both administration methods testing began 30 mins after the delivery of CNO to allow activation of the DREADDs receptors by the ligand. The 10 mg/kg dose was chosen as it was at the higher end of the effective range reported in the literature (Roth, 2016) and I wanted to ensure successful activation of the receptors for the duration of the ASST testing, which can take upwards of three hours (Gilmour et al., 2013).

3.3.3 Behavioural Testing

Two weeks following surgery, all rats underwent the standard 7-stage ASST (as described in Chapter 2: General Methods) to familiarise them with stimuli and

discriminations and ensure they could complete the task within the time course of CNO-mediated DREADDs activation (Roth, 2016). Following baseline testing, the rats were tested in the 7-stage ASST twice more in an AB design with half of the rats receiving 10 mg/kg CNO i.p. and half of the rats receiving saline in each test. Next, to validate the effectiveness of oral CNO administration, the rats were tested twice more in an AB design. In each test half of the rats received 10 mg/kg CNO orally, while the other half received 'jelly' alone.

➤ *4-stage simple task*

Testing took place in the same chamber as the ASST, and followed the same procedure for stimuli presentation, counterbalancing, and criterion to stage completion (see ASST methods for details; Chapter 2), with few changes to account for the 'simple' method of stimulus pairing. During the first stage, rats were required to perform a simple discrimination (SD) between two stimuli either (O_A vs O_B ; or M_A vs M_B). The second stage introduced a redundant stimulus into the irrelevant dimension however, the redundant stimuli were 'simple' paired with only one of the stimuli from the SD (for example, the rat was always presented with $O_A M_A$ vs $O_B M_B$). During the first compound discrimination (CD) normal rats should not associate the redundant stimulus with reward as their attention to the correct stimulus from the previous stage (the SD), should induce the 'blocking' phenomenon (Kamin, 1968; Sharpe and Killcross, 2014). After acquisition of the CD the rats were required to reverse to the previously incorrect stimulus pair (e.g. from $O_A M_A$ to $O_B M_B$). At this reversal stage (REV) the normal rats should continue to ignore the redundant stimulus and associate the stimuli within the dimension from the SD with the reward. For example, if the rat's SD were between two odours, then the redundant stimuli would be the media paired with the odours during the CD and REV stages. This hypothesis is tested at the final stage (probe), as the redundant stimulus paired with the REV stage rewarded stimulus, is paired with the now correct stimulus, a novel stimulus yet to be presented during the task (e.g. O_C). Additionally, the redundant stimulus from the incorrect REV stimulus is now paired with a novel incorrect stimulus from the other

dimension (e.g. O_D). A representation of this procedure is depicted in Figure 3.1. If the rat has been attending to the stimuli within the dimension from the SD (e.g. odour) then the pairing of the redundant stimulus from the REV stage with the correct stimulus for probe, should not provide any additional information. However, if the rat has attended to the redundant stimulus during the REV stage then acquisition of probe should occur in fewer trials.

➤ *6-stage simple-probe ASST*

Testing took place in the same chamber as the ASST (see ASST methods for details). All stages required a 6-in-row-correct criterion to be reached before the rat could advance to the next stage. The first four stages (SD, CD, REV, ID) of the task followed the same method as the standard 7-stage ASST. After reaching criterion at the ID, rats were required to complete a 'simple probe'. During this stage only two bowls were presented, one with the correct stimulus from the ID paired with the to-be-correct stimulus for the upcoming ED, one with the incorrect stimulus from the ID paired with the to-be-incorrect stimulus from the ED. To complete the simple probe rats were required to reach criterion digging in the bowl containing the correct stimulus from the ID. This stage was designed to allow $mPFC_{in}$ -rats an opportunity to exploit their inability to reduce attention to the irrelevant dimension stimuli. The procedure is shown in Figure 3.1. Control rats (which have formed an attentional set) should not associate the introduction of a 'novel' redundant stimulus during the 'simple' ID with reward, as it should be blocked (Kamin, 1968). On the other hand, if $mPFC_{in}$ -rats cannot reduce attention to redundant information, they should associate the redundant stimulus with reward. This hypothesis is tested in the final stage, a partial change ED (ED_{PC}), during which the stimulus paired with the correct stimulus from the 'simple' ID is rewarded. For control rats, a positive shift-cost should be present, suggesting set-formation, set-shifting, and successful blocking during the simple probe. However, $mPFC_{in}$ -rats should require fewer trials than controls to complete the ED_{PC} , evidence of performance enhancement reflective of their inability to reduce attention to the redundant irrelevant stimulus during the simple probe.

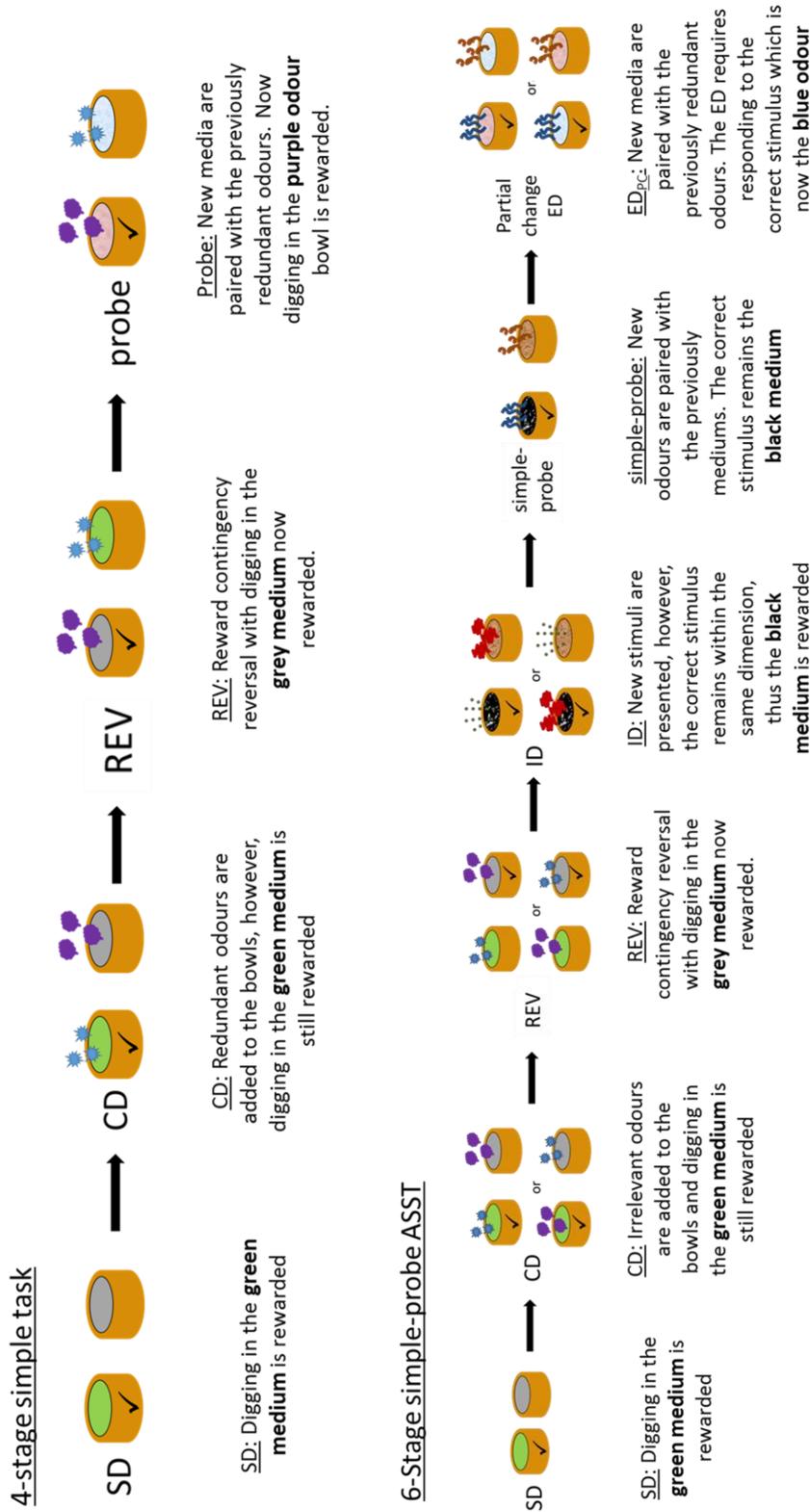
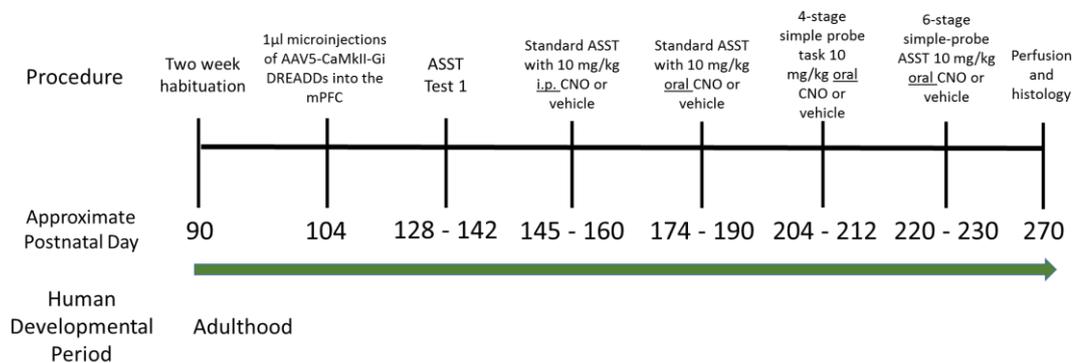


Figure 3.1) Diagram of the procedure for the 4-stage (top), and 6-stage simple-probe ASST (bottom). SD, simple discrimination; CD, compound discrimination; REV, reversal; ID, intradimensional discrimination; ED, extradimensional discrimination.

➤ *Order of Testing*

Two weeks following surgery, all rats underwent the standard 7-stage ASST to familiarise them with stimuli and discriminations and ensure they could complete the task within the time course of CNO-mediated DREADDs activation (Roth, 2016). For all following testing, the rats were tested in an AB design with half of the rats receiving 10 mg/kg CNO and half of the rats receiving saline in each test. First, the rats were tested in the 7-stage ASST following i.p. injection of 10 mg/kg CNO. Two weeks later the rats were retested in the 7-stage ASST however this time following oral administration of 10 mg/kg CNO suspended in a jelly or jelly alone. After validation of oral administration for the effect of interest, all following testing was done with oral administration of CNO. The 4-stage partial reinforcement task occurred two weeks following the last 7-stage ASST. One week afterwards the rats were all tested in the 6-stage partial reinforcement ASST.

Timeline for mPFC_{in} experiments



3.4 Results

3.4.1 mPFC_{in}-rats have set-shifting deficits in the standard 7-stage ASST

The first experiment tested the effect of mPFC_{in} on performance in the standard ASST by administering 10 mg/kg CNO, or vehicle, first by i.p. injection and then orally. The mean TTC across all stages was overall slightly higher in the first tests, with i.p. injections, compared to the second tests, with oral administration

(17.14, \pm 1.5 versus 15.72, \pm 1.33; main effect of Route: $F_{(1, 11)} = 16.7$, $p < 0.05$). All interactions with Route were not significant and, in particular, Route x CNO x Stage: $F_{(6,66)} < 1$). Irrespective of route of administration, CNO-treated rats required more trials to complete the ED stage than when they were vehicle-treated (TTC ED: mPFC_{in} = 26.04, \pm 1.26; control = 15.21, \pm 0.66). There was also a small increase in TTC when the rats were CNO-treated in the REV 1 stage (TTC REV 1: mPFC_{in} = 23.0, \pm 1.11; control = 19.71, \pm 1.48). These effects were confirmed by a significant CNO x Stage interaction ($F_{(1, 6)} = 7.87$, $p < 0.05$; Figure 3.2) and subsequent restricted comparisons (ED: $F_{(1, 11)} = 49.39$, $p < 0.05$; REV 1: $F_{(1, 11)} = 5.25$, $p < 0.05$).

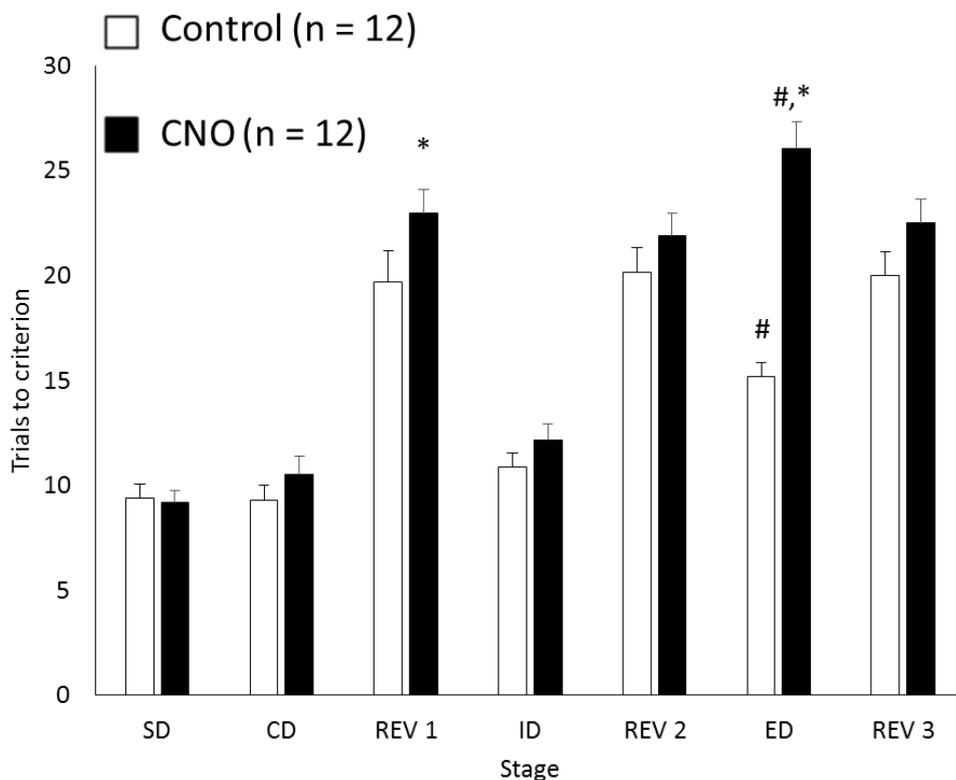


Figure 3.2) Mean trials to criterion data (+ SEM) on the standard 7-stage task following i.p. and oral administration. mPFC_{in}-rats required significantly more trials to complete the ED-stage than controls, but do not significantly differ from controls at any other stages (#, ID significantly different than ED, both controls and mPFC_{in}-rats; *, REV 1 & ED significantly different than controls).

3.4.2 Behavioural pattern analysis reveals increased irrelevant dimension responding in mPFC_{in}-rats

To further examine the ID/ED deficit mPFC_{in}-rats, an analysis of the rats behavioural patterns during the ASST was performed (Figure 3.3; see Chapter 2: General Methods section for the Bayesian method description). Analysis of the pattern of bowl choices suggested that mPFC_{in}-rats were more likely to respond based on irrelevant dimension stimuli during reversal stages as evidenced by more quartiles with a Bayes factor of 3 or higher for the irrelevant dimension behavioural pattern (mPFC_{in} = 6, controls = 1). Responding to irrelevant dimension stimuli was also evidenced at the ED in mPFC_{in}-rats (mPFC_{in} = 2, controls = 0). Analysis of behavioural patterns further suggested that while both group of rats displayed spatial patterns (e.g. place match or win-stay), mPFC_{in}-rats did so less than controls (mPFC_{in} = 6, controls = 11; Figure 3.3), and instead chose bowls based on the irrelevant dimension stimuli (mPFC_{in} = 8, controls = 2; Figure 3.3). These data suggested rather than solely being cognitively inflexible resulting in impaired set-shifting, mPFC_{in}-rats were actually shifting responding across stimulus dimensions (relevant/irrelevant) through ASST stages which did not require dimensional-shifts. Of final note, the analysis found only one instance of perseveration. This occurred during the first quartile of REV 3 for mPFC_{in}-rats (Figure 3.3). Given the findings of a behavioural pattern suggesting increased irrelevant dimension responding during the ASST, the next experiments sought to directly test if indeed mPFC_{in}-rats were still attending to irrelevant dimension stimuli, despite the evidence that they had formed an attentional set.

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

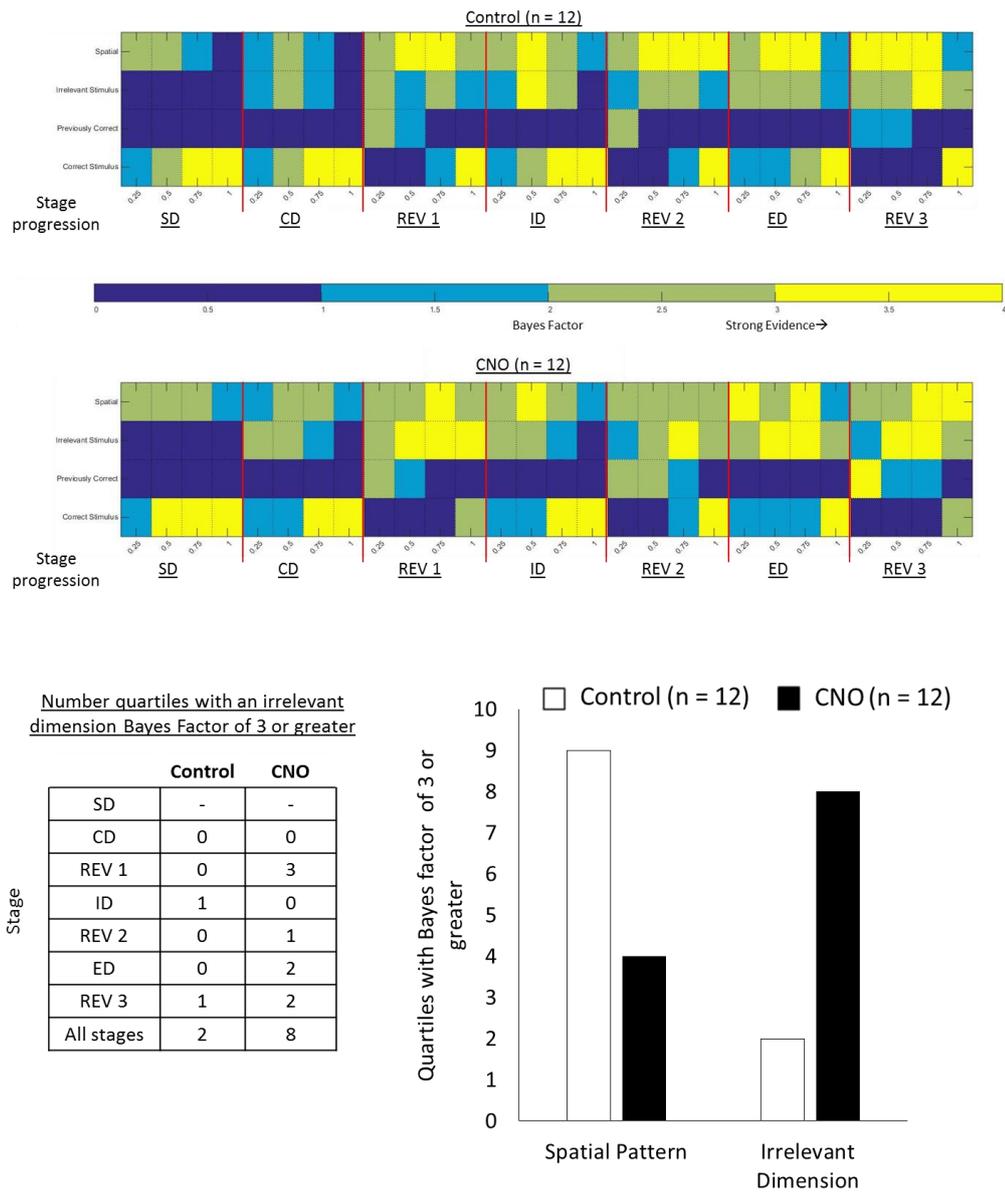


Figure 3.3. *Top.* Heatmap of evidence for behavioural patterns during the standard ASST (oral and i.p. CNO administration). *Bottom left.* Table of sum quartiles per stage of irrelevant dimension responding. Across all stages mPFC_{in}-rats had more evidence for increased responding to irrelevant dimension. *Bottom right.* Bar graph showing total spatial and irrelevant dimension quartiles by group across all stages. mPFC_{in}-rats showed less evidence of spatial pattern use compared to controls.

3.4.3 mPFC_{in}-rats attend to stimuli in the irrelevant dimension

The next set of experiments tested the hypothesis that mPFC_{in}-rats were impaired at the ED stage due to an inability to reduce attention to the irrelevant dimension stimuli. To examine this, I designed two tasks which would enhance performance at the probe stage if the subject were attending to irrelevant dimension stimuli during previous stage. In the first task (simple 4-stage ASST; top diagram of Figure 3.1), a significant CNO x Stage interaction was found ($F_{(1,6)} = 3.86$, $p < 0.05$; Figure 3.4) as compared to control rats (TTC probe: $11, \pm 1$), mPFC_{in}-rats performed significantly better at the probe (TTC probe: $7, \pm 0$). This difference was confirmed by ANOVA restricted to the probe stage ($F_{(1,33)} = 14.42$, $p < 0.05$) No other significant effects were found across any other stages ($F_s < 1$). Following these results the next experiment sought to further support the findings of mPFC_{in}-rats attending to irrelevant dimension stimuli by testing them in a 'true' ID/ED test where only one stage was 'simple'.

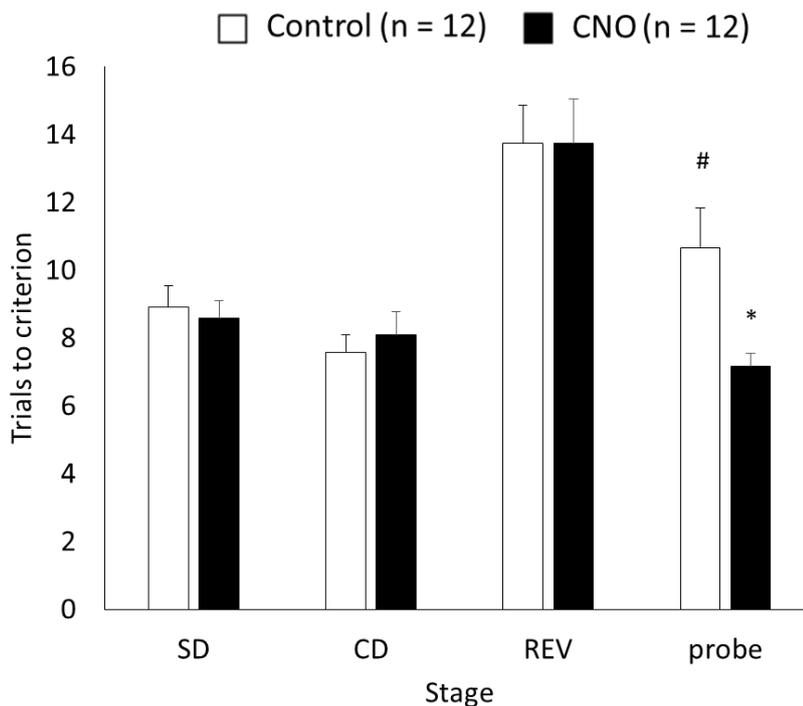


Figure 3.4. Mean trials to criterion (+ SEM) for the 4-stage simple task. mPFC_{in}-rats are faster to acquire a probe when the to-be-correct stimulus for the probe is paired with the correct stimulus from the ID.

In the 6-stage, simple probe version of the ASST (bottom diagram Figure 3.1), regardless of CNO-treatment, the rats did not appear to notice when the stimuli in the irrelevant dimension changed during the simple-probe stage and they continued to respond to the previously-rewarded stimulus. When vehicle was administered, the rats showed an ED shift-cost, taking more trials to complete the ED stage than to complete the ID stage. However, when CNO-treated, there was no difference between ID and ED stages: the rats appeared to have benefitted from what should have been unattended-to information. This was confirmed by an interaction of CNO x Stage ($F_{(1, 5)} = 3.08, p < 0.05$; Figure 3.5) and verified by restricted comparison (ED: $F_{(1, 55)} = 12.54, p < 0.05$; all other stages $F_s < 1$).

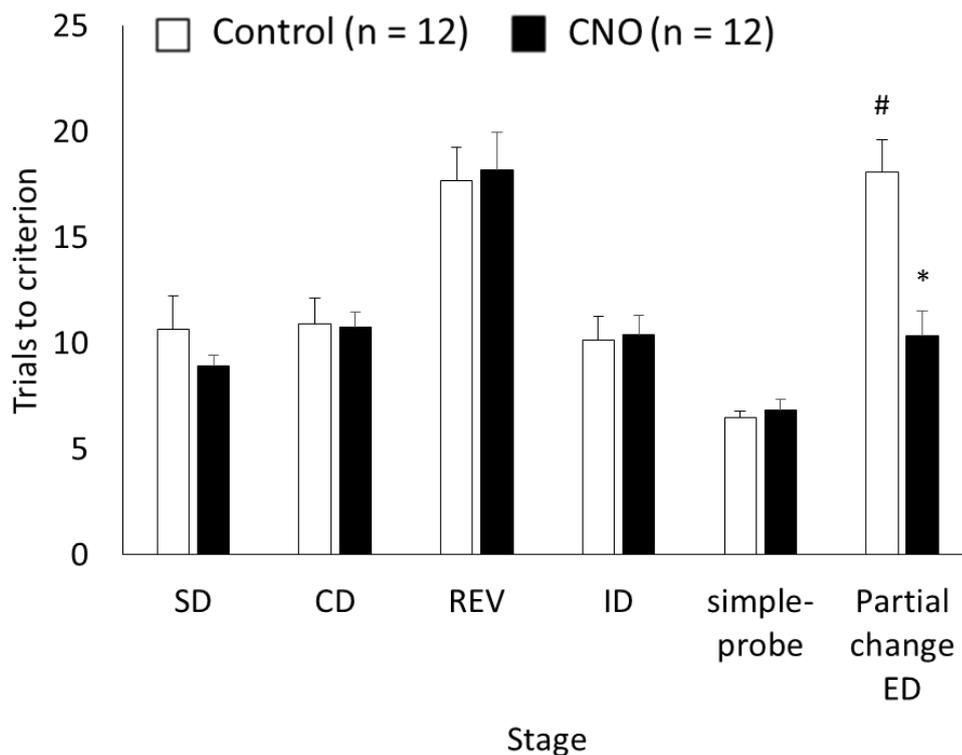


Figure 3.5) Mean trials to criterion data (+ SEM) on the 6-stage simple-probe task. mPFC_{in}-rats were faster to acquire an ED when the to-be-correct stimulus for the ED was paired with the correct stimulus from the ID (#, ID significantly different than ED; * ED significantly different than controls).

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

Bayesian analysis of the 6-stage simple-probe revealed a slight change in the pattern of behaviour for CNO-treated rats (Figure 3.6). Rather than evidence of irrelevant dimension responding during the REV, this pattern was evidenced during the CD (see table in Figure 3.6), whereas control rats did not. Furthermore,

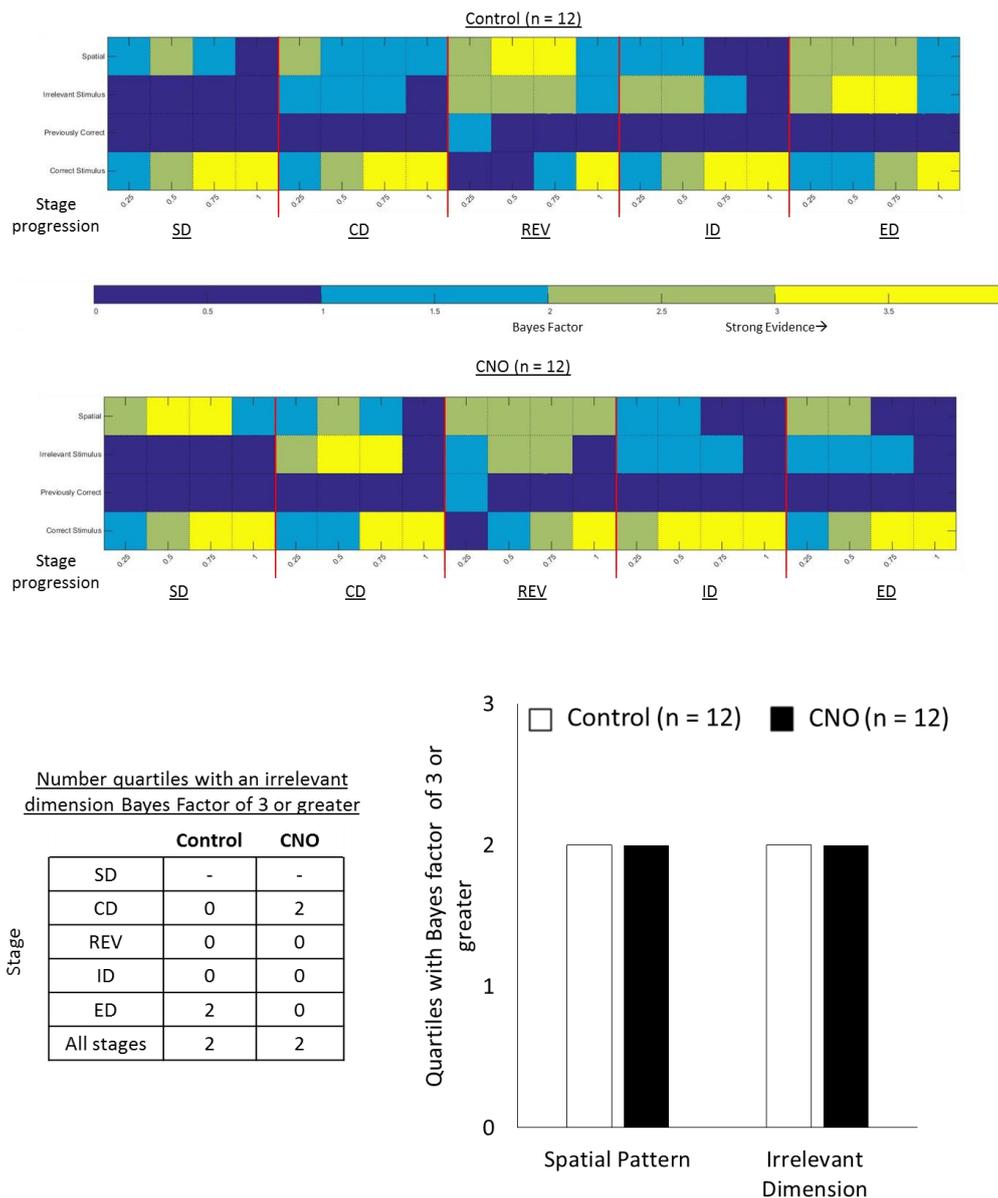


Figure 3.6) *Top*. Heatmap of evidence for behavioural patterns during the standard ASST. *Bottom left*. Table of sum quartiles per stage of irrelevant dimension responding. There was strong evidence for mPFC_{in}-rats responding to the irrelevant dimension during the CD as opposed to during the ED as controls did. *Bottom right*. Bar graph showing total spatial and irrelevant dimension quartiles by group across all stages.

whereas control rats showed responding to the previously relevant dimension during the ED_{PC}, mPFC_{in}-rats did not have substantial evidence for any incorrect behavioural patterns.

To further illustrate the effect of mPFC_{in} on performance, Figure 3.7 shows the difference in TTC shift-cost (ED minus ID) for both the 7-stage (both i.p. and oral CNO administration data) and the 6-stage simple probe tasks. In the standard 7-stage task, control and mPFC_{in}-rats formed and shifted an attentional set as indicated by a positive shift-cost in both conditions, however, mPFC_{in}-rats showed a greater cost than controls ($t_{(10)} = -4.84, p < 0.05$), reflecting their impaired ED performance. Control rats also show a shift-cost in the 6-stage 'simple'-probe task, but mPFC_{in}-rats do not ($t_{(10)} = 2.65, p < 0.05$), reflecting enhanced performance at the ED stage arising because mPFC_{in}-rats attended the to-be-correct-stimulus during the simple-probe stage, while it was irrelevant to control rats. Collectively, these data suggest that although mPFC_{in}-rats can form and shift an attentional set, they simultaneously attend to irrelevant/redundant stimuli.

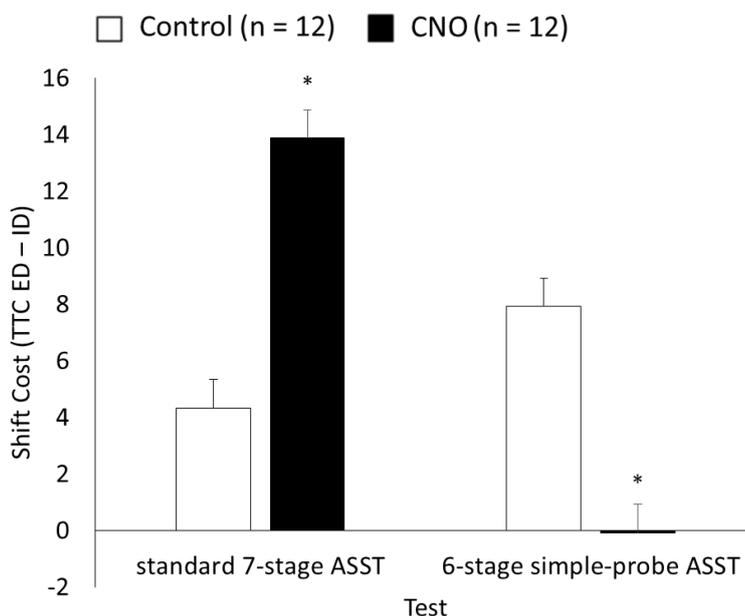


Figure 3.7) Mean trials to criterion data (+ SEM) on the 6-stage simple-probe task. mPFC_{in}-rats were faster to acquire an ED when the to-be-correct stimulus for the ED was paired with the correct stimulus from the ID (*, ED significantly different than controls).

3.5 Discussion

The first finding of the experiments within this chapter is that DREADDs-mediated inhibition of the mPFC induces a deficit at the ED stage of the standard ASST. This finding is consistent with the previous reports described in the introduction showing ED deficits following disruption of the mPFC by ibotenic acid, acute and sub-chronic NMDAR antagonism, and chronic stress. There was also evidence of a REV 1 deficit in CNO-treated rats, however, this increase in TTC was small (3 trials), only present at one of the three reversal stages, and not present in the later tests (4- or 6-stage modified tasks). However, that is not to say it is not a potential result of the manipulation as previous studies in the lab with ibotenic acid mPFC-lesioned rats have also sometimes seen a small but significant increase at REV 1 (David Tait personal communication).

Furthermore, in a modification of the ASST, a partial change ED (ED_{PC}) stage is performed in fewer TTC than when the rats were CNO-treated. Critically, this modification provided information on learning of irrelevant dimension stimuli in $mPFC_{in}$ rats and controls. During the simple-probe, through attentional regulation mechanisms (blocking/attentional set), vehicle-treated rats did not learn about the reward contingency of the redundant stimuli within the irrelevant dimension. This was evidenced by a robust shift-cost at the subsequent ED_{PC} relative to the ID. However, when the rats were administered CNO, the addition of the simple-probe enhanced performance at the ED_{PC} as they showed no shift-cost (ID/ ED_{PC} shift-cost = 0, ± 2) – evidence that CNO-treated rats had learned the predictive nature of the redundant stimulus. That is to say, the redundancy of the irrelevant dimension stimulus during the probe facilitated performance at the ED_{PC} following CNO-treatment.

3.5.1 Irrelevant dimension responding during set-shifting inducing enhanced learned irrelevance.

The analysis of the behavioural patterns of $mPFC_{in}$ -rats during the standard ASST suggested increased irrelevant dimension responding during reversal stages.

Essentially, early in the reversal stage, once responding to the previously correct stimulus began to be inhibited (reinforced by the exploratory trials), whereas control rats utilised a spatial pattern until they determined the correct stimulus and began responding optimally, mPFC_{in}-rats were more likely to respond based on stimuli within the irrelevant dimension. The detection of this anomalous behaviour via the behavioural pattern analysis highlights the utility of the approach for assessing actions which are undetectable by standard TTC and errors analysis. The analysis further suggested that while control rats were behaving optimally and responding to the correct stimulus by the completion of each stage, there was not strong evidence of mPFC_{in}-rats responding based on the correct stimulus during REV 1 and REV 3.

Based on the behavioural pattern analysis, an explanation for the behaviour of mPFC_{in}-rats during the standard ASST is as follows: although acquisition stages do not appear different from controls, if indeed mPFC_{in} induces a deficit in sensory gating (failure to block), than CNO-treated rats 'learn' that the reward contingency rate of the irrelevant dimension stimuli is 50%, however, their behaviour is guided by the 100% reward-contingent correct stimulus. It follows, that during REV 1 mPFC_{in}-rats attempt to obtain the reward based on one of the irrelevant dimension stimuli, which they learned are partially contingent on reward. This choice behaviour would then reward them 50% of the time when they chose the irrelevant stimulus that was paired with the now correct stimulus. Thus the behaviour of mPFC_{in}-rats is guided by the irrelevant dimension stimuli until they learn that the previously incorrect stimulus is now contingent with reward. This is in contrast to their behaviour when vehicle-treated, where they instead show spatial based responding (also 50% chance of reward), until they learn the new reward contingency. By the end of REV 1 under both conditions (vehicle or CNO-treated) the rats are able to reach criterion (6-in-a-row; although the Bayes factor for the CNO-treated rats suggests they are undertrained). However, compared to control rats, CNO-treated rats may have received 'enhanced learned irrelevance' (Baker and Mackintosh, 1977), due to their

attempt to obtain reward based on irrelevant dimension stimuli. During REV 2, the irrelevant dimension responding is again present but less so as mPFC_{in}-rats learn that the stimuli in the irrelevant dimension are not 100% predictive of reward (likely coincident with set-formation), and complete the stage with strong evidence they are responding to the correct stimulus.

Under these conditions mPFC_{in}-rats would have enhanced learned irrelevance of the irrelevant dimension compared to control rats, as for two stages (REV 1 and REV 2) they attempted to obtain reward based on the irrelevant dimension stimuli. Analysis of the ED suggests that this is in fact the case, as the mPFC_{in}-rats attempt to obtain reward based on stimuli from the previously relevant dimension. Eventually, mPFC_{in}-rats complete the shift, indicated by the strong evidence for the correct stimulus at the end of the ED. Finally, at REV 3 they are challenged again and perhaps due to a combination of enhanced learned irrelevance and reversal cost, they perseverate on the previously correct stimulus before responding to the previously relevant dimension (from the initial 5 stages: SD - REV 2) again and although reaching criterion (6-in-a-row-correct), fail to show strong Bayesian evidence for responding to the correct stimulus.

This explanation based on the behavioural pattern analysis is consistent with a deficit in sensory gating arising for mPFC-hypofunction as previously reported (Koch and Bubser, 1994; Sharpe and Killcross, 2014). In the next section I explore the neural mechanisms associated with mPFC-hypofunction which generate this abnormal performance the ASST.

3.5.2 Model of neural mechanisms of the mPFC-inhibition on ASST performance.

There is evidence for the involvement of dopamine, in the form of reward prediction errors, signalling which stimuli within the environment are most predictive of reward during operant conditioning (Schultz, 1997). In rats, a majority of dopaminergic neurons within the ventral tegmental area (VTA), exhibit phasic responses indicative of prediction errors signals and these VTA neurons project heavily to the mPFC through the mesocortical pathway (Thierry et al., 1973;

Hoover and Vertes, 2007). Stimulation of VTA neurons (at a burst firing rate similar to prediction error phasic responses) induces a prolonged 'up state' during which the membrane potential of mPFC neurons are closer to action potential threshold (Lewis and O'Donnell, 2000). An early finding in macaques revealed that depletion of dIPFC dopamine impaired working memory performance (Brozoski et al., 1979). Additional accumulated evidence has supported theorising that the role of dopaminergic synapses within dIPFC/mPFC is to stabilise working memory representation of reward-relevant stimuli through VTA burst firing (for review see: Seamans and Yang, 2004).

However, dopamine is only one of two neuromodulatory signals known to affect ED performance. In rats, lesions of the dorsal noradrenergic bundle, or deafferentation of mPFC noradrenergic fibres also induces a deficit at the ED (Tait et al., 2007; McGaughy et al., 2008). Indeed the locus coeruleus also exhibits phasic responses to reward cues however, this is considered to be derived from PFC inputs (Hoover and Vertes, 2007). Thus, the manipulation herein where the mPFC was inhibited, might induce the ED deficit through loss of signalling from either dopamine or noradrenaline. Importantly, these neuromodulatory systems are known to interact during normal mPFC functioning. For example noradrenaline levels are known to regulate dopamine levels within the mPFC, such that antagonism of noradrenergic α_2 receptors increases extracellular noradrenaline *and* dopamine, with the converse being true for agonism of noradrenergic α_2 receptors (Gresch et al., 1995). For sake of simplicity, the focus here remains on dopamine as dopamine neuron responses occur earlier in response to reward than noradrenergic neuron responses which are more associated with effort to perform the action once the decision has been made (Varazzani et al., 2015).

Dopamine signalling within the mPFC of rats is critically involved in sensorimotor gating such as prepulse inhibition (Koch and Bubser, 1994; Arime et al., 2012). Similarly, dIPFC dopamine signalling has also been found to mediate prepulse inhibition in humans (Giakoumaki et al., 2008). Predictably, lesions of either the mPFC or dIPFC also produces impairments in prepulse inhibition (Knight

et al., 1989; Lacroix et al., 2000). The origin of such deficits are likely multi-fold due to several interconnected functions of the mPFC/dIPFC.

The first relates to the working memory function of mPFC/dIPFC. In task with delays (of seconds to minutes), the recurrent (intra-cell layer III) activity of glutamatergic cells within dIPFC/mPFC functions to maintain the task relevant information (for review see: Arnsten et al., 2012). In support of this function, dopamine projections from the VTA signalling reward-relevant information supports the activity of the neurons maintaining the reward-relevant information in working memory (Brozoski et al., 1979; Arnsten et al., 2012). Current models suggest that the activity of dopamine projections to dIPFC/mPFC increase the signal to noise for the relevant dIPFC/mPFC network processing (Miller and Cohen, 2001; van Schouwenburg et al., 2010). By biasing dIPFC/mPFC network activity, a second function, the tonic inhibition of dIPFC/mPFC on sensory processing, is also biased to gate irrelevant information, and enhance processing of relevant information. Indeed, the deficits in sensory gating are likely due to loss of dIPFC/mPFC tonic inhibition of sensory processing through excitatory synapses on inhibitory neurons within sensory cortices, as well as projections to inhibitory thalamic reticular nucleus (Barbas and Zikopoulos, 2007; but for review see: Zikopoulos and Barbas, 2007). Critically, the network is filled with feedback loops. For example, the mPFC projects directly to the VTA and can modulate the activity of dopamine neurons (Lodge, 2011), as well as additional feedback from the dIPFC/mPFC biasing of cortical and thalamic sensory processing which may modulate the VTA through a network of glutamatergic afferents to the VTA still under investigation (Geisler et al., 2007; but for review see: Zellner and Ranaldi, 2010). Lastly, the dIPFC/mPFC is also involved in reciprocal connectivity with the dorsal and ventral striatum, which may act as an intermediary between the dIPFC/mPFC and the VTA. Activity in the ventral striatum (nucleus accumbens), has been shown to also increase working memory activity within the dIPFC (Fallon and Cools, 2014; perhaps through thalamic feedback projections), while mPFC glutamatergic afferents to ventral striatum inhibit dopamine-induced increases in

activity (Ferenczi et al., 2016): dopamine signals which the dorsal striatum also uses to perform goal-directed behaviour such as in the 'strategy-switching' task (Goto and Grace, 2005). Also, mPFC projections to the dorsal striatum provide goal-directed information, such that lesions of the dorsomedial striatum, can induce similar impairments in attention as mPFC lesions (Rogers et al., 2001). This neural network model of attention is diagrammed in Figure 3.8.

Within the framework of this model, I theorise that the mPFC, dopamine-dependent, biasing of sensory processing is disrupted such that CNO-treated rats learned equally about reward contingency of the stimuli within the relevant and irrelevant dimensions. However, control rats effectively lessened attention to the irrelevant dimension stimuli, therefore not learning about their reward-contingencies, such as occurs during blocking. In CNO-treated rats this 'aberrant salience'-induced learning resulted in an increase in TTC at the ED, where novel stimuli are presented, and thus the higher order dimension-based discrimination guides behaviour. Conceptually, this is enhanced learned irrelevance resulting in perseverative-like responding to the previously relevant dimension. However, this attentional regulation deficit is beneficial when, learning about a redundant stimulus within the irrelevant dimension can be transferred to a subsequent partial change ED. Indeed a similar phenomenon has recently been reported in Parkinson's patients who exhibited both perseveration to the previously irrelevant dimension, as well enhanced learned irrelevance (Fallon et al., 2016). Thus the origin of the set-shifting deficit here is likely a disruption of neuromodulatory signals within mPFC (i.e. reward prediction error signals), resulting in abnormal attentional biasing (sensory gating), and stimulus-reward contingency learning within the ASST.

As a caveat for generalisation to other manipulations inducing mPFC-disruption, it should be stated that the exact mechanisms underlying the ED deficit following acute inactivation may differ from the other causes of mPFC-hypofunction. For example, acute mPFC_{in} by GABAR agonists, induces a

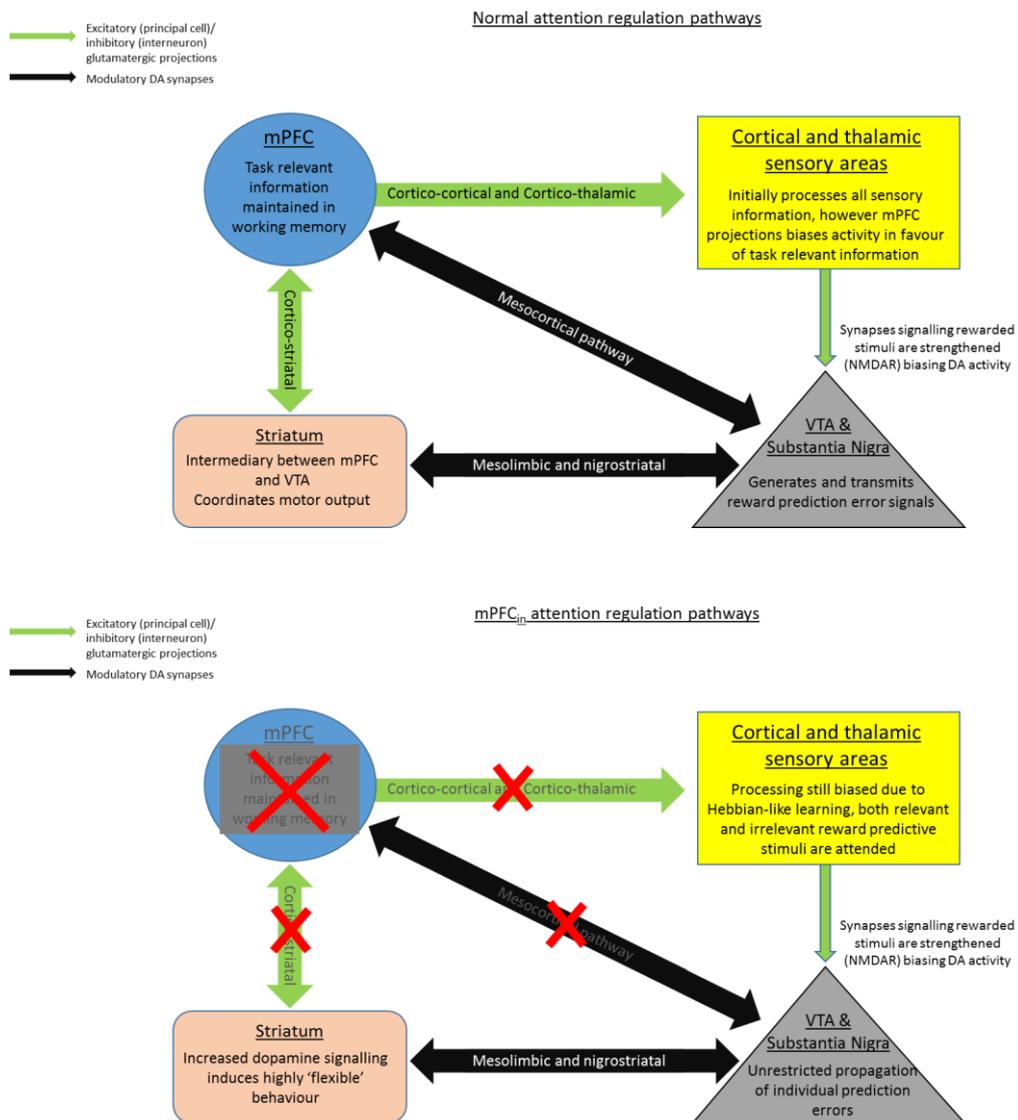


Figure 3.8) *Top*. When the mPFC is functioning normally, its sustained representation of the task relevant information in working memory, results in biasing of sensory processing, i.e. attention, and prediction error signalling. *Bottom*. When the mPFC is not functioning properly, the downstream biasing of sensory processing and prediction errors. In this scenario non-selective predictive errors drive behaviour through dopamine signalling within the striatum.

hyperactive subcortical dopamine system (Jo et al., 2013), whereas mPFC-ibotenic acid lesions induce hypoactive dopamine signalling (Shim et al., 1996).

Does this model and its focus on dopamine hold up empirically, within the context of set-shifting? While there is a clear role for dopamine in the dlPFC/mPFC for set-shifting, results are mixed, with some researchers reporting no ID/ED shift-cost (and thus no evidence of set-formation), and others reporting an ED deficit (Table 3.1). Dopamine depletion (6-OHDA lesions) within the dlPFC of marmosets (Roberts et al., 1994) resulted in fewer errors to criterion at the ED stage compared to controls such that there was no difference between the ED and the previous ID stage (i.e. no shift-cost). In the same study, the 6-OHDA-lesioned animals also had poorer working memory performance, consistent with a previous report (Brozoski et al., 1979). However, results in rodents have found an ED deficit following manipulations which reduce mPFC dopamine signalling. In transgenic mice, reducing mPFC dopamine signalling via enhanced catechol-o-methyltransferase (COMT; a catecholamine degrading enzyme) activity selectively impaired the ED shift and working memory (Papaleo et al., 2008). Furthermore, amphetamine sensitisation in rats (which induces mPFC-hypofunction; Homayoun and Moghaddam, 2006) also impaired set-shifting by increasing TTC at the ED, a deficit which was ameliorated by D1R agonism (Fletcher et al., 2005). In contrast, increasing mPFC dopamine signalling via inhibition of COMT resulted in no ID/ED shift-cost (Tunbridge et al., 2004), a similar finding as the 6-OHDA-lesioned marmosets (Roberts et al.). As a positive shift-cost is the only index of set-formation (Eimas, 1966; Durlach and Mackintosh, 1986), it is not possible to ascertain whether the data from 6-OHDA-lesioned marmosets (Roberts et al.) and COMT-inhibited rats (Tunbridge et al.), indicates enhanced set-shifting or an inability to form an attentional set.

Given that the marmoset task is substantially different from the rodent task in regards to training, feedback (no exploratory trials), and mechanisms of stimulus selection (energy intensive digging vs touchscreen), if the focus is placed on results from the rodent tasks, then the conclusion for the role of dopamine in

set-shifting is that mPFC hypo-dopaminergic impairs set-shifting. This conceptualisation for reduced mPFC dopamine easily fits with the model (Figure 3.8). The increased dopamine signalling is more complicated to explain. Further examination of the data from Tunbridge et al. (2004) reveals that a substantial increase in TTC for all ASST stages was clear for all animals (most stages required more than double the TTC normally reported) perhaps due to the presence of the guide cannulas (lesions).

Thus, while the role of dopamine in set-shifting is clear from two (Fletcher et al., 2005; Papaleo et al., 2008) of the four studies, the results of the remaining two studies are harder to interpret as either species/task (Roberts et al., 1994) or manipulation differences (Tunbridge et al., 2004) make a direct comparison between these studies less valid. An additional concern is that while both studies that report no shift-cost were specifically targeted to the dlPFC/mPFC (Roberts et al.; Tunbridge et al.), the two reporting an ED deficit were more global manipulations (i.e. transgene expression (Papaleo et al.) or i.p. amphetamine treatment (Fletcher et al.)).

Study	Species	Dopamine/Manipulation	Result
Roberts et al., 1994	Marmoset	↓ /6-OHDA lesions	no ID/ED shift-cost
Tunbridge et al., 2004	Rat	↑ / inhibited COMT	no ID/ED shift-cost
Fletcher et al., 2005	Rat	↓ /amphetamine sensitisation	increased ID/ED shift-cost
Papaleo et al., 2008	Mouse	↓ /enhanced COMT	increased ID/ED shift-cost

Table 3.1. Studies with dopamine manipulation during the ASST.

3.5.3 Implications for theories of attention and associative learning

These findings (as well as those of: Sharpe and Killcross, 2014; Fallon et al., 2016) support Mackintosh's theory of attention (Mackintosh, 1975), which posits that the ability to selectively attend and choose based on one stimulus dimension does not prohibit the additional learning of stimuli which may be irrelevant but still present. In this context, controls rats appear to behave optimally, and not only selectively attend to the relevant dimension stimuli, but also greatly reduce their attention to the irrelevant dimension to the extent that providing redundant

information during the simple-probe does not benefit them in the ED_{PC}. However, in mPFC_{in} rats, an inability to reduce attention to the irrelevant dimension, does not noticeably guide behaviour, until the irrelevant dimension contains information that is reward predictive. Thus while, the higher-order attentional set-shifting is impaired in mPFC_{in} rats, the lower-order inability to block redundant stimuli, is sufficient to alter behaviour at a stage (the ED_{PC}) where mPFC_{in} rats would otherwise show a deficit to controls.

Further support for Mackintosh's theory, arises from the comparison of our simple-probe to ED_{PC} manipulation, to that of McGaughy et al. (2008). Pearce et al. (2012) found that a redundant stimulus in a blocking paradigm (A+ → AX+ → X+), compared to a redundant stimulus in a simple discrimination (AY+, BY-), elicited greater responding when presented alone. The simple-probe to ED_{PC} manipulations presented here reflects the blocking phenomenon as the irrelevant dimension stimulus is reward-contingent (e.g. the X in the A+ → AX+ → X+ paradigm). In McGaughy et al. (2008) the irrelevant dimension stimulus (e.g. texture as "Y" in the AY+, BY- paradigm) is no more predictive of reward than the relevant stimuli dimension, thus even if the rats were attending to that dimension, it would be a poor behavioural pattern for obtaining reward, compared to choosing based on stimuli which were previously rewarded.

A caveat of Mackintosh's theory is that prediction error signals must represent multiple stimuli. That is to say, individual error terms govern the associative strengths of stimuli. In this scenario, the intermittent rewarding of the irrelevant dimension stimuli would result in lower associative strength compared to the correct stimulus. Critically, this also predicts that the redundant stimulus paired with the correct stimulus during the simple-probe would also develop greater associative strength than either of the novel (non-reward contingent) previously relevant dimension stimuli presented during the ED_{PC}. While seemingly contradictory, there is considerable evidence for both global and individual error terms. As a result, a number of hybrid models have been developed to account for the accumulating evidence (Haselgrove et al., 2010; Barrot et al., 2012).

In conclusion, these behavioural data have shown that DREADDs-mediated mPFC_{in} induces a set-shifting deficit consistent with previous methodologies. Furthermore, the data show that this deficit is likely linked to a deficit in the sensory gating (and working memory functions) of mPFC, consistent with findings in sensory gating paradigms (i.e. PPI or blocking) following mPFC- or dlPFC-disruption. Perhaps somewhat controversially, the fact that mPFC_{in}-rats were able to 'shift' faster during the ED_{PC}, counters the concept that the mPFC is the universal shifter. Indeed, the data here support a deconstruction of the mPFC as a 'homunculus' with mysterious executive functioning capacities, and instead supports its functions of 'working memory' as an emergent property of sensory (primarily multisensory) processing, where the inputs into the mPFC (i.e. the dopamine reward prediction signals), decide the output of the mPFC (biasing sensory processing based on the information stabilised by reward signals). Thus, working memory (dopamine-stabilised representations) and executive function (which can be reduced to sensory biasing) are essentially, the same set of processes. Indeed, this theory has been proposed by Hazy et al. (2006). In their biologically constrained mathematical model, 'executive functions' can be accomplished by simple reinforcement learning, thus demystifying the mechanisms underlying mPFC processes in abstract representation processing, such as attentional set. Applying this theory to the data presented here, although the shift-cost suggests that mPFC_{in}-rats formed an attentional set, the irrelevant dimension responding suggests that this set was 'weak'. It is possible that while mPFC-function helps in the formation of attentional set (through working memory and biasing of attention), it is actually the OFC which maintains the 'representation' of the attentional-set. This is congruent with the findings that lesions of the OFC impairs set-formation, as OFC-lesioned animals require more TTC for IDs than controls (Dias et al., 1996a; McAlonan and Brown, 2003; Chase et al., 2012).

3.6 Histological Analysis of mPFC DREADDs

Rationale

The behavioural experiments in Chapter 3 suggested effective and robust inhibition of the mPFC in the tested rats. We validate those results by providing evidence for DREADDs expression within the target region as well as a measure of neuronal activity (c-Fos) following administration of CNO. Additionally, the trafficking of DREADDs receptors through the axons of infected neurons allowed for investigation of where, outside of the mPFC, CNO activation of DREADDs may induce changes in behaviour.

3.7 Methods and materials

Tissue was collected as described in the General Methods section (Chapter 2). For mCherry and c-Fos immunoreactivity detection every 4th section of the frontal cortex was collected. Sections were placed in 9-hole netwells and petri dishes and washed four times for 5 min each in phosphate buffered saline (PBS: 158 M NaCl in 50% distilled H₂O and 50% 0.2 M phosphate buffer) on an automated rotator. Next the netwells were placed in blocking solution (1:5 normal goat serum, 1:100 10% Triton, in PBS) and rotated for an hour at room temperature. Sections were then washed in PBS 3 times for 5 min each. Sections were placed in histology pots before being incubated with 5 ml of anti-mCherry (rabbit anti-mCherry 1:2000, Abcam) or anti- c-Fos (rabbit c-fos 1:10000), suspended in antibody diluting solution (ADS; 1:100 normal goat serum, 1:100 10% triton, in PBS) overnight at room temperature. The following day sections were washed in PBS 3 times for 5 min each. Sections were then switched into histology pots and incubated in 5 ml biotinylated anti-rabbit antibodies (goat anti-rabbit, 1:500, Vectorlabs, in ADS) for one hour at room temperature. After incubation, sections were washed 3 times for 5 min in PBS, then placed in histology pots containing 5 ml of the biotinylation solution (Vectastain ABC KIT, Vectorlabs) and

incubated for one hour. Sections were then washed 3 times for 5 min in PBS and parvalbumin immunoreactivity was detected by staining with 3,3'-Diaminobenzidine (DAB; one tablet per 20 ml, Sigma, in distilled H₂O). The sections were determined to be stained when landmark anatomical structures were clearly identifiable or until they had been incubated for 20 min. Following DAB staining sections were washed 3 times for 5 min in PBS, and stored in 9-hole netwells at 4°C until (up to 72 hours) they were mounted to treated glass slides and coverslipped with DPX.

Fluorescent mCherry detection was performed on a subset of the brains to examine nuclear vs axonal mCherry expression. The procedure was the same as for DAB immunoreactivity up until the sections were removed from the primary (rabbit anti-mCherry 1:2000, Abcam) incubation. Sections were removed from the primary and washed 5 times for 5 min each, then incubated for one hour in foil-covered histology pots containing the secondary (goat anti-rabbit IgG H&L, Alexa Flour 594, Abcam). After secondary incubation, sections were washed 3 times for 5 min in PBS, then mounted to slides. Vectashield anti-fade mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vectorlabs), was applied then slides were cover-slipped and sealed.

3.8 Results

3.8.1 All rats exhibited clear DREADDs expression within the mPFC

Figure 3.9 shows a representation image of DAB-stained mPFC neurons infected with AAV5-CamKII-hM4Di-mCherry. All rats (n = 12) showed mCherry tagged DREADDs expression within the mPFC target region (approximate distance from Bregma 4.2 - 2.2 mm). Additional DREADDs expression was evident in parts of the medial OFC ventral to the target area. However, in all cases the most prominent staining was within the pre- and infralimbic cortex, and the anterior cingulate cortex (ACC). It is possible that this spread into the medial OFC or the ACC could underlie the transient reversal impairment.

3.8.2 DREADDs are trafficked throughout the projections of the infected mPFC neurons.

Fluorescent images were collected to determine axonal compared to cellular DREADDs expression as well as the extent of DREADDs expression through mPFC projections (Figure 3.10). Fluorescent detection of mCherry indicated reduced infection compared to the DAB-staining with less lateral extent. However, only a sample (n = 3) of the brains were analysed with this method. As can be seen in Figure 3.10, the DREADD receptors were trafficked within axons from mPFC cell bodies through the striatum. Implications for the expression of the receptor outwith mPFC cell bodies is considered further in the discussion section.

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

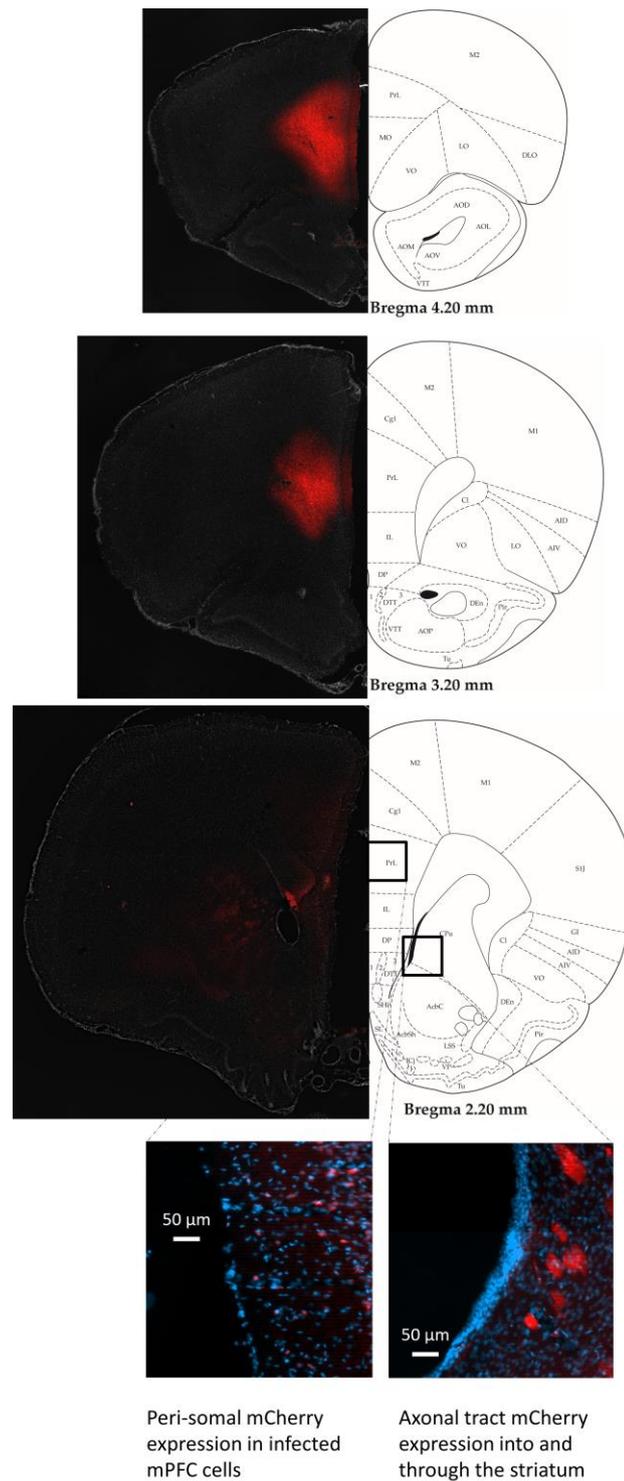


Figure 3.10) Representative images of mPFC-glutamatergic neurons infected by microinjection of AAV5-CamKII-hM4Di-mCherry as detected by anti-mCherry immunohistochemistry. Inset shows non-cellular (DAPI-stained; blue) mCherry expression. PrL = prelimbic, IL = infralimbic, Cg1 = cingulate, MO = medial orbital, LO = lateral orbital, M1 = primary motor cortex.

3.8.3 No difference in c-Fos immunoreactivity following clozapine-*N*-oxide administration.

We sought to validate our CNO administration protocol with measurement of the neuronal activity marker c-Fos. Figure 3.11 is a representative grayscale image of sections stained for c-Fos immunoreactivity. No significant difference was found in the expression of c-Fos positive cells (mm^2) between rats treated with 10 mg/kg CNO orally or vehicle ($t_{(10)} = 0.38, p = 0.71$). This finding is explored further in the discussion section.

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

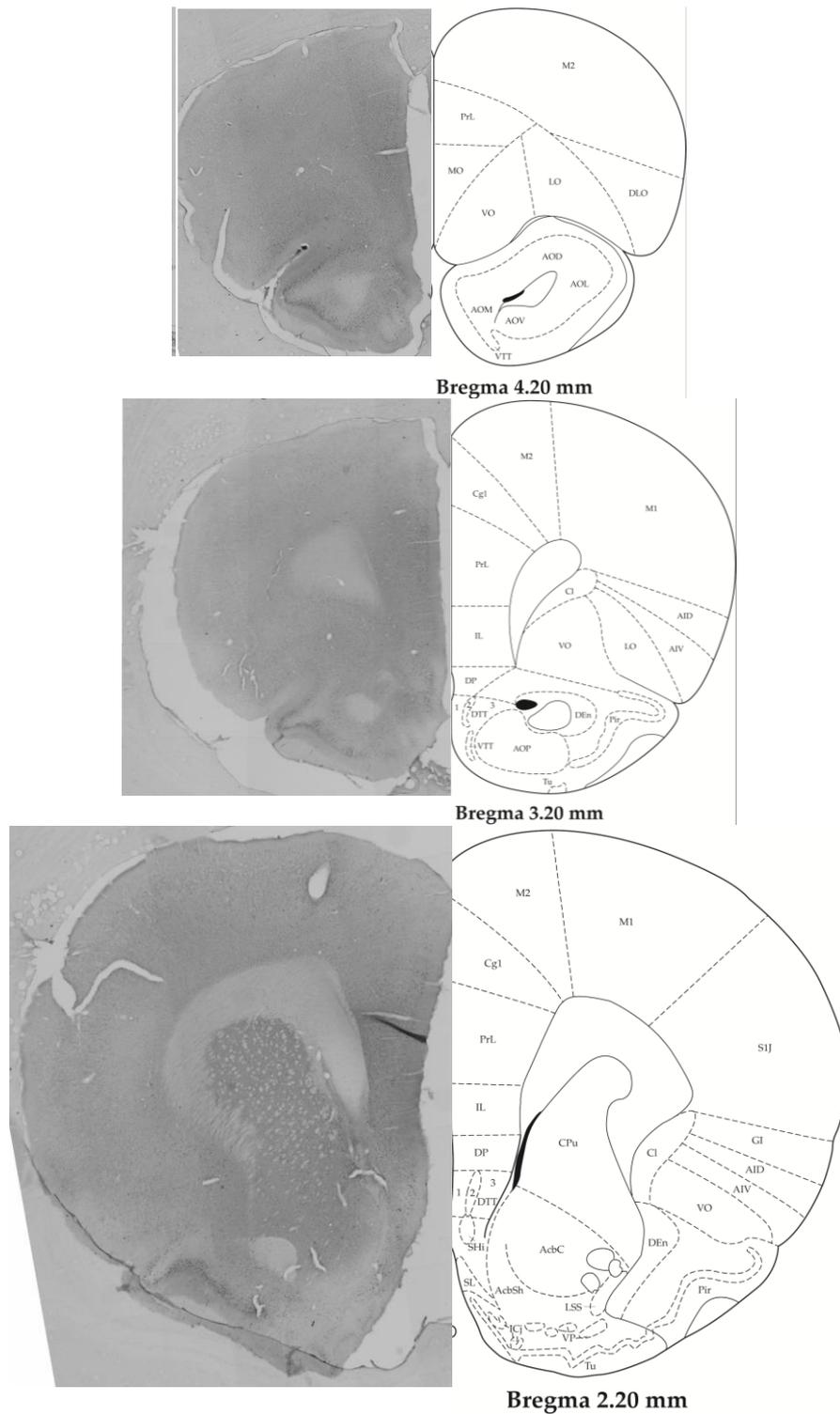


Figure 3.11. Gray-scale images showing representative c-Fos immunoreactivity throughout the PFC (estimated distance from Bregma: 2.2 - 4.2 mm). PrL = prelimbic, IL = infralimbic, Cg1 = cingulate, MO = medial orbital, LO = lateral orbital, M1 = primary motor cortex.

Chapter 3. Investigation of Medial Prefrontal Cortex (mPFC) Mediated Attentional Set-Shifting Deficits

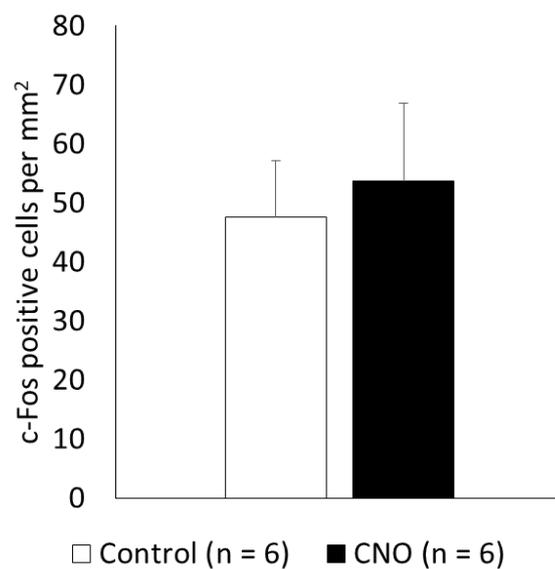
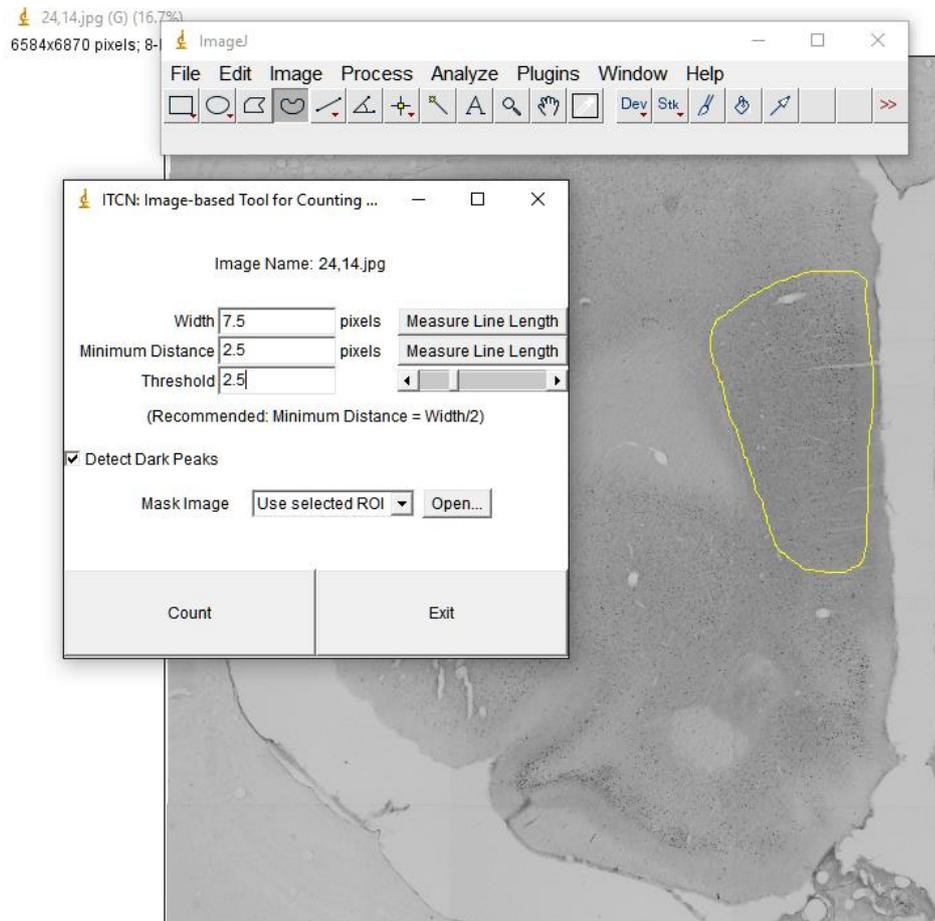


Figure 3.11. cont. *Top*. Image showing method for automated cell counting and size of region analysis. *Bottom*. Bar graph showing mean c-Fos expression per square mm (+ SEM) throughout the mPFC (estimated distance from Bregma: 2.2 - 4.2 mm). No significant differences were found between rats treated orally with 10 mg/kg or vehicle.

3.9 Discussion

3.9.1 Analysis of DREADDs expression.

The level of DREADDs expression I report supports our observed behaviour effects and is in line with our previous findings with ibotenic acid lesions (Birrell and Brown, 2000; Tait et al., 2009). Whilst the target area is the prelimbic cortex (PL), unfortunately the ability to limit the infection solely to that area eluded me. However, despite the clear infection of collateral areas, no evidence of confounding occurred as the only deficit to manifest was the ED shift deficit. Given the proximity of the medial OFC and ACC to the target areas and the clear infection, it is not possible to rule out inhibition of these areas in the observed effects.

3.9.2 Infected projections are consistent with the pattern of mPFC neuronal projections

The immunohistochemistry results suggest that infection of mPFC neurons results in DREADDs expression at terminal synapses within the medial striatum, nucleus accumbens, and other areas consistent with anterograde tracer (*Phaseolus vulgaris leucoagglutinin*) injections into the PL and infralimbic cortices (IL; Vertes, 2004). This expression within the medial striatum suggests that all rats had infection of the target area (PL). Again however, the spread into the ACC and medial OFC, and their corresponding target projections cannot be ruled out (and may underlie the increase in trials at REV 1). Additional experiments can further separate infection in the targeted PL compared to the IL by examining downstream areas where the innervation varies by region such as the nucleus accumbens or the sub-nuclei of the amygdala (Vertes, 2004). Given that the behaviour was consistent with ibotenic acid lesions into the same PL-targeted coordinates (Birrell and Brown, 2000; Tait et al., 2009), it is most likely that the effect was primarily mediated by the same population of neurons (within PL) as opposed to offsite effects. Previous lesion studies using the same coordinates have shown that the size of the lesion (either localised to the PL or spread into medial OFC or ACC),

does not obscure or confound the PL-mediated ED deficit (Birrell and Brown; Tait et al.).

3.9.3 Inhibitory DREADDs can be effective without reduction in activity at the cell body

Despite no physiological or anatomical evidence of the CNO-induced DREADDs inhibition of mPFC neurons, the behavioural data suggest the binding of CNO to DREADDs receptors was potent and effective at disrupting the target neuron population. CNO is orally bioavailable and has been shown to have physiological effects when administered orally such as through the drinking water (Jain et al., 2013; Cassataro et al., 2014). However, the oral bioavailability is reduced (Bryan Roth, personal communication, 2016). Furthermore, the extent to which the DREADDs receptors expression continued for the duration of experiments (approximately 4 months) could cause substantial increases in the receptor-ligand ratio. While most studies are done within one month of DREADDs infection, significant DREADDs expression throughout axons and into synapses can occur throughout at eight weeks or more (Stachniak et al., 2014; Kerstetter et al., 2016). In this instance, the effective dose to induce neuronal inactivation in the presence of more receptors spread along the axon may be greater.

As c-Fos is merely a measure of activity at the cell body, it does not inform, the number of action potentials generated following CNO administration, or on the propagation of generated action potentials along the axon, nor the subsequent induction of neurotransmitter release from the post-synaptic terminal. Given that these experiments employed a non-localised delivery of CNO it is highly likely that DREADDs receptors in the terminals were also activated by the ligand. Indeed, it has been shown that behavioural effects can be instigated in DREADDs infected animals following CNO administration directly onto the post-synaptic terminals of a target region. Stachniak et al. (2014), reported robust effects on feeding behaviour following selective silencing of hypothalamic projections into midbrain. This effect on behaviour came with no reduction in the

action potential generation of the cell body of the afferent neurons, evidence that inhibition occurs at multiple sites following CNO administration.

One last point is the fact that I did not attempt to raise the activity of mPFC neurons prior to CNO administration. Low baseline c-Fos activation can mask CNO-mediated inhibition (Sasaki et al., 2011; Koch et al., 2015; Koike et al., 2016). Koike, et al., reported no difference in c-Fos in ACC DREADDs infected neurons following 10 mg /kg (i.p.) CNO despite a clear behavioural effect in a task dependent on ACC function. However, when c-Fos expression was pharmacologically increased via treatment of a GABA_A receptor antagonist, a significant reduction in c-Fos positive cells was found in mice treated with CNO.

Chapter 4. Effects of Neonatal-Phencyclidine (PCP) Treatment on Attentional Regulation in Set-Shifting and Novel Object Recognition

The experiments in this chapter utilised developmental disruptions targeting (primarily medial prefrontal cortex; mPFC) glutamatergic synapse maturation in neonatal rats. Findings from testing in adulthood revealed that whereas neonatal-PCP-treatment only caused subtle non-significant deficits in the standard ASST, which appeared to worsen with age, significant deficits in extradimensional set-shifting were revealed through repeat testing in a modified ASST with maximal separation of stimuli. Behavioural pattern analyses revealed elevated spatial and irrelevant dimension-based responding in neonatal-PCP-treated rats across all tests. Additional age-dependent effects were found for novel object recognition (NOR) such that, young neonatal-PCP-treated rats had deficits in the 3-min delay NOR, but they did not have those deficits in adulthood. However, additional testing in a 24-hour delay NOR found a deficit in neonatal-PCP-treated rats consistent with dysfunction of the mPFC.

4.1 Introduction to neonatal-NMDAR antagonism rats

Due to a short gestational period in rats, the first two weeks of postnatal development, also known as the neonatal stage of development, roughly corresponds to the second trimester of pregnancy in human fetuses (Bayer et al., 1993; Clancy et al., 2001). In both species, this is a critical timepoint for development of glutamatergic synapses. Experiments in rodents have shown that particularly during postnatal days (PND) 1 - 14, NMDARs are hypersensitive to glutamate signalling to strengthen key synaptic connections (Ikonomidou et al., 1989). Blockade of NMDARs during this two-week period induces apoptosis in key areas for cognition, such as the hippocampal formation (CA1 and dentate gyrus), the caudate nucleus, and the cingulate and frontal cortices (Ikonomidou et al., 1999). Although the apoptosis induced by NMDAR blockade is greatest during PND 0 - 7, the phenotype of animals treated with an NMDAR antagonist during that time point is overly severe and not consistent behaviourally with schizophrenia patients. Instead, selective NMDAR blockade during PND 7 - 14, despite inducing less apoptosis, generates a phenotype more consistent with the neural and behavioural perturbations reported in patients with schizophrenia.

While the immediate effect of NMDAR blockade during the first two weeks of development is apoptosis in multiple brain regions (Ikonomidou et al., 1999; Wang et al., 2001; Wang et al., 2003), the surviving glutamatergic neurons undergo additional disruptions, with long-lasting effects. Throughout the lifespan, calcium (Ca^{2+}) signalling through NMDAR activation alters the NMDAR subunit composition of the activated cells (Scheetz and Constantine-Paton, 1994). Blockade of Ca^{2+} signalling by NMDAR antagonist phencyclidine (PCP) during PND 5 - 10, induces select reductions in cortical NR2B subunit mRNA expression (Sircar et al., 1996; Anastasio and Johnson, 2008a) as well as overall reductions in NMDAR expression (Sircar et al., 1996). The actions of NMDAR antagonism on NR2B levels are the proposed mechanism for many of the subsequent cognitive and behavioural phenotypes of neonatal-PCP-treated rats. Reductions in NR2B subunit expression reduces excitatory long-term potentiation (LTP), the mechanism by

which cells strengthen their connections, allowing for efficient communication and information processing (Luscher and Malenka, 2012). Either genetically or pharmacologically induced acute reduction of NR2B expression in frontal cortex can not only reduce LTP within the region, but also impair contextual fear memory, a behaviour known to be regulated by frontal cortex activity (Zhao et al., 2005). All together the data indicate direct dysregulation of NMDAR signalling, throughout the time period that roughly corresponds to adolescence in rats following NMDAR antagonism two-weeks postnatal.

In the next section the long-term effects of neonatal-PCP administration on brain function and behaviour in adolescent and adult rats are reviewed in relation to symptoms present in human schizophrenia patients.

4.1.1 Neurochemistry

As described in the general introduction (Chapter 1), the neurochemical basis of schizophrenia remains a key target for studies and many rodent manipulations with relevance for schizophrenia are validated based on the presence of neurochemistry that mirrors the alterations seen in patients with schizophrenia.

To begin with, the neonatal-NMDAR antagonism manipulation recapitulates several of the dopamine-related findings. Neonatal-NMDAR antagonism induces decreases in the density of tyrosine hydroxylase (an enzyme involved in the biosynthesis of dopamine) within the axons of mPFC-neurons (Wedzony et al., 2005a). Perturbation of dopaminergic signalling can be further demonstrated following treatment with compounds which increase dopamine at the synapse such as amphetamines and methamphetamines. Semba et al. (2001) found that neonatal-PCP treatment reduced methamphetamine-induced c-Fos expression in the nucleus accumbens and VTA compared to control rats, suggesting abnormal dopaminergic signalling in neonatal-PCP rats.

Schizophrenia-related abnormalities are also found in the glutamatergic system. Although direct evidence of reduced NR2B expression past PND 21 in rats

or mice has not yet been reported, there is neurochemical evidence of dysregulated glutamate signalling through NMDARs. From PND 21 to PND 56, neonatal-PCP-treated rats, exhibit increased seizure activity in response to NMDA administration (Brooks et al., 1997b,) suggesting that the neonatal treatment induces hypersensitivity in the glutamatergic system. This increased sensitivity to NMDA may be due to continued overexpression of NR1 and NR2A (Anastasio and Johnson, 2008a, b) and reduced expression of NR2B (Sircar et al., 1996; Anastasio and Johnson, 2008a). There is evidence that increased expression of the NR1 subunit persists up to PND 180 (Baier et al., 2009). Indeed, increased overall binding of NMDAR within the mPFC (suggestive of upregulation of receptor expression, likely due to reduced glutamate at the synapses) has been found in adult neonatal-NMDAR blockade rats compared to controls (Sircar, 2003; Anastasio and Johnson, 2008b; du Bois et al., 2009). This overactive glutamatergic signalling in neonatal-PCP-treated rats is also evidenced following acute administration of PCP. Measurement of relative cerebral blood volume shows persistent (25 - 30 mins post injection) increase in the frontal cortex (amongst other regions) following intravenous administration of 0.5 mg/kg PCP in neonatal-PCP-treated rats. Rats treated with vehicle also exhibited increased blood flow in response to acute PCP, albeit for a shorter duration (Broberg et al., 2013).

GABAergic neurotransmission is also altered in neonatal-PCP-treated rats. Wang et al. (2008) reported that a single dose of PCP during PND 7 caused reductions in markers of GABAergic interneurons (parvalbumin, calretinin, and calbindin) in cortical tissue. Repeated neonatal-PCP treatment is reported to selectively reduce PV mRNA expression within the mPFC (Redrobe et al., 2012; Kaalund et al., 2013). Consistent with the reduction in GABAergic neuronal markers, neonatal-PCP administration diminishes inhibitory signalling within the mPFC, specifically in layer 2/3 (Kjaerby et al., 2014). In direct relation to known dysfunction of the GABA transporter in humans, neonatal-PCP administration also compromises sensitivity of the transporter. Also, in direct reference to the human condition, disruptions in neureglin-1 and its primary receptor ERB4 have recently

been evidenced in neonatal-PCP-treated rats (du Bois et al., 2012; Radonjic et al., 2013).

4.1.2 Brain structural abnormalities

Neonatal-NMDAR blockade in rats also results in brain structural abnormalities reminiscent of schizophrenia. At the neuronal level, expression of myelin basic protein, a marker of myelination, is reduced for more than two weeks following exposure to PCP (Zhang et al., 2012). Additional reduction in a marker of oligodendrocytes (the cell type primarily responsible for myelination) is also evident. Both of these changes were reported to be specific to the frontal cortex. These changes in myelination are likely associated with the select reduction in the length of layer 2/3 mPFC pyramidal neuron basilar dendrites, evidenced through PND 60 (Wedzony et al., 2005b). Post-mortem analysis of (occipital) cortical tissue from neonatal-PCP-treated rats at PND 20 shows reduced number of synapses and increased synaptic length, while at PND 30 - 40, the neonatal-PCP-treated rats exhibit an increased number of synapses, and synaptic length no longer differs from controls (Brooks et al., 1997). However, by PND 60 no differences are present between groups. Given the vast loss of inhibitory interneurons and microglia, in combination with the effects on mPFC dendrites, it is unsurprising that many reports on neonatal-NMDAR blockade find lower adult brain weight in treated animals compared to controls (Mouri et al., 2007; Boctor and Ferguson, 2010; Lim et al., 2012).

4.1.3 Schizophrenia-related innate behaviour in neonatal-NMDAR blocked rats

Observation of locomotor activity is a low-level measurement of behaviour, however distance travelled while freely exploring an open chamber is known to be sensitive to dysregulation of neural systems, in particular the dopaminergic and glutamatergic systems. Manipulation of the dopaminergic system via methamphetamine or amphetamine induces hyperlocomotion in neonatal-NMDAR blockade rats (Uehara et al., 2010; Ingallinesi et al., 2015). Acute treatment with PCP also causes hyperlocomotion in these rats (Wang et al., 2001;

Anastasio and Johnson, 2008b; Boctor and Ferguson), suggesting increased sensitivity in both glutamatergic and dopaminergic systems. While one study reports increased basal locomotor activity (Wedzony et al., 2008), baseline activity generally appears to be unaffected (Kawabe et al., 2007; Anastasio and Johnson, 2008b; Boctor and Ferguson, 2010; Uehara et al., 2010; Gaskin et al., 2014), and differences between neonatal-NMDAR blockade rats and controls only consistently appear following a challenge to homeostasis (such as acute drug exposure). It is clear that the method induces long-lasting physiological changes with implications for behaviour in adult rats, in particular susceptibility of the glutamatergic and dopaminergic systems.

Continuing on with innate behavioural responses, the PPI task (see Chapter 1: Introduction for review) has also been used to assay neonatal-NMDAR blockade rats. The earliest report showed reduced basal PPI at PND 24 - 26 in neonatal-PCP rats compared to controls (Wang et al., 2001). This effect was replicated by Wang et al. (2003) and Anastasio and Johnson (2008b), as well as recently being shown in PND 32 rats (Zhang et al., 2012). However, other attempts to find PPI deficits in neonatal-PCP-treated rats failed at PND 25 (Boctor and Ferguson, 2009) and PND 32 - 33 (Rasmussen et al., 2007). The apparent inconsistencies in results are not easily explained away by strain differences (all of the neonatal-PCP studies used Sprague Dawley rats), nor PPI calculations (Boctor and Ferguson), therefore it would appear that the effectiveness of PCP to induce PPI is, in fact, not reliable. A recent paper which used Lister Hooded rats reported a deficit in PPI, however electrophysiological recordings did not replicate the schizophrenia findings in these rats, suggesting that the effect was not aetiologically identical (Boctor and Ferguson, 2010).

Evidence is also mixed for other neonatal-NMDAR blockade induced PPI deficits. In the MK801 manipulation, no PPI deficit was found following acute methamphetamine treatment at PND 34 - 35. However, when tested later (PND 62 - 63), significant deficits were found across a range of pulse decibel levels (Uehara et al., 2010). Although baseline deficits in PPI have also been reported for

PND 56+, neonatal-MK801-treated rats (Harris et al., 2003), this result also failed to be replicated (Lyll et al., 2009). It is important to note that despite the failure to replicate the main PPI effect, the researchers did report a subtle but significant effect in the delay in prepulse-induced startle.

Novel object recognition (NOR) primarily measures memory and relies on innate increases in attention to unfamiliar (novel) stimuli in the environment. Originally developed by Ennaceur and Delacour (1988), the test has proven to be highly sensitive to dysfunction in glutamatergic neurotransmission (Warburton et al., 2013). Interestingly, despite the numerous reports of disruptions to the glutamatergic system, most reports of neonatal-NMDAR blockade effects on NOR, during PND 50 - 60, did not find differences between treated and controls rats (Stefani and Moghaddam, 2005; Gaskin et al., 2014). While one study does report NOR deficits (5-hour delay), the analysis required pooling of data across six time points (PND 30 - 180) to reach significance, suggesting the effect was not robust at any given developmental period (Baier et al., 2009). Further studies show that, combined with social isolation, a deficit in NOR discrimination (2 and 24 hour delay periods) can manifest during PND 60 - 65 (Gaskin et al.; Watson et al., 2016), although this may be primarily driven by the social isolation manipulation (Gaskin et al.). Thus, despite the physiological effects reported earlier in the chapter, the effects on measures of innate adult behaviours appears to be inconsistent.

4.1.4 Schizophrenia-related attentional set-shifting behaviour in neonatal-PCP-treated rats

Of most interest to this dissertation are the reported effects of neonatal-NMDAR blockade on ASST performance. To date three reports have examined ASST deficits in neonatal-PCP-treated rats, all from Lundbeck research groups in Denmark. According to these publications, neonatal-PCP administration consistently induces an impairment at the ED stage (Broberg et al., 2009 2008; Redrobe et al., 2012), although it should be noted that the most recent report also includes a deficit at the reversal stage following the ED (Redrobe et al.).

4.2 Rationale

The repeated findings of an ED impairment are entirely expected given the clear pattern of mPFC-specific physiological disruption. Following on these anatomical, sensory gating, and ASST findings, this chapter sought to replicate the ED impairment in neonatal-PCP-treated rats and further explore the effects of the treatment on attentional regulation and behavioural flexibility.

4.3 Methods and materials

4.3.1 Animals

Adult male (n = 3) and female (n = 6) Lister Hooded rats were purchased from Charles River UK for breeding. Once the females had reached a body weight of 250 g (approximately 4 weeks after arrival), they were separated into pairs and placed with a single male in the cage. Approximately 3 weeks after pairing, 5 of the 6 paired dams gave birth to litters. Day of birth was counted as PND 0. Each dam was housed separately with her litter until weaning at PND 21. On PND 7, 9, and 11, male rat pups were injected subcutaneously with either 20 mg/kg/ml PCP (Sigma; Lot No 113M4924V) in saline or saline as a control. The first two litters (1 and 2) received saline injections, while litters 3 & 4, were born a day later, and received PCP injections. Of the 15 pups that received the PCP injections, only 6 survived. We believe this may have been due to a combined effect of the PCP and abnormally large litter sizes (12 - 15 pups). For the later-born fifth litter, all of the pups were treated with PCP regardless of sex. Litter 5 was also much smaller to begin with (just 6 pups), and all of these pups survived to weaning without incident. Post-weaning, male pups (and female pups from litter 5) were housed in standard housing conditions. Rats were allowed ad libitum access to food except during ASST testing, for which they were placed on a mild food control regimen of 15 - 20 g per day to increase their motivation in the task. Behavioural testing began at PND 35 with the novel object recognition task. Following completion of this

testing, rats continued being fed ad libitum until PND 63 at which they were placed on the food control regimen one week prior to the start of ASST testing.

4.3.2 Behavioural Testing

➤ *Attentional set-shifting test*

As described in General Methods (Chapter 2).

➤ *Maximal separation of stimuli (ASST-MSS)*

In this variant of the ASST, rather than a pseudo-random probability of any particular set of bowls being presented, on every trial the stimulus pairs are orthogonal. That is to say, the pairing of the irrelevant stimuli with the correct, rewarded, stimulus changed on every trial. The purpose of this was (1) to maximally separate the stimuli to encourage faster learning and (2) to enable greater confidence in judging which component (exemplar) of the compound stimulus the animal was selecting.

Training and testing occurred in a different type of standard ASST chamber, which had 2 holes, each 4-cm wide, in the floor of each test chamber (see Figure 4.1). The apparatus was elevated, with runners (attached to the underside of each test chamber) that allowed plastic panels (38 cm x 9 cm x 2 mm) to be freely pulled forwards via the holes in the test chamber floors. The day before testing, rats were trained to pull unscented velvet-covered panels forward to obtain a cereal reward located at the end of the panel, with one rewarded panel in each chamber. The pot containing the reward was located 4 cm from the end of each panel, thus each rat needed to pull a panel approximately 34 cm to obtain the reward. The distance the rat had to pull the panel forward to obtain the reward was steadily increased after each successful (reward obtained) response. Once the panels were extended to their full length the training continued until the rat regularly made complete responses to each side (generally 3 to each side). Next, rats were trained on a simple odour discrimination. One velvet panel was scented with cumin, and the other with coriander. The first two pulls were deemed exploratory meaning the

Dimension	Training Pairs	Pair 1	Pair 2	Pair 3
Odours	Cumin / Coriander	Cinnamon / Ginger	Fenugreek / Paprika	Turmeric / Cloves
Media	Coarse Cork / Fine Cork	Straws / Velcro	Sand Paper/ Grit	Plastic beads / Plastic hatches



Figure 4.1) *Left*. Table of exemplars used. Exemplars were presented in these pairs. Presentation order during testing was counterbalanced (e.g. 1 → 2 → 3 or 2 → 3 → 1). *Right*. Image of modified testing chambers for panels based testing.

response was noted as correct/incorrect but the rat was allowed to explore the unchosen panel after an incorrect response. Following these exploratory trials, incorrect responses resulted in the lowering of the barrier to the chamber containing the correct panel then progression to the next trial. Odour discrimination training continued until the rat made six consecutive correct responses. Next rats were trained on a simple texture discrimination. One panel was covered in a cork tile, and the rewarded panel was covered in ground cork. Testing occurred the following day. First, the rat was presented with a simple discrimination (only odour or texture stimuli presented). Next the rats performed a compound discrimination during which the rewarded stimulus remained the same, however irrelevant dimension stimuli were added (both odour and medium stimuli presented; see Figure 4.1 for stimuli and pairs). The third stage was an intradimensional discrimination during which new stimuli pairs were presented however the rewarded stimulus remained within the same dimension as the previous stages. The final stage was an extradimensional shift during which new pairs of stimuli were presented and one of the stimuli in the previously irrelevant dimension was the rewarded stimulus. If a rat had formed an attentional set then the ED stage would take more trials than the ID, similar to the standard 7-stage task. All rats were tested twice in this task.

*Compared to the bowl digging task, the use of the panels apparatus resulted in more overall TTC for each stage. This may have been due to the reduced 'effort' required to pull the panel, compared to digging for the reward.

➤ *Adolescent Novel Object Recognition Test*

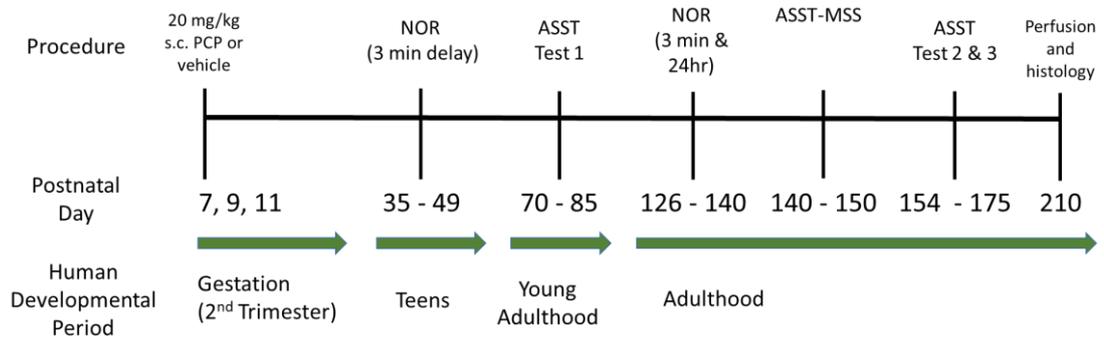
At PND 35, rats were assayed for novelty preference in the NOR. The rats were habituated to the chamber (65 x 65 x 50 cm) for 3 days (30 mins each day) with sample objects placed in the same locations where test objects would be located. Following habituation, rats were tested twice to rule out side/object preferences. On the test day, rats were allowed to acclimate to the chamber for 3 mins. Then they were placed in the holding cage while a pair of identical objects were placed in the test chamber. After a 1 min delay, the rat was placed back in the test chamber to explore the familiar object pair for 3 mins (acquisition phase). Following acquisition the rat was placed in the holding cage while the test chamber was cleaned of odours, the explored familiar objects were removed and replaced with a third identical familiar object and a novel object. After a 1 min delay, the rat was placed back into the test chamber to explore the familiar and novel objects for 3 mins (test phase). The discrimination index (DI) was calculated as time spent exploring the novel object minus time spent exploring the familiar object, divided by the total exploration time. This calculation allows for sensitivity to individual differences in total exploration time. All objects were counterbalanced across condition, side, and presentation as the familiar object (during acquisition phase), or as the novel object (during test phase). The repeat tests were performed within 48 hours of the previous test, and the novel object presentation side was counterbalanced across tests. For all rats, all phases (habituation, acquisition, and test) of all tests were video recorded. Videos were scored blind and data were recorded using the Observe video recording software (Jackie Macpherson, University of St Andrews). Tests were first analysed by repeated measures ANOVA. If no main effect of test was found then, DIs were averaged across the tests and the conditions were compared using t-test.

Litter 5 (n = 3, male pups) was not of age for the adolescent NOR studies thus were not included. Video from one control was corrupted and therefore not scored.

➤ *Adult Novel Object Recognition Test*

Once the rats had matured to adulthood (PND 126) they were assayed again in the novel object recognition task (NOR). The rats were habituated to the chamber for 3 days (30 mins each day) with sample objects placed in the same locations where test objects would be located. Following habituation, rats were tested twice to rule out side/object preferences. The first pair of tests were done with a 1 min delay. A week later, a second pair of tests were done with a 24-hour delay. Video for the 1 min delay from one control rat was corrupted and therefore not scored.

Timeline for neonatal-PCP-treated rats experiments



4.4 Results

4.4.1 Neonatal-PCP-treated rats show no clear deficits in the ASST

All rats were tested three times in the standard 7-stage ASST. The first was performed at PND 70 - 85, while the second and third tests were performed at PND 154 – 175 (Figure 4.2). A main effect of Test ($F_{(2, 36)} = 4.89, p < 0.05$) was found, suggesting performance changed with age/experience. Compared to the first test,

performance improved slightly for controls, while neonatal-PCP-treated rats performed slightly worse, as indicated by trend level significance for the interaction of Test x Group ($F_{(2, 1)} = 3.1, p = 0.057$). However, there was no interaction of Stage x Group ($F_{(6, 1)} = 1.2, ns$), nor an overall interaction of Test x Stage x Group ($F_{(2, 6)} < 1, ns$), therefore no stage specific differences could be determined. Both neonatal-PCP-treated rats and controls formed and shifted an attentional set regardless of age (Test 1: $F_{(1, 19)} = 24.63$; Test 2 & 3: $F_{(1, 19)} = 40.17, p < 0.05$).

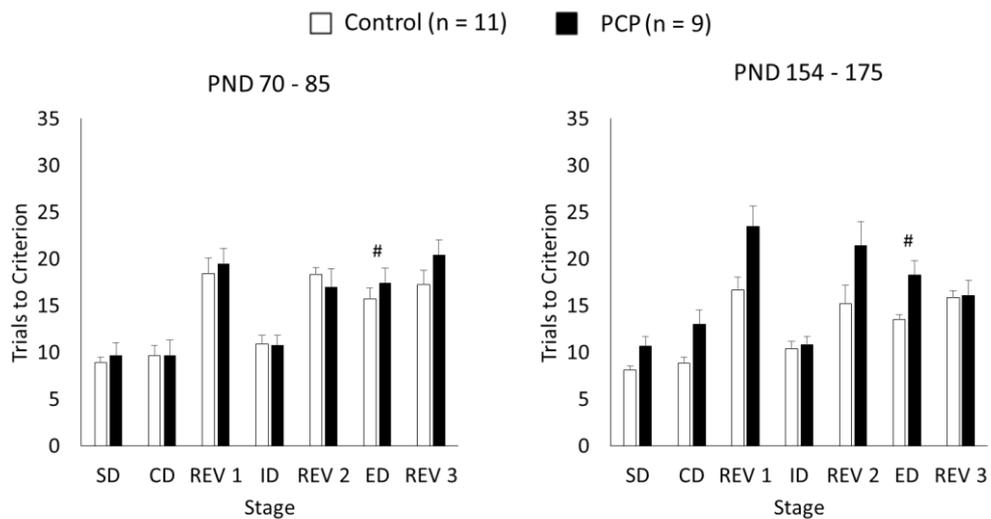


Figure 4.2) Mean TTC (+ SEM) for neonatal saline- and PCP-treated rats in the standard ASST. Compared to their performance as young rats (PND 70 – 85: *left*), adult (PND 154 – 175: *right*) control rats appeared to perform better, while neonatal-PCP-treated rats, appear have worsening performance. However, there was only a trend level Test x Group interaction ($p = .057$; #, ID significantly different than ED both groups).

Bayesian analysis of the data (for details of the analysis see Chapter 2: General Methods), did not reveal clear differences between groups at the stage of interest (the ED; Figure 4.3). However, although both groups of rats completed each stage of the ASST with a Bayes factor greater than 3, indicating that they were eventually responding to the correct stimulus, neonatal-PCP-treated rats had more quartiles with strong evidence of non-random systematic incorrect behavioural patterns (i.e., spatial patterns, irrelevant dimension, or previously correct stimulus responding) than controls across the entirety of the task (controls

= 7; PCP = 11). Specifically, there was evidence of perseveration in neonatal-PCP-treated rats during REV 1 (see the heatmap in Figure 4.3), as well as more irrelevant dimension responding during REV 1 and REV 2 than controls (table in Figure 4.3). This increased irrelevant dimension-based responding was not *in lieu* of spatial-based responding, as neonatal-PCP-treated rats, actually had one more quartile than controls, with strong evidence for this behavioural pattern (Figure 4.3). These data suggest that although there were no statistically significant effects by stage, throughout the second and third tests, there was more evidence for incorrect behavioural patterns, than controls.

Chapter 4. Effects of Neonatal-Phencyclidine (PCP) Treatment on Attentional Regulation in Set-Shifting and Novel Object Recognition

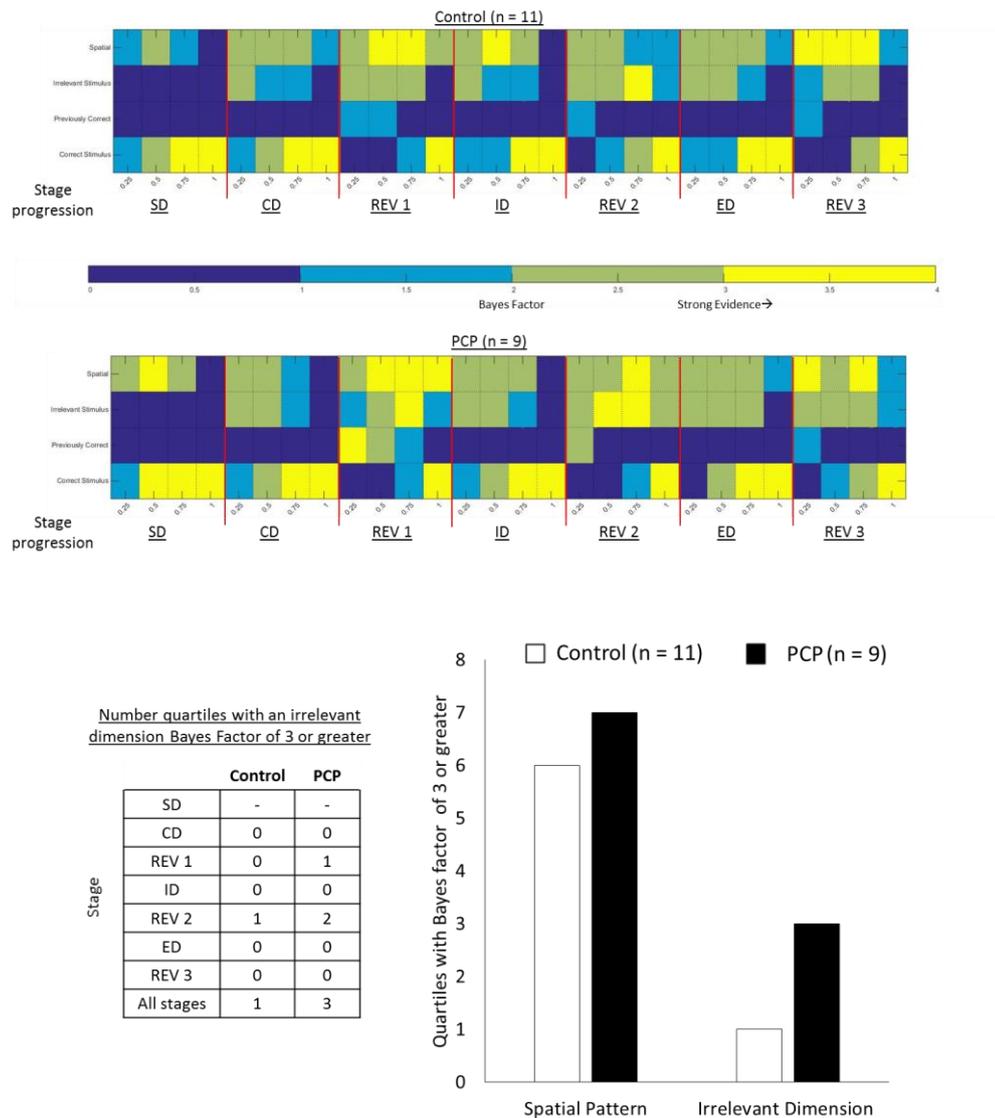


Figure 4.3. *Top.* Heatmap of evidence for behavioural patterns during the standard ASST. Compared to controls neonatal-PCP-treated rats show more evidence for all incorrect behavioural patterns. *Bottom left.* Table showing sum number of quartiles by stage for irrelevant dimension-based responding by group. Neonatal-PCP-treated rats showed more irrelevant dimension-based responding than control rats. *Bottom right.* Bar graph showing sum number of quartiles with strong evidence for spatial- or irrelevant dimension-based responding in control and neonatal-PCP-treated rats.

4.4.2 Neonatal-PCP-treated rats have an ED deficit in the ASST-MSS

At PND 140 - 150, all rats were tested twice in the ASST-MSS. As expected both groups of rats required more trials to complete the ED compared to the ID indicating that an attentional set was formed (ID/ED main effect of stage $F_{(3, 54)} = 30.78, p < 0.05$; Figure 4.4). Unlike in the standard ASST, compared to controls, neonatal-PCP-treated rats required more trials to complete the ID/ED shift (restricted comparison ED: $F_{(1, 54)} = 16.3, p < 0.05$; Stage x Group interaction: $F_{(3, 1)} = 2.92, p < 0.05$). Analysis of the ID/ED shift-cost revealed that neonatal-PCP-treated rats required a mean of 10 additional trials than controls to complete the ID/ED shift ($t_{(18)} = 2.73, p < 0.05$). In the second test, all rats completed the SD with more trials compared to the first test (controls: Test 1 SD: , 12.64 (± 1.61), Test 2 SD: 23.64 (± 4.53); neonatal-PCP-treated rats: Test 1 SD: 15.22 (± 3.76), Test 2 SD: 24.89 (± 2.91)). This was confirmed in a significant Test x Stage interaction ($F_{(1, 3)} = 4.03, p < 0.05$). However, the main effect of Test ($F_{(1, 54)} = 1.2$) and all other interactions with Test ($F_s < 1$), were not significant.

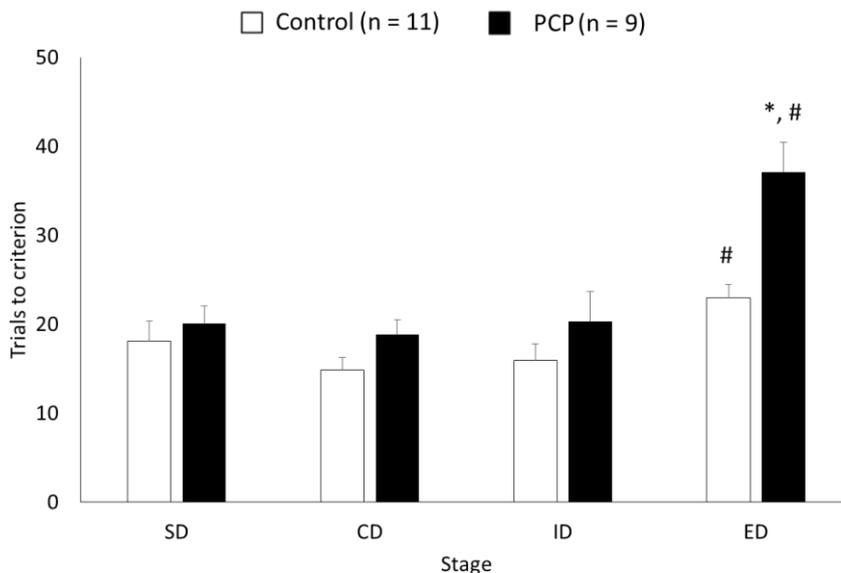


Figure 4.4) Mean TTC (+ SEM) for control and PCP-treated rats in the modified ASST-MSS. Neonatal-PCP-treated rats, required more TTC for the ED stage than controls.

Analysis of the behavioural patterns evidenced during the ASST-MSS, revealed that neonatal-PCP-treated rats were more likely to have patterns of responding to the irrelevant dimension than controls across all stages (controls = 3; PCP = 8; Figure 4.5). In this modified task, the evidence for irrelevant dimension based responding from neonatal-PCP-treated rats diminished from the CD to the ID in both groups (controls: from 2 to zero; neonatal-PCP-treated from 3 to 1), as would be expected with the formation of an attentional set. At the ED, there was strong evidence for neonatal-PCP-treated rats responding to stimuli within the previously relevant dimension for all quartiles, and there was only weak evidence of them responding to the correct stimulus by completion of the stage. Again, this was not *in lieu* of spatial based-responding and neonatal-PCP-treated rats also had more quartiles with strong evidence for spatial-based responding than controls (controls = 1; PCP = 3). Interestingly, three quartiles with strong evidence for spatial-based responding were evident in neonatal-PCP-treated rats during the SD, whereas this was not found for controls.

Chapter 4. Effects of Neonatal-Phencyclidine (PCP) Treatment on Attentional Regulation in Set-Shifting and Novel Object Recognition

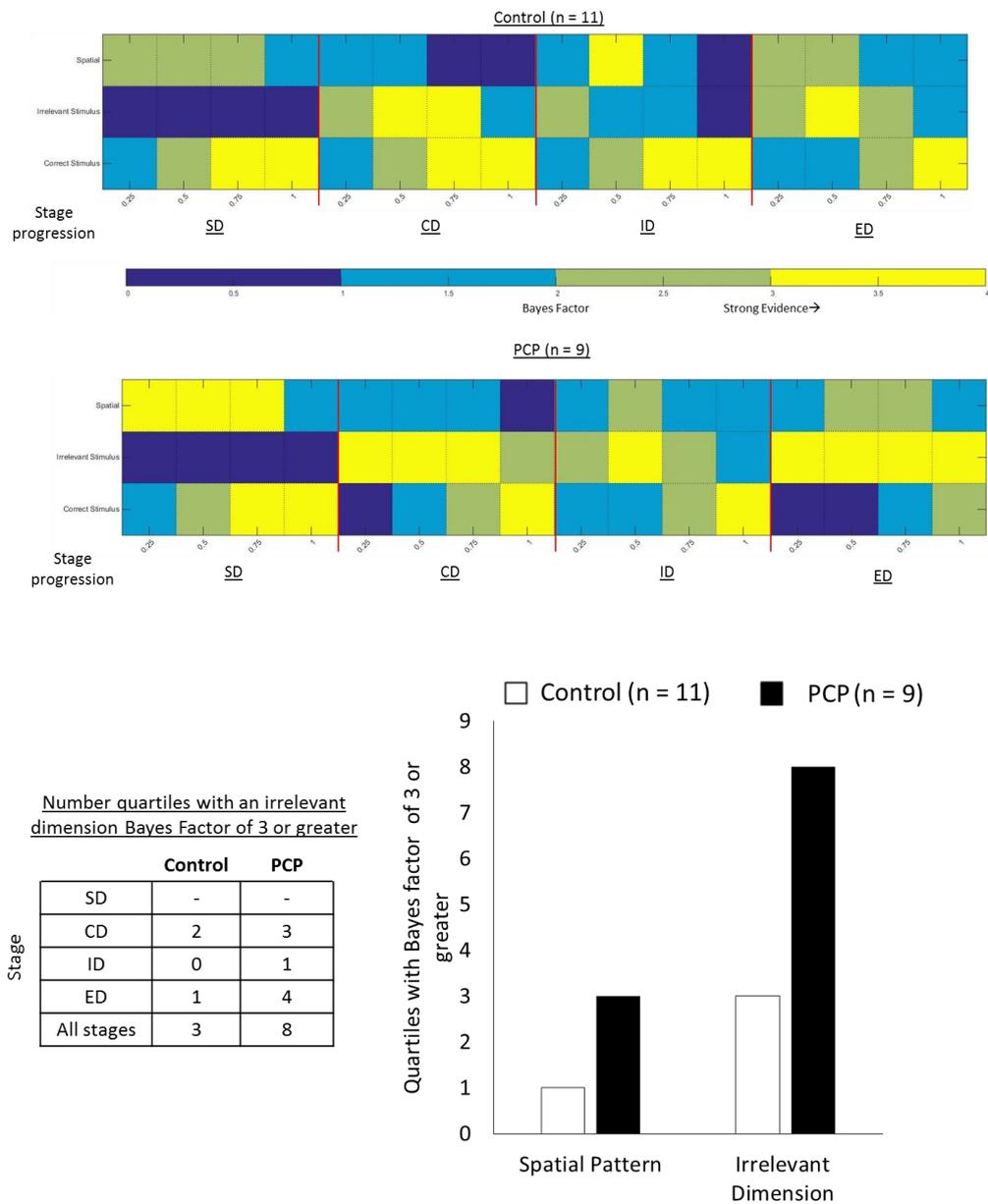


Figure 4.5) *Top*. Heatmap of behavioural patterns evidenced during the modified ASST-MSS. There was substantial evidence for neonatal-PCP-treated responding to irrelevant dimension stimuli during the CD, ID, and ED compared to controls. *Bottom left*. Table of sum quartiles per stage of irrelevant dimension responding. Across all stages the evidence suggested neonatal-PCP-treated rats responding to irrelevant dimension stimuli. *Bottom right*. Bar graph showing total spatial and irrelevant dimension quartiles by group across all stages except for the ED. Neonatal-PCP-treated rats showed more evidence of incorrect behavioural patterns (irrelevant dimension or spatial-based responding than controls).

4.4.3 Young neonatal-PCP-treated rats show deficits in the short delay novel object discrimination, however this deficit is not present in adult neonatal-PCP rats

At PND 35 - 49, all rats were tested in the NOR task following a 1 min delay. Compared to controls, neonatal-PCP-treated rats exhibited reduced novel object exploration, as measured by the DI ($t_{(14)} = 2.42, p < 0.05$; Figure 4.6). This reduction in time spent exploring the novel object was not due to an overall decrease in exploration time (controls = 48.9 secs (± 3.22), PCP = 46.78 secs (± 10.29)). At PND 126 - 140, rats were again tested in the NOR. However, at this timepoint no significant differences were found in the DI between controls and neonatal-PCP-treated rats ($t_{(17)} = 0.85, ns$). The loss of a difference between groups was due to a reduction in the DI for controls (-0.10) and an increase in the DI for neonatal-PCP-treated rats (+0.13). No significant differences were found between groups in object exploration times.

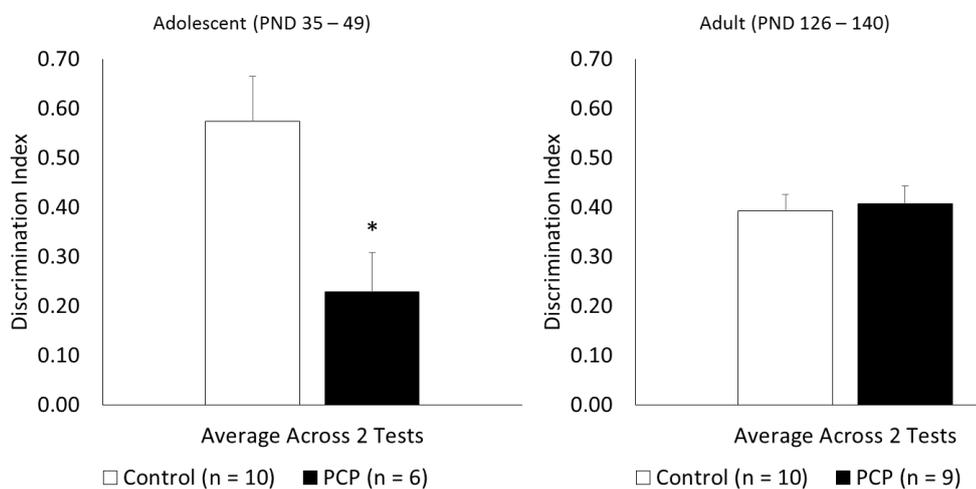


Figure 4.6) Mean discrimination index (+ SEM) for the 1 min delay NOR. Although adolescent neonatal-PCP-treated rats show a deficit in exploration of the novel object (*left*), this deficit is not present in adulthood (*right*).

4.4.4 Adult neonatal-PCP-treated rats show deficits in the 24-hour delay novel object discrimination

The adult neonatal- PCP-treated rats were also tested in the 24-hour delay NOR task. Here, neonatal-PCP-treated rats again showed a deficit in discrimination of the novel object compared to controls ($t_{(18)} = 2.35, p < 0.05$; Figure 4.7). Compared to control rats, neonatal-PCP-treated rats had greater overall exploration time ($t_{(18)} = 3.64, p < 0.05$; controls = 63.14, (± 2.45); PCP = 79.11, (± 3.83).

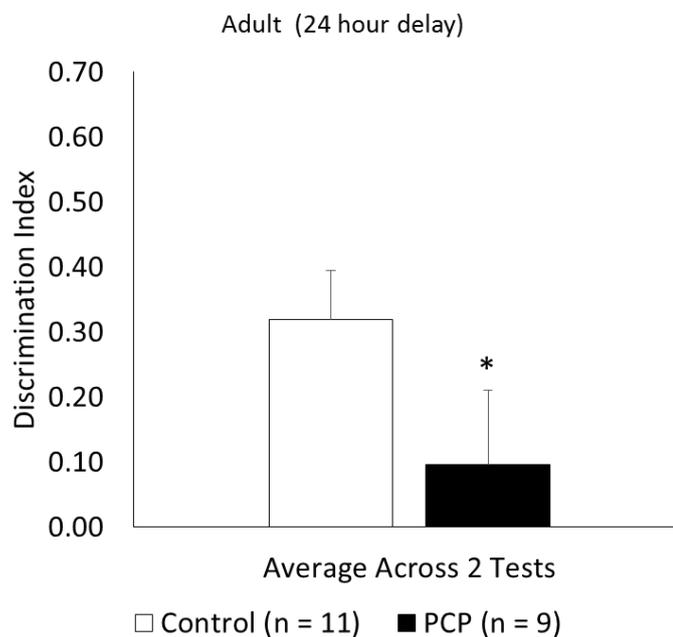


Figure 4.7) Mean discrimination index (+ SEM) for the 24-hour delay NOR. Compared to controls, neonatal-PCP-treated rats show a deficit in exploration of the novel object, following the long delay.

4.5 Discussion

4.5.1 Failure to replicate the ASST deficits in neonatal-PCP-treated rats

Several previous reports suggested a robust ED deficit in the standard ASST in neonatal-PCP-treated rats (Broberg et al., 2008; Broberg et al., 2009; Redrobe et al.), however the experiments in this chapter did not replicate these previous

reports. In addressing potential causes of these conflicting findings, the foremost consideration is the differences in ASST protocol. All three previous reports used the same protocol as each other, but different to the protocol used here, as described below.

First, in the previous reports the odour stimuli (scented oils) were placed around the rim of the pots containing the media and reward. As described in the general methods section, all ASST experiments in this thesis were performed with the odour discriminanda intermixed within the media. As such both the odour and medium within each bowl are simultaneously experienced, whereas when the odour is around the rim the rat may independently experience either the odour or the medium. Thus, placement of the odour around the rim potentially separates not only the 'compound' nature of the stimuli, but can also result in dissociation of the reward from the odour cue.

Second, the shift-direction was not counterbalanced in the previously published studies (Broberg et al., 2008; Broberg et al., 2009; Redrobe et al., 2012). All rats performed medium discriminations for the first five stages and the ED shift was always to an odour stimulus. Given the potential for differential difficulty between odour-medium and medium-odour shifts, arising from the placement of the odours on the rim, the failure to counterbalance stimulus dimensions for the ED shift may artificially, increase the TTC for the stage. The TTC for control rats during the all stages outwith the ED are broadly similar to published data from our lab (e.g. Birrell and Brown, 2000; Tait et al., 2009; Chase et al., 2012). However, the number of TTC for the ED is markedly greater for control and neonatal-PCP-treated rats.

While other differences in protocol also exist (the published papers also report: sprinkling cheerio dust over the media in the bowls to 'mask' the reward, only one exploratory trial, and replacement of the post-CD reversal with an additional ID stage), as there are no apparent differences between performance at the other stages, the evidence points to the lack of counterbalancing between dimension shift-direction, and placement of the odour around the rim of the bowl,

as a likely cause of differences in the previously reported ED deficit, and the lack of a clear deficit in this report. However, as reviewed in the introduction section to this chapter, other behaviours (hyperlocomotion and PPI) are also reportedly inconsistent in neonatal-PCP-treated rats. Therefore, the ASST findings here may also reflect this variability in the behavioural phenotype resulting from the manipulation.

Despite the lack of a clear deficit in the ASST, the behavioural patterns analysis suggested suboptimal performance in neonatal-PCP-treated rats compared to controls. In particular, neonatal-PCP-treated rats were more likely to show patterns of responding to the irrelevant dimension during reversal stages. The pattern of results from the second and third tests also suggest that while control rats improved, neonatal-PCP rats' task performance deteriorated. While this was only a trend level interaction, I sought to further examine the possible set-shifting deficit in a task designed to enhance the set-formation, and also more consistently detect responding to irrelevant dimension stimuli.

4.5.2 The ASST-MSS task revealed an ED deficit in neonatal-PCP-treated rats

In the ASST-MSS, the stimulus presentation order was changed with the intent of maximally separating the stimulus pairs. The task was limited to 4 stages: SD, CD, ID, and ED, to control for potential interference from reversal stages. In addition, the test was performed in a novel testing apparatus with stimuli (odour and haptic) presented on panels as opposed to within bowls. The results showed that both control and neonatal-PCP-treated rats were able to form and shift attentional set in this task design, supporting its use for testing of set-shifting. In this task-setup, there was an ED deficit in neonatal-PCP-treated rats compared to controls. In addition, the behavioural patterns analysis supported the hypothesis that neonatal-PCP-treated rats were responding based on irrelevant dimension stimuli, as the evidence for this pattern was consistently present across all stages. Indeed, the evidence for irrelevant dimension-based responding during the CD suggests that the task design successfully, maximised stimulus presentation to

detect which element of the compound stimuli rats were likely choosing based on. Interestingly, control rats also showed strong evidence for irrelevant dimension-based responding during the CD, evidence that they too failed to block the novel stimuli within that dimension. However, control rats only showed two quartiles of strong evidence for irrelevant dimension-based responding, one of which co-occurred with strong evidence for responding based on the correct stimulus, compared to neonatal-PCP-treated rats which showed evidence for irrelevant dimension-based responding from the first quartile. Furthermore, whereas there was strong evidence for control rats responding to the correct stimulus by the third quartile of the CD, this evidence was not present until the fourth quartile for neonatal-PCP-treated rats. At the ED, controls also showed responding to the previously relevant dimension early in stage performance (second quartile), however the evidence suggests this pattern was quickly abandoned and control rats completed the ED with strong evidence for responding to the correct stimulus. This was not the case for neonatal-PCP-treated rats, as there was strong evidence for irrelevant dimension-based responding throughout the ED, and they did not have strong evidence for responding based on the correct stimulus by the end of the stage.

However, the task was not without its flaws. The ED deficit was only detectable following repeat testing to increase statistical power. Additionally, as the task did not use reversals, during the first test several stimuli were never rewarded. This lack of reward for those stimuli likely drove the elevated TTC for the SD. For example, the rat with the highest increase in trials for the SD between Test 1 and Test 2 (44 trials) was rewarded for odour 2 (ginger) during the ED of Test 1. The SD for Test 2 was odour 1 (cinnamon). This increase in TTC at the SD highlights an important concern in regard to repeat testing with the task. A potential resolution for this is exposure to all stimuli (not in compound) and to allow the reward to be obtained from each following each test to remove the potential for this bias. Chase et al. (2012), utilised this 're-exposure' procedure with the 4ID task. In their report, while there was a main effect of test, with

improved performance from test one to test two, the pattern of performance for both groups of rats at each stage was stable across tests.

4.5.3 Developmental period dependent effects in neonatal-PCP-treated rats in the novel object recognition task

The results from the young and adult neonatal-PCP NOR testing are both consistent with previous reports in neonatal-NMDAR blockaded rats (Stefani and Moghaddam, 2005; Baier et al., 2009; Gaskin et al., 2014). These results further previous findings by showing that there is also no effect of neonatal-PCP administration with a short (1 min) delay, whereas the previous studies all used 2 - 5 hour inter-trial intervals. However, the presence of a deficit after a 24-hour delay has never been reported previously.

The role of the mPFC (specifically, glutamatergic signalling within the region) in NOR has only recently begun to be elucidated (Warburton et al., 2013). Traditionally, memory has been the responsibility of medial temporal lobe regions, such as the hippocampus, and perirhinal and entorhinal cortices. While the mPFC has been shown to be involved in appetitive and aversive memory processes (for review see Euston et al., 2012), a role in basal memory processes is less evidenced. Typically, some form of 'cognitive load' must be in place prior to the requirement for mPFC function. For example, Barker and Warburton (2011) compared the effect lesions of the hippocampus, perirhinal cortex, and mPFC on the standard NOR as well as three variants of the task: object location, object-in-place, and temporal order. The researchers also used three different delay periods: 1 min, 3 hours, and 24 hours. In the short delay tasks (5 min or 3 hours), the mPFC-lesioned rats significantly differed from controls in the high load tasks (object-in-place and temporal order). In the published report, mPFC-lesioned rats did not differ from controls at short delay NOR, and showed equal exploration of the novel object, as controls. However, the authors noted that mPFC-lesioned rats performed significantly worse at the NOR following the 24-hour delay, and did not successfully discriminate the novel from the familiar object as they had following

the shorter delays. This finding suggests a role for the mPFC in either retrieval of a 'remote memory' or consolidation of the memory/experience.

The most direct evidence suggests a PCP-induced disruption in mPFC-mediated consolidation. Akirav and Maroun (2006) found that micro-infusion of NMDAR antagonist, (2R)-amino-5-phosphonovaleric acid, into the mPFC immediately following the acquisition phase caused a deficit in the test phase 24 hours later. The same treatment had no effect on discrimination after a 3-hour delay highlighting the disruption of memory consolidation as the mechanism underlying the deficit. Given the reported mPFC-glutamatergic signalling disruptions in neonatal-PCP-treated rats (Sircar, 2003; Anastasio and Johnson, 2008a; du Bois et al., 2009; Broberg et al., 2013) this is the most parsimonious explanation. Specifically, the hypo-glutamatergic state within the mPFC induced by neonatal-PCP administration (indicated by the abnormal expression of mPFC NMDAR subunits) can result in reduced NMDAR signalling much like the direct inhibition of mPFC NMDAR by Akirav and Maroun.

4.5.4 Reproducibility of set-shifting results

The failure to evidence clear and robust deficits in either of the set-shifting tasks (i.e. statistically robust findings from one round of testing) suggests that the results here and elsewhere are not highly replicable. Indeed, the neonatal-NMDAR blockade manipulation has been criticised for reproducibility not just in high-level cognitive tasks, but also in the innate behaviours such as PPI and locomotor activity (Boctor and Ferguson, 2009; Lim et al., 2012). Sircar (2003) suggested that the irreproducibility of the hyperlocomotor activity, may be due to differences in the cohorts' environments, such as handling, housing, cage size/population, and maternal care. In this case, ameliorating the reduction in body weight commonly found following neonatal-PCP administration can prevent the deficits in locomotor behaviour in adulthood. Supporting this hypothesis, comparison of the two reports suggests this is the case. When PCP-treated pups are underweight compared to controls, a deficit in dopaminergic-related locomotor activity can be

found (Semba et al., 2001). However, when the environment is sufficient to eliminate the body weight differences, the underlying dopaminergic system is no longer as affected (Sircar).

It would seem that while neonatal-PCP may predispose the system to disruptions in glutamatergic and dopaminergic signalling, with relevance to schizophrenia, the most effective and reliable manipulations rely not only on the drug treatment but also on additional environmental disruptions. For example, Gaskin et al. (2014) compared neonatal-PCP vs post-weaning social isolation, and a combination of the two. The “dual-hit” manipulations which employs both neonatal-PCP and social isolation resulted in a more robust behavioural phenotype than either manipulation alone. For both the NOR and PPI tests, the “dual hit” manipulation generated a greater deficit compared to controls, than neonatal-PCP alone. It should be noted that this study also failed to find any effect of neonatal-PCP (either alone or in combination with social isolation) on locomotor behaviour (either basal or in response to acute PCP treatment (3 mg/kg)). Future experiments will likely continue to use this dual approach, as it has proven effective and the behavioural deficits appear responsive to antipsychotics (Watson et al., 2016).

Last, it seems that many of the reported “cognitive” deficits in neonatal-NMDAR-blockade rats are subtle. In the delayed spatial alternation task (performed during PND 34 -70), deficits compared to controls are present for the initial acquisition phases, however equivalent performance is found between groups by the end of testing (Wang et al., 2001; Wiley et al., 2003). Once the criterion accuracy was reached by both groups, no differences between groups were found with increasing delay periods, or acute treatment with PCP, ketamine, or amphetamine (Wiley et al.). Similarly, in the Morris water maze, neonatal-PCP-treated rats show increased escape latencies during the initial acquisition trials, however perform equivalent to controls for the remaining trials. This finding was replicated by Andersen and Pouzet (2004), who furthered the finding by showing that neonatal-PCP-treated rats also had deficits in spatial reversal learning. The

Chapter 4. Effects of Neonatal-Phencyclidine (PCP) Treatment on Attentional Regulation in Set-Shifting and Novel Object Recognition

authors concluded that while spatial reference memory was an inconsistent and slightly measured impairment, the impairment in spatial reversal learning was consistent and robust.

4.6 Histological analysis of neonatal-PCP-treated rats

Rationale

Given the failure to find a robust ED deficit in the standard ASST, and only finding an ED deficit in the ASST-MSS task following repeat testing, this chapter sought to verify PCP-induced neural and anatomical disruptions. The extant literature (reviewed in the introduction Chapter 4.1) indicates body and brain weight reductions, in addition to alterations to the neuronal composition of the mPFC (primarily reduced PV-expressing interneurons). Additionally, given the apparent ‘time point sensitivity’, this section sought to highlight the ‘state’ of the neonatal-PCP-treated rats.

4.7 Methods and materials

All procedures in this section were performed as described in Chapter 2: General Methods.

4.8 Results

4.8.1 Neonatal-PCP-treated rats exhibit a deficit in weight gain and post-mortem brain weight

Compared to controls, neonatal-PCP-treated rats showed reduced weight gain throughout development (Week x Group $F_{(14, 1)} = 5.89, p < 0.05$). Specifically, during weeks 3 - 10, neonatal-PCP-treated rats weighed less than controls (Figure 4.8). This difference between groups was no longer present after week 10, and both control and neonatal-PCP-treated rats continue to gain weight at a normal rate. Despite no difference in body weight at the end of testing, the brain weight of neonatal-PCP-treated rats was significantly less than controls ($t_{(17)} = 2.13, p < 0.05$; Figure 4.8), suggesting that neonatal-PCP-induced neuronal development

Chapter 4. Effects of Neonatal-Phencyclidine (PCP) Treatment on Attentional Regulation in Set-Shifting and Novel Object Recognition

disruption produced some transient (body weight) and some permanent (brain weight) changes.

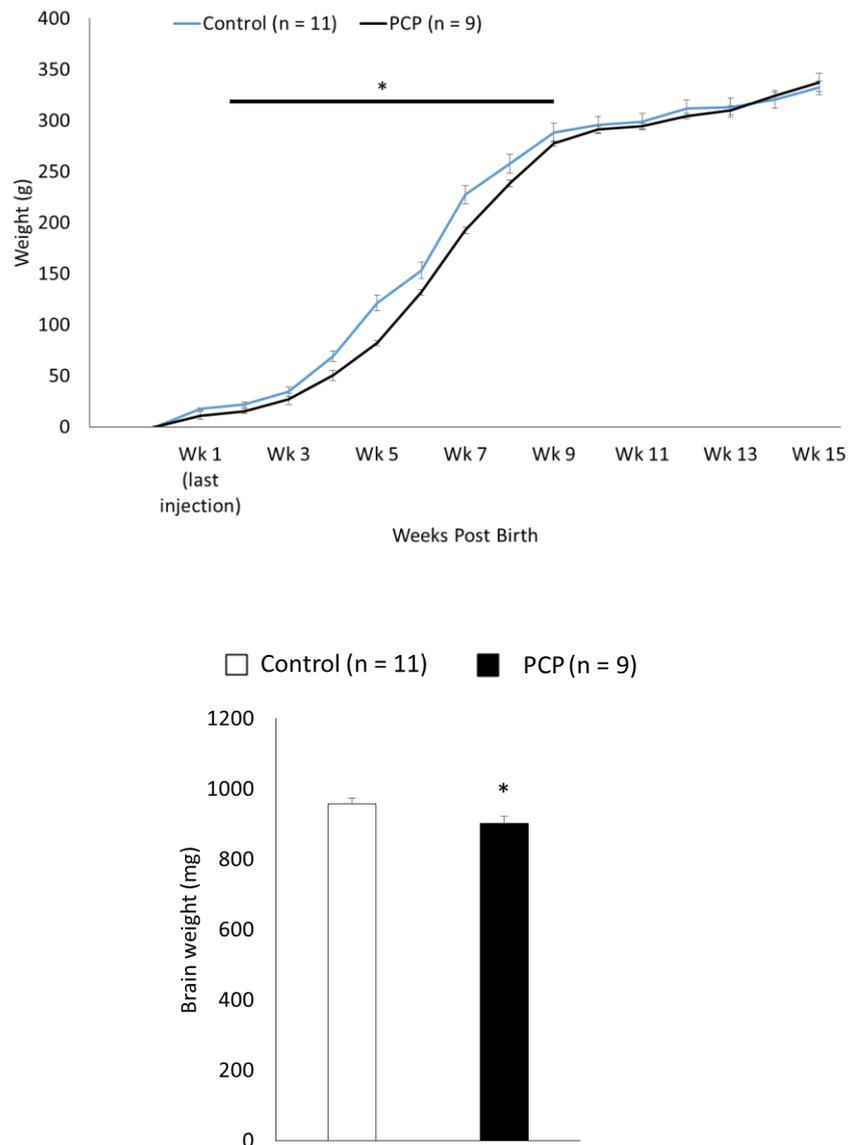


Figure 4.8) *Top*. Mean weight gain (+ SEM) throughout the duration of experiments. Early in development neonatal-PCP-treated rats show reduced weight gain, however, this remedies by early adulthood. *Bottom*. Mean brain weight (+ SEM). A post-mortem reduction in brain weight was found for neonatal-PCP-treated rats.

4.8.2 Neonatal-PCP-treated rats have less parvalbumin-expression within the mPFC

Quantification of PV-expressing cells revealed significantly fewer PV-expressing cells within the mPFC of neonatal-PCP-treated rats compared to controls ($t_{(17)} = 2.11, p < 0.05$; Figure 4.9).

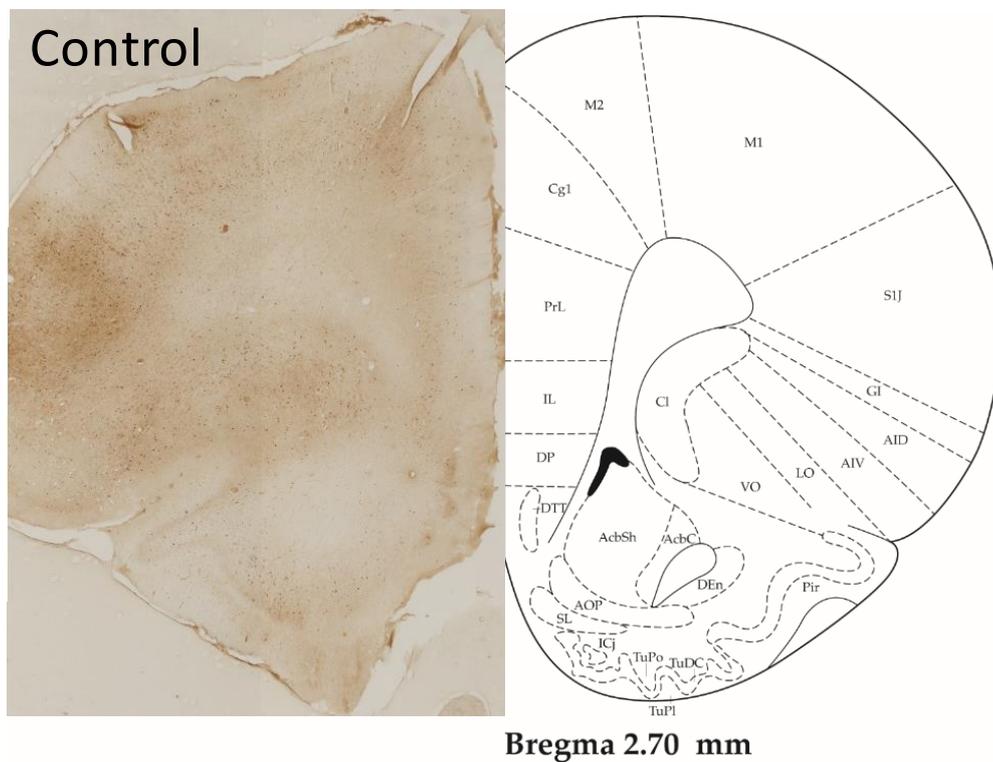


Figure 4.9) Representative images of staining for PV-expressing neurons within the mPFC (PL and IL) of a vehicle-treated rat.

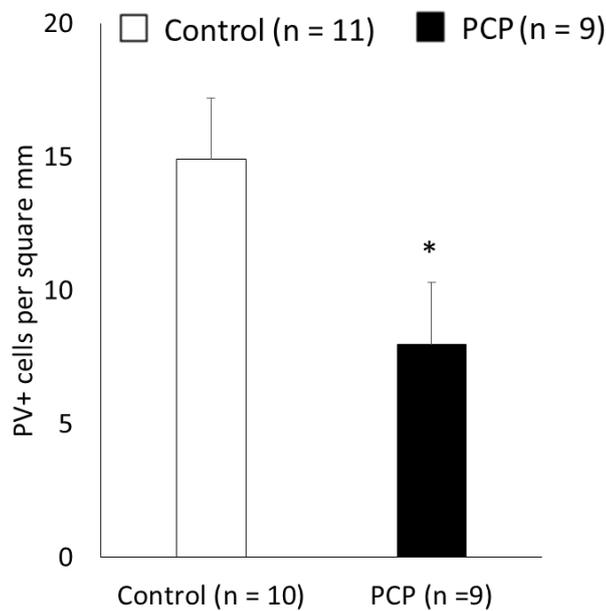
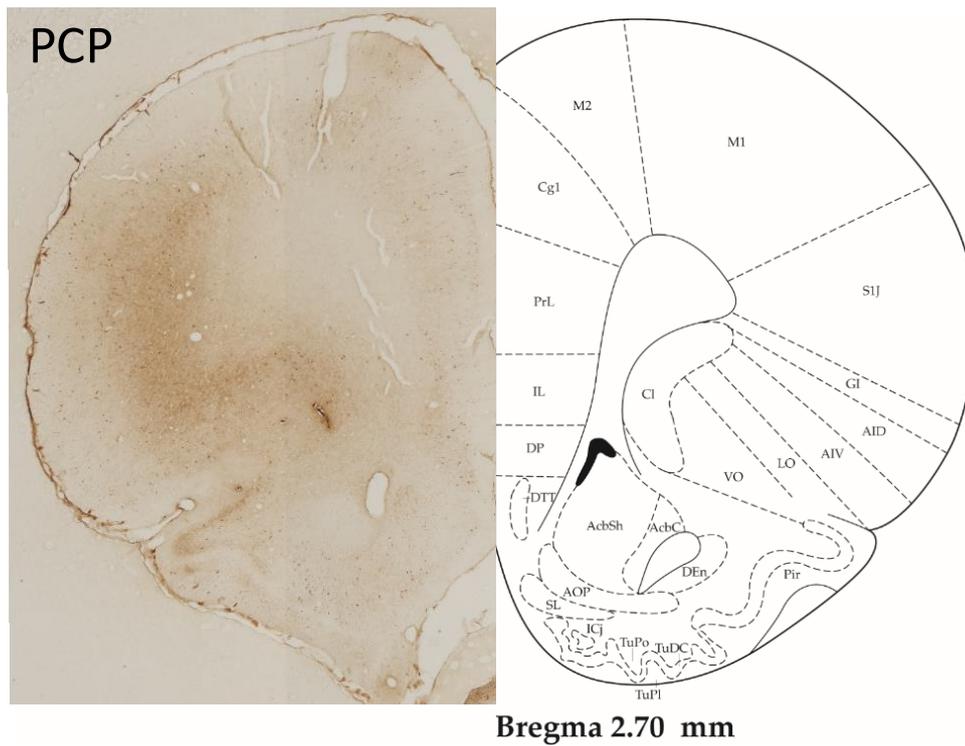


Figure 4.9 continued) Top. representative image of staining for PV-expressing neurons within the mPFC (PL and IL) of a PCP-treated rat. *Bottom* Image-J based quantification of PV-expressing neurons within the mPFC. A significant reduction in PV+ cells was found in neonatal-PCP-treated rats. PrL = prelimbic, IL = infralimbic, Cg = cingulate, MO = medial orbital, LO = lateral orbital, M1 = primary motor cortex.

4.9 Discussion

4.9.1 Successful replication of neonatal-PCP induced anatomical disruption

The cohort of neonatal-PCP-treated rats used in these experiments, replicated many of the anatomical changes reported previously including reductions in: body weight (Boctor and Ferguson, 2009), brain weight (Boctor and Ferguson, 2009 2008), and PV-expressing neurons in the mPFC (Wang et al., 2008; Redrobe et al., 2012; Kaalund et al., 2013). The brain anatomy changes are hallmarks of 'schizophrenia-related' alterations and likely underlie the behavioural deficits reported in the previous section (Chapter 4.1).

4.9.2 Role of the mPFC disruption in the novel object recognition deficits

While the effects of neonatal-PCP induced disruptions of the mPFC are easily linked to the performance in the set-shifting task, the NOR (24-hour delay) may also be temporal lobe-dependent (Barker and Warburton, 2011; Warburton et al., 2013). This analysis did not explore potential alterations to the temporal lobe (specifically the perirhinal cortex), as the PND 7, 9, and 11 neonatal-PCP literature largely indicates that the effects of neonatal-PCP treatment (primarily the induction of apoptosis), do not extend to this region immediately following the last treatment with PCP (Wang et al., 2001; Wang et al., 2003 2004; Anastasio and Johnson, 2008b; Liu et al., 2011). Specifically, in adult neonatal-PCP-treated rats, acute PCP-induced MRI-measured brain activity (Broberg et al., 2013) changes in PV-expressing neurons (Kaalund et al., 2013), as well as dysregulated GABAergic inhibitory activity (Kjaerby et al., 2014), have only been evidenced for the mPFC.

Again, however, the alterations (cellular and molecular) to the brains of neonatal-PCP-treated rats appears to be transient and more complicated than measurements at a single time point can ascertain. The first evidence for less obvious disruptions was presented by du Bois et al. (2009), who showed downregulation of muscarinic receptor 1 (M1/4) in CA1 and CA3 on PND 12 (24 hour after last PCP-treatment). Downregulation of M1/4 was also present in the mPFC on PND 12, but the receptor was subsequently over-expressed at juvenile

and adult time-points. The upregulation was greatest at PND 32 (47% compared to controls) until control expression of M1/4 increased to a similar (but still significantly less) level at PND 96 (10% compared to controls). Examination at different time-points also revealed changes in expression of proteins implicated in schizophrenia, NMDAR, Nrg1/erb4, and PSD 95 (see Chapter 1: Introduction, for descriptions of these proteins) within the hippocampus. The researchers found increased levels of PSD 95 and NMDAR at PND 12 and PND 35 compared to controls. However, at PND 140 the expression of these proteins (and Nrg1/erb4) was reduced compared to controls. While long-term (over 4 months old rats) examination of GABAR expression has yet to be done in neonatal-PCP-treated rats, at PND, 18, 32, and 96, GABAR expression within the hippocampus is higher than in controls (du Bois et al., 2009).

Thus, although the data collected here strongly support the mPFC-mediated behavioural dysfunctions, without further analysis, a role for the temporal lobe cannot be eliminated. Indeed, it is possible that an interaction between the regions allow for the clear NOR deficit, whereas the primarily mPFC-dependent ED deficit was not as robust due to neural compensation during development.

Chapter 5. Effects of Gestational Methylazoxymethanol Acetate (MAM)-treatment on Attentional Set-Shifting Task Performance

The experiments in this chapter utilised a developmental disruption targeting hippocampal and cortical maturation in rats during their gestational period (corresponding to first trimester in humans). Testing in the standard ASST during adulthood revealed a dearth of dysfunction as MAM-treated rats only displayed a deficit at REV 1. Histological analysis indicated robust disruption of brain development with MAM-treated rats displaying several 'schizophrenia-related' anatomical markers such as enlargement of the lateral ventricles. The medial prefrontal cortex, although smaller than that of controls, was not found to exhibit other schizophrenia-related disruptions.

5.1 Introduction to the MAM manipulation

Maternal infection during pregnancy is a potent risk factor for the development of schizophrenia in offspring (Boksa, 2008). Many animal manipulations have been developed to examine the sensitive nature of the foetus during the first trimester, which corresponds to the gestational days 15 - 18 in rats (Bayer et al., 1993; Clancy et al., 2001). Of the manipulations designed to test the increased risk for development of schizophrenia following an insult during this period, prenatal methylazoxymethanol acetate (MAM) is one of, if not the, most reported, with 66 PubMed indexed publications dating from 1998 - 2016. The MAM toxin targets DNA and was originally implicated as the cause of Guam neurodegenerative disease (Escalire, 1999; Kisby and Spencer, 2011). Following injection, MAM breaks down into reactive molecules and methylates guanine residues, essentially lesioning the DNA, with a strong preference for neurons (Matsumoto, 1966; Spencer et al., 2012). MAM manipulations in rodents have identified multiple clinically relevant phenotypes resulting from MAM administration, including cancer, susceptibility to epileptic seizures, and 'schizophrenia-related' abnormalities (Lodge and Grace; Colciaghi et al., 2011; Spencer et al.). Interestingly, the resultant phenotype is largely dependent on the timing of MAM exposure such that the schizophrenia-related rats are best generated via exposure at gestational day 17 (MAM), a time point that coincides with cortical development.

5.1.1 MAM-treated rats anatomical and histological abnormalities

MAM-treated rats have been reported to have brain structural and anatomical abnormalities which parallel findings in human schizophrenia patients. Whole brain weight in MAM-treated rats is significantly reduced with reports ranging from 7 - 11% compared to saline exposed age-matched controls (Flagstad et al., 2004; Moore et al., 2006), as well as reduced brain surface area and circumference (Matricon et al., 2010). Most astonishingly, specific regional 'schizophrenia-related' alterations are also paralleled in MAM-treated rats, with

reductions in hippocampal volume (Matricon et al.; Chin et al., 2011), thickness (Moore et al.), and area (Moore et al.); and reductions in size of medial dorsal and medial geniculate thalamus (Moore et al.; Matricon et al.), and mPFC (PL, IL, and ACC; Moore et al.). In line with these reductions in regional sizes, the commonly found ventricular enlargement in schizophrenia patients, is also evident in MRI images of MAM-treated rats (Chin et al.), and ventricular enlargement has been observed in post mortem MAM-treated brains (Le Pen et al., 2006; Moore et al.; Matricon et al.).

Also, consistent with schizophrenia patients, in addition to the gross size reduction, MAM-treated rats show increased cell expression per square mm (Moore et al., 2006; Matricon et al., 2010). These neurons are often reported as abnormal morphologically, with reduced spine density (Singh, 1980; Xing et al., 2016), reduced soma size (Matricon et al.), and disrupted organisation in hippocampal cell layers (Matricon et al.). The latter is likely induced by the increased methylation (silencing activation) of the reelin glycoprotein promoter region in MAM-treated rats (Matricon et al.). Reelin is linked to neuron organisation and commonly reported as functioning abnormally in schizophrenia patients (see Chapter 1: Introduction). Specific alterations to interneurons are also reported, as MAM-treated rats display reductions in PV-expressing interneuron density (Penschuck et al., 2006; Lodge et al., 2009; Gastambide et al., 2012). PV-expressing GABAergic interneurons are known to be selectively dysregulated in patients with schizophrenia, and are key targets for 'schizophrenia-related' behavioural deficits (Lewis et al., 2004; Lewis, 2013). In MAM-treated rats, PV-expressing neurons are reduced in the hippocampus (Penschuck et al.; Gastambide et al.), mPFC (Lodge et al.; Gastambide et al.), and OFC (Gastambide et al.), all regions with major implications for behaviour.

Overall, MAM-treated rats exhibit structural and anatomical abnormalities which parallel the biomarkers found in patients with schizophrenia. These biomarkers are a large benefit of the MAM manipulation, over acute drug-induced manipulations, in particular since schizophrenia is believed to largely be a

developmental disorder arising from progressive changes in neural networks (Lewis and Levitt, 2002). Taking these structural and anatomical abnormalities into consideration, the next section will explore neurochemical deficits in MAM-treated rats.

5.1.2 Neurotransmitter & electrophysiological abnormalities in MAM-treated rats

Concurrent with structural abnormalities, schizophrenia is associated with dysregulation in basal dopaminergic, glutamatergic, and GABAergic neurotransmission (Guidotti et al., 2005; Laruelle, 2014; for review see Chapter 1: Introduction). Animal manipulations allow further investigation of these biological phenotypes and MAM-treated rats have been proven as useful tools, as they exhibit many of the same neurotransmitter dysregulations as patients with schizophrenia.

Dopaminergic system aberrations found in MAM-treated rats include basal hyperactivity of dopamine neurons (Flagstad et al., 2004; Lodge and Grace, 2007; Gill et al., 2011). Specifically, Lodge and Grace, reported an increase in the number of spontaneously firing dopamine neurons in the VTA of MAM-treated rats compared to control rats. The hyperactive VTA dopamine neuron activity is exacerbated by administration of amphetamine, leading to increased dopamine release in the nucleus accumbens (Flagstad et al.). Additionally, stimulation of the VTA results in increased spike firing in the mPFC of MAM-treated rats, while controls display reduced firing in response to the same stimulation (Goto and Grace, 2006). These alterations in the dopaminergic system are not limited to the activity of VTA projections alone as local infusion of amphetamine into the mPFC of MAM-treated rats also results in increased extracellular dopamine in the nucleus accumbens (Flagstad et al.). This increased extracellular dopamine may be interpreted as MAM dopaminergic synapses containing excess dopamine packaged into the readily releasable pool of vesicles, and it is this dopamine that is displaced by amphetamine; or that the enzyme which breaks down extracellular dopamine (monoamine oxidase) is not maximally functional. This latter hypothesis

might better explain why the significant difference in extracellular dopamine is not evidenced until 30 min later; however evidence for monoamine oxidase dysregulation in schizophrenia is inconsistent (Li and He, 2008). At any rate, application of dopamine to superficial layer (1-2) mPFC neurons in MAM-treated rats, does not alter layer 5 mPFC cell activity, while the same treatment in control rats causes a reduction in firing in deep layer mPFC neurons (Lavin et al., 2005).

Reports also indicate alterations in glutamatergic and GABAergic neurotransmission in adult MAM-treated rats. The ventral hippocampus projects heavily to the VTA and inactivation of the region (via tetrodotoxin) blunts the spontaneous activity of VTA dopaminergic neurons in MAM-treated rats (Lodge and Grace, 2007). This finding has led researchers to propose that the elevated dopamine neuron activity is secondary to elevated glutamatergic activity in the ventral hippocampus (Lodge and Grace, 2007, 2009). Further changes in the glutamatergic signalling pathway can be evidenced earlier in development. PND 11 - 45 MAM-treated rats display reduced expression of NMDAR subunits within the dorsal hippocampus (Snyder et al., 2013). The abnormal expression of NMDAR subunits in MAM-treated rats may lead to the reduced LTP evidenced in CA1 (Snyder et al.), as well as the attenuated extracellular glutamate and dopamine release in the mPFC following MK-801 treatment (Lena et al., 2007).

As mentioned earlier, GABAergic PV-expressing interneurons are also dysregulated in schizophrenic patients (see Chapter 1: Introduction). In MAM-treated rats the reduction in PV-expressing interneurons (Penschuck et al., 2006; Lodge et al., 2009; Gastambide et al., 2012) has been proposed as the driver of the hyperactive hippocampus (Gill et al., 2011) and subsequent hyperactive dopaminergic tone. Either of these hypotheses are consistent with the hippocampal anatomical disruptions reported in both humans with schizophrenia (Jaaro-Peled et al., 2010; Takayanagi et al., 2010) and MAM-treated rats (Gourevitch et al., 2004; Moore et al., 2006). The combination of these developmental biological neurochemical abnormalities underlies the behavioural deficits described in the next section.

5.1.3 Innate and behavioural flexibility deficits in MAM-treated rats

Locomotor activity (LMA) is a highly sensitive to neurochemical phenotypes (reviewed in Chapter 4). Basal LMA in MAM-treated rats is elevated compared to controls consistent with a hyperactive dopamine system (Le Pen et al., 2006; Lodge and Grace, 2007; Ratajczak et al., 2015). Hyper-sensitivity of both the dopaminergic and glutamatergic systems is also found in MAM-treated rats as they show greater increases in LMA following treatment with either amphetamine (Flagstad et al., 2004; Perez et al., 2013; Chen et al., 2014), the D2/3R agonist quinpirole (Perez and Lodge, 2012), or NMDAR antagonists PCP (Moore et al., 2006; Penschuck et al., 2006), ketamine (Phillips et al., 2012), and MK-801 (Lena et al., 2007). Although one publication failed to find MK-801-induced hyperlocomotion in MAM-treated rats, that may be due to strain differences as the experiment used Wistar rats (Goda et al., 2015) compared to the more commonly used Sprague-Dawley MAM-treated rats (Lena et al.).

MAM-treated rats also exhibit 'schizophrenia-related' symptoms in other tests of innate behaviour. Of these, the most regularly reported is a deficit in the PPI response, where MAM-treated rats are found to have a greater startle response to the auditory stimulus than controls (Le Pen et al., 2006; Moore et al., 2006; Mackowiak et al., 2014). A deficit in latent inhibition (measured by conditioned stimulus-induced LMA changes) has also been reported for MAM-treated rats (Lodge et al., 2009).

Schizophrenia symptoms normally emerge post-puberty, with few significant differences between adolescents who develop the disorder and adolescents who do not. The MAM manipulation also replicates this delayed emergence of symptoms in that certain behavioural deficits appear pre-puberty, for example, reduced social interaction time and increased LMA (Le Pen et al., 2006), and reduced spontaneous alternation (Hazane et al., 2009). Additional behavioural deficits are evident in adult MAM-treated rats, such as the PPI deficit (Le Pen et al.; Hazane et al.; Mackowiak et al., 2014). Furthermore, the deficits are not limited to male MAM-treated rats. Despite a male bias for development of

schizophrenia (Tandon et al., 2008) either sex can develop the disorder. Hazane et al., reported that female MAM-treated rats also display select 'schizophrenia-related' phenotypes pre-puberty and with the full array of behavioural deficits emerging in adulthood, suggesting that the MAM-manipulation may also serve for translation of schizophrenia in female patients.

Of the available 'animal manipulations with relevance to schizophrenia', the MAM-manipulation is consistently reported to have the most 'schizophrenia-related' deficits in the ASST. The first report by Featherstone et al. (2007), showed that MAM-treated rats had deficits at both reversal stages (REV 1 and REV 3) and the ED stage. These findings were mostly replicated by (Gastambide et al., 2012) and Perez et al. (2013), who both report deficits at REV 1, REV 2, and the ED. Most recently however, Gomes et al. (2015) found deficits at all reversal stages but no difference between MAM-treated and control rats at the ED stage.

5.1.4 MAM-based translational evidence for schizophrenia treatments

Many pharmacotherapies, already in use for human patients with psychosis, are also effective at treating 'schizophrenia-related' phenotypes in MAM-treated rats. Specifically, D2R antagonists such as haloperidol and sertindole can acutely reduce dopaminergic neuron activity in the VTA of MAM-treated rats but not controls, reflective of the fast actions of antipsychotics in schizophrenia patients (Valenti et al., 2011). Behaviourally, acute treatment with haloperidol, risperidone, and clozapine, have been shown to attenuate spontaneous, and MK-801-induced, hyperactivity in MAM-treated rats (Le Pen et al., 2011).

A primary purpose of a preclinical animal manipulation is to test potential treatments for the disorder the manipulation recapitulates, many studies have been done with MAM-treated rats to explore putative therapies. To this end, the MAM-manipulation has generated some interesting discoveries. Diazepam, a GABA_A receptor PAM, has been found to enhance GABAergic neurotransmission in MAM-treated rats after sub-chronic administration during the puberty stage of development (PND 31 - 40), resulting in normal VTA dopamine neuron activity,

and amphetamine induced hyperlocomotion, when assayed in adulthood (PND 60 - 80; Du and Grace, 2013)). Recently, this approach was extended to MAM-treated rat amygdala hyperactivity with a similarly positive result of attenuated neuronal activity and reduced measures of anxiety in the elevated plus maze (Du and Grace, 2016). A common adjunct treatment for schizophrenia, valproic acid, has also been shown to be effective at reducing 'schizophrenia-related' phenotypes (PPI and histone deacetylase 2 expression) in MAM-treated rats when administered in early adolescence (PND 23 – 29) or in adulthood (Bator et al., 2015). Interestingly, a study utilizing a combination of forward and reverse translation, found that a history of chronic D2R antagonists treatment in MAM-treated rats resulted in a blunted response to selective enhancement of GABA_Aα5 receptors (Gill et al., 2014), a finding with powerful implications for treatment approaches in schizophrenia patients.

MAM-based studies on enhancement of GABAergic signalling have also found that acute treatment with an α5-GABAR PAM also reduces the hyperactivity of dopaminergic neurons and amphetamine induced hyperlocomotion (Gill et al., 2011). The authors of the study were also able to show that increasing inhibitory activity within the ventral hippocampus via local administration of the PAM had the same effect, indicating ventral hippocampus hyperactivity as a primary target for therapeutics. Additional studies with local administration of tetrodotoxin (Lodge and Grace, 2007), GABAergic interneuron cell grafts (Perez and Lodge, 2013), and deep brain stimulation (Perez et al., 2013), targeted to the ventral hippocampus, also proved therapeutic for hyperdopaminergic activity in MAM-treated rats.

5.2 Rationale

The empirical evidence supporting the MAM-manipulation is compelling. While, D2R antagonists have proven effective at treating the positive symptoms associated with the disorder, novel pharmacotherapies are required to reduce the cognitive deficits (Conn et al., 2009). A 2007 clinical trial found that patients with

schizophrenia had reduced positive and negative symptoms in response to a glutamatergic pharmacotherapy (Patil et al., 2007). Indeed, Gastambide et al. (2012) found that enhancement of glutamatergic signalling via an mGluR5 PAM normalised the ASST reversal learning performance in MAM-treated rats. Unfortunately, the compound used in the study was unfit for long-term human consumption. However, the potential for glutamatergic enhancement of ASST performance in MAM-treated rats (and hopefully eventually patients) drove the studies in this experimental chapter.

The NMDAR PAM ORG49209 is a synthetic steroid which our lab has preliminary evidence supporting it as putative cognitive normaliser (Chase dissertation 2013). With the discovery of novel steroid binding sites and steroid-mediated regulation of NMDARs (Park-Chung et al., 1997; Paul et al., 2013), interest has emerged in directly targeting NMDARs via synthetic derivatives of these compounds. The cholesterol metabolite 24 (S)-hydroxycholesterol is an endogenous neuroactive steroid which has its own allosteric NMDAR binding site. 24 (S)-hydroxycholesterol can potently enhance NMDAR-mediated Ca^{2+} influx as measured by increased NMDA-stimulated excitatory postsynaptic currents (EPSCs) in cultured mouse hippocampal neurons (Paul et al.). Derived from 24 (S)-hydroxycholesterol, the synthetic neurosteroid ORG49209 (SGE201) also enhances NMDAR activity as measured by increased EPSCs, in addition to enhanced high frequency stimulation-induced long-term potentiation in rat hippocampal slices (Paul et al.). Experiments from our lab found that 15 days of treatment with 10 mg/kg ORG49209 improved reversal learning performance in aged rats (12 - 18 months old; Chase, 2013). Since ORG49209 directly targets the NMDAR, it is of interest to discover if the compound may be effective at ameliorating 'schizophrenia-related' behaviours. The aims of this chapter were to: 1) replicate previous findings of reversal learning and set-shifting deficits in MAM-treated rats, and 2) to explore the potential for ORG49209 to normalise any deficits found.

5.3 Methods and materials

5.3.1 Animals

Male Sprague Dawley rats exposed to MAM (22 mg/kg/ml) or saline vehicle on gestational day 17 were obtained from (Lilly, Wokingham, UK). A total of 28 male rats were obtained (control = 14, MAM = 14). Rats were housed in standard housing conditions (two to three per cage, 0700 - 1900 hr light phase, controlled temperature and humidity, and ad libitum water). Animals were at least 5 months old at the beginning of behavioural testing. One month prior to behavioural testing rats were placed on a food control regimen of 15 - 20 g per day, to increase their motivation to work for food reward. All animals continued to gain weight at a normal rate and were handled during weighing and general husbandry procedures.

5.3.2 Baseline 7-Stage ASST testing

Training and testing were performed as described in Chapter 2: General Methods however, following acquisition of the digging for reward response, the rats were 'pre-exposed' to stimuli that would be used in the task. All odours that were to be tested were presented mixed in with sawdust. Medium exemplars to be tested were presented without any additional odour. Rats were allowed to retrieve rewards from both to-be-tested exemplars on all trials (i.e. no discriminations were made). Pre-exposure continued until rats were reliably digging in both exemplars (typically two trials). Presentation order of exemplars was counterbalanced pseudorandomly. This pre-exposure was performed following attempted testing of two rats which exhibited abnormally long latencies to dig following presentation of novel exemplars (neophobia), which significantly disrupted testing. Following pre-exposure, all testing proceeded as normal. All rats completed the pre-exposure and training stage however, three control and five MAM-treated rats were unable to complete the entire battery of tests as they did

not reliably dig and thus were excluded from further experiments. All rats were tested twice to increase statistical power.

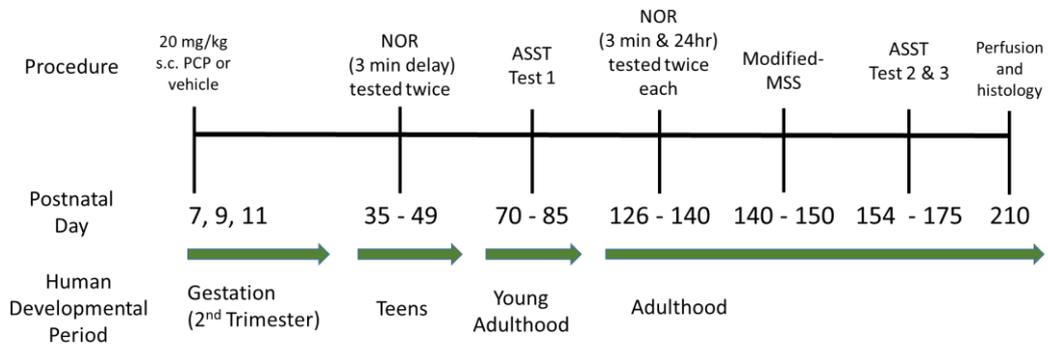
5.3.3 ORG49209 ASST testing

Each rat was tested a total of three times in the standard ASST to assess the effects of ORG49209 on ASST performance. A Latin square design was used to analyse vehicle (10% hydroxypropyl beta-cyclodextrin; HPBCD, Sigma, UK) and two doses (3 & 10 mg/kg/ml in solution in 10% HPBCD) of the NMDA receptor PAM ORG49209. Prior to injection, ORG49209 was sonicated in vehicle for 30 min. All injections were administered i.p. 30 min prior to behavioural testing.

5.3.4 4-ID testing

The 4-ID task (Chase et al., 2012) allows investigation of set-formation/shifting in the absence of reversal stage. The first stage is an SD with the correct stimulus being either an odour or medium exemplar; next is a CD in which the correct stimulus from the SD is now paired with stimuli from an irrelevant dimension; then ID 1 in which the correct stimulus remains within the same dimension as the correct stimulus from the CD, however all new stimulus pairs are presented. Each of the next three stages (ID 2, ID 3, ID 4), repeat this process of presenting new stimulus pairs but maintaining the correct stimulus within the same dimension. Next, the rat performs an ED in which the correct stimulus is now switched to an exemplar from the previously irrelevant dimension. Last, the rats are presented with the SD again to control for effects at the ED stage being caused by fatigue and/or satiety. This testing was performed, blind to condition, by lab member Rudi Stanislaus-Carter.

Timeline for neonatal-PCP-treated rats experiments



5.4 Results

5.4.1 MAM-treated rats have a deficit at REV 1 of the standard ASST

Analysis of TTC in the first and second tests, revealed a significant Condition x Stage interaction ($F_{(1,6)} = 3.65, p < 0.05$; Figure 5.1). Restricted analysis revealed a REV 1 impairment in MAM-treated rats compared to controls ($F_{(1,108)} =$

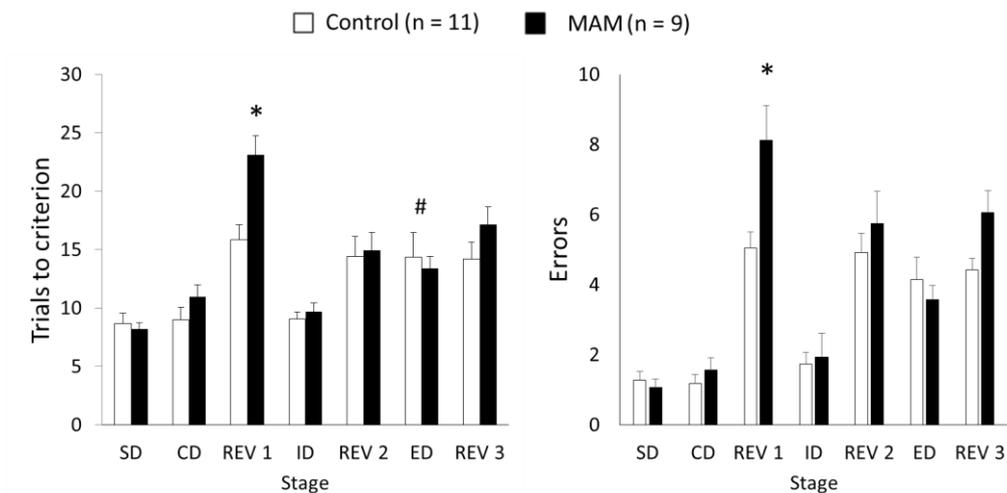


Figure 5.1) *Left*. Bar graph showing mean TTC (+ SEM) for the standard ASST. Compared to control rats, MAM-treated rats required more TTC for REV 1. *Right*. Analysis of the errors committed during the ASST revealed that MAM-treated rats committed more errors during REV 1.

28.83, $p < 0.05$). No other significant differences were found at any other stage. Although there was a significant effect of Test ($F_{(1, 18)} = 12.32$, $p < 0.05$) evidenced by more overall TTC in the second test compared to the first, it did not significantly interact with any other factor indicating the effect of test was the same across all stages and conditions (Test x Condition x Stage, Test x Stage interaction, and Test x Condition interactions, $F_s < 1$).

To compare these results to a previously published report (Gastambide et al., 2012), errors to criterion were also analysed. Consistent with the TTC measurement, a significant Condition x Stage interaction was found ($F_{(1,6)} = 2.43$, $p < 0.05$). Restricted analysis revealed that MAM-treated rats committed more errors at REV 1 compared to controls ($F_{(1, 108)} = 15$, $p < 0.05$; Figure 5.1). No other stage significant differences were found. Further analysis qualifying the errors committed at this stage as perseverative or regressive (Gastambide et al., 2012), did not yield any significant differences between the types of errors committed by MAM-treated rats and controls.

5.4.2 MAM-treated have increased evidence for irrelevant dimension-based responding

Compared to control rats, there was more evidence for MAM-treated rats responding to the irrelevant dimension across all stages (heatmap in Figure 5.2; for Bayesian analysis description see Chapter 2: General Methods). The bar graph in Figure 5.2 shows the overall difference in evidence for spatial- vs irrelevant dimension based responding between groups. Whereas control rats more quartiles with strong evidence for spatial-based responding (total quartiles = 5) compared to irrelevant dimension responding (total quartiles = 3), MAM-treated rats showed the opposite behavioural pattern with less evidence of spatial based responding (total quartiles = 3), in favour of more irrelevant dimension responding (total quartiles = 7). This increased evidence for irrelevant dimension responding (compared to controls) occurred at stages of interest including: REV 1, REV 2, and the ED.

Chapter 5. Effects of Gestational Methylazoxymethanol Acetate (MAM)-treatment on Attentional Set-Shifting Task Performance

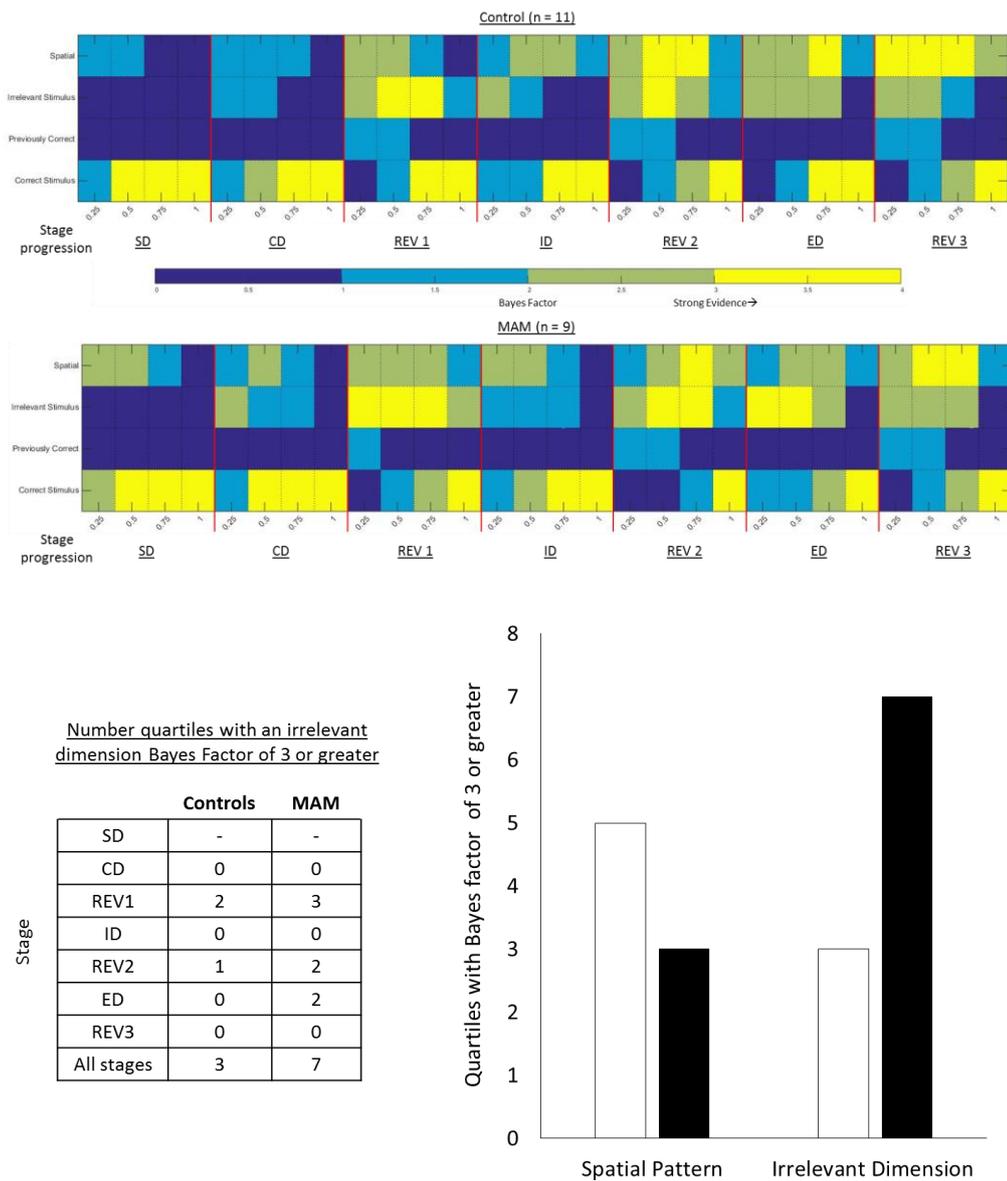


Figure 5.2. *Top*. Heatmap of evidence for behavioural patterns during the standard ASST. Compared to controls, MAM-treated rats show more evidence for irrelevant dimension responding (REV 1, REV 2, and the ED), as well as perseveration during REV 3. *Bottom left*. Table showing sum number of quartiles by stage for irrelevant dimension-based responding by group. MAM-treated rats showed more irrelevant dimension-based responding than control rats. *Bottom right*. Bar graph showing sum number of quartiles with strong evidence for spatial- or irrelevant dimension-based responding in control and neonatal-PCP-treated rats.

5.4.3 No evidence of a MAM-induced ED deficit in the 4 ID task

To further examine the lack of increased TTC at the ED shift in MAM-treated rats, the rats were tested in the 4-ID version of the ASST. The 4-ID task removes a potential confound caused by the effect of reversal impairments on set-formation (Chase et al 2012). There are no reversal stages and the original set dimension (odour or medium) is maintained for the first 6 of 8 stages. Even in this test however, MAM-treated rats still did not have an ED shift deficit compared to controls (Condition x Stage interaction: $F_{(1,7)} < 1$; Figure 5.3). Both control and MAM-treated rats formed an attentional set as indicated by increased TTC to complete the ED stage compared to the previous ID (main effect of Stage: $F_{(7, 119)} = 10.5, p < 0.05$).

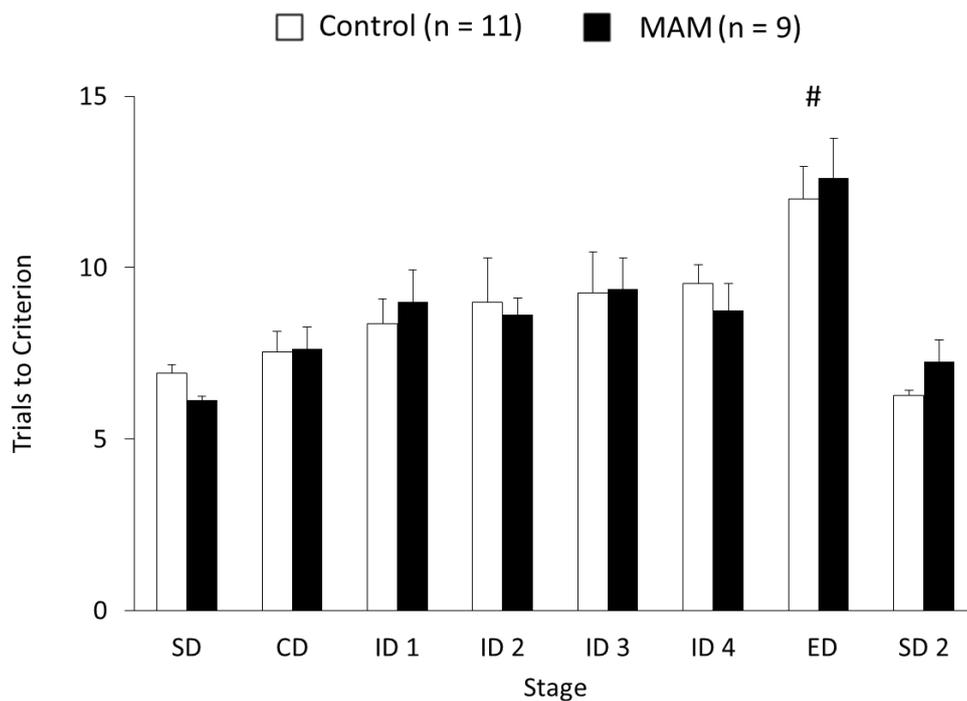


Figure 5.3. Bar graph showing mean TTC (+ SEM) for the 4-ID ASST. No significant differences were found between groups. Both control and MAM-treated rats, required more trials for the ED than the previous ID.

5.4.4 ORG49209 effect on REV 1 performance in controls and MAM-treated rats

The effect of ORG49209 at REV 1 was of most interest, as this was the stage that was impaired in the MAM-treated rats in the baseline tests. An effect of ORG49209 was to abolish the difference between the conditions at REV 1. However, the effect of the drug was apparent in both conditions: a slight decrease in TTC for the MAM-treated rats and an increase in TTC for control rats which

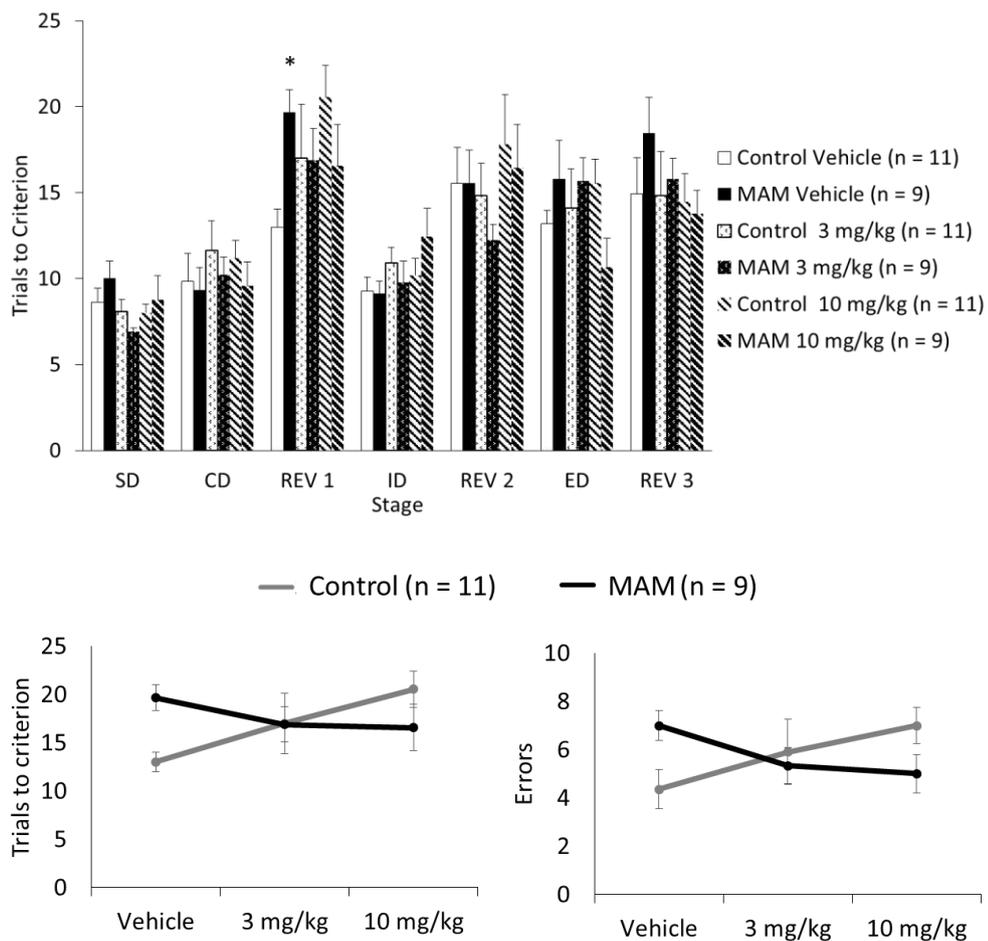


Figure 5.4. *Top.* Bar graph showing mean TTC (+ SEM) following treatment with 0, 3, or 10 mg/kg ORG49209. Treatment with ORG49209 did not produce any clear overall between group effects on ASST performance across stages. ANOVA restricted to REV1 confirmed that when vehicle treated, controls still required fewer TTC than MAM-treated rats. *Bottom.* Line graphs showing the effects of ORG49209 on mean (\pm SEM) for TTC (*left*) and errors (*right*). A pattern of disrupting performance in controls and improving performance in MAM-treated rats during REV 1 was only trend level ($p = .07$).

approached but did not reach significance (REV 1: restricted ANOVA, Dose x Condition interaction: $F_{(1, 2)} = 3.53$, $p = 0.07$; Figure 5.4). ORG49209 appeared drive an increase in TTC for controls, reflected by a slight increase in response to 3 mg/kg (mean TTC increase: + 4 (\pm 3)); and a large increase following treatment with 10 mg/kg (mean TTC increase: + 7.5 (\pm 2.5)). Whereas the effect in MAM-treated rats was \sim 3 TTC less regardless of dose and did not reach control vehicle performance (line graphs in Figure 5.4).

5.5 Discussion

5.5.1 Reversal learning impairment in MAM-treated rats

Consistent with all previous reports (Featherstone et al., 2007; Gastambide et al., 2012; Perez et al., 2013; Gomes et al., 2015), MAM-treated rats displayed marked impairments in REV 1 (Figure 5.1). The REV 1 MAM deficit was the largest in magnitude in that report, while the REV 2 and ED deficits were reduced (albeit significantly worse than controls). Another recent report also found the largest magnitude deficit at REV 1 (Gomes et al., 2015). Therefore, replicating the REV 1 deficit here is unsurprising, and suggests that any other deficits may be less robust. However, the earliest ASST MAM report had the largest magnitude differences at the ED and REV 3, and not REV 1 (Featherstone et al.). It should be noted that Featherstone et al., used a texture/fabric around the rim of the bowl which can dissociate the reward (located in the bowl) from the discriminanda (texture). A more thorough critique of a similar design (odours placed around the rim), has been discussed in Chapter 4 in regards to neonatal-PCP-treated rats.

5.5.2 Irrelevant dimension responding and perseveration in MAM-treated rats

Gastambide et al. (2012) reported perseveration in MAM-treated rats, as measured by “digging in the incorrect bowl for 3 or more trials in consecutive blocks of 4 trials each”. An attempt to replicate these findings with REV 1 and REV 3 errors here, did not reveal any significant differences between the conditions.

Overall, the rats made few errors and so there were possibly floor effects that would compromise further analysis of this measure. The behavioural pattern analysis did however indicate strong evidence of perseveration (as responding to the previously correct stimulus) in MAM-treated rats during the first quartile of REV 3. Furthermore, the analysis found increased irrelevant dimension responding (and reduced spatial-based responding) in MAM-treated rats compared to controls, suggesting abnormal decision making behaviour in MAM-treated rats. As perseveration and dysfunctional regulation of attention are two hallmark schizophrenia symptoms, these behavioural findings support the 'schizophrenia-related' behaviour of the MAM-treated rats, despite the TTC and errors analysis not being as robust as previous reports.

5.5.3 Protocol based explanations for failures to replicate

We hypothesise the small number of errors, particularly as compared to Gastambide, et al., (2012) may be due to differences in the testing protocols (e.g. the Brown lab protocol allows 10 min for exploration per trial, while the previous report allowed 3 min). Our statistical analysis of TTC revealed MAM-induced impairments at REV 1 with no significant differences in other stages. This varies slightly from the impairments in REV 1 & REV 2, as well as at the ED stage, reported previously by Gastambide et al. Additionally, our results differ slightly from the REV 1, REV 3, and ED impairments reported by Featherstone, et al. (2007). Addressing the ED impairments in Featherstone et al., one may hypothesise that the use of 'texture' as opposed to medium (i.e. sand paper wrapped around the bowl, as opposed to our use of sand as the digging medium), is an explanation for these differences. However, Gastambide et al., reported ED impairments in MAM-treated rats using, digging medium as a dimension. Thus, it would appear that the texture vs medium argument does not explain the lack of an ED impairment in our MAM-treated rats. It is also possible that the ED impairment is only evident following a history of stress. Both of the previous attentional set-shifting reports in MAM-treated rats (Featherstone et al.; Gastambide et al.) utilised much stricter

food control regimens than those employed by our lab; our rats were fed 15 - 20 g per day, a diet which allows steady weight gain, whilst in the previous two studies rats were food restricted up to 85% of their original body weight. It is possible that MAM-treated rats which are known to have abnormal weight gain through PND 90 (Flagstad et al., 2004), a deficit which has also been evidenced in our rats (PND 74 MAM-treated rats had reduced body mass at the start of food restriction; a consistent 4-5% reduction was recorded), may have an exacerbated response to the food restriction employed by the previous reports, and that this response manifested as the ED. Chronic psychological stress has been shown to selectively impair ED performance in rats (Bondi et al., 2008; Naegeli et al., 2013). However, Gomes et al. (2015) also food restricted to 85% body weight and did not find an ED deficit in MAM-treated rats. Last, it also possible that there is batch-to-batch variability in MAM-treated rats underlies the lack of an ED impairment in our report. Additional concerns regarding variability in developmental manipulations are discussed in the general conclusions section.

5.5.4 ORG49209 failed to improve REV 1 performance in MAM-treated rats

Treatment with ORG49209 yielded interesting, albeit confusing, results. While the treatment abolished the REV 1 difference between control and MAM-treated rats, this was accomplished by raising the TTC in control rats and slightly lowering TTC in MAM-treated rats suggesting that the drug may be detrimental to control rats as well as therapeutic for MAM-treated rats. However, the findings were not statistically robust enough to support either of these hypotheses. Outside of REV 1 the drug had no obvious effect in control rats suggesting that there is something inherently unique and (perhaps demanding) about REV 1 that makes this stage sensitive to interventions. Other reports have also shown select deficits at only REV 1 (Lapiz-Bluhm et al., 2009; Donegan et al., 2014), and when deficits arise across different stages in multiple reports, the REV 1 impairment is often the most consistent (Featherstone et al., 2007; Gastambide et al., 2012; Perez et al., 2013; Gomes et al., 2015).

5.6 Histological analysis of MAM-treated rats

Rationale

MAM-treated rats are reported to have numerous anatomical and histological abnormalities. To verify these differences within our cohort, I decided to examine post-mortem brain tissue for size and dimensions, and to stain brain sections for cresyl violet and PV. Cresyl violet stains acidic parts of cells such as ribosomes and rough endoplasmic reticulum, allowing for visualisation of cell layer structure, which is commonly reported as abnormal in schizophrenia. Additionally, PV-expression is commonly reported as abnormal, thus these two stains were used to visualise the mPFC, OFC, and hippocampus of MAM-treated rats and controls.

5.7 Methods and materials

PV immunostaining was performed as described in General Methods (Chapter 2). For cresyl violet staining every 4th section of the frontal cortex was collected, and every 8th section of the striatum through to the hippocampus. Sections (50 µm thick) were mounted onto treated glass slides, and left for 48 hrs in a formaldehyde desiccator. Once sections were fixed to the slides they were dipped in a xylene bath for 2 min, followed by a 100% ethanol (EtOH) bath for 2 min, a 50% EtOH bath (diluted in H₂O) for 1 min, and then an H₂O bath for 2 min, before being placed in a cresyl violet bath for 2 min. Next slides were washed in a running water bath for 5 min. Then differentiated through ascending concentration EtOH baths (50% - 100% - 100%), before being placed in a xylene bath and coverslipped with DPX (Sigma). One brain was excluded from analysis due to a faulty dissection. For both cresyl and parvalbumin Image J analyses 7-10 sections per brain were used for the PFC and 3-4 for the hippocampus, see Chapter 2 General Methods.

5.8 Results

5.8.1 MAM-treated rats exhibit abnormal brain and body weights

Body weights prior to the start of food control were 7% lower in MAM-treated rats compared to controls ($t_{(18)} = 2.11, p < 0.05$; Figure 5.5). For the first month of food control, MAM-treated rats showed reduced weight gain compared to controls (Condition x Week interaction: $F_{(1, 3)} = 2.81, p < 0.05$). However, over the full course of the experiments (42 weeks), MAM-treated rats eventually reached and maintained equivalent weights as controls (Figure 5.5). Following behavioural experiments, rats were anaesthetised and perfused for anatomy and histology. Brains were dissected out and the brain stem and cerebellum were removed to eliminate the variability generated through differences in dissection. Compared to controls, MAM-treated rats displayed a consistent and robust reduction in brain weight of ~181 mg (mean MAM ($n = 9$) = 949 mg, ± 14.31 , controls ($n = 11$) = 1143 mg, ± 25.45 ; $t_{(18)} = 6.27, p < 0.05$; Figure 5.5).

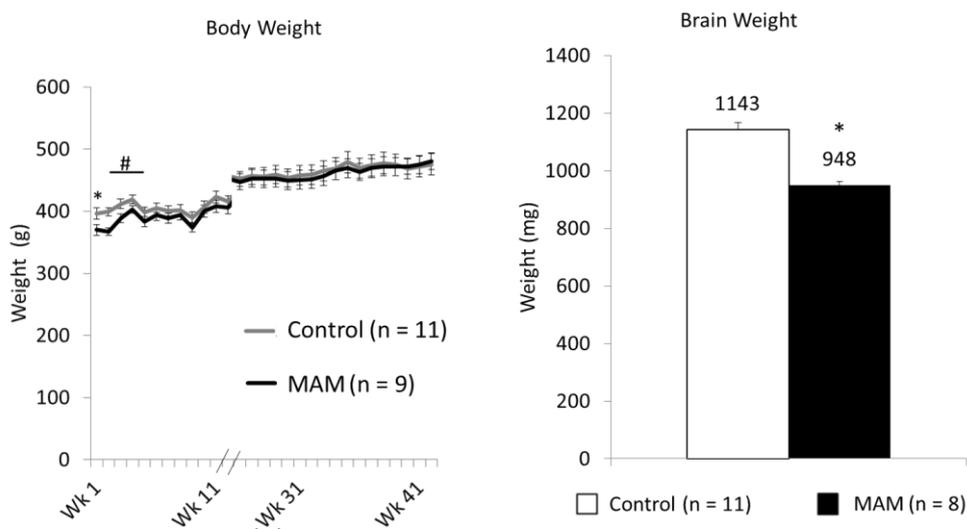


Figure 5.5) *Left*. Line graph showing mean body weight through weeks of experiments. MAM-treated rats showed an initial reduction in body weight compared to controls, however this difference was abolished over the course of experiments and both groups gained weight as normal. *Right*. Despite no difference in body weight, post-mortem brain weight was significantly reduced in MAM-treated rats.

5.8.2 Gross observation reveals schizophrenia-related anatomy in MAM-treated rats

Given the reduction in brain weight in MAM-treated rats, the areas of regions of interest were calculated using cresyl violet stained sections. Gross observation also revealed enlargement of the lateral ventricles in *all* MAM-treated rats compared to controls (Figure 5.6). Furthermore, the areas of all

Control

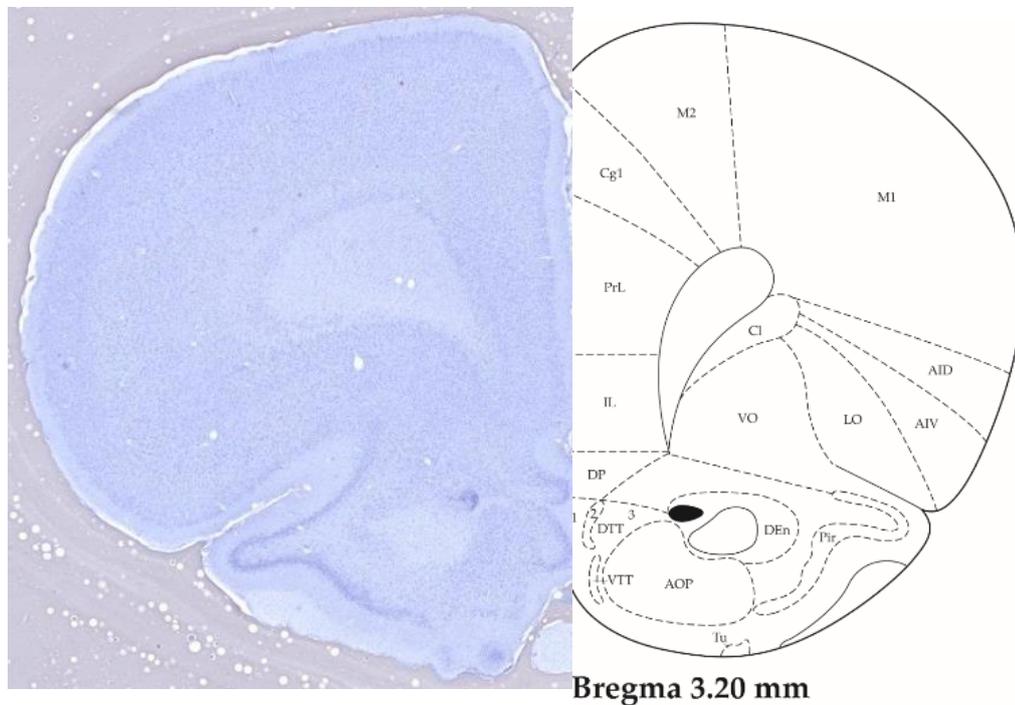


Figure 5.6) Representative cresyl violet stained mPFC and OFC sections in controls. *Bottom.* Mean (+ SEM) total area (approximate distance from Bregma: 4.7 mm – 2.7 mm). MAM-treatment induced robust reductions in the size of both the mPFC and the OFC

MAM-treated

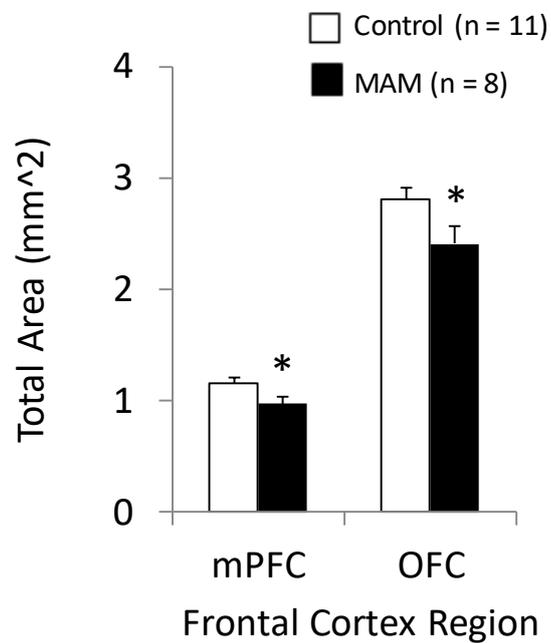
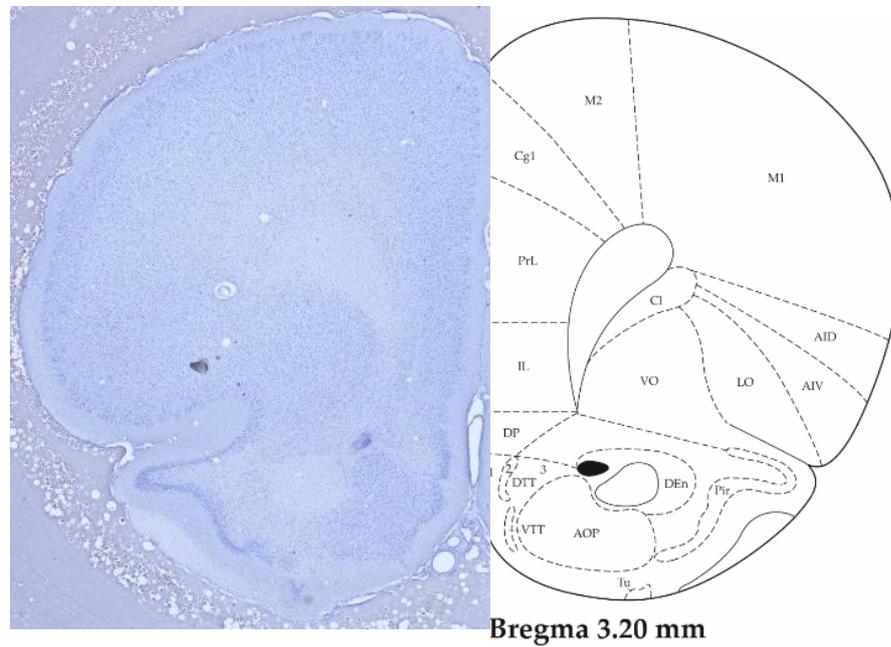


Figure 5.6 continued) *Top*. Representative cresyl violet stained mPFC and OFC sections from MAM-treated rats. *Bottom*. Mean (+ SEM) total area (approximate distance from Bregma: 4.7 mm – 2.7 mm). MAM-treatment induced robust reductions in the size of both the mPFC and the OFC.

regions of interest were significantly smaller in MAM-treated rats compared to controls PFC (bar graph in Figure 5.6; mPFC, $F_{(1,17)} = 4.49$, $p < 0.05$; OFC, $F_{(1,17)} = 4.83$, $p < 0.05$), and the hippocampus (CA1, $F_{(1,17)} = 14.87$, $p < 0.05$; CA2/3, $F_{(1,17)} = 84.97$, $p < 0.05$; DG, $F_{(1,17)} = 34.45$, $p < 0.05$). Additional disruption of cell layering of CA2/3 was evident in all MAM-treated rats.

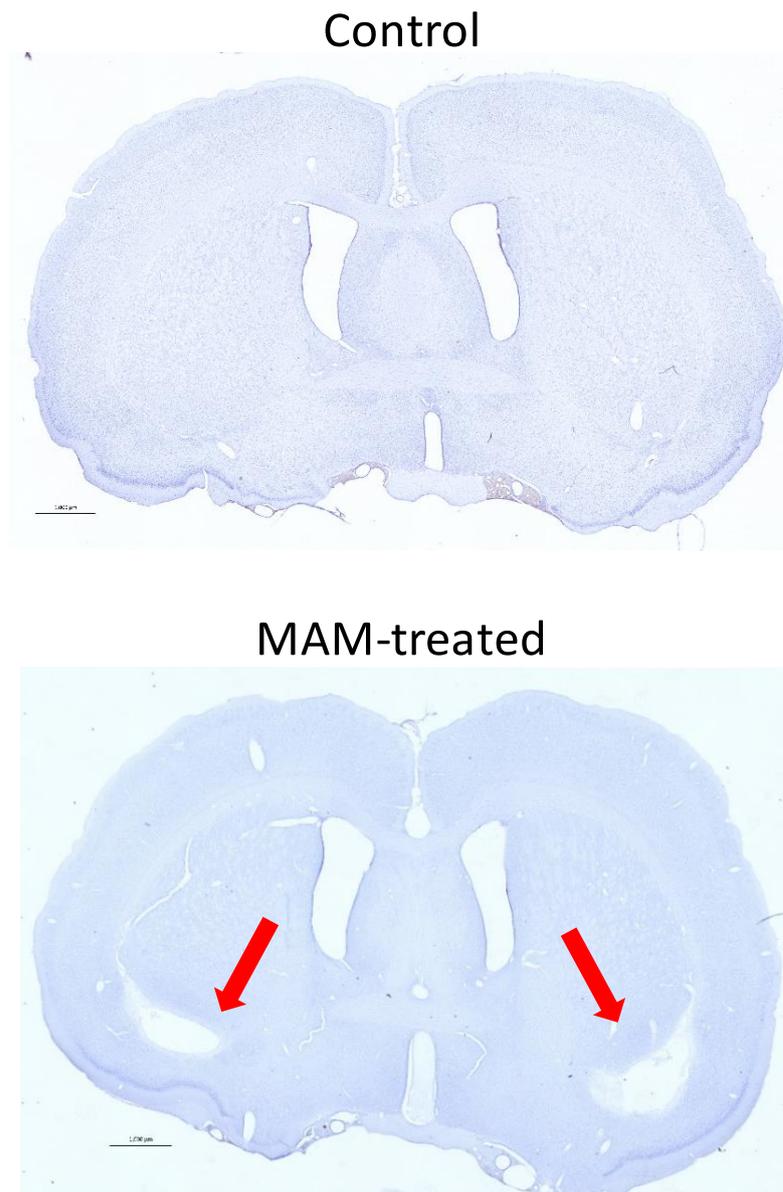
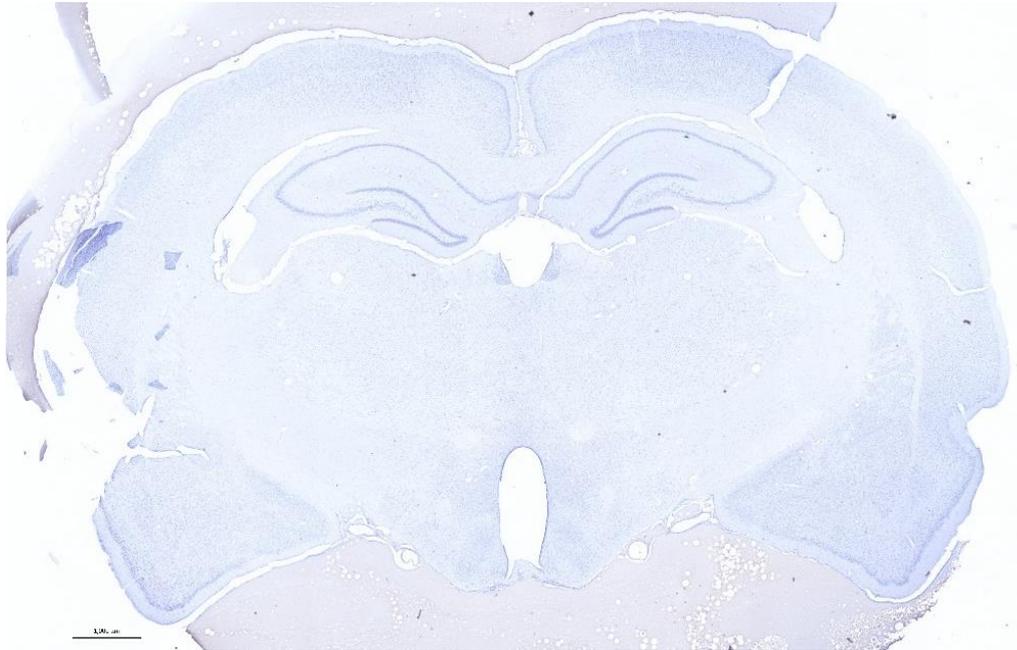


Figure 5.7) Representative cresyl violet stained striatal sections in controls (top) and MAM-treated rats (bottom). Lateral ventricle enlargement (red arrows) was evident at the level of the striatum in all MAM-treated rats.

Control



MAM-treated

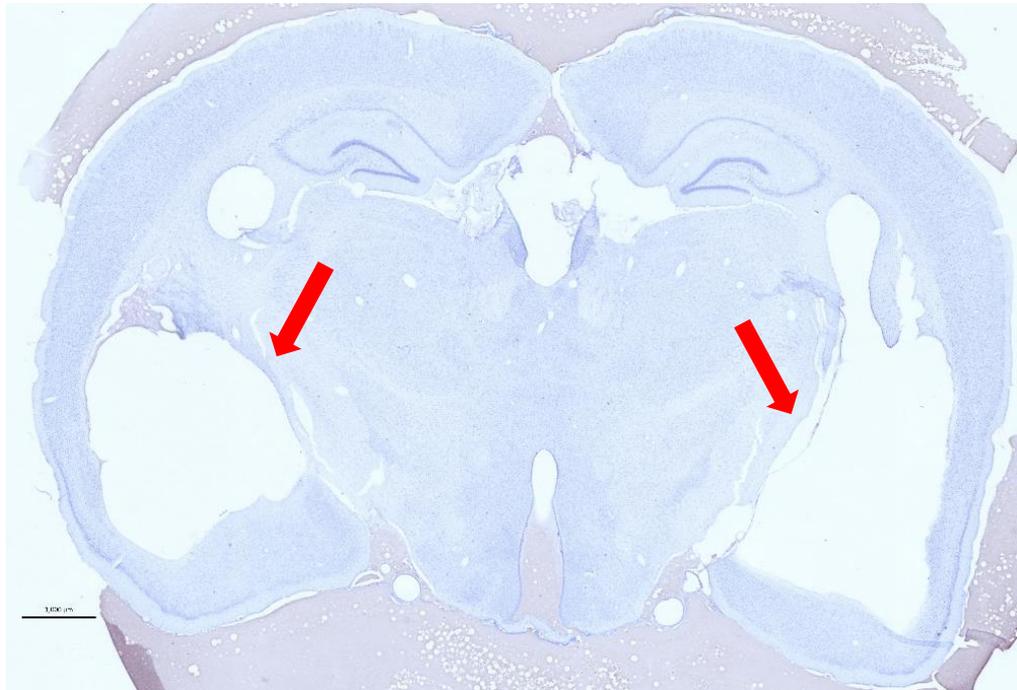


Figure 5.7 continued) Representative cresyl violet stained hippocampal; sections in controls (top) and MAM-treated rats (bottom). Lateral ventricle (red arrows) enlargement was evident in all MAM-treated rats.

5.8.3 Parvalbumin expression is altered in the hippocampus but not mPFC of MAM-treated rats

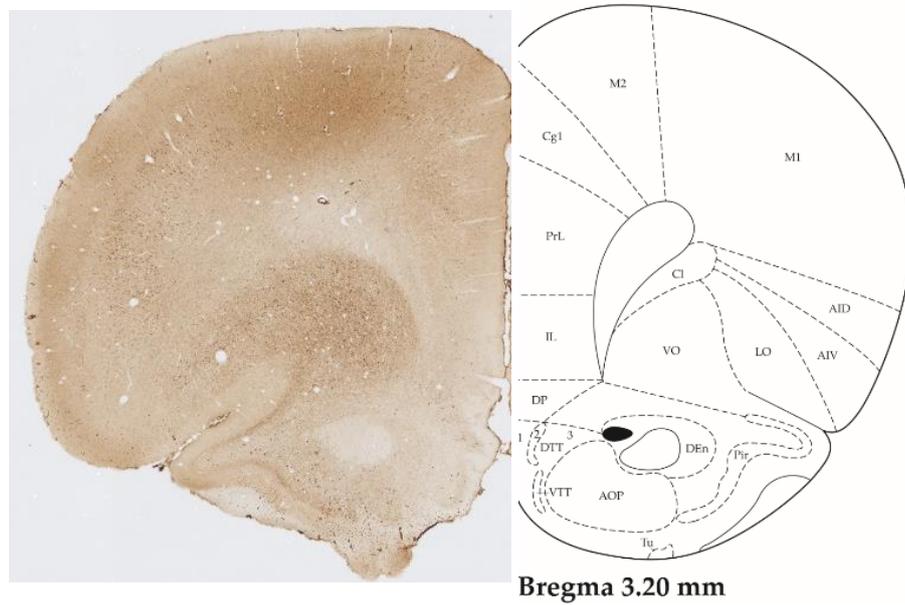
➤ *PFC analysis*

Analysis of PV-expressing interneurons in the mPFC revealed no significant differences in either total number of PV-expressing cells ($F_{(1,17)} = 2.03$, *ns*), or density of PV-expressing cells ($F_{(1,17)} = 1.3$, *ns*). Analysis of the OFC also revealed no significant differences in total number of PV-expressing cells ($F_{(1,17)} < 1$), or density of PV-expressing cells ($F_{(1,17)} < 1$).

➤ *Hippocampus analysis*

As no significant differences were found in the mPFC or OFC, I next analysed the PV-immunoreactivity in the hippocampus. The total number of PV-expressing cells was significantly less in the dentate gyrus (DG; $F_{(1,17)} = 10.89$, $p < 0.05$), but not within CA1 ($F_{(1,17)} < 1$) or CA2/3 ($F_{(1,17)} < 1$, *ns*). The density of PV-expressing neurons was significantly higher in CA2/3 ($F_{(1,17)} = 9.32$, $p < 0.05$), and the DG ($F_{(1,17)} = 6.5$, $p < 0.05$), but not within CA1 ($F_{(1,17)} = 1.17$, *ns*).

Control



MAM-Treated

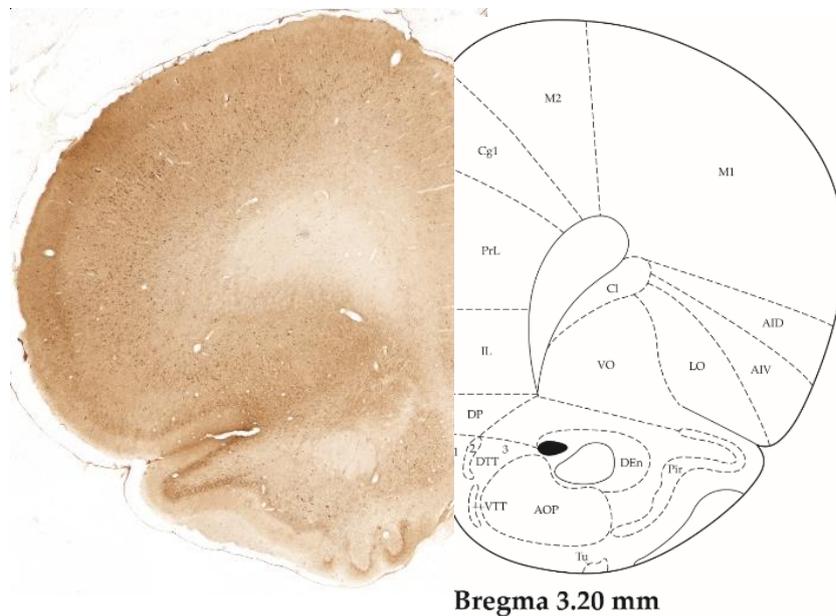
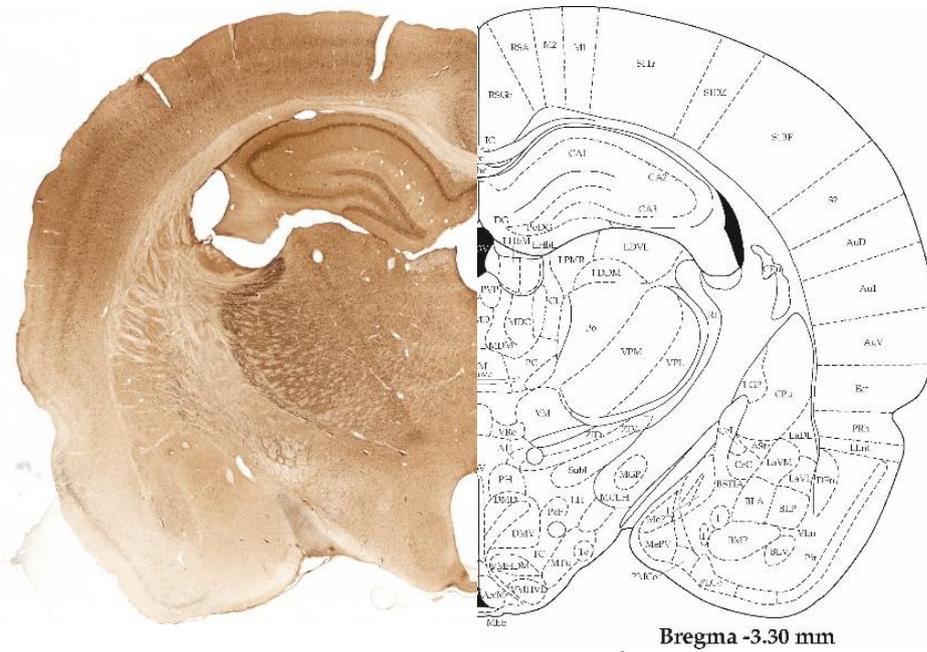


Figure 5.8) Representative PV stained PFC sections. *Top right.* Mean (+ SEM) PV-expressing cells (approximate distance from Bregma: 4.7 mm – 2.7 mm). No significant differences were found between MAM-treated rats and controls.

Control



MAM-Treated

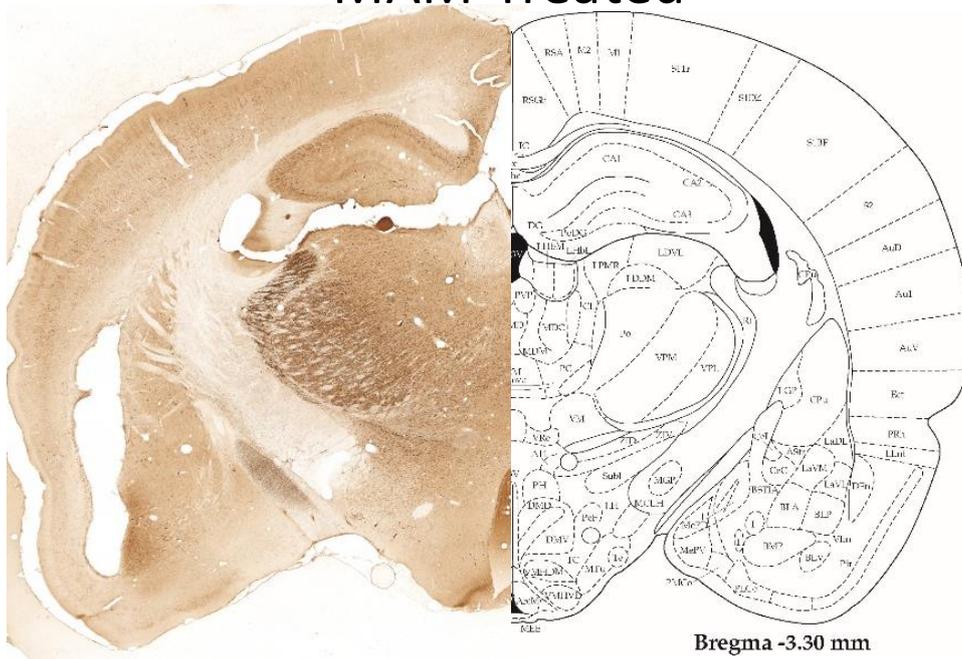


Figure 5.8) Representative PV-stained hippocampal sections. MAM-treatment increased the density of PV-expressing neurons in the CA2/3 region, while reducing the density in the DG (*, $p < 0.05$). No effect was found for CA1 PV-expression.

5.9 Discussion

5.9.1 Failure to replicate reduced parvalbumin expression within the PFC

The lack of robust attentional set-shifting deficits in MAM-treated rats suggest that the cohort tested in this thesis lacked the biological phenotype to induce the previously reported ID/ED deficits. The anatomy and histology analysis is consistent with this rationale. Gross observation of brain sections suggested that the largest anatomical abnormalities were manifested in the striatum (abnormally rostral localisation of the lateral ventricles) and the hippocampus (microcephaly and disarray of hippocampal cell layers). Specific observation of the mPFC suggested that this region was intact and spared from the effects of the toxin. Despite the overall reduction in the size of the mPFC and OFC no significant differences were found in PV-expressing neurons. However, in the hippocampus formation both significant reductions in size, and PV-expressing neurons were discovered suggesting that this region was most affected by the MAM-treatment.

We cannot rule out alternative histology methodology as a cause for our findings differing from previous studies. While Gastambide et al. (2012), reported a reduction in PV-expressing cells in the mPFC and OFC, Penschuck et al. (2006), reported no significant difference in PV-expressing cell counts in the mPFC but significant differences in hippocampus cell counts. Lodge et al. (2009), also reports PV reduction in mPFC and in the ventral subiculum. Gastambide et al., used paraffin embedded 6 μm sections, an antigen retrieval step with 100° C citrate buffer, a different brand of anti-PV primary antibody (Swant), and cells were counted with the ImageScope software, which allowed for the determination of density through all three dimensions (volumetric density analysis). Lodge et al. used the same thickness for brain sections, however they also used a different anti-PV primary antibody (Swant), and method for cell counting (z-stack collapsed sampling). Penschuck et al., did not find significant differences in the mPFC, although the authors reported using the same anti-PV primary antibody, and similar tissue thickness (40 μm). However, despite these differences in

methodology it is still possible that batch-to-batch variability accounts for the differences in findings.

5.9.2 MAM treatment primarily affects the hippocampus

Despite the few behavioural differences between control and MAM-treated rats, anatomical and histological experiments revealed severe abnormalities in the brains of MAM-treated rats. The brains of MAM-treated rats consistently displayed neuroanatomical pathologies that mirror those found in patients with schizophrenia. We confirmed previous reports of reductions in gross brain weight, hippocampal, prefrontal microcephaly, enlargement of the lateral ventricles, CA 2/3 cell layer disarray, and reduction PV-expressing interneurons within the DG. However, the failure to replicate the PV-expressing interneuron reduction within the mPFC or OFC, suggest that our assessment of cognitive flexibility in the ASST may have failed to replicate previous reports due to an incomplete replication of the MAM biology.

From the data presented here, it would appear that the ability of the MAM treatment to induce 'schizophrenia-related' biological abnormalities may be limited to only reliably affecting the hippocampal formation. Proteomic and metabolomic analysis of MAM-treated rats found that the primarily affected area was the hippocampus glutamatergic signalling, and not the mPFC (Hradetzky et al., 2012). If the mPFC abnormalities and subsequent mPFC-dependent behavioural deficits are downstream of the MAM treatment altering the hippocampus then it is probable that not all cohorts of MAM-treated rats have the same second-order mPFC effects, perhaps due to biological compensation during development. Recently, it has been reported that variation in the pregnant dams' response (indicated by weight gain or weight loss) to the toxin polyinosinic: polycytidylic acid (Poly I:C) can differentially determine the behavioural deficits in the offspring (Missault et al., 2014). Behavioural deficits are reflective of alterations in the underlying neurocircuitry, such that performance in the ASST reversal stages reflects OFC functionality (Chase et al., 2012; although there is also

a role for the oFC in set-formation), and performance in the ID/ED shift reflects mPFC functionality (Birrell and Brown, 2000). Thus, if the desired behavioural deficits do not manifest following the manipulation the assumption can be made that the targeted area was not sufficiently disrupted. This appears to be the case with our MAM-treated rats. Furthermore, MAM-treated rats rarely present with the full range of cognitive deficits, despite almost each cohort having the 'schizophrenia-related' biological phenotype (Hugh Marston, SFN Symposium, 2014). The reports in the literature regarding the deficits in the ASST may likely be a reflection of selective reporting, rather than a statement of a consistent and robust behavioural phenotype resulting from MAM treatment.

Consistent with the hypothesis that MAM treatment affects hippocampal development more consistently and robustly than it does mPFC development, other schizophrenia-related manipulations which target gestational day 15 - 17 also largely report hippocampal disruptions (Dickerson et al., 2014; Zhang and van Praag, 2014) and to a lesser extent mPFC disruptions (Wischhof et al., 2015). Indeed, it may be possible that the hippocampal alterations reported contributed to the REV 1 impairment reported here. PV-expressing neurons within the DG have been reported to be involved in an odour discrimination digging task. Morellini et al. (2010) reported that mice with increased inhibitory signalling (by ablation of tenascin-R glycoprotein) within the DG performed better in an odour-based digging reversal task as indicated by reduced latency to choose the correct stimuli and increased numbers of correct approaches to the relevant and rewarded stimuli. Lesions to the DG are known to disrupt spatial working memory. Jeltsch et al. (2001) reported that bilateral colchicine-induced lesions of the DG resulted in impairments in the radial arm maze task as measured by increased visits to previously visited correct arms and previously visited incorrect arms. Hernandez-Rabaza et al. (2007) found that similar lesions impaired performance in a spatial-tactile T-maze working memory task. Tying in both the wide reports of hippocampal dysregulation in rats with relevance for schizophrenia, and the role of the DG in reversal learning, Savanthrapadian et al. (2013) reported that Poly I:C

rats had increased LTP within the DG as well as impaired reversal learning measured as increased time spent in the previously rewarded arm during a plus maze task. Performance of the rats however improved as the task progressed and was identical to that of controls. Given the reduction in evidence for spatial pattern based responding in MAM-treated rats it is possible that impaired hippocampus-dependent spatial processing leads to their increased irrelevant dimension based behavioural pattern.

Chapter 6. General Discussion and Conclusions

Attentional regulation is integral in navigating a changing environment. Unfortunately, persons with schizophrenia suffer from deficits in attentional regulation which impairs their cognition and subsequent quality of life. The three empirical chapters in this thesis were designed to explore attentional regulation in three rat manipulations with relevance to schizophrenia. The findings indicated that during goal-directed behaviour, a primary cause of deficits in extradimensional set-shifting and reversal learning is a deficit in sensory gating resulting in abnormal learning of irrelevant stimuli. I posit that these data support theories which implicate dl/mPFC working memory biasing of attention as the core of the cognitive deficits in schizophrenia. Furthermore, the data here indicate that normal mPFC function mediates prediction error signals such that only relevant stimuli gain associative strength, whereas mPFC dysfunction causes loss of sensory gating, and non-selective prediction errors to guide learning and behaviour.

6.1 Experimental summary

The experiments in this thesis focused on attentional regulation in rats following manipulations with relevance for schizophrenia. The manipulations involved targeted disruption at three distinct developmental periods (gestational, neonatal, or adulthood). The primary measures of attentional regulation were performance at the reversal and ED stages of the ASST, stages where patients with schizophrenia perform poorly. As described in Chapter 1: Introduction, patients with schizophrenia have remained wanting for treatment of the cognitive deficits induced by the disorder. The aims of the thesis were two-fold: 1) validate ASST deficits in neurodevelopmental manipulations with relevance to schizophrenia (neonatal-PCP and MAM), and subsequently explore putative pharmacotherapies for those deficits; and 2) across all three manipulations (mPFC_{in}⁻, neonatal-PCP-treated, and MAM-treated rats) determine the underlying neurobiological and psychological mechanisms for any deficits evidenced, thus increasing knowledge on the potential nature of deficits in patients and provide new target mechanisms for treatments.

In Chapter 3, findings from acute mPFC_{in} revealed that an apparent deficit in set-shifting may arise from enhanced learned irrelevance (due to deficient sensory gating), rather than perseveration alone. While it had previously be shown that acute manipulations resulting in mPFC-hypofunction induced a robust deficit at the ED stage, the experiments here replicated that finding and expanded it to explore the nature of the ED deficit through the use of two modified ASSTs. The modifications allowed comparisons to be made to findings on the role of the mPFC in sensory gating, primarily the role of the mPFC in the attenuation of attention to irrelevant stimuli within the environment. From the results, the conclusion can be drawn that rather than 'cognitive flexibility' in general being impaired in mPFC_{in} rats, there is an inability to regulate attention through mPFC processes (likely dopamine mediated), which induces a state where mPFC_{in} rats learn equally about relevant and irrelevant dimension stimuli, producing what appears under different conditions as both behavioural flexibility and inflexibility. Furthermore, the results

suggest that the apparent contradictory evidence that mPFC-hypofunction induces a deficit in sensory gating, although the same manipulations do not impair formation of attentional set, may be due to the double dissociation between the mPFC and OFC, with the latter being more responsible for set-formation, and the former being more involved in the regulation of attention.

The experiments in Chapter 4, with neonatal-PCP-treated rats also supported findings that mPFC-hypofunction induces a deficit in attentional regulation that manifests as an ED deficit. While the standard ASST results failed to meet statistical significance, this conclusion can be drawn from the results generated from the ASST-MSS. Additional validation for the mPFC-hypofunction aetiology of the ED deficit was provided by deficits in the 24-hour delay NOR task, as well as histological confirmation of reduced PV-expression within the mPFC of neonatal-PCP-treated rats.

The last experiments presented in Chapter 5, explored ASST performance in MAM-treated rats, and while evidence was found for a reversal learning impairment, at the first reversal stage (REV 1), the data failed to replicate previous reports of deficits across several reversal learning stages and, critically, the ED. The failure to find an ED deficit was not limited to the standard task alone, as MAM-treated rats were also tested in a multiple ID ASST which measures set-formation and set-shifting without potential interference from reversal stages. In this task, MAM-treated rats appeared to both form, and shift attentional set, parallel to control performance.

6.2 Reliability of acute versus developmental manipulations and the relevance to schizophrenia

Comparing the experiments among the three manipulations in rats, the results from the experiments with neonatal-PCP-treated rats (Chapter 3) and MAM-treated rats (Chapter 4), suggests that overall, developmental manipulations may not reliably generate the deficits in ASST performance

previously reported, whereas the acute manipulations and perhaps manipulations in adult rats in general, are more reliable and consistent within the published literature. While both developmental manipulations induced deficits in task performance, with neonatal-PCP-treated rats having poor performance at the ED stage of the ASST-MSS, and MAM-treated rats having a deficit at REV 1 of the standard ASST, both of these results were only due to repeat testing to increase statistical power. The DREADDs-mediated mPFC_{in} however, produced a reliable and robust deficit that was present without repeat testing, aside from the AB design.

This difficulty in reproducing results with the developmental manipulations may lie in the considerable methodological differences between labs regarding, housing and food restrictions regimes, in combination with the brain's inherent plasticity and compensation mechanisms. The literature is rife with reports of discordant results from both developmental manipulations (neonatal-PCP and MAM) suggesting that the failure to replicate the findings within these experiments are not uncommon. The issues surrounding a failure to replicate are plentiful, however the methodological differences (e.g. the medium to odour only shift in the neonatal-PCP ASST reports; see Chapter 4) should be well noted as the robustness of the findings are then drastically reduced (Steward, 2016). Therefore, while the developmental manipulations may induce a degree of mPFC-hypofunction, the extent to which that disruption affects behaviour may be overestimated by less rigorous testing procedures.

In relation to the face validity and how well each model recapitulated schizophrenia-related biology and behaviour, while the MAM-model generated the most consistent anatomical pathology, the behavioural deficits were not consistent with schizophrenia. Indeed, it is possible that additional manipulations in conjunction with the MAM-treatment (such as an environmental stressor), may be needed to generate the behavioural deficits, similar to the gene x environment interaction findings described in Chapter 1: Introduction. The remaining two manipulations (neonatal-PCP treatment and DREADDs mPFC inhibition) both

generated clear behavioural phenotypes but did not induce robust anatomical disruption consistent with schizophrenia. Rather these manipulations better captured the behavioural deficits that can arise from specific disruptions of the mPFC rather than the gross anatomical disruptions induced by schizophrenia. Indeed, while the MAM-model may be best in studies of schizophrenia-related biology such as disrupted dopaminergic signalling and hippocampal pathology, the manipulations later in development may be better utilised for behavioural studies.

6.3 Utility of the Bayesian analysis for characterisation of behavioural pattern

The narrative at the beginning of this thesis referred to my experience of the ‘cocktail party effect’ (Cherry, 1953). Ironically, the experience of this phenomenon is more likely in healthy persons with ‘low’ working memory capacity than those with ‘high’ working memory capacity. Conway et al. (2001), reported that while the majority (65%) of participants who classified as having a low working memory capacity, reported hearing their name in an unattended irrelevant message during a selective listening procedure; only a minority (20%: of which I assume I would have been one of) of the high working memory capacity participants had the same experience. The Bayesian analysis consistently implicated abnormal behavioural patterns in the form of irrelevant dimension-based responding during performance of the ASST in all three schizophrenia-related rat manipulations. This common finding suggests that while performance in the ASST may not have been consistently impaired across all three groups, an underlying deficit in sensory gating, perhaps due to impaired working memory, may have manifested in all three. In direct contrast, the pattern for control rats was consistently the opposite with more spatial-based responding during challenging stages, such as reversals. Without the additional behavioural pattern analysis, this common finding would have eluded basic ASST TTC and errors measurements, and may have only been uncovered by additional testing such as

PPI or blocking experiments. Indeed, this is the first thesis from this lab to document a methodological analysis that performs consistently across set-shifting tests, can empirically differentiate between behavioural patterns, and can provides unifying results across behavioural phenotypes.

As described in Chapter 2: General Methods, the Bayesian analysis is constrained by only two parameters, the likelihood ratio, 'c', and the experimenter defined number of hypotheses tested by the analysis. There was consistent corroboration of 6-in-a-row criterion based stage completion, with the Bayesian analysis also indicating strong evidence for responding based on the correct stimulus. Indeed, this was evident across all stages for control rats. This finding supports the validity of the analysis with the parameters used, and if anything suggests that the frequentist based criterion may inadvertently be overtraining control rats, as during several stages, the Bayesian-derived evidence suggested responding based on the correct stimulus during the third quartile of that stage. On the other hand, the behavioural pattern analysis suggests that the frequentist criterion may be too lenient for experimental manipulations as the Bayes factor was not substantial for neonatal-PCP-treated, and mPFC_{in} rats at the completion of several stages. Indeed, if a Bayesian criterion had been used, it is likely that deficits which did not manifest in the TTC data by measurement with the 6-in-a-row criterion, may have been detected.

The Bayesian analysis was pivotal in the development of the modified tasks in this thesis as the simple-4 stage, and 6-stage simple-probe ASSTs developed for the mPFC_{in} experiments (Chapter 3), as well as the ASST-MSS task employed with the neonatal-PCP-treated rats (Chapter 4), were generated to address the Bayesian-derived hypothesis that the rats were responding to stimuli within the irrelevant dimension. These tasks successfully confirmed this hypothesis, further validating use of the analysis with the current parameters.

6.4 Modifications to the standard ASST allow exploration of additional phenomenon

In addition to experiments utilising the standard ASST, this thesis describes three new additional tasks to investigate responding based on irrelevant dimension stimuli. The adaptability and direct translatability of the rodent and human versions of the ASST to such modifications, is a primary strength it possesses over other assays, such as the WCST which are less amenable to stage substitutions and insertions. For example, the early modifications to the human ASST by Owen et al. (1993), which aimed to separate ED deficits arising from perseveration versus learned irrelevance, were directly translated into the rodent task by McGaughy et al. (2008). Within this thesis, the stimulus presentation of the Houses and Faces task designed for human testing (Hampshire and Owen, 2006), was partially back translated for use in rats, with the critical maximal separation between relevant and irrelevant stimuli across trials preserved, as well as the use of a novel panels-based ASST apparatus for testing. Additionally, the standard ASST, was amenable to the insertion of a blocking stage (Kamin, 1968; but see: Sharpe and Killcross, 2014), in the 6-stage simple-probe task. Both of these manipulations were implemented without requiring lengthy additional training of the rats overall, and did not hinder performance in control rats, while providing critical information on task performance in both mPFC_{in} and neonatal-PCP-treated rats.

Despite the obvious benefits of the rodent ASST, it remains critically flawed due to the reliance on an observer's subjective scoring of the rat's choice (digging), as well as the labour involved in the observer also preparing and presenting the stimuli for each trial. The potential for human error and bias is considerable, and there is variability amongst labs not only in the non-organic components of testing (apparatus, stimuli used, testing procedure), but also in the training of observers. While the panels modification presents some advancement in testing, in that the scoring of a 'pull' is based on a specific length marked on the panel itself, the

panels modification still remains susceptible to human error in stimulus preparations (such as too little or too much odour on the panels), and ultimately, the task needs to be automated. Unfortunately, thus far automation attempts have failed to generate a viable alternative. Implementation of a visual discrimination ASST, using an automated touchscreen, has been explored in mice. However, the task required several days (median of 13) for a single mouse to complete all stages of a single test (Brigman et al., 2005). Thus, to address the questions of set-formation and set-shifting, the researchers split the mice into two groups, one which performed an additional ID on the final day of testing, and one which performed an ED. Brigman et al., found no difference in performance for mice which were required to perform an ED shift compared to those performing an additional ID, thus there was no indication of set-formation. Similar results have been found with rats within the same visual discrimination touchscreen apparatus (Johan Alsiö personal communication) and thus far no studies have been published with either species showing viability of the method.

This 'rock and a hard place' scenario with subjectivity concerns regarding the bowl digging methods, and lack of evidence for attentional set-formation in the objective automated procedure, remains a primary concern for the future of the ASST paradigm. Ideally, an automated procedure utilising stimuli rats readily discriminate (i.e. odours and media), will be developed as a middle ground, however, this has yet to be successfully accomplished, and the Birrell and Brown (2000) ASST remains the most common measure of attentional set-shifting in rodents (Gilmour et al., 2012).

6.5 Relevance of findings for theories of attention

In Chapter 3, I introduced the conclusion that rather than performing ambiguous 'higher order cognitive processes', the role of the mPFC was to maintain a representation of the relevant information (e.g. the correct stimulus), and bias sensory processing (attention) and dopamine signalling, in favour of that information while 'ignoring' the irrelevant information. The consistent Bayesian

derived evidence for irrelevant dimension-based responding, concurrent with the deficit in sensory gating reported for all three manipulations, supports the recent findings that individual error terms (Mackintosh, 1975) exist for stimuli presented in associative learning assays, rather than a single global error term (Rescorla and Wagner, 1972; Pearce and Hall, 1980). Indeed, the mPFC is likely the site of multisensory integration and abstract rule representation that reduces processing of the less predictive prediction errors, resulting in phenomena such as ‘Kamin blocking’.

One of the earliest descriptions of schizophrenia, described that at its core was “loosening of associations” (Bleuler, 1911). All three schizophrenia-related manipulations support this as a core deficit arising from ‘schizophrenia-related’ biology. Furthermore, the findings of this thesis and the recent evidence from animal behaviour experiments supporting individual prediction error terms (Haselgrove et al., 2010; Pearce et al., 2012; Sharpe and Killcross, 2014), indicates that earlier ASST manipulations designed to disentangle perseveration to the previously relevant dimension, compared to learned irrelevance in patients with frontal lobe damage (Owen et al., 1993), schizophrenia (Elliott et al., 1995), or in rats with deficient mPFC-noradrenergic signalling (McGaughy et al., 2008), may have failed to detect learned irrelevance contributions due to the lack of associative strength between the tested irrelevant dimension and reward feedback. In both the primate (where a novel dimension is introduced), and the rodent (which uses a never discriminable dimension), learned irrelevance modifications, the tested dimension would not have elicited individual prediction error signals, to the extent of the irrelevant dimension stimuli in the 4-stage, and 6-stage simple probe ASSTs. In the modifications in this thesis, the irrelevant dimension stimuli were either 50% predictive of reward during the standard discriminations, or 100% predictive of reward during the probe stages. Thus, further investigation into the nature of the ED deficit in patients with dlPFC damage, or schizophrenia, is warranted. Ideally, by taking advantage of the associative learning theories posits of individual prediction error terms.

A recent implementation of a design which allowed individual prediction error to influence a learned irrelevance manipulation, re-examined perseveration versus learned irrelevance in Parkinson's patients (Fallon et al., 2016). Using visual compound discriminations with two superimposed stimuli from three possible dimensions (e.g. fruit, houses, faces), the researchers found that Parkinson's patients were impaired at stages that required responding to stimuli in a novel dimension presented in compound with stimuli from the previously relevant dimension, in addition to being impaired in responding to stimuli in previously irrelevant dimension compounded with a novel dimension. This was in contrast to a previous finding not using a design with an individual prediction error component (Owen et al., 1993). Thus, patients had both perseveration to the previously relevant dimension, in addition to enhanced learned irrelevance. Both schizophrenia and Parkinson's disease induce dysfunctional dopamine signalling, and it is possible that if indeed individual prediction error signals contribute to the aberrant processing of irrelevant dimension stimuli, resulting in enhanced learned irrelevance, then similar findings may be found in patients with chronic schizophrenia. Indeed recent studies have returned to the dopaminergic dysfunction in schizophrenia as a target for remediation of the cognitive symptoms, with researchers focusing on agonism of D1Rs, as enhancement of this signalling pathway has been evidenced to improve working memory in monkeys (recently reviewed by: Arnsten et al., 2016). However, there is also potential for enhancement of D2Rs to enhance dlPFC function (Ott and Nieder, 2016). Given this yet mysterious relationship between D1/2Rs and working memory, and the extant efficacy of D2R antagonists for treatment of schizophrenia's positive symptoms, it is possibly more pragmatic to pursue glutamatergic, GABAergic or other transmitter signalling pathways to improve cognition in patients already treated with atypical antipsychotics.

6.6 General Conclusion

The major conclusion of this thesis is that dysfunctional sensory gating, arising from mPFC-hypofunction, is the mechanism by which ASST performance measures, primarily ED set-shifting, and potentially, reversal learning are derived. The 'working memory' function of the mPFC (and dlPFC in primates), is critically important in the regulation of attention through biasing of sensory processing to task relevant stimuli, and inhibiting processing of irrelevant stimuli. Indeed, this function of mPFC is likely an emergent property of its multisensory processing. The role of dopamine and individual prediction errors, in the irrelevant dimension-based responding evidenced in this thesis warrants further investigation. Future experiments can target dopamine projections to the mPFC and determine if inhibition of those synapses disrupts working memory, and sensory gating, resulting in irrelevant dimension-based responding in the ASST. Experiments in the mechanistic basis of ASST deficits provide additional neural circuit targets for remediation of the debilitating cognitive symptoms of neuropsychiatric disorders such as schizophrenia.

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