The Cognitive Profiling of the Methylazoxymethanol Acetate Model of Schizophrenia

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Abstract

Schizophrenia is a lifelong disorder that affects 1% of the population worldwide and often begins in late adolescence or early adulthood. It is characterised by positive, negative and cognitive symptoms. Negative and cognitive deficits are more difficult to measure in rodents but may be a better predictor than positive symptoms of the long-term outcome following treatment of patients with schizophrenia. One of the major challenges in schizophrenia research is the development of suitable models for these aspects of the disorder.

In this regard, one model we have studied in some detail is the methylazoxymethanol (MAM) neurodevelopmental model. MAM is a neuro-specific antimitotic agent that prevents cells from dividing for a short time after injection. It can be administered intraperitoneally to pregnant dams on gestational day 17, theoretically causing neuroanatomical and behavioural alterations in offspring that are akin to core symptoms seen in schizophrenia.

Our findings confirm that MAM model offspring exhibit several neurodevelopmental and pathological changes that bear similarities to schizophrenia. These include reductions in cortical thickness and hippocampal size, and enlargement of ventricles. Behavioural consequences of MAM, largely emergent after puberty, include increased locomotor responsiveness to NMDA antagonist administration, and also pre-pulse inhibition and cognitive flexibility deficits.

However, the robust neuropathological and neurophysiological findings found in the MAM E17 model do not always translate into behavioural deficits. This is of critical consequence for model validation and use in discovery research. The variability in behaviour effects will be discussed, taking into consideration factors such as litter effects, study design and statistical analysis.

Overall, our data suggests that maternal treatment with MAM on embryonic day 17 leads to persistent alterations in the adult offspring of SD-CD rats that are relevant for modelling aspects of schizophrenia - but revised methods for study design and statistical analysis are crucial to avoid misinterpretation of findings.

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Contents

Chapter 1: General Introduction

The main aims of the thesis were to investigate the validity of the MAM E17 model as a model for schizophrenia and to utilise the model to investigate the effects of a potential pro-cognitive compound in cognitive tasks to evaluate the use of the model as a tool for drug discovery. Therefore, this introduction will cover the background to schizophrenia, its treatment and how the disorder can be modelled in animals.

1.1 Schizophrenia

Modern notions of schizophrenia are mainly based on the work of two people, Kraepelin and Bleuler. In 1896, in the 5th edition of his Textbook of Psychiatry, Emil Kraepelin brought together the formerly disparate concepts of hebephrenia, catatonia and paranoia into a single entity that he called 'dementia praecox'. This name reflected his belief that its distinctive feature was mental deterioration usually occurring in young people. Eugen Bleuler, however, felt that the process in the disorders described by Kraepelin represented a splitting of psychic functions and, in 1908, he renamed the disorder 'schizophrenia' (Black and Boffeli 1989). Bleuler regarded the primary symptom as cognitive dysfunction. It provided links to Kraepelin's 'dementia' and to the biological origins of the disease but also to disorders of affectivity, ambivalence, attention and will. These essential symptoms could be observed in every case and, according to Bleuler, schizophrenia could be

identified by the presence of these essential symptoms alone, while the accessory symptoms were not caused by the biological process or processes. These accessory symptoms consisted of psychotic features, such as hallucinations and delusions, catatonia, somatisation, changes in speech and writing, and certain mnestic disturbances.

Many attempts have been made to carry forward, refine or even break up the syndromes described by Kraepelin and Bleuler. In the latest iteration, schizophrenia is thought to have three key symptom domains, positive, negative and cognitive. Positive symptoms are the presence of certain phenomena which are not present in normal individuals. These include delusions and hallucinations which can be auditory, olfactory, visual or even tactile (Cutting, 2003). Negative symptoms are described as the absence of certain functions or aspects of the mind which should be present in a normal individual. Common negative symptoms include anhedonia (lack of pleasure), apathy (diminished ability to follow through or even initiate plans) and alogia (reduced quantity of speech). The third category, cognitive impairment, includes problems in attention, learning and memory and executive functions such as problem solving.

Schizophrenia typically has an age of onset between 16 and 30 years typically beginning with the emergence of negative and depressive symptoms, followed shortly by cognitive and social impairment and then the emergence of psychotic symptoms several years later. The whole process takes on average 5 years (Mueser

and McGurk, 2004). The etiology of schizophrenia is still unclear but studies suggest that genetics, early environment, neurobiology, psychological and social processes are important contributory factors and some recreational and prescription drugs appear to cause or worsen symptoms. There is increasing evidence suggesting that early insults during brain development are associated with an increased risk of schizophrenia (Rapoport *et al.,* 2005). Due to the many possible combinations of symptoms, there is debate about whether the diagnosis represents a single disorder or a number of discrete syndromes.

Although there are similar rates of incidence and prevalence of schizophrenia between genders, differences exist in terms of manifestation and course. Female patients present more affective and paranoid symptoms, whereas males show more negative symptoms (Lewine, 1981). Furthermore, females tend to have a 3–4-year delay of symptom onset and first hospitalization compared with males, and a second peak at the onset of menopause (Hafner *et al.,* 1993). Women also show better outcomes regarding social skills (Angermeyer *et al.,* 1990) which may be because they have later symptom onset and are, therefore, more likely to establish relationships, marry, and start professional careers. Male patients tend to deteriorate more rapidly, especially close to symptom onset (Larsen *et al.,* 1996).

The most frequently confirmed neurobiological finding in schizophrenia is enlargement of the lateral and third ventricles which is also accompanied by overall reductions in brain volume and cortical grey matter (Honea *et al.,* 2005; Zakzanis *et*

al., 2000). The frontal lobes, amygdala, hippocampus, medial temporal lobe and thalamus also have decreased volumes in patients with schizophrenia compared to controls (Harrison and Weinberger, 2005). The ventricular enlargement and brain volume reductions are also present in newly diagnosed patients and occur in unaffected at risk relatives (Pantelis *et al.,* 2007; Rapoport *et al.,* 2005). Neuroimaging studies show structural and functional abnormalities in first-episode, never treated patients suggesting that these abnormalities are not secondary to treatment or duration of illness (Rapoport *et al.,* 2005).

1.2 Dopamine Hypothesis

For the past 30 years, the predominant hypothesis for the pathophysiology of schizophrenia has been that excessive dopamine (DA) neurotransmission in the forebrain contributes significantly to psychosis. It is derived from pharmacological evidence that DA agonists, such as amphetamine, have psychotomimetic effects and that the clinical potencies of antipsychotics correlate with their ability to block DA D_2 receptors (D_2R) (Seeman, 2001). The consistent finding that all antipsychotic drugs, occupy high levels of D2 receptors, suggests that the blockade of D2 is an essential minimal requirement for clinical antipsychotic action in those patients who respond to neuroleptics (Seeman, 2001). Excessive DA transmission has also been supported by functional imaging studies showing that schizophrenia patients exhibit greater amphetamine-induced increases in synaptic DA in the striatum, associated with the worsening of psychotic symptoms (Laruelle *et al.,* 1996).

Although the dopamine theory of schizophrenia is supported by the correlation between the clinical potencies of the antipsychotic drugs and their affinity for the dopamine D2 receptor, there are inconsistencies that do not support the theory. While antipsychotic drugs can often control the positive symptoms of schizophrenia, such as hallucinations and delusions in the acute setting, the same antipsychotics tend to be less effective in patients who have been symptomatic for many years. It is also true, however, that many schizophrenia patients may not clinically improve despite high occupancy (>75%) of their D2 receptors (Seeman, 2001). In addition, the antipsychotics are much less effective at addressing negative symptoms or the cognitive impairments, after a year of treatment which coincides with the emergence of reduction of PFC volume (Paz *et al.,* 2008). There has also been little evidence of dopamine receptor abnormality in schizophrenia. Post-mortem studies initially suggested that there could be an increased density of striatal dopamine D2 receptors in schizophrenia (Owen *et al.,* 1978; Mackay *et al.,* 1980a; Seeman *et al.,* 1984), but there has been controversy as to whether these results arose as a result of chronic antipsychotic treatment (Clow *et al.,* 1980; Mackay *et al.,* 1980b). Studies using single photon emission tomography and positron emission tomography methods in drug naive patients with schizophrenia have also failed to find any difference in dopamine D2 receptor density in vivo compared to controls (Farde *et al.,* 1990; Martinot *et al.,* 1991; Pilowsky *et al.,* 1994). A recent meta-analyses (Laruelle, 1998; Zakzanis and Hansen, 1998) suggested that there may be a small elevation in dopamine D2 receptors in drug-free patients with schizophrenia; however; the degree of overlap between patients

and controls makes it unlikely that this abnormality is clinically meaningful (Zakzanis and Hansen, 1998).

1.3 Glutamate Hypothesis

The N-methyl-D-aspartic acid (NMDA) receptor hypofunction (Glutamate) hypothesis of schizophrenia was developed in part to explain some of these inconsistencies. Glutamate is the major excitatory neurotransmitter in the central nervous system. Its receptors are classified into two broad categories: ionotropic and metabotropic receptors. Ionotropic glutamate receptors (iGlu), which include NMDA, kainate, and AMPA subtypes, initiate rapid depolarization by facilitating sodium or calcium entry into neurons through channels formed by the receptor itself. Metabotropic glutamate receptors (mGlu) modulate neurotransmission by activating G-protein coupled synaptic transduction mechanisms. Some mGlu receptors, in particular the mGlu5 subtype, interact closely with NMDA receptors and may directly modulate the function of the NMDA receptor channel. Executive functions of the prefrontal cortex, including working memory and behavioural flexibility are impaired by NMDA antagonists and are therefore thought to be critically dependent on NMDA receptors (Verma and Moghaddam, 1996; Dalton *et al.*, 2011; Featherstone *et al.*, 2012). They also play an essential role in the development of neural pathways, particularly for neuronal migration during brain development, making them a critical component of developmental processes (Haberny *et al.*, 2002).

The idea of a glutamatergic abnormality in schizophrenia was first proposed by Kim, Kornhauber, and colleagues in 1980 (Kim et al., 1980) based on their findings of low cerebrospinal fluid (CSF) glutamate levels in patients with schizophrenia, however these findings could not be replicated at the time. In the last two decades, however, basic and clinical evidence has been accumulating to support the idea that aberrant NMDA receptor function subserves many aspects of molecular, cellular, and behavioural abnormalities associated with schizophrenia. Clinical observations of abusers of the NMDA receptor antagonist phencyclidine (PCP) showed that PCP exposure resulted in disordered thought and confusion, emotional blunting, auditory hallucinations, impulsiveness and working memory deficits (Javitt and Zukin., 1991; Luby *et al.,* 1959). Ketamine another NMDA receptor antagonist, also induces a clinical state similar to schizophrenia including both positive and negative symptoms, and can worsen symptoms in clinically stable patients (Jentsch and Roth., 1999) further supporting the theory. PCP and Ketamine can also lead to cognitive deficits as shown by impaired performance in the Wisconsin Card Sort and Vigilance tasks and it has also been shown that the pro-psychotic effects of phencyclidine and ketamine can be effectively treated by haloperidol (Giannini *et al.,* 2000). Other behavioural studies show that hyperlocomotion and working memory impairments induced by PCP are correlated with increased glutamate levels in the PFC (Adams and Moghaddam., 1998) and microdialysis studies have shown that glutamate levels are increased in the striatum (Bustos *et al.,* 1992) and PFC (Moghaddam *et al.,* 1997) of animals acutely treated with NMDA antagonists.

Simply stated, the hypothesis proposes that NMDA receptor hypofunction, the condition induced by an NMDA antagonist, might also be viewed as a model for a disease mechanism which could explain the symptoms and natural course of schizophrenia. Several lines of evidence have also suggested that a typical consequence of NMDAR hypofunction is reduced glutamatergic excitation of subcortical gamma-amino-butyric acid (GABA) interneurons, which results in disinhibition of glutamate (as well as dopamine and acetylcholine) neurotransmission to the cortex (Homayoun and Moghaddam., 2007; Krystal *et al.,* 2003; Moghaddam, 2003). In addition, increased glutamate signalling mainly at AMPA receptors has also been found to be a possible downstream effect of NMDAR blockade (Moghaddam and Javitt 2011; Moghaddam *et al.,* 1997]. It has been proposed that this excessive release of excitatory transmitters and consequent overstimulation of postsynaptic neurons might explain the cognitive and behavioural disturbances associated with schizophrenia (Olney and Farber, 1995, Moghaddam et al., 1997 and Adams and Moghaddam, 1998). Hence, refinements of the glutamate hypothesis postulate that behavioural and cognitive symptoms of schizophrenia appear to be caused by a dysregulation of glutamatergic neurotransmission, characterised by NMDAR hypofunction and subsequent excess glutamatergic activity.

In schizophrenic patients, postmortem studies have shown changes in glutamate receptor binding and subunit protein expression in the prefrontal cortex, thalamus, and hippocampus (Akbarian *et al.,* 1996, Clinton and Meador-Woodruff., 2004) and

significant reductions in NMDA receptor binding have also been found in medication-free, but not antipsychotic-treated schizophrenic patients compared to healthy subjects (Pilowsky *et al.,* 2006). Levels of amino acids N-acethylaspartate (NAA) and N-acethylaspartylglutamate (NAAG), and the activity of the enzyme that cleaves NAA to NAAG and glutamate are also altered in the CSF and postmortem tissue from individuals with schizophrenia (Tsai et al., 1995). NAAG is an endogenous ligand for the mGlu3 subtype of glutamate receptor, the gene for which has been implicated in increased propensity to develop schizophrenia. Furthermore, reduced NAA levels are thought to reflect decreased glutamate availability.

Glutamate neurons regulate the function of other neurons that have been strongly implicated in the pathophysiology of schizophrenia. These include GABA interneurons whose morphology has been altered in schizophrenia (Lewis et al., 2005), and dopamine neurons, which are the target of antipsychotic drugs. For example, bursting of dopamine neurons, which is thought to be an integral component of their proper response to environmental stimuli, is dependent on activation of NMDA receptors on these neurons (Johnson et al., 1992). Along the same lines, it is noteworthy that two key pharmacological clues to the pathophysiology of schizophrenia—clinical efficacy of D2 receptor antagonist and increased probability of developing schizophrenia after cannabis use during adolescence—are consistent with deficient NMDA receptor function in schizophrenia. Cannabinoid CB1 receptor and D2 receptors are localized

presynaptically on glutamate terminals and work to inhibit the release of glutamate. Cannabis, therefore, reduces glutamate release, in particular in corticostriatal regions (Gerdeman and Lovinger, 2001), leading to deficient activation of NMDA receptors, whereas reduced D2 receptor function produces modest increases in glutamate release (Cepeda et al., 2001; Yamamoto and Davy, 1992).

These lines of evidence have led to the current thinking that disruptions in any of the numerous mechanisms that influence the function of NMDA receptors, by either modifying the kinetics of the NMDA channel itself, or the function of proteins that link NMDA receptors to signal transduction pathways, can compromise behaviour in a manner suggested by the symptoms of schizophrenia. This theory is consistent with the complex genetic predisposition feature of schizophrenia because it predicts that any of the genes that encode or regulate the large number of proteins that influence the function of NMDA receptors would be plausible susceptibility genes for this disease. In addition to theoretical implications, these findings have been the basis for identification of novel therapeutic targets for treatment of schizophrenia (Moghaddam and Adams, 1998). A recent proof-of-concept clinical trial paper by Eli Lilly shows promising results with one of these compounds (Patil et al., 2007). This double-blind placebo-controlled study showed that after four weeks of treatment, an agonist for the metabotropic glutamate 2/3 receptor (mGlu2/3R) has similar efficacy as olanzapine in ameliorating positive and negative symptoms of schizophrenia.

Several approaches have been made in recent years to integrate the evidence for glutamatergic abnormalities and dopamine in the pathogenesis and current treatment of schizophrenia (Stone *et al.,* 2007; Javitt 2007; Laurelle *et al.,* 2003). A current, widely supported theory states that dopaminergic imbalances in striatal and cortical areas are preceded and modulated by NMDAR hypofunction in the PFC. In turn, the arising dopaminergic dysregulation might further disrupt glutamatergic signalling at NMDA receptors (Stone 2011; Laurelle *et al.,* 2003; Toda and Abi-Dargham., 2007). Dopaminergic dysregulation might therefore be the result of impairments of glutamate neurotransmission and a subsequent reinforcing factor in maintaining these impairments (Coyle., 2006). In fact, lower glutamate levels in hippocampal areas of individuals in the prodromal states of schizophrenia, but not in healthy controls, have been found to be linked to increased dopaminergic neurotransmission (Stone *et al.,* 2010).

Glutamatergic theories of schizophrenia account for negative symptoms and cognitive dysfunction, as well as positive symptoms, and thus may lead to new treatment approaches specifically targeting this unmet medical need. Improving the future treatment of schizophrenia and increasing our biological understanding of the disease will be contingent on development of appropriate models and biomarkers for glutamatergic drug development. Although clinical drug development progresses slowly, the field has now progressed to the point where treatment predictions of the glutamate model can be tested.

1.4 Neurodevelopmental Hypotheses

The emergence of psychosis several years after early structural brain changes led to the hypothesis that schizophrenia is a neurodevelopmental disorder. The hypothesis states that schizophrenia, or a predisposition to the disease, results from pre- and/or peri-natal disturbances that affect normal CNS development (Harrison., 1997). As previously mentioned, structural changes are apparent before first-episode and in first-degree relatives indicating that the abnormalities are a expression of familial risk factors, the most likely being genes affecting neurodevelopment. Further evidence that neuropathological changes in schizophrenia are prenatal rather than postnatal comes from the absence of glial reactions in the brains of schizophrenics. Glial reactions (gliosis) are a reactive cellular process that occurs after injury to the CNS. As with scarring in other organs and tissues, the glial scar is the body's mechanism to protect and begin the healing process in the nervous system. The process of gliosis involves a series of cellular and molecular events that occur over several days (Fawcett et al., 1999). Typically, the first response to injury is the migration of macrophages and local microglia to the injury site. This process, which constitutes a form of gliosis known as microgliosis, begins within hours of the initial CNS injury. 3–5 days later, oligodendrocyte precursor cells are also recruited to the site and may contribute to remyelination. The final component of gliosis is astrogliosis, the proliferation of surrounding astrocytes, which are the main constituents of the glial scar (Fawcett et al., 1999). Gliosis is common to most adult-onset brain injuries and neurodegenerative disorders such as Alzheimer's disease but not with neuropathological events

occurring in early development. Furthermore, post-mortem histological studies have shown cortical cytoarchitectural abnormalities which can only occur during early brain development. Findings in this area indicate defective cellular organisation where the laminar distribution of cortical neurons is displaced inwards. The normal migration of neurons intended for cerebral cortex proceeds outwards during the second trimester of gestation; a defect in the cortical layers of schizophrenics suggests a defect in the neuronal migration during this developmental stage. These findings are particularly important because they are difficult to interpret in other than neurodevelopmental terms (Weinberger., 1995).

It has long been recognised that schizophrenia is highly familial suggesting a possible genetic aetiology. Lifetime risk to siblings of schizophrenic patients is about ten times the population risk and using twin data a heritability of 0.6-0.8 has been estimated (Owen *et al.*,2000). It is generally accepted that there are multiple susceptibility genes and according to two recent meta-analyses, approximately 12 regions of the genome are now thought to contain schizophrenia susceptibility genes (Badner *and Gershon.*,2002; Lewis et al 2003). These genes include dysbindin (6p22), neuregulin 1 (8p12), G72 (13q34), RGS4 (regulator of G protein signalling 4) (1q21, q22), COMT (catechol-o-methyltransferase (22q11) and DISC 1 (disrupted in schizophrenia 1) (1q42). It is thought that the different genes may have differential relevance at different stages of development and under different environmental conditions and, therefore, interact with each other as well as with environmental risk factors to produce the clinical condition.

Many groups have now examined prenatal exposure to influenza as a candidate risk factor for schizophrenia. Maternal exposure to viral infection has been associated with an increased risk of schizophrenia, and it has been suggested that the maternal immune response may interfere with normal foetal brain development. A study suggested that foetuses exposed during the second trimester to the 1957 type A2 influenza pandemic have an increased risk of schizophrenia (Mednick *et al.,* 1988). Those exposed to the viral epidemic during their second trimester of foetal development were at elevated risk of being admitted to a psychiatric hospital with a diagnosis of schizophrenia. This was true for both males and females and independently in several psychiatric hospitals. The second-trimester effect was seen in the elevated proportion of schizophrenics among those admitted to a psychiatric hospital and also in higher rates of schizophrenia per 1000 live births in the city of Helsinki (Mednick *et al.,* 1988). Another group identified that a population of males who, as foetuses, were exposed to the 1969 Hong Kong influenza epidemic had a significantly elevated schizotypal personality scores compared to controls. Further analysis revealed that these differences were accounted for by those exposed to the influenza virus in week 23 of the gestation cycle (Machon *et al.,* 2001). Although studies in rodents have shown that perinatal viral infections can lead to neuropathological and behavioural abnormalities considered relevant to schizophrenia (Fruntes and Limosin., 2008; Romero *et al.,* 2008), it is not clear whether the consequences of prenatal infection are due to the infection itself or to the maternal immune response to infection. One theory is that the maternal cytokine-associated inflammatory response to infection may be a

crucial link as the identity of the pathogen seems to be irrelevant (Romero *et al.,* 2008). Maternal serum levels of the cytokine tumour necrosis factor–α or interleukin are elevated in mothers of patients with schizophrenia. In addition to their immunological roles, cytokines have been shown to affect survival, differentiation and morphology of developing neural cells. Therefore, variations in the levels of inflammatory cytokines in the foetal environment may therefore adversely affect the development of the nervous system and contribute to the development of future psychobehavioural and/or cognitive impairment (Romero *et al.,* 2008).

There has also been considerable interest in recent years of the impact of prenatal malnutrition. Susser *et al.,* (1996) identified an increased risk of schizophrenia in the offspring of women who were pregnant during a famine in Holland during World War II. The risk of schizoid personality disorder, as defined by ICD-6 to ICD-9, in men at age 18 years was compared in birth cohorts that were conceived during the Dutch Hunger Winter famine and in unexposed birth cohorts. The exposed cohort had a significantly greater risk of schizoid personality disorder (Hoek *et al.,* 1996). Another group assessed the effects of nutritional deficiency during the first trimester of pregnancy on brain morphology in patients with schizophrenia exposed during the first trimester of gestation to the Dutch Hunger Winter. Prenatal famine exposure in patients with schizophrenia was associated with decreased intracranial volume and an increase in brain abnormalities, predominantly white matter hyper-intensities in controls (Hulshoff *et al.,* 2000).

Low prenatal vitamin D has also been proposed as a candidate for a risk factor for schizophrenia (McGrath., 1999). In a review by Bouillon *et al.,* (1995), evidence was presented linking vitamin D with cell growth and proliferation, immune response and foetal development. It has been shown to be a potent inducer of nerve growth factor synthesis but the precise links between vitamin D and the central nervous system are still poorly understood. One of the most robust findings in schizophrenia epidemiology is that a high percentage of patients with schizophrenia are born in winter. Moskovitz (1978) suggested that the marked seasonal variations in the serum level of vitamin D may be linked to the seasonality of schizophrenia births. Low vitamin D levels are frequently reported during winter and more vitamin D is required during pregnancy due to the rapid growth of the foetus, especially in the third trimester. It is therefore hypothesised that the high number of winter births seen in schizophrenia may be related to low maternal vitamin D levels associated with reduced light during the winter period (McGrath., 1999).

The most extensively studied of early developmental markers are obstetric complications (OCs). Many obstetric risk factors have been linked with schizophrenia including pre-eclampsia (Kendell *et al.,* 1996), low birth weight (Jones *et al.,* 1998), rhesus factor incompatibility (Hollister *et al.,* 1996) and maternal bleeding during pregnancy (Hultman *et al.,* 1999). It has been reported that the relative risk for schizophrenia was increased up to 2.5 times by pre-eclampsia, gestational age below 33 weeks, respiratory illness and low birth weight. Pre-eclampsia, an indicator of foetal malnutrition emerged as the strongest risk factor,

increasing the risk for schizophrenia between 2 and 2.5 times (Dalman *et al.,* 1999). Sacker *et al.,* (1995) found that schizophrenia was associated with a higher likelihood of risk behaviour during pregnancy by the mothers, including smoking, drinking and poor prenatal care but these behaviours do not qualify as OCs and therefore are not used in most studies.

1.5 Treatment of Schizophrenia

Current antipsychotics are divided into typical (e.g haloperidol and chlorpromazine) and atypical (e.g. clozapine, olanzapine and risperidone) groups based on their affinity for dopaminergic receptors (Meltzer *et al.,* 1989). Both classes of drug act on the dopaminergic system with more or less affinity toward D2 receptors. The typical antipsychotics are effective in reducing positive but not negative symptoms of psychosis but also have several side effects due to chronic blockade of D2 receptors in the striatum. The newer atypical antipsychotic drugs are usually preferred for initial treatment as they are often better tolerated and associated with lower extrapyramidal side effects as well being more effective in reducing the negative and cognitive symptoms. However they are also more likely to induce weight gain and obesity related diseases (olanzapine) (Liberman *et al.,* 2005) or agranulocytosis (clozapine) (Haas *et al.,* 2007). Due to the large number of antipsychotics available, many have not been compared head to head until recently. Leucht et al (2013) however performed a multiple-treatment meta-analysis to compare the efficacy and tolerability of 15 different antipsychotic drugs

in schizophrenia. They compared two first generation antipsychotics (haloperidol and chlorpromazine) with 13 second generation antipsychotic drugs. They found that Clozapine was the top-ranking antipsychotic in terms of efficacy (figure 1) with amisulpride, olanzaoine and risperidone also more effective than the rest of the drugs. However, Leucht et al emphasise that the differences in efficacy between drugs were small (standardised mean differences 0·11–0·55, median 0·24), but, for perspective, the efficacy differences compared with placebo were of only medium size (0·33–0·88, median 0·44), so the differences in efficacy between drugs are possibly substantial enough to be clinically important. Also, because most clozapine studies were done in refractory patients, clozapine is thought to be superior only in this subtype, but in our analysis of non-refractory patients it was also more effective than all the other drugs. However, this result has the limitation that it was mainly based on older comparisons of clozapine with first-generation drugs. There was no clear winner in terms of side effects (all cause discontinuation, extrapyramidal side-effects, prolactin increase, QTc prolongation and sedation) with the differences among drugs being much larger than the changes observed for efficacy (Leucht et al., 2103).

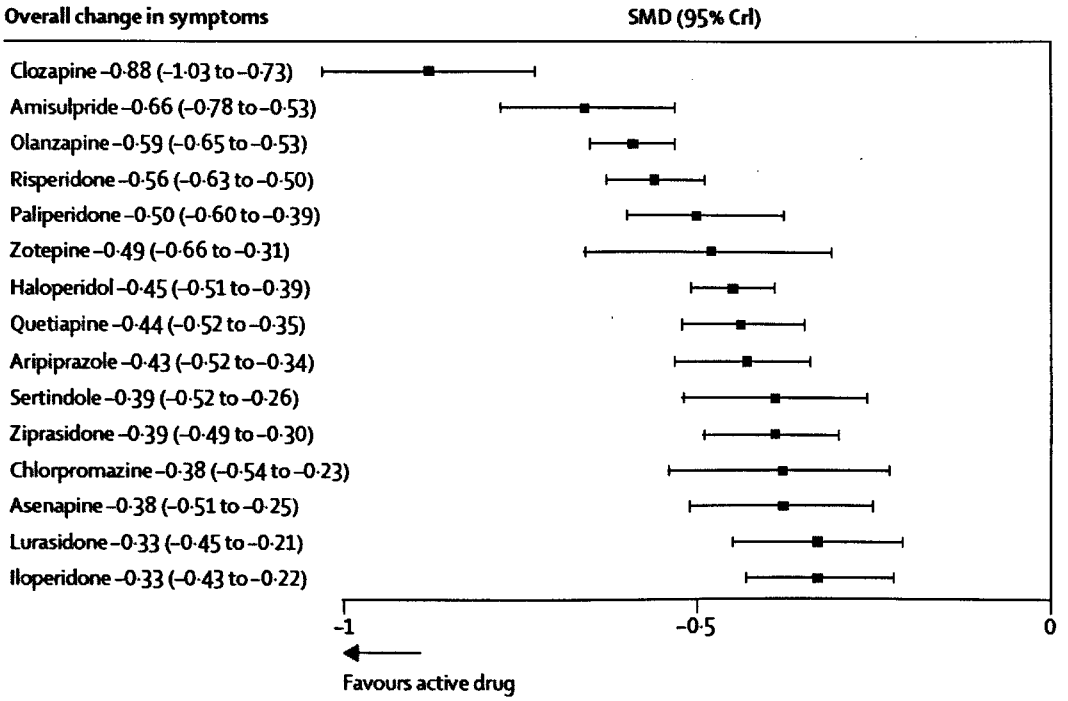


Figure 1: Forest plot for efficacy of antipsychotics drugs compared with placebo (Leucht et al 2013)
Treatments are ranked according to their surface under the cumulative ranking (SUCRA) values (appendix p 98).SMD=standardised mean difference. CrI=credible interval.

It is not clear which neurobiological mechanisms beyond D2 receptor antagonism is the final therapeutic target responsible for the beneficial effects seen in schizophrenia. Many of the atypical antipsychotics have been further characterised with respect to their affinities for different receptors (table 1) (Horacek *et al.*, 2006). The hypothetical mechanisms of action of atypical antipsychotics can be classified into dopaminergic, sertonergic or combined modulation effects.

Antipsychotic	Classification	Criteria for atypical antipsychotic[a]	Receptor affinity (K_i)[a]									
			D_2	D_2 dissociation (k_{off})[b]	5-HT_{2A}	5-HT_{2A}/D_2	5-HT_{1A}	5-HT_{2C}	α_2	α_1	H_1	M_3
Amisulpride	D_2/D_3 antagonist	0EPS (?), 0PRL	1.3	0.02	2000	1539	<10 000	<10 000	1600	7100	<10 000	<10 000
Aripiprazole	Partial dopamine receptor agonist[c]	0EPS, 0PRL	2.3	0.037	4.6	2	5.6	181	74	25	23	4677
Clozapine	MARTA	0EPS, 0PRL	187	1.396	130	0.07	140	6.1	142	1.6	0.23	20
Fluphenazine	Conventional	NA	0.6	0.01	80	133	2829	658	304	9	67	<10 000
Haloperidol	Conventional	NA	2.4	0.017	50	20	2832	4475	1130	12	4160	<10 000
Chlorpromazine	Conventional	NA	6.7	0.02	12	1.8	3115	6.1	194	0.3	0.18	67
Iloperidone	SDA	0EPS	37	0.59	5.6	0.19	93	146	162	0.31	12.3	<10 000
Loxapine	Conventional	NA	22	0.444	4	0.18	2900	17	2400	28	2.8	390
Melperone	Conventional[d]	0EPS, 0PRL	143	2.27	102	0.71	2200	1342	150	180	580	<10 000
Olanzapine	MARTA	mEPS, mPRL	31	0.039	3.5	0.11	2720	14	314	109	0.65	51
Quetiapine	MARTA	0EPS, 0PRL	700	3.013	96	0.13	320	1184	3630	22	2.2	1942
Remoxipride	D_2/D_3 antagonist	0EPS	51	1.23	<10 000	<500	<10 000	5500	<10 000	<10 000	<10 000	<10 000
Risperidone	SDA	mEPS	1.65	0.026	0.55	0.33	420	33	151	4.5	27	<10 000
Sertindole	SDA	0EPS, 0PRL	7	0.11	0.35	0.05	280	0.7	640	3.9	130	<5000
Sulpride	D_2/D_3 antagonist		0.21–78	0.003	<10 000	<500	<10 000	<10 000	4300	<10 000	<10 000	<10 000
Thioridazine	Conventional	NA	8.3	0.14	60	7.2	NA	46	453	5	16	15
Ziprasidone	SDA	0EPS	4.6	0.073	1.4	0.30	112	4.1	160	18	130	<10 000

a Affinity constants (K_i) for individual receptors involved in antipsychotic action are reviewed by Roth et al.[31] and National Institute of Mental Health databases (http://pdsp.cwru.edu/pdsp.asp).

b Reflects the rate of unbinding from D_2 receptors.[32]

c Also has 5-HT_{2A} receptor antagonist properties.

d Melperone is classified as a conventional antipsychotic but its low affinity for D_2 receptors gives it a clinical profile similar to that of atypical agents.

0EPS = none or low induction of EPS; 0PRL = no prolactin elevation; EPS = extrapyramidal syndrome; MARTA = multi-acting receptor-targeted antipsychotics; mEPS = moderate induction of EPS; mPRL = moderate prolactin elevation; NA = not applicable; SDA = serotonin-dopamine antagonists.

Table 1: Pharmacodynamic characteristics and classification of selected antipsychotics and clinical criteria characterising atypical antipsychotics (Horacek *et al.*, 2006)

As previously mentioned, D2 receptor blockade in the brain is a general pharmacodynamics property of all antipsychotics, and without it, a drug will not show any antipsychotic properties (Seeman, 2002). With typical antipsychotics, the level of D2 blockade is directly related to the antipsychotic effect, but this is not the case with atypicals. It has also repeatedly been confirmed that D2 receptor occupancies >80% are associated with extra-pyramidal side effects (EPS) (Farde *et al.*, 1988; Farde *et al.*, 1992; Goyer *et al.*, 1996; Kapur *et al.*, 1999). The substituted benzamines antipsycotics (table 1), for example, remoxipride and amisulpride, have a higher affinity for D3 than D2 receptors (Scatton *et al.*, 1997). D3 receptors are localised in the limbic cortex, therefore, blocking these receptors would result in

regionally selective anti-dopaminergic activity, resulting in an accentuated effect on positive symptoms (Bressan *et al.*, 2003; Leucht *et al.*, 2002).

Partial agonism of D2 receptors is another model for explaining the properties of atypical antipsychotics. Partial agonism is when binding to the receptor, the drug, blocks the effects of the extracellular physiologically active substance (e.g. dopamine) but at the same time has an agonistic effect on the receptor. Aripiprazole is an example of an antipsychotic which uses this model. This partial agonism results in high D2 occupancy at therapeutic doses with low EPS (Burris *et al.*, 2002).

Interest in serotonergic modulation for the treatment of schizophrenia arose from the finding that $5HT_{2A}$ receptor agonists (e.g. LSD) are strong psychedelic drugs that can elicit psychotic symptoms. $5HT_{2A}$ receptors are localised on hippocampal and cortical pyramidal cells, as well as on GABA neurons. The highest density of receptors is in the fifth neocortex layer where the inputs of various cortical and subcortical brain areas are integrated (Willins *et al.*, 1997; Jacob *et al.*, 1998). As $5HT_{2A}$ agonism induces depolarisation of pyramidal cells, it has been speculated that $5HT_{2A}$ blockade is responsible for normalisation of pyramidal cell activity which leads to the therapeutic activity of atypical antipsychotics (Martin *et al.*, 1998).

Some atypicals (clozapine and risperidone) and some typicals (e.g. chlorpromazine, fluphenazine) have high affinities for 5-HT_{2C} receptors. $5HT_{2C}$ receptors have been

found in cortical areas and in the hippocampus, striatum, septal, thalamic and midbrain nuceli and brain stem. In cortical areas, these receptors appear to be mostly expressed on glutamatergic pyramidal cells and it has been suggested that that they control monoaminergic and cholinergic neurons. In the substantia nigra, 5-HT_{2C} receptors are co-localised with GABA, indicating that they yield indirect control of dopaminergic transmission (Leyson, 2004). The blockade of $5ht_{2C}$ receptors on GABA cells in the substantia nigra would potentiate D2 receptor mediated tonic inhibitory control with a protective effect against EPS (Di Matteo *et al.*, 2001).

Agonism of $5HT_{1A}$ receptors is also considered a possible mechanism associated with the activity of some atypical antipsychotics (aripiprazole, clozaline, qutiapine, risperidone) (Meltzer *et al.*, 2003). $5HT_{1A}$ receptor blockade prevents the increase in dopamine in the prefrontal cortex induced by these drugs, even with olanzapine which does not express $5HT_{1A}$ receptor affinity (Ichikawa *et al.*, 2001). Generally it may be concluded that a simple effect at serotonergic receptors is probably not sufficient for an antipsyhotic effect in schizophrenia but that it works in combination with D_2 receptor blockade.

Meltzer *et al.*, (1989) defined atypical antipsychotics drugs as those showing a higher affinity for 5-HT_{2A} receptors than for D2 receptors and a lower affinity for D_2 receptors than that seen with typical antipsychotics. For the nigrostriatal pathway, a model has been suggested in which blockade of $5HT_{2A}$ receptors leads to

increased output of dopaminergic neurones into the striatum. Such increased extracellular activity of dopamine in the striatum displaces the antipsychotic from its binding to D2 receptors and thus decreases the risk of EPS development (Kapur and Remington, 1996). $5HT_{2A}$/D2 receptor antagonism on the mesolimbic dopaminergic pathway into the nucleus accumbens is linked with greater efficacy against the positive symptoms of schizophrenia (Yan, 2000; Pehak *et al.*, 2001). On dopaminergic mesocortical projections, $5HT_{2A}$ receptor antagonism increases dopamine release in the prefrontal cortex. This effect is thought to be linked with antipsychotic effects on cognitive and negative symptoms (Seeman, 1994; Ichikawa and, Meltzer., 1990).

Some atypicals, such as olanzapine and clozapine, have a marked affinity for cholinergic muscarinic (M1 and M4) receptors. These are predominantly expressed in the frontal and limbic areas of the brain (Levey, 1993; Bymaster *et al.*, 2003). The main effect of antimuscarinics is to antagonise antipsychotic0induced EPS, and the affinity of antipsychotics for muscarinic receptors has been shown to be inversely correlated with EPS effects (Snyder et al., 1974).

Taking into consideration the side effect problems with current treatments, it is necessary to look for new psychotropic drugs which have a better efficacy, a faster onset of action and fewer adverse side effects. As altered glutamate neuronal transmission has been suggested to be important in the etiology of schizophrenia,

drugs which target metabotropic glutamate receptors and enhance NMDA function may be alternative efficacious antipsychotics.

MGlu2 and mGlu3 receptors are highly localized within limbic and forebrain areas associated with schizophrenia including the hippocampus, and prefrontal cortex (Ohishi et al. 1993a and b) and in recent years, several lines of evidence have shown that mGlu2/3 receptor agonists may be effective for the treatment of schizophrenia in both animal models and in humans. mGlu2/3 receptor agonists have been reported to attenuate locomotor hyperactivity induced by an NMDA receptor antagonist (Moghaddam and Adams., 1998, Rorick-Kehn *et al.,* 2007) and amphetamine (Rorick-Kehn *et al.,* 2007, Galici *et al.,* 2005) in rodents. Moreover, mGlu2/3 receptor agonists have also been shown to inhibit conditioned avoidance responding (Rorick-Kehn *et al.,* 2007, Takamori *et al.,* 2003] and reverse the effects of stress and anxiolytic-like and antipsychotic-like effects in a variety of preclinical rodent in vivo paradigms including fear- potentiated startle in rats and marble burying in mice (Rorick-Kehn *et al.,* 2007). LY354740, an mGlu2/3 agonist, improved PCP-impaired performance in a T-maze discrete-trial delayed alternation task (Moghaddam and Adams., 1998) and reversed the deficits of social discrimination induced by neonatal treatment with PCP (Harich *et al.,* 2007). Decreased levels of glutamate and changes in several markers of glutamatergic function also occur in schizophrenic brain. As previously mentioned, administration of PCP or ketamine to rodents elicits an increase in locomotion and stereotypy, this is also accompanied by an increase in glutamate efflux in several brain regions. Systemic administration

of mGlu2/3 receptor agonists suppresses PCP-induced behavioural effects and the increase in glutamate efflux. Thus, drugs such as mGlu2/3 receptor agonists which might correct or normalize glutamatergic functions may have therapeutic promise in treating schizophrenia.

mGlu5 receptor agonists have been reported to attenuate PCP, ketamine, amphetamine, and apomorphine induced increases in locomotor hyperactivity (Chan *et al.,* 2008, Kinney *et al.,* 2005, Liu *et al.,* 2008), improve PPI disruption and object recognition memory impairment induced by ketamine (Chan *et al.,* 2008) and to reduce conditioned avoidance responding in rats (Liu *et al.,* 2008). These findings suggest that mGlu5 receptor potentiation exerts antipsychotic activity in animal models and may be effective for the positive symptoms of schizophrenia. In a novel object recognition test, mGlu5 recpetor agonists have also improved the impaired recognition capability that was induced by MK-801 or ketamine (Liu *et al.,* 2008, Uslaner *et al.,* 2009) and attenuated MK-801-impaired task performance in a set-shifting paradigm (Darrah *et al.,* 2008). They have also been found to enhanced object recognition memory in a novel object recognition test (Liu *et al.,* 2008) and spatial memory in Ymaze spatial alternation (Balschun *et al.,* 2006) and Morris water maze task (Ayala *et al.,* 2009), indicating that mGlu5 receptor potentiators may have memory-enhancing effects, even for normal conditions. Therefore, enhancement of mGlu5 receptor activity with mGlu5 receptor potentiators may be effective for treating the dysfunctions of several cognitive domains associated with schizophrenia. The involvement of the mGlu5 receptor on cognition is further

supported by a study of mGlu5 receptor-null mice. These mice exhibited reduced long-term potentiation in the CA1 region and dentate gyrus of the hippocampus (NMDA receptor dependent pathways), but not in the CA3 region (an NMDA receptor-independent pathway). mGlu5 receptor-null mice also displayed impaired spatial learning in a Morris water maze task, impaired contextual learning during fear-conditioning (Lu *et al.,* 1997) and disrupted PPI (Kinney *et al.,* 2003).

1.6 Modelling schizophrenia in animals

Despite fifty years of drug development research, one of the biggest challenges in schizophrenia-related drug discovery is to find an appropriate animal model of the illness so that novel hypotheses can be tested at the basic science level. As more knowledge of the pathophysiology of schizophrenia accrues, it is essential that appropriate animal models of the illness be developed that have better translational value. A number of pharmacological, genetic, and neurodevelopmental models have been introduced; however, none of these models has been rigorously evaluated for translational relevance or to satisfy requirements of "face," "construct" and "predictive" validity. Figure 2 shows a diagram of the key behavioural, neurochemical and structural changes expected be present, and to have translational relevance to the three core symptom domains of schizophrenia, in an animal model of the disorder (Jones *et al.*, 2011). The degree of phenomenological similarity between the animal model and the human condition it is meant to simulate is known as face validity. In the context of schizophrenia,

challenges to face validity immediately arise due to the nature of the symptoms of the illness. For examples, some features of schizophrenia are uniquely human (language disorders) and others (hallucinations, delusions) are impossible to determine in animals. However, several symptoms known to have an important impact on illness outcome in schizophrenia can be modelled successfully in animals. These include deficits of information processing (e.g., prepulse inhibition) and several domains of cognition (e.g., attention, working memory). Other symptoms that can be modelled successfully in animals include hyperactivity, sensitivity to psychostimulants, and deficits in latent inhibition. The level of homology between the animal model and the human illness that underlies disease symptoms is known as construct validity. Demonstrating construct validity for animal models of schizophrenia is difficult since neither the underlying neurobiological substrates of the behavioural symptoms nor the cognitive deficits have been clearly established. However, several animal models exhibit some pathophysiological features that have been detected in schizophrenia (e.g., neurotransmitter deficits, enlarged ventricles, decreased hippocampal volume). Predictive validity in drug discovery is primarily defined by the degree to which the model can be used to predict efficacy of a new therapeutic agent in humans. Typically, in drug discovery research, assessing the effects of positive-control compounds provides this type of validation. In schizophrenia, positive controls for some of the symptoms of schizophrenia (e.g., efficacy in prepulse inhibition, impairment models, amphetamine locomotor assays) have been identified but this has been more difficult for the negative and cognitive symptoms. Given the apparent polygenic nature of schizophrenia and the limited

translational significance of pharmacological models, neurodevelopmental models may offer the best chance of success.

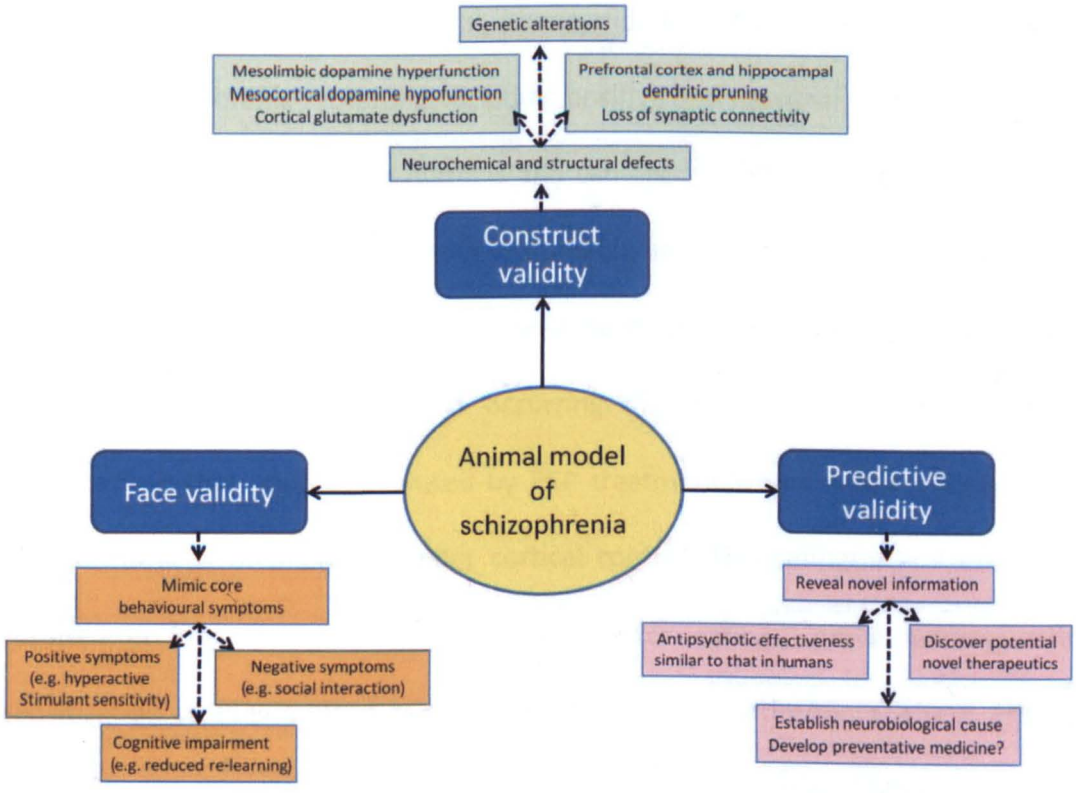


Figure 2: Schematic diagram of the key behavioural, neurochemical and structural changes expected be present and to have translational relevance to the three core symptom domains of schizophrenia in an animal model of the disorder. (Jones *et al.*, 2011)

Many approaches have been taken to represent schizophrenia in animals including pharmacological, i.e.; perinatal PCP and neonatal ventral hippocampal lesions, genetic via knock-out mice and neurodevelopmental, which include social isolation and disruption of neurogenesis using immune activation or the antimitotic Methylazoxymethanol Acetate (MAM). Table 2 shows a table from Jones et al., (2011) comparing different animal models of schizophrenia, however this

introduction will concentrate on models that target the glutamatergic system and the hippocampus.

NMDA receptor antagonists such as phencyclidine (PCP) and ketamine mimic a range of schizophrenia symptoms including positive and negative symptoms as well as thought disorder in normal humans and can elicit a prolonged reoccurrence of the acute psychotic state in patients with stable chronic schizophrenia (Javitt and Zukin., 1991). PCP was administered on post natal days (PND) 7, 9 and 11 (10mg/kg) and as neurodevelopment is still occurring during this time (figure 3) it is hypothesised that apoptosis caused by PCP treatment would result in deficits in function directly or indirectly under cortical control. The perinatal PCP exposure resulted in long lasting deficits in sensorimotor gating, spatial learning and sensitisation to PCP-induced hyperlocomotor activity, furthermore, these effects are blocked by the atypical antipsychotic, olanzapine (Wang *et al.,* 2001).

Table 2: Comparative overview of the changes in basal and psychostimulant-induced locomotor activity in an open-field arena; sensorimotor gating; learning and memory; social interaction with a conspecific, structural and neurochemical changes in cortical and hippocampal areas and the reversal of these changes with antipsychotic and related drugs for selected animal models of schizophrenia. (Jones *et al.*, 2011)

Animal model	Basal- and drug-induced locomotor activity	Sensorimotor gating	Cognition	Social interaction	Structure and neurochemistry	Antipsychotic reversal
Gestational MAM (GD17) (Moore *et al.*, 2006; Lodge *et al.*, 2009)	Spontaneous hyperactivity in novel arena emerging at puberty. Enhanced amphetamine- and NMDA antagonist-induced locomotion.	Deficit in PPI appears at puberty.	Normal acquisition, but impaired re-learning in the Morris water maze; impaired extra-dimensional shift in attentional set-shifting task	Reduced total social interaction appears prior to puberty.	Reduced PFc and hippocampal size, enlarged ventricals, reduced hippocampal soma size and neuropil; enhanced nAcc DA release; spontaneously hyperactive VTA DA neurones; decreased PFc Parvalbumin GABA interneurones	No pharmacological reversal of behaviour attempted. CLZ does not reverse change in BDNF.
Post-weaning social isolation (Lapiz *et al.*, 2003; Fone and Porkess, 2008)	Hyperactivity in a novel arena appearing 2–3 weeks after commencing isolation; hyper-responsivity to amphetamine and cocaine together with increased nAcc DA release	Persistent, but strain-dependent reduction in PPI to acoustic startle appearing about 6 weeks after isolation	Deficit in novel object recognition; no effect on acquisition of spatial learning by impaired reversal learning in water maze, extradimensional shift in the attentional set-shifting task and fear-motivated conditioned emotional response	Increased aggression and increase in total social Interaction	Reduced PFc volume; reduced dendritic spine density, cytoskeletal alteration and loss of parvalbumin-containing interneurones and reelin in the hippocampus; reduced PFc D1 binding, no change in striatal D2 density, but increased proportion of striatal D2 High; increased spontaneously active VTA DA neurones	PPI reversed by atypical antipsychotics, D2 antagonists, a7-nicotinic agonists; novel object discrimination impairment reversed by 5-HT6 antagonists and mGluR2/3 agonist
Amphetamine models (Featherstone *et al.*, 2007a; Featherstone *et al.*, 2008; Sarter *et al.*, 2009)	Sensitization of locomotor response to amphetamine	Persistent deficit in PPI dependent on dosage regimen	Deficits in attention and the attentional set-shifting task; hippocampal- dependent memory unimpaired	No reduction in social interaction	Enhanced mesolimbic DA response; altered Ach function in PFc	Locomotor sensitization blocked by CLZ and HLP; moderate attenuation of attention impairment by CLZ and HLP
PCP models (Jentsch and Roth, 1999; Phillips *et al.*, 2001; Mouri *et al.*, 2007; Neill *et al.*, 2010)	Sensitization of locomotor response to PCP; hyper-responsive locomotor response to amphetamine and mild stress	No sustained deficit in PPI	Deficits in novel object recognition, attentional set shifting and T-maze delayed alternation	Reduced frequency and duration of primate social behaviour	Reduced basal and stress-induced PFc DA and glutamate release; decreased synaptic spines on Fc neurones and cortical and hippocampal parvalbumin-positive neurones	Deficits in reversal learning reversed by atypical antipsychotics but not HLP; locomotor sensitization attenuated by CLZ and HLP

Neonatal ventral hippocampal lesion (Lipska, 2004; Tseng *et al.*, 2009)	Locomotor hyper-responsivity to stress, amphetamine and NMDA receptor antagonists; enhanced apomorphine-induced stereotypy	Adult onset deficit in PPI	Impaired acquisition of T-maze delayed alternation and water maze; impaired radial arm maze choice accuracy; selective deficit in extra-dimensional shift and reversal in the attentional set-shifting task	Deficits in social interaction with increased aggression at all developmental ages	Unaltered basal nAcc DA release, but enhanced response to stress or amphetamine; reduced mPFc NAA levels and GAD67 mRNA expression	Amphetamine-induced hyperactivity reversed by acute or chronic antipsychotic injection; social interaction deficit not reversed by CLZ
DISC-1 knock-out (Jaaro-Peled, 2009)	Hyperactivity seen in L100P, CaMK-DC mutants, but not in others; no data available regarding psychostimulant-induced locomotor activity to date	Deficits in PPI seen in some (e.g. constitutive CaMK-DC, L100P, Q31L), but not all mutants (e.g. inducible CaMK-DC, D25 bp); PPI not tested in CaMK cc or BAC DC mutants	Impaired T-maze performance seen in most strains; impaired spatial working memory only seen in female CaMK-DC inducible mutants	Reductions in social activity seen in some strains (e.g. Q31L) and some CaMK- DC transgenics	Reduced brain volume in most strains; enlarged lateral ventricles, reduced hippocampal and PFc dendritic density, structure and complexity in some strains; reduced hippocampal parvalbumin immunoreactivity in some, but not all mutants	PPI deficits in L100P mice reversed by HLP and CLZ
Neuregulin1 and ErbB4 knock-out (Harrison and Law, 2006a; Mei and Xiong, 2008)	Most, but not all, neuregulin and ErbB4 mutants show spontaneous locomotor hyperactivity, but inconsistent responses to psychostimulants	PPI deficits seen in most neuregulin mutants reviewed; ErbB4 mutants show normal PPI	Impaired contextual fear and mismatched negativity performance in some mutants	Some deficits in social interaction, increased aggression and reduced responses to social novelty	Increased lateral ventricles and reduced hippocampal spine density; reduction in functional forebrain NMDA receptors	Spontaneous and psychostimulant induced Locomotor hyperactivity reversed by CLZ in Nrg1(DTM)+/- and Nrg1(BACE)-/- mutants
Dysbindin knock-out (Karlsgodt *et al.*, 2011; Papaleo *et al.*, 2010)	Spontaneous locomotor hyperactivity and hyper-responsivity to amphetamine challenge	Increased PPI and startle response shown to be reversed by quinpirole, but not eticlopride	Increased acquisition of T-maze task; impaired spatial reference memory and novel object recognition performance	Reduced social contact during social interaction task	Hyperexcitability of PFc pyramidal neurones; altered synaptic structure and formation; elevated HVA/DA ratio in cortico-limbic regions	No data on antipsychotic reversal
Reelin knock-out (Krueger *et al.*, 2006; Tueting *et al.*, 2006)	Reduced locomotion in an open field; enhanced response to methamphetamine	Variable PPI responses, highly dependent on strain, environment and testing protocol	Few memory deficits reported; normal reversal learning and inhibitory control, normal MWM performance; some learning deficits in acquisition of operant tasks	Some modulation of social activity in novelty and/or interaction tasks	Increased neuronal packing and decreased dendritic spine density in PFc and hippocampal neurones	Normalization of reduced spontaneous activity by OLZ

The neonatal ventral hippocampal lesion (NVHL) model reproduces several behavioural abnormalities observed in schizophrenia, including hypersensitivity to stimulants, hyperactivity, reduced social interactions, and impaired working memory (Lipska and Weinberger., 2000). In this model, ibotenic acid is infused directly into rat ventral hippocampus on PND 7 which leads to a number of dopamine related behaviours that resemble behaviour seen in animals sensitised to psychostimulants. Chronic Clozapine and Risperidone treatment reverse the locomotor hyperactivity and PPI deficits, but not social interaction deficits seen, in this model (Rueter *et al.,* 2004). However this model lacks construct validity as schizophrenia brains do not manifest a lesion analogous to the ventral hippocampal lesion in this model.

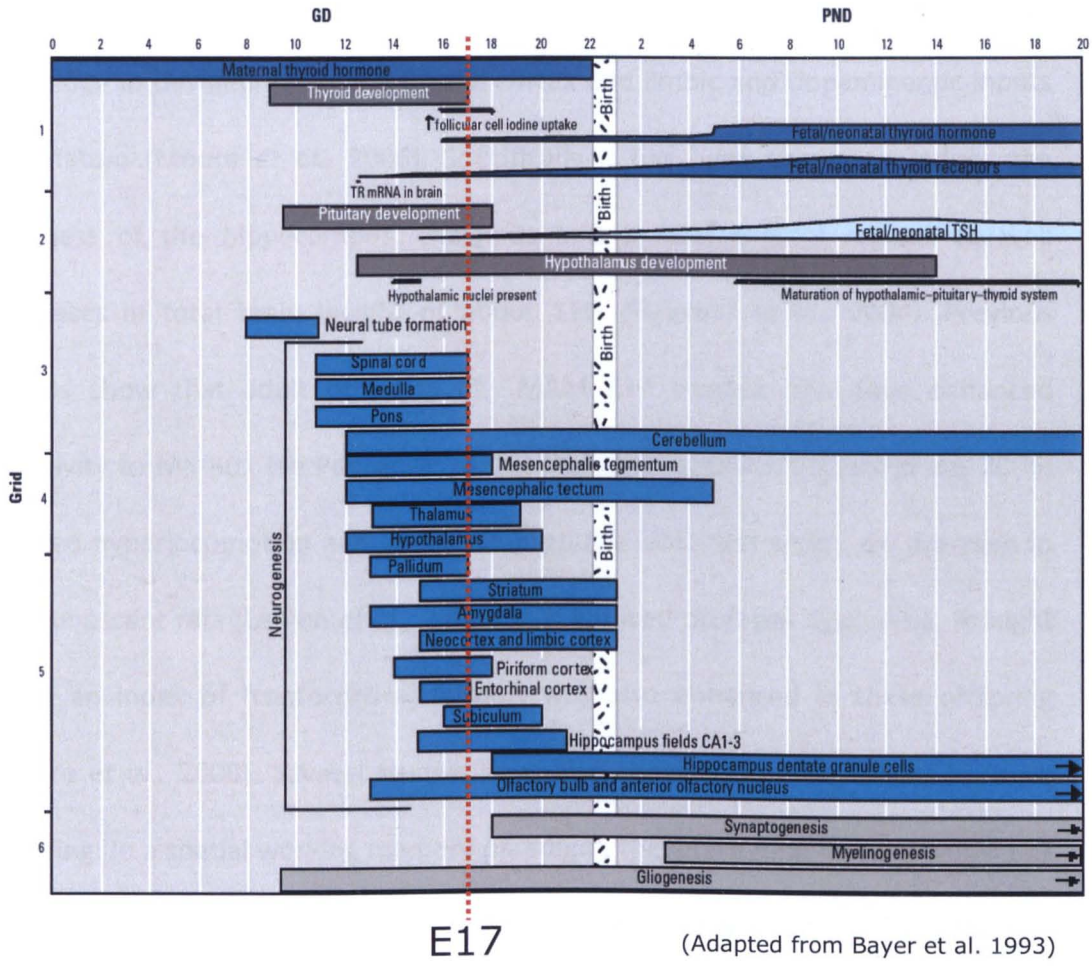


Figure 3: Timeline of brain development from conception to PND20.
Conception = GD0 and birth = PND1.

Methylazoxymethanol (MAM) is a neurospecific antimitotic agent that prevents cells from dividing for a short time after injection (Balduini *et al.,* 1991; Cattabeni and Luca., 1997). Injection on embryonic day 17 (E17) is therefore likely to have a major impact on the brain structures that are actively developing at this time (see figure 3), including the cortical areas, hippocampus and limbic system which have been shown to be deficit in function in schizophrenia patients (Liddle at al 2006). MAM-E17 exposure also leads to a pattern of histopathology similar to that

observed in schizophrenia and behavioural abnormalities with translational homology to dysfunction of the frontal cortex and limbic and dopaminergic inputs to striatum (Moore *et al.,* 2006). Specifically it has been shown to reduce the thickness of the hippocampus, thalamus and several cortical regions as well decreases in total brain weight of about 11% (Flagsted *et al.,* 2004). Previous studies show that adult offspring of MAM E17 treated rats have enhanced sensitivity to MK-801 (Le Pen *et al.,* 2006) and amphetamine (Moore *et al.,* 2006) induced hyperlocomotion and deficits in prepulse inhibition which are not seen in pre-pubescent rats (Le Pen *et al.,* 2006). PCP induced orofacial dyskinesia, thought to be an index of frontocortical lesions, was also enhanced in these offspring (Moore *et al.,* 2006). Several studies have also shown cognitive deficits in these offspring. In a spatial working memory paradigm, the alternating Y-maze, MAM E17 rats learnt the initial rule quicker than the controls but too longer to learn the rule reversal (Moore *et al.,* 2006). In a radial arm maze task, MAM E17 rats were again able to learn the task but were impaired in the spatial working memory component (Gourevitch *et al.,* 2004). In the attentional set-shifting bowl digging task, MAM E17 rats took significantly more trials to reach criterion in the reversal and extra-dimensional shift trials (Featherstone *et al.,* 2007, Gastambide *et al.,* 2012). MAM E17 injected rats may therefore be an important neurodevelopmental model for schizophrenia.

1.7 Measuring changes in animal models of schizophrenia

Because of the complexity of schizophrenia the approach to the development of animal models relies on focusing on specific phenotypic signs or symptoms associated with the disorder rather than mimicking the entire syndrome. By focusing on specific signs and symptoms rather than the syndrome it increases the confidence in the cross species validity of the model. Here specific observations that have been identified in schizophrenic patients provide a focus for study and therefore the validation of any model can only be as sound as the information available in the relevant clinical literature. Another approach is based more on theoretically on psychological constructs believed to be affected in schizophrenia. These include attention, perseveration, sensorimotor gating and working memory. Behavioural measures have been used extensively for establishing the validity of animal models in schizophrenia. Hyperlocomotion is used primarily as a functional measure of dopaminergic activity in response to neuroleptics but antagonism of locomotor activity is thought to be comparable with the positive symptoms of schizophrenia. Other behavioural measures, such as disruption of prepulse inhibition of acoustic startle or impaired attentional set shifting also resemble characteristics of schizophrenia. These measures are useful for establishing the construct and predictive validity of putative animal models (Willner., 1986).

Changes in locomotor activity in rodents have often been used to assess both models of schizophrenia and the effects of antipsychotic treatments. Cross species studies in animals treated with psychostimulants show hyperlocomotor activity and at higher doses stereotypic and perseverative behaviours which are thought to have face validity with the stereotyped behaviours induced by amphetamine, PCP and ketamine in humans.

Clinical observations in schizophrenic patients have also identified deficiencies in the processing of information including inability to filter or 'gate' irrelevant thoughts and sensory stimuli. Numerous studies have observed deficits in the habituation of startle responses in schizophrenic patients which may reflect failures of sensory filtering leading to disorders of cognition. Prepulse inhibition (PPI) is a test of pre-attentional sensorimotor gating and is based on the fact that a weak prestimulus presented 30-50 milliseconds before a startling stimulus reduces or 'gates' the amplitude of the startle response (Geyer and Swerdlow., 1998). This phenomenon is robust, is observed in many species and is evident both within and between multiple sensory modalities when a variety of stimulus parameters are used. These stimulus-evoked changes in PPI are similar in humans and rats, and DA agonists, such as apomorphine, and NMDA antagonists such as PCP disrupt PPI in both species, so mimicking the PPI deficits observed in patients with schizophrenia. The administration of antipsychotic drugs can also restore this psycho stimulant induced PPI deficit (Geyer *et al.*, 2001).

Cognitive deficits are reported across all subtypes of schizophrenia including impairments of working memory, attention, verbal memory and set shifting. The limited cognitive capacity of rodents hinders the development of cognitive tasks that can be considered to be entirely analogous to human tasks such as the Wisconsin Card Sorting Task and Continuous Performance Test. Some common rodent cognitive assays, such as delayed matching to position, delayed alternation, fear conditioning, reversal learning and the five choice serial reaction time task have been developed to look at specific aspects of learning and memory and it is important to utilise a battery of such tasks when evaluating any new animal model of the disorder.

1.8 Aims of the thesis

This thesis aims to investigate the validity of the MAM E17 model as a model for schizophrenia.

The first aim was to characterise the model in different strains of rats and to also assess any potential sex and age effects. This initially involved establishing the MAM E17 pup generation protocol within the institution. A battery of tests was used to measure different aspects of behaviour: locomotor activity and prepulse inhibition of acoustic startle.

The second aim was to assess the effect of MAM treatment on cognition using an operant reversal learning task and a fear conditioning task and to examine whether any deficits could be reversed using a potentially cognition enhancing compound.

Chapter 2: Validation

2.1 Introduction

The aetiology of schizophrenia is still unclear but studies suggest that genetics, early environment, neurobiological and psychological processes are all important contributory factors (Rapoport *et al.,* 2005). There are similar rates of incidence and prevalence of schizophrenia between genders, however differences exist in terms of manifestation and course. Female patients present more affective and paranoid symptoms, whereas males show more negative symptoms (Lewine., 1981). Furthermore, females tend to have a 3–4-year delay of symptom onset and first hospitalization compared with males, and a second peak at the onset of menopause (Hafner *et al., 1993;* Maurer *et al.*, 1993), thus any animal model needs to include sex comparisons.

Preschizophrenic individuals exhibit minor deviations in motor, cognitive, and social development, suggesting that abnormalities in brain function are present very early in life in individuals who later develop schizophrenia (Rapoport *et al.,* 2005). Consistent with this, post-mortem studies have revealed reduced volume, decreased neuronal size, and a loss and/or malpositioning of cells in the hippocampus and prefrontal, entorhinal and cingulate cortices of schizophrenic patients (Harrison and Weinberger., 2005).

As mentioned in the previous chapter Methylazoxymethanol (MAM), when given on embryonic day 17 (MAM E17), it leads to a pattern of histopathology similar to that observed in schizophrenia, namely reduced thickness of the hippocampus, thalamus and several cortical regions as well decreases in total brain weight of about 11% (Flagstad *et al.*, 2004).

Only a basic assessment of MAM in behaviour has been researched so far, and the aim of the studies was to firstly establish the model on site and then to further behaviourally characterise the model. A strain comparison will be performed as there is no literature on the MAM model in Lister Hooded rats and there are literature reports of strain differences in behavioural tasks. Weiss *et al.,* (2000) investigated the effects of isolation rearing on acoustic startle response, PPI and locomotor activity in Sprague Dawley (SD) and Lister Hooded (LH) rats and showed that LH rats had increased locomotor activity compared to the SD strain and less sensitivity to apomorphine in PPI. Another laboratory looked at the effect of dosing LH and SD rats daily with nicotine and then microdialysis studies were performed on day 9 with a challenge dose of nicotine of 0.4 mg/kg. SD rats pre-treated with nicotine showed increased basal overflow of DA in the accumbal core and pre-treatment with 0.1 and 0.3mg/kg also resulted in sensitisation of the response to a 0.4 mg/kg nicotine challenge. In LH rats, pre-treatment with nicotine reduced basal overflow in the accumbal core and did not cause sensitisation to a subsequent challenge with nicotine (Iyaniwura *et al.,* 2001). Mirza and Bright (2001) tested LH and CD rats in the 5CSRT task and their responses to nicotine. CD rats only reached

an accuracy of approximately 60% whereas LH rats reached 80%. Higher doses of nicotine improved the accuracy in the task in SD rats but not the LH rats.

We also performed sex comparison of MAM treatment since the age of onset and symptoms of schizophrenia depends on gender, and all previous MAM studies exposure have been on male pups. Animal neurochemical data shows there are sex differences in central dopamine levels (Castner *et al.,* 1993) as well as sensitivity to amphetamine (Beatty and Holtzer., 1978). Male rats also seem to be affected to a greater extent by neonatal ventral hippocampal lesions than females. In tests of social interaction, the number of encounters were significantly decreased in female lesioned rats whereas male lesioned rats showed a significantly reduced duration of active social interactions. Furthermore, a deficit of spatial learning and memory was only shown in male lesioned rats in the Morris Water Maze test rats (Silva-Gomez 2003).

The effects of dosing MAM on E15 as well as E17 was also be examined in the current project. Prenatal exposure to MAM at E15 affects primarily telencephalic (endbrain) areas such as cortex and hippocampus. Treatment at this time also leads to gross motor impairments, including ataxia and a blunted startle reflex. A marked impairment in rule learning, reversal learning and passive avoidance tasks is observed in these rats (Moore *et al.,* 2006; Balduini *et al.,* 1991). MAM treatment at E15 was therefore used as a positive control.

The effect of MAM treatment on locomotor activity, prepulse inhibition of the acoustic startle response and brain and body weight was measured together with the effect of MK-801 on locomotor activity.

2.2 Methods

2.2.1 Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals

2.2.1a Subjects

Twenty four timed-pregnant Sprague Dawley (Crl:CD(SD)) (SD) and Lister Hooded (Crl:LIS) (LH) dams, were obtained from Charles River, Margate, UK on E10 and randomly split into 4 groups. Each group was treated with either MAM or saline (SHAM group) on either embryonic day 15 (E15) or day 17 (E17). 4 days after birth the litters were culled to 10, keeping equal numbers of males and females where possible and only those litters born on E22 were kept. On postnatal day 28 the pups were weaned and re-housed in groups of 3 or 4 with non-littermates of the same strain, sex and treatment. The cages of rats were then randomly assigned to two groups, juvenile (tables 1 and 2) and adult (tables 4 and 5) and those animals tested at juvenile age were not retested at adulthood. The timeline for testing is depicted in figure 1 and the n numbers are in shown in table 3.

All experiments were conducted in full compliance with the Home Office Guidance (Animals (Scientific Procedures) Act 1986, project licence 70/6560) and the ethical policies of Eli Lilly. Facilities were also AAALAC (Association for Assessment and

Accreditation of Laboratory Animal Care) accredited. Housing rooms were temperature-controlled (21± 1° C) and maintained on a 12: 12 h light/dark cycle (lights on at 07:00). Food and water were available *ad libitum*.

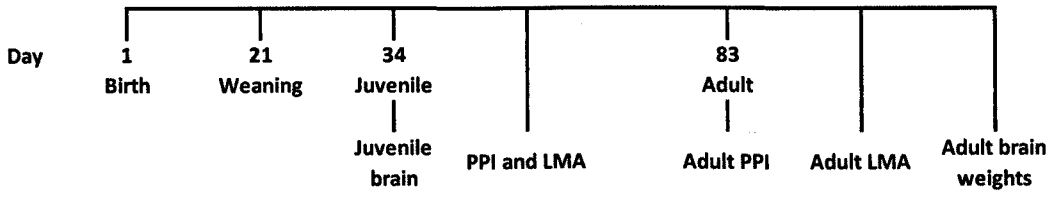


Figure 1: Timeline of events and studies performed for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals

2.2.1b Drugs

Methylazoxymethanol acetate (MAM) was obtained from Midwest Research Institute (Kansas City, Missouri, http://www.mriresearch.org) and was administered intraperitoneally (i.p.) at a dose of 28 mg/kg, expressed as salt weight. MAM was dissolved in 0.9% saline and administered in a volume of 1 ml/kg. MAM is light sensitive and was stored, formulated and maintained in light-restricting bottles.

MK-801 Maleate (Sigma Aldrich, UK) was administered subcutaneously (s.c.) at a dose of 0.1 mg/kg, as base, in 5% glucose in a volume 1 mg/ml.

2.2.1c Neuroanatomy

2.2.1c(i) Brain weights and Tissue

Animals were terminally anaesthetised using isoflurane and then trans-cardially perfused with 0.9% saline followed by 10% buffered formalin. The brain was

removed and stored in the 10% buffered formalin and weighed 24hrs later. The brains were then placed on clean absorbent tissue and photographs taken. Perfused brains were kept in formalin before being cut into segments using an ASI Rodent Brain Matrix Coronal RBM-8000C (pup matrix) for the juvenile samples and ASI Rodent Brain Matrix Coronal RBM-4000C (adult matrix) Bilaney Consultants Ltd, UK). The juvenile brains were cut into two segments (forebrain and hindbrain) and the adult brains cut into 3 segments (bregma 6.12 to bregma -1.32mm, bregma -1.32 mm to bregma -6.84 mm and bregma -6.84 mm to bregma – 13.20 mm) and placed in mega-cassettes (Medim, UK) of the TISSUE-TEK VIP2000 vacuum infiltration processor (Miles Scientific, Bayer Diagnostics, UK) and processed overnight (see (O'Neill and Clemens 2001). Brain segments were then embedded in blocks of paraffin wax for subsequent histopathology. Coronal sections (8μm) were taken from bregma 6.12mm to bregma -9.96 mm. A second cohort of brains were processed using multibrain technology (NeuroScience Associates (NSA), Knoxville, TN, USA, www.neuroscienceassociates.com) and 40μm sections cut using a freezing microtome.

2.2.1c(ii) Neuropathology and image analysis

Sections from selected stereotaxic levels throughout the brain were stained with cresyl violet using an X-Y Varistainer (ThermoShandon, UK). On completion of staining the slides were cover slipped with Consul-mount™ using an automated Shandon Consul® coverslipper (ThermoShandon, Cheshire, UK). The slides were cleaned and the samples from the first study were scanned using SprintScan 35

hardware (model CS2700, Polaroid) and the images analysed using Optimus 5.2 software. For all subsequent studies, slides were scanned using Aperio Scanscope XT hardware and digital images generated using ImageScope software (Aperio Technologies Inc.). To confirm the key pathological changes in the MAM tissues, the areas of the medial prefrontal cortex (mPFC) at bregma 4.68mm, the dorsal hippocampus at bregma -3.12 and the lateral ventricles at bregma -4.08 was calculated by tracing around the structure and compared to the area of the same structure in SHAM treated rats.

Table 1: Detail of CD Sprague Dawley pups tested in PPI, LMA and used for histology at the juvenile timepoint for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals.

CD							
Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage
E276	MAM E15	Male	1	C263	MAM E17	Female	30
K340	MAM E15	Male	1	F292	MAM E17	Female	30
P381	MAM E15	Male	1	S412	MAM E17	Female	30
Q389	MAM E15	Male	1	X467	MAM E17	Female	30
B245	MAM E15	Male	10	D272	MAM E17	Female	32
K339	MAM E15	Male	10	M358	MAM E17	Female	32
L350	MAM E15	Male	10	U435	MAM E17	Female	32
T420	MAM E15	Male	10	X464	MAM E17	Female	32
B252	MAM E15	Female	12	H308	SHAM E17	Male	54
K342	MAM E15	Female	12	I316	SHAM E17	Male	54
P387	MAM E15	Female	12	R400	SHAM E17	Male	54
Q394	MAM E15	Female	12	W450	SHAM E17	Male	54
E279	MAM E15	Female	15	H309	SHAM E17	Female	57
L353	MAM E15	Female	15	I320	SHAM E17	Female	57
Q392	MAM E15	Female	15	R406	SHAM E17	Female	57
T427	MAM E15	Female	15	W455	SHAM E17	Female	57
C257	MAM E17	Male	23	A234	SHAM E15	Male	41
F284	MAM E17	Male	23	G297	SHAM E15	Male	41
N362	MAM E17	Male	23	J327	SHAM E15	Male	41
U429	MAM E17	Male	23	V441	SHAM E15	Male	41
D264	MAM E17	Male	26	A243	SHAM E15	Female	49
F289	MAM E17	Male	26	G303	SHAM E15	Female	49
N364	MAM E17	Male	26	J332	SHAM E15	Female	49
X458	MAM E17	Male	26	V446	SHAM E15	Female	49

Table 2: Detail of Lister hooded pups tested in PPI, LMA and used for histology at the juvenile timepoint for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals.

LH							
Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage
C19	MAM E15	Male	2	F45	MAM E17	Female	33
D28	MAM E15	Male	2	I79	MAM E17	Female	33
G55	MAM E15	Male	2	M115	MAM E17	Female	33
P144	MAM E15	Male	2	O141	MAM E17	Female	33
A3	MAM E15	Male	7	F49	MAM E17	Female	35
E38	MAM E15	Male	7	H69	MAM E17	Female	35
L106	MAM E15	Male	7	M118	MAM E17	Female	35
X220	MAM E15	Male	7	V209	MAM E17	Female	35
D30	MAM E15	Female	10	K94	SHAM E15	Male	41
L107	MAM E15	Female	10	N123	SHAM E15	Male	41
P148	MAM E15	Female	10	S175	SHAM E15	Male	41
C26	MAM E15	Female	10	U191	SHAM E15	Male	41
D34	MAM E15	Female	13	K99	SHAM E15	Female	46
G61	MAM E15	Female	13	N131	SHAM E15	Female	46
X223	MAM E15	Female	13	S177	SHAM E15	Female	46
A7	MAM E15	Female	13	U198	SHAM E15	Female	46
B13	MAM E17	Male	18	J85	SHAM E17	Male	50
I73	MAM E17	Male	18	Q153	SHAM E17	Male	50
O134	MAM E17	Male	18	W210	SHAM E17	Male	50
T184	MAM E17	Male	18	R163	SHAM E17	Male	50
B9	MAM E17	Male	21	J89	SHAM E17	Female	59
I72	MAM E17	Male	21	Q156	SHAM E17	Female	59
M114	MAM E17	Male	21	R169	SHAM E17	Female	59
V204	MAM E17	Male	21	W216	SHAM E17	Female	59

Table 3: n numbers for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals.

		CD					LH				
Group	Sex	PPI		LOCO			PPI		LOCO		
		Juvenile	Adult	Juvenile	Adult Vehicle	Adult MK-801	Juvenile	Adult	Juvenile	Adult Vehicle	Adult MK-801
E15 MAM	Male	8	16	8	8	8	8	16	8	8	8
E15 MAM	Female	8	16	8	8	8	8	16	8	8	8
E17 MAM	Male	8	16	8	8	8	8	16	8	8	8
E17 MAM	Female	8	16	8	8	8	8	16	8	8	8
E15 SHAM	Male	4	12	4	6	6	4	12	4	6	6
E15 SHAM	Female	4	12	4	6	6	4	12	4	6	6
E17 SHAM	Male	4	12	4	6	6	4	12	4	6	6
E17 SHAM	Female	4	12	4	6	6	4	12	4	6	6
		48	112	48	56	56	48	112	48	56	56

Table 4: Detail of CD Sprague Dawley pups tested in PPI, LMA and used for histology at the adult timepoint for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals.

CD											
Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage
B249	MAM E15	Male	2	C260	MAM E17	Female	33	A241	SHAM E15	Female	47
L346	MAM E15	Male	2	N365	MAM E17	Female	33	G299	SHAM E15	Female	47
P378	MAM E15	Male	2	S415	MAM E17	Female	33	J330	SHAM E15	Female	47
T422	MAM E15	Male	2	U437	MAM E17	Female	33	V443	SHAM E15	Female	47
K335	MAM E15	Male	4	D273	MAM E17	Female	34	G298	SHAM E15	Female	48
K338	MAM E15	Male	4	M357	MAM E17	Female	34	G300	SHAM E15	Female	48
Q388	MAM E15	Male	4	S414	MAM E17	Female	34				48
Q390	MAM E15	Male	4	U436	MAM E17	Female	34				48
B247	MAM E15	Male	5	D270	MAM E17	Female	36	I318	SHAM E17	Male	50
K336	MAM E15	Male	5	F291	MAM E17	Female	36	R399	SHAM E17	Male	50
P380	MAM E15	Male	5	U433	MAM E17	Female	36				50
T421	MAM E15	Male	5	X463	MAM E17	Female	36				50
E274	MAM E15	Male	6	D269	MAM E17	Female	38	R402	SHAM E17	Male	51
L347	MAM E15	Male	6	M359	MAM E17	Female	38	W451	SHAM E17	Male	51
P379	MAM E15	Male	6	U432	MAM E17	Female	38				51
Q391	MAM E15	Male	6	X465	MAM E17	Female	38				51
E277	MAM E15	Female	13	E282	MAM E15	Female	17	H304	SHAM E17	Male	52
K341	MAM E15	Female	13	O373	MAM E15	Female	17	I315	SHAM E17	Male	52
P386	MAM E15	Female	13	P382	MAM E15	Female	17	R398	SHAM E17	Male	52
T423	MAM E15	Female	13	Q395	MAM E15	Female	17	W449	SHAM E17	Male	52
E281	MAM E15	Female	14	A239	SHAM E15	Male	40	H305	SHAM E17	Male	53
L352	MAM E15	Female	14	J328	SHAM E15	Male	40	I317	SHAM E17	Male	53
O376	MAM E15	Female	14	V440	SHAM E15	Male	40	R401	SHAM E17	Male	53
T426	MAM E15	Female	14				40	W448	SHAM E17	Male	53
B253	MAM E15	Female	16	A236	SHAM E15	Male	42	H310	SHAM E17	Female	55
E280	MAM E15	Female	16	G295	SHAM E15	Male	42	R403	SHAM E17	Female	55
O374	MAM E15	Female	16	J326	SHAM E15	Male	42				55
Q396	MAM E15	Female	16	V438	SHAM E15	Male	42				55
D266	MAM E17	Male	22	A237	SHAM E15	Male	43	I319	SHAM E17	Female	56
F287	MAM E17	Male	22	G296	SHAM E15	Male	43	R404	SHAM E17	Female	56
S410	MAM E17	Male	22				43				56
X460	MAM E17	Male	22				43				56
C254	MAM E17	Male	24	A235	SHAM E15	Male	44	H311	SHAM E17	Female	58
F285	MAM E17	Male	24	G294	SHAM E15	Male	44	I322	SHAM E17	Female	58
S411	MAM E17	Male	24	J325	SHAM E15	Male	44	R405	SHAM E17	Female	58
U428	MAM E17	Male	24				44	W456	SHAM E17	Female	58
D267	MAM E17	Male	27	A242	SHAM E15	Female	45	H313	SHAM E17	Female	59
M354	MAM E17	Male	27	G302	SHAM E15	Female	45	I321	SHAM E17	Female	59
N360	MAM E17	Male	27				45	W452	SHAM E17	Female	59
X461	MAM E17	Male	27				45	R407	SHAM E17	Female	59
D265	MAM E17	Male	29	A240	SHAM E15	Female	46				
M355	MAM E17	Male	29	G301	SHAM E15	Female	46				
S408	MAM E17	Male	29	J333	SHAM E15	Female	46				
U430	MAM E17	Male	29	V447	SHAM E15	Female	46				

Table 5: Detail of Lister Hooded pups tested in PPI, LMA and used for histology at the adult timepoint for Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals.

LH											
Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage	Pup ID	Group	Sex	Cage
A1	MAM	Male	1	B14	MAM E17	Male	26	K98	SHAM E15	Female	47
C22	MAM	Male	1	H62	MAM E17	Male	26	N129	SHAM E15	Female	47
G52	MAM	Male	1	T183	MAM E17	Male	26	U195	SHAM E15	Female	47
X219	MAM	Male	1	O136	MAM E17	Male	26	U194	SHAM E15	Female	47
C21	MAM	Male	4	F50	MAM E17	Fem	28	U197	SHAM E15	Female	48
L104	MAM	Male	4	I78	MAM E17	Fem	28	K101	SHAM E15	Female	48
P145	MAM	Male	4	M121	MAM E17	Fem	28				48
X222	MAM	Male	4	V205	MAM E17	Fem	28				48
A2	MAM	Male	6	B16	MAM E17	Fem	29	K100	SHAM E15	Female	49
C23	MAM	Male	6	H70	MAM E17	Fem	29	N130	SHAM E15	Female	49
G54	MAM	Male	6	O140	MAM E17	Fem	29				49
P142	MAM	Male	6	V206	MAM E17	Fem	29				49
A4	MAM	Male	8	B15	MAM E17	Fem	31	W213	SHAM E17	Male	51
G53	MAM	Male	8	H68	MAM E17	Fem	31	J82	SHAM E17	Male	51
L105	MAM	Male	8	M116	MAM E17	Fem	31				51
X221	MAM	Male	8	T188	MAM E17	Fem	31				51
C25	MAM	Female	11	F48	MAM E17	Fem	32	J83	SHAM E17	Male	52
L109	MAM	Female	11	I76	MAM E17	Fem	32	R161	SHAM E17	Male	52
P151	MAM	Female	11	M119	MAM E17	Fem	32				52
D31	MAM	Female	11	V207	MAM E17	Fem	32				52
C24	MAM	Female	12	K93	SHAM E15	Male	39	J84	SHAM E17	Male	53
D29	MAM	Female	12	N124	SHAM E15	Male	39	R162	SHAM E17	Male	53
G60	MAM	Female	12	U190	SHAM E15	Male	39	W211	SHAM E17	Male	53
P149	MAM	Female	12	S176	SHAM E15	Male	39	R166	SHAM E17	Male	53
A6	MAM	Female	14	U193	SHAM E15	Male	40	R164	SHAM E17	Male	54
D35	MAM	Female	14	K92	SHAM E15	Male	40	Q152	SHAM E17	Male	54
L110	MAM	Female	14				40	R165	SHAM E17	Male	54
X225	MAM	Female	14				40	W212	SHAM E17	Male	54
A5	MAM	Female	15	K95	SHAM E15	Male	42	J87	SHAM E17	Female	55
D33	MAM	Female	15	S170	SHAM E15	Male	42	Q157	SHAM E17	Female	55
G59	MAM	Female	15	S174	SHAM E15	Male	42				55
P150	MAM	Female	15	U192	SHAM E15	Male	42				55
F43	MAM	Male	19	K96	SHAM E15	Male	43	J88	SHAM E17	Female	56
I75	MAM	Male	19	N125	SHAM E15	Male	43	R167	SHAM E17	Female	56
O132	MAM	Male	19				43	W214	SHAM E17	Female	56
V202	MAM	Male	19				43	Q155	SHAM E17	Female	56
B10	MAM	Male	22	K97	SHAM E15	Male	44	J86	SHAM E17	Female	57
H63	MAM	Male	22	N122	SHAM E15	Male	44	J91	SHAM E17	Female	57
O135	MAM	Male	22				44	Q159	SHAM E17	Female	57
T185	MAM	Male	22				44	W215	SHAM E17	Female	57
B12	MAM	Male	24	N126	SHAM E15	Fem	45	J90	SHAM E17	Female	58
I74	MAM	Male	24	N127	SHAM E15	Fem	45	Q158	SHAM E17	Female	58
T181	MAM	Male	24	S179	SHAM E15	Fem	45				58
V203	MAM	Male	24	U199	SHAM E15	Fem	45				58

2.2.1d Behavioural Analyses

2.2.1d(i) Locomotor Activity

Locomotor activity was measured in complete darkness (LUX value of zero using Testo 435 meter) in 16 clear Perspex boxes (40x40x30cm) located on infrared fields. Four boxes were placed on each field, which were monitored using overhead infrared cameras. The cameras fed into a Quad compressor unit, which in turn fed a PC running the image analysis application Ethovision (Noldus). Ethovision digitizes the path made by animals at a rate of 25 frames per second and uses this to calculate various different parameters.

At the beginning of a trial, rats were placed in the centre of the arena and the behaviour exhibited during a 30 min habituation period was recorded. For the adult rats, at the end of this period they were removed, given an injection of vehicle or MK-801 (0.1 mg/kg) and returned to the same arena, and behaviour was recorded for a further 120 min. The distance travelled in the arena by the animals was analyzed in 5-min intervals.

2.2.1d(ii) Prepulse Inhibition of the Acoustic Startle Response

Testing was conducted in eight ventilated sound-attenuated startle chambers (San Diego Instruments, CA), controlled by SDI software (SR-LAB, San Diego Instruments, CA). Each chamber contained a clear Plexiglas tube mounted on a platform in a ventilated and illuminated chamber. Acoustic pulses and prepulses were delivered via a speaker located above the tube. Movement inside the tube was detected by a

piezoelectric accelerometer below the frame. The amplitude of the whole body startle to an acoustic pulse was defined as the maximum of 100 one-millisecond accelerometer readings collected from pulse onset. The volume of the loud speakers had been previously measured using a sound level meter (Radioshack) and the speakers in all 8 boxes were calibrated to be within 3dB of each other.

The procedure comprised two sessions: the habituation and the test sessions (adapted from (Swerdlow and Geyer 1998), separated by 24 hours. On day one, the rats were placed in startle chambers (San Diego Instruments, Inc., San Diego, California) and habituated to a background noise level of 67 dB for 15 minutes, followed by eight startle trials (40 ms, 120 dB); the intertrial interval (ITI) averaged 15s (range 10-20s). The total session lasted seventeen minutes.

For the test session, a five minute acclimation period (white noise 67dB) preceded a sequence of six startle trials. Prepulse inhibition of startle (PPI) was quantified using background noise (white noise 67dB), 120 dB startle tone (white noise burst, Pulse (P)) of 40 milliseconds duration, prepulses (ppP) ranging from 4 to 16 dB above background of 40 milliseconds duration, and inter-trial intervals of 10-15 seconds. Prepulses preceded 75% of the startle trials with an inter-stimulus interval of 120 milliseconds. A sequence of six startle tones ended the session.

Percentage prepulse inhibition was calculated at each of the four prepulse intensities using the following formula:

% PPI = 100 * [(P – ppP) / P] where P = pulse and ppP = prepulse

2.2.1e Statistical analysis

All statistical analysis were calculated using STATISTICA v.7 (Statsoft Inc.) and all data are expressed as mean ± standard error of the mean (s.e.m.). In general, data were analysed using analysis of variance, followed, when appropriate, by planned comparisons (univariate test of significance) with between-subject factors of group (MAM; SHAM) vs. treatment (E15; E17) or sex (M; F). Significant interactions were further submitted to analyses of simple effects and, if appropriate, to planned comparisons. In all cases, $P<0.05$ indicated significance.

2.2.1e(i) Litter Size and Brain and Body Weight

As body weights between albino (Sprague Dawley, Wistar) and Lister Hooded rats differ considerably at the same age, brain and body weights were analysed separately for each strain using two-way analyses of variance with between subject factors of group (MAM; SHAM), treatment (E15; E17) and sex (M; F). Litter size was analysed using two-way analyses of variance with between subject factors of strain (SD; LH) so they could be directly compared.

2.2.1e(ii) Locomotor Activity

A general linear model approach was used for all multivariate ANOVAs calculated for Ethovision parameters.

Spontaneous locomotor activity in juvenile animals – Data were submitted to a two-way between-subjects analysis of variance with factors of sex (M; F) and MAM treatment (E15; E17). This analysis was conducted for each strain separately.

MK-801-induced changes in locomotor activity - Separate analyses were conducted on the 'Habituation' phase and 'Run' phases of the experiment. Data generated during the habituation phase was submitted to two-way between-subjects analysis of variance for each strain. Data generated for each strain during the run phase was submitted to a three-way ANOVA with factors of sex (M; F), treatment (MAM E15; SHAM E15; MAM E17; SHAM E17) and drug (MK801; vehicle). Planned comparisons were conducted by comparing equivalent MAM and SHAM treated groups.

2.2.1e(iii) Prepulse Inhibition

Prepulse Inhibition (dB) and maximal startle amplitude (arbitrary units) were compared across SHAM and MAM-exposed rats using three-way mixed-repeated measures ANOVA. The factors were treatment (MAM-E15; MAM-E17; SHAM), sex (M; F) and prepulse intensity (+4, +8, +12, +16 dB over baseline) in juvenile and adult rats independently. These analyses were repeated for both LH and CD-SD rats separately. Startle amplitude was calculated as the average of the six pulse (P) trials. Significant main effects were followed with planned comparisons against control; significant interactions were followed by analysis of simple effects and, if significant, planned comparisons against control.

2.3 Results

2.2.1 Experiment 1: Strain Comparison in Methylazoxymethanol Acetate (MAM) treated animals

2.3.1a Body Weights, litter size.

Analysis showed no significant difference in the body weight gain (repeated measures_ between MAM and SHAM treated rats in the male or female Charles Derived Sprague Dawley and Lister Hooded rats ($F(3,53) = 0.012$, $p>0.5$) ($F(3,52) = 0.268$, $p>0.5$) ($F(3,53) = 0.046$, $p>0.5$) ($F(3,52) = 1.422$, $p>0.1$) respectively. There was also no effect of strain ($F(1,40) = 1.688$, $p>0.1$) or group ($F(3,40) = 0.980$, $p>0.1$) on litter size (MAM E15, 11.44 ± 0.55, MAM E17, 12.18 ± 0.64, SHAM E15, 12.38 ± 0.63, SHAM E17, 13.00 ± 0.76).

2.3.1b Brain Weights

2.3.1b(i) Juvenile Brain Weights

There was a significant effect of MAM administration on brain weight in both Lister Hooded and Charles Derived Sprague Dawley rats (Figure 1). The analyses revealed that treatment with MAM on either E15 or E17 significantly decreased brain

weights in both male and female Lister Hooded rats ($F(3,40) = 17.80$, $p<0.0001$). There was an overall effect of sex ($F(1,40) = 18.26$, $p<0.001$) with brains from female rats weighing less than those from males. However these factors did not interact ($F(3,40)<0.1$). Planned comparisons showed that both E15 and E17 MAM LH rats had significantly decreased brain weights compared to their SHAM equivalents and E15 MAM treated rats had significantly smaller brains compared with E17 MAM treated rats. In Charles Derived SD rats, there was an effect of sex ($F(1,39) = 19.17$, $p<0.0001$) in that female brains weighed less, and of treatment ($F(3,39) = 82.33$, $p<0.0001$) where MAM treated brains weighed less but there was no interaction between MAM treatment and sex ($F(3,39) = 0.18$, $p>0.5$). Planned comparisons showed that E15 MAM CD-SD rats had significantly decreased brain weights compared to their SHAM equivalents (male, $p<0.001$, female, $p<0.001$). Male rats exposed to MAM on E17 did not differ from their SHAM counterparts. As body weight was not altered by treatment, brain weights were not expressed in relation to total body weight.

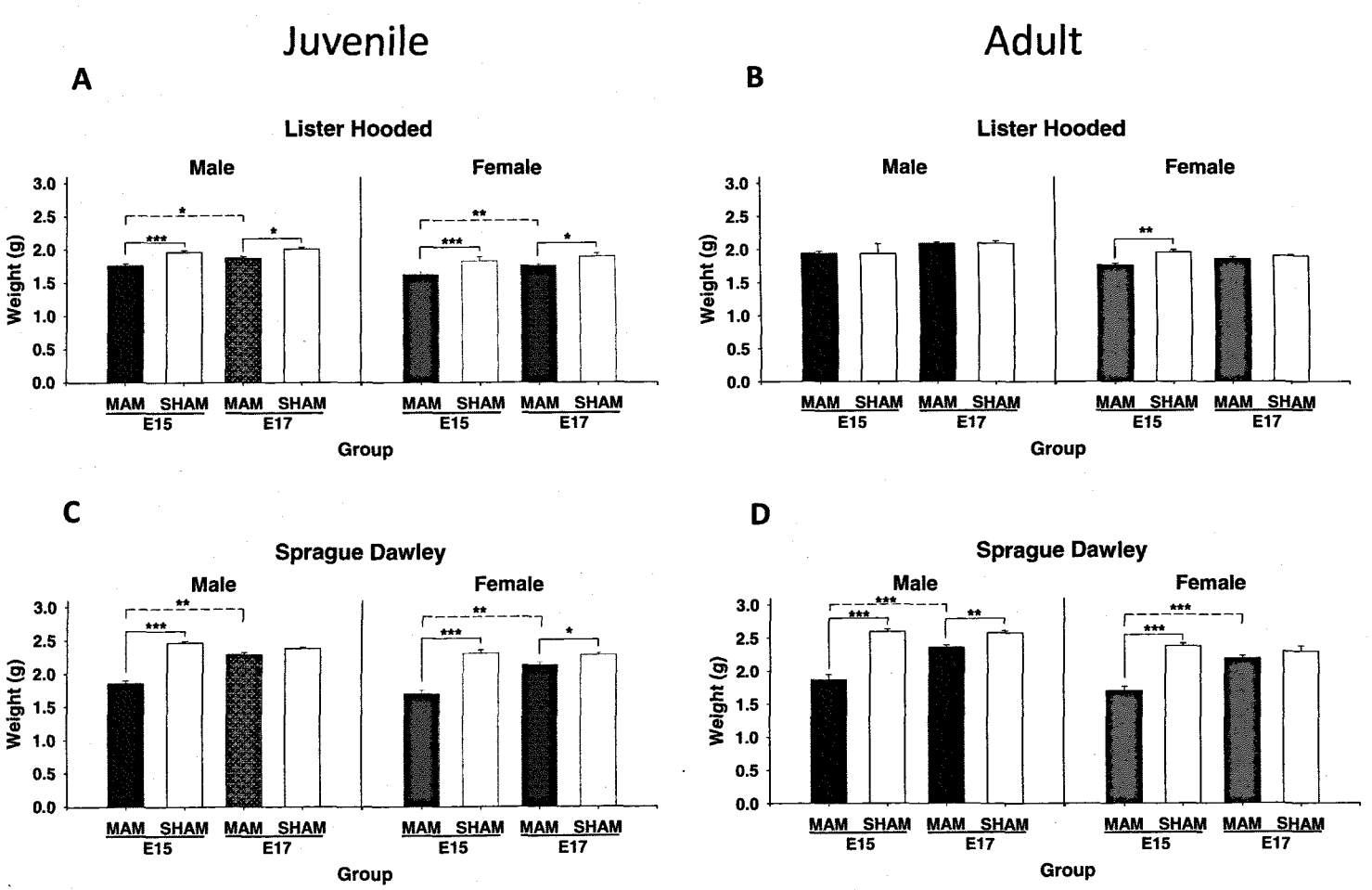


Figure 1: Brain weights in (a) juvenile and (b) adult Lister Hooded and (c) juvenile and (d) adult Charles Derived Sprague Dawley rats treated with MAM on E15 or E17

Effect of MAM treatment on days E15 or E17 in (a) juvenile (MAM n = 8; SHAM n = 4) and (b) adult Lister Hooded rats (MAM n = 8; SHAM n = 8) or (c) juvenile (MAM n = 8; SHAM n = 4) and (d) adult Charles River derived Sprague Dawley SD rats (MAM n = 8; SHAM n = 8) on brain weights (g). Data are presented as mean ± standard error of the mean. Significant differences are indicated on the Figure as follows:* P<0.05; ** P<0.01; *** P<0.001.

2.3.1b(i) Adult Brain Weights

For the Lister Hooded rats, there was an effect of sex ($F(1,48) = 11.804$, $p<0.01$) and treatment group ($F(3,48) = 2.96$, $p<0.05$) (Figure 1B) but no interaction between the two ($F(3,48) = 1.765$, $p>0.1$). Planned comparisons showed that only female E15 MAM LH rats had significantly decreased brain weights compared to their SHAM equivalents (Figure 1B). In Charles Derived SD rats, there was an effect of sex ($F(1,48) = 23.89$, $p<0.0001$) and treatment group ($F(3,48) = 61.47$, $p<0.0001$) (Figure 1D) but no interaction between the two ($F(3,48) = 0.37$, $p>0.5$). Planned comparisons showed that E15 MAM CD-SD rats had significantly decreased brain weights compared to their SHAM equivalents (Fig 1D) but only male rats exposed to MAM on E17 differed from their SHAM counterparts with no difference being seen in the E17 females. As body weight was not altered by treatment, brain weights were not expressed in relation to total body weight.

2.3.1b(iii) Neuropathology

The macroscopic photographs taken of the dorsal surface of the rat brains indicated that the E15 MAM produced large changes in brain structure in both male and female juvenile (Figure $_{2A}$) and particularly in adult CD Sprague Dawley rats (2B). In contrast, E17 MAM produced much smaller changes in juvenile (Figure 2B) and adult Lister Hooded rats (Figure 3B).

Initial studies on SHAM and MAM treated male CD Sprague Dawley E17 rats indicated that there was a decrease in mPFC area and thickness, a reduced area at

the level of the dorsal hippocampus and a large increase in the size of the lateral ventricles. This was followed up with a larger study using a second cohort of animals.. There was an effect of treatment group on the mPFC ($F(1,14) = 6.008$, $p<0.05$) (Figure 3a), the dorsal and ventral hippocampi, ($F(1,14) = 21.170$, $p<0.001$) and ($F(1,14) = 11.452$, $p<0.01$), respectively, (Figure 3b,c) and a significant increase in the area of the lateral ventricles ($F(1,14) = 19.16648$, $p<0.001$) (Figure 3d). Some of the images at the level of the Prefrontal cortex, dorsal hippocampus and lateral ventricles are illustrated in Figure 3E.

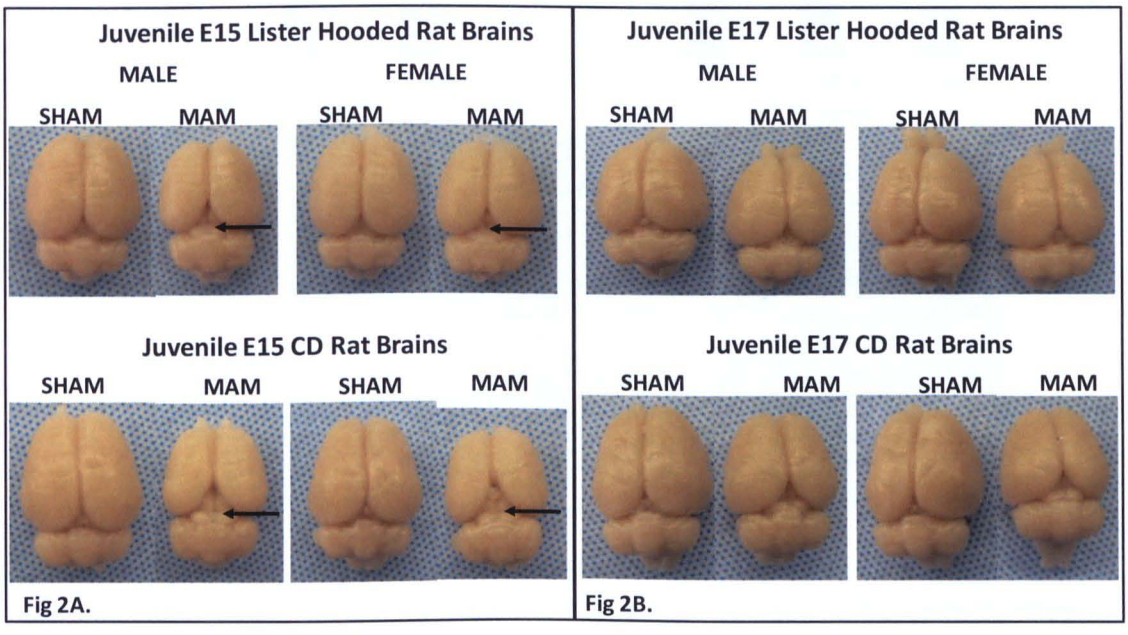


Figure 2: Representative photographs of juvenile Lister Hooded and Charles Derived Sprague Dawley rat brains.
Effect of MAM treatment on days E15 ($_{2A}$) or E17 (2B) in juvenile Lister Hooded rats or juvenile Charles River derived Sprague Dawley rats. There is clear overall shrinkage of the brains after E15 MAM and this is most pronounced in E15 Charles Derived SD rats. There are also some changes in brain structural after E17 MAM, but this is much less severe that was observed with E15 MAM.

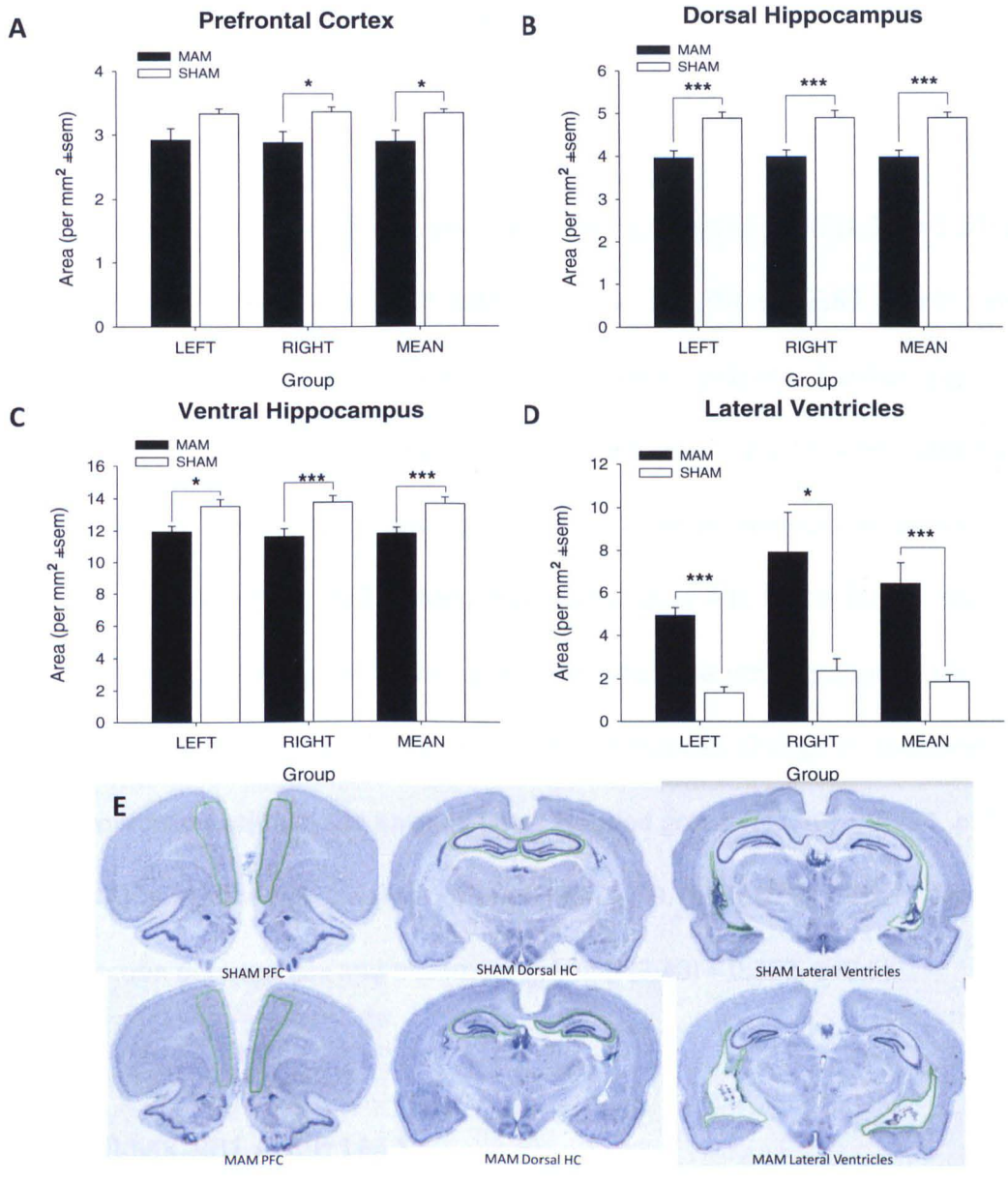


Figure 3. Structural analysis of quantification of the size of various anatomical brain structures in SHAM and E17 MAM treated Charles Derived Sprague Dawley rats.

The area of A. prefrontal cortex, B. dorsal hippocampus, C. ventral hippocampus and D. lateral ventricles and E. Representative cresyl violet stained coronal sections in SHAM and E17 MAM Charles Derived Sprague Dawley rats. Data are expressed as mm2 from the left and right hemispheres of the rat brain and the mean of both hemispheres is also shown. There were clear decreases in the size of the area of the prefrontal cortex, dorsal and ventral hippocampus and a large increase in the area of the lateral ventricles. Data are presented as mean ± standard error of the mean. Significant differences are indicated on the Figure as follows:* P<0.05; ** P<0.01; *** P<0.001.

2.3.1c Locomotor Activity (LMA)

2.3.1c(i) Juvenile LMA

In the juvenile CD Sprague Dawley rats there was no effect of sex ($F(1,39) = 2.8156$, $p>0.1$), but there was an effect of treatment group ($F(1,39) = 4.0389$, $p<0.05$) and an interaction between the two ($F(3,39) = 4.1608$, $p<0.05$). Further planned comparisons showed that the MAM E15 treated male rats showed significant hyperactivity in a novel arena throughout the 30 minute habituation period but there was no effect in the E17 treated male rats (Figure 4B). In the female rat, the E15 SHAM rats seemed to be more hyperactive than the other groups at the first time point only (Figure 4B). There was no MAM-induced change in spontaneous exploratory locomotion in the juvenile Lister Hooded group ($F(3,40) = 1.234$, $p>0.1$) (Figure 4A) but there was an effect of sex ($F(1,40) = 9.195$, $p<0.01$). There was also no interaction between sex and treatment group ($F(3,40) = 0.362$, $p>0.5$).

2.3.1c(ii) MK-801 Adult LMA

There was an effect of treatment group and sex on LMA during the habituation phase in LH rats ($F(3,94) = 6.156$, $p<0.001$) and ($F(1,94) = 18.060$, $p<0.0001$) respectively. As expected, MK-801 produced a marked hyperactivity in the two hours after administration such that post-injection of MK-801 caused an effect of treatment group, sex and drug ($F(3,93) = 7.1817$, $p<0.001$), ($F(1,93) = 76.7405$, $p<0.0001$) and ($F(1,93) = 82.8638$, $p<0.0001$) respectively on the total LMA over 2 h. Further comparisons showed there was no significant difference between male LH

E15 MAM and SHAM or LH E17-MAM or E17-SHAM rats treated with MK-801 (Figure 5A). There were however significant differences between E15 and E17 LH female MAM rats treated with MK-801 compared to their equivalent SHAMS (Figure 5B). Treatment with MK-801 increased hyperactivity in male MAM E17 treated rats and female rats treated with MAM on both E15 and E17 (treatment group $F(3,96) = 3.094$, $p<0.05$), (sex $F(1,96) = 33.595$, $p<0.0001$) and (drug group $F(1,96) = 66.507$, $p<0.0001$). Further comparisons showed significant differences between E15 and E17 CD female MAM rats treated with MK-801 compared to the equivalent SHAMS (Figure 5D).

There was an effect of treatment group and sex during the habituation phase in SD rats in that the females and MAM E15 treated rats were more hyperactive ($F(3,96) = 25.643$, $p<0.0001$) and ($F(1,96) = 7.417$, $p<0.01$) respectively and there was no effect of drug group ($F(1,96) = 0.880$, $p>0.1$) (Figure 5).

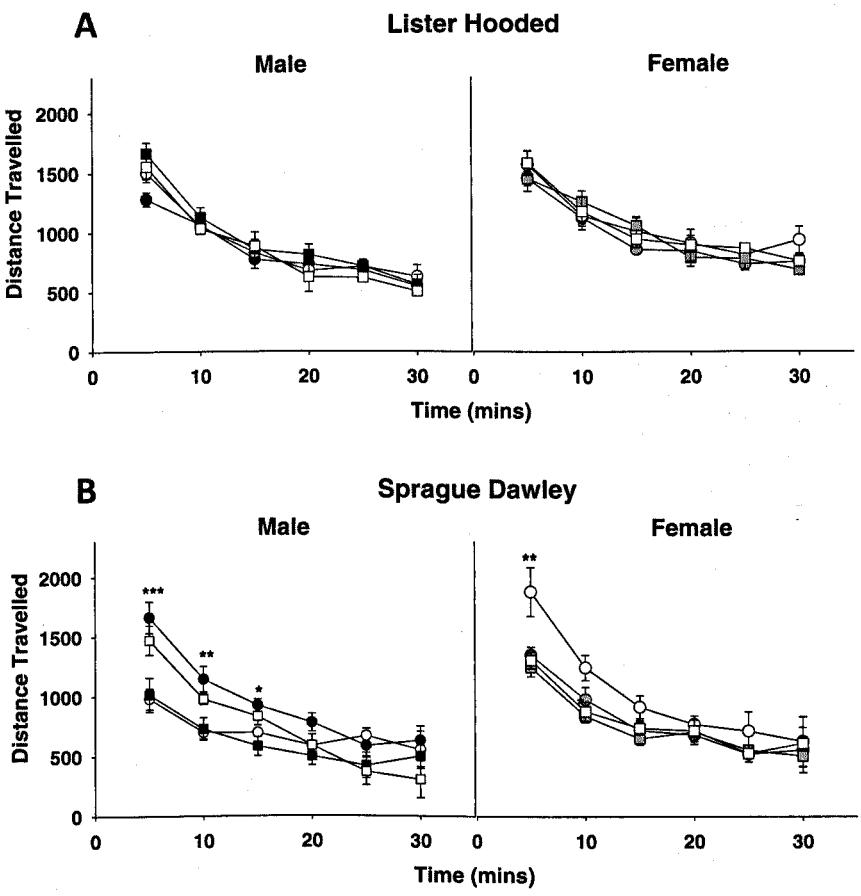


Figure 4: Locomotor activity in juvenile Lister Hooded and Charles Derived Sprague Dawley rats treated with MAM on E15 or E17

Effect of MAM treatment on days E15 or E17 in (a) juvenile male and female Lister Hooded rats or (b) juvenile male and female Charles River derived Sprague Dawley rats on locomotor activity. Data are presented as mean ± standard error of the mean of total distance travelled (cm) (n = 8 for all MAM groups and n = 4 for all SHAM groups). Symbols as follows: male MAM E15 treated rats (●); male SHAM E15 treated rats (○); male MAM E17 treated rats (■); male SHAM E17 treated rats (□); female MAM E15 treated rats (●); female SHAM E15 treated rats (○); female MAM E17 treated rats (■); female SHAM E17 treated rats (□). Significant pair-wise comparisons between SHAM and MAM E15 groups are indicated on the Figure as follows:* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

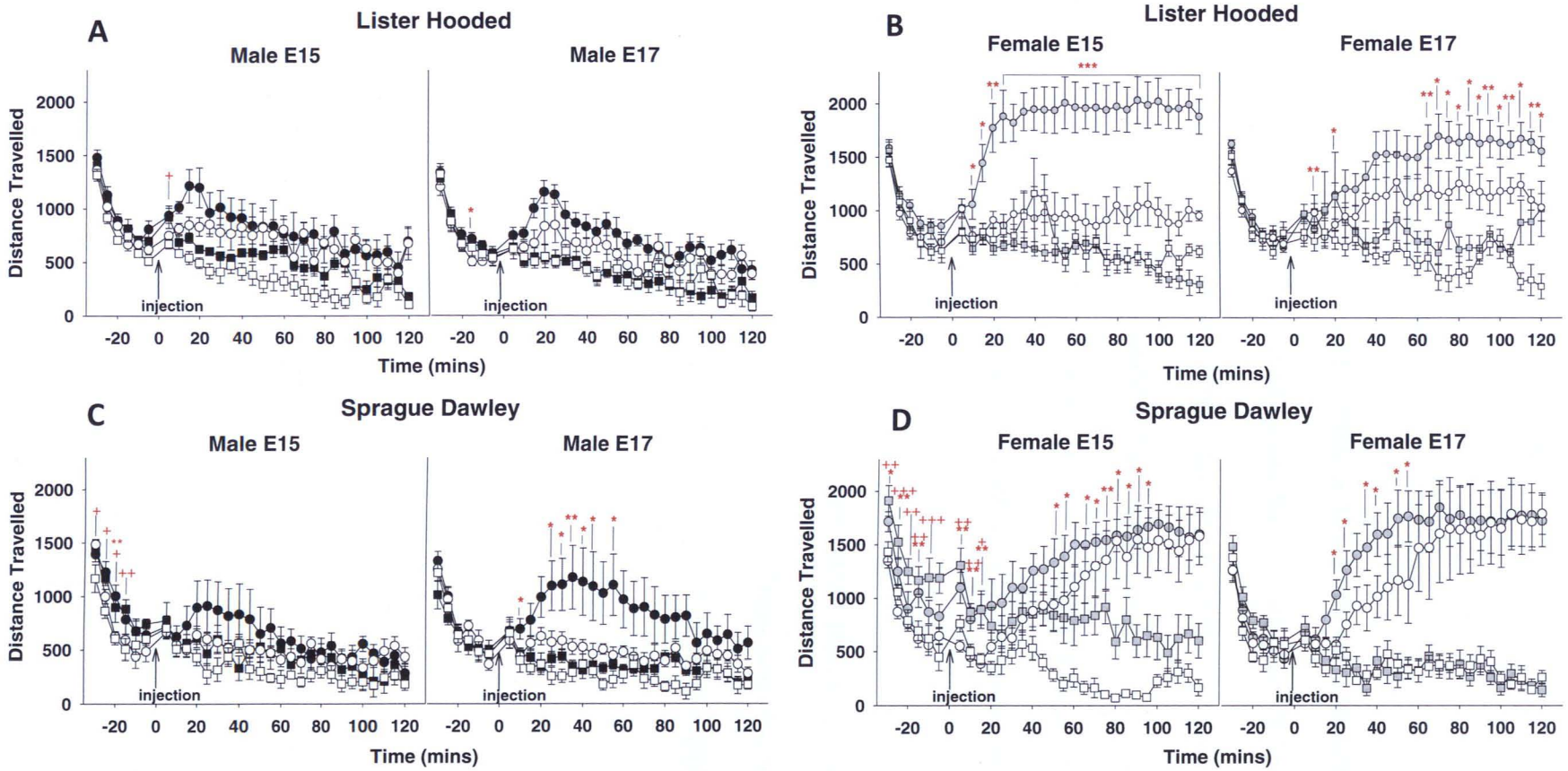


Figure 5: Locomotor activity in adult Lister Hooded and Charles Derived Sprague Dawley rats treated with MAM on E15 or E17

Effect of MAM treatment on days E15 or E17 in (a) adult male, (b) adult female Lister Hooded rats, (c) adult male and (d) adult female Charles River derived Sprague Dawley rats on locomotor activity. Data are presented as mean ± standard error of the mean of total distance travelled (cm) (n = 8 for all MAM groups and n = 6 for all SHAM groups). Symbols as follows: male MAM rats treated with MK801 (●); male SHAM rats treated with MK801 (○); male MAM rats with vehicle (■); male SHAM rats with vehicle (□); female MAM rats with MK801 (●); female SHAM rats with MK801 (○); female MAM rats with vehicle (■); female SHAM rats with vehicle (□). Significant pair-wise comparisons between are indicated on the figure as follows: MK-801 treated SHAM vs. MK-801 treated MAM * P<0.05; ** P<0.01; *** P<0.001.

2.3.1d Prepulse Inhibition

2.3.1d(i) Juvenile PPI

As group sizes for the SHAM group in this study were limited (n = 4 for the SHAM groups; n = 8 for the MAM group for both LH and CDSD rats), statistics were performed to determine if the data from SHAM E15 and SHAM E17 could be collapsed into a single SHAM group for comparison purposes. There was no effect of group in the Lister hooded ($F(1,48) = 1.893$, $p>0.1$) or the Charles Derived Sprague Dawley rats ($F(1,48) = 1.092$, $p>0.1$), therefore the SHAM groups were combined to give an n = 8.

There was no effect of treatment with MAM ($F(2,206) = 1.5577$, $p>0.1$) or sex ($F(1,206) = 0.0263$, $p>0.5$) in the LH group. There was also no effect of treatment group or sex on prepulse inhibition in juvenile CDSD rats ($F(2,210) = 0.417$, $p>0.5$) and ($F(1,210) = 2.178$, $p>0.1$), respectively (Figure 6B).

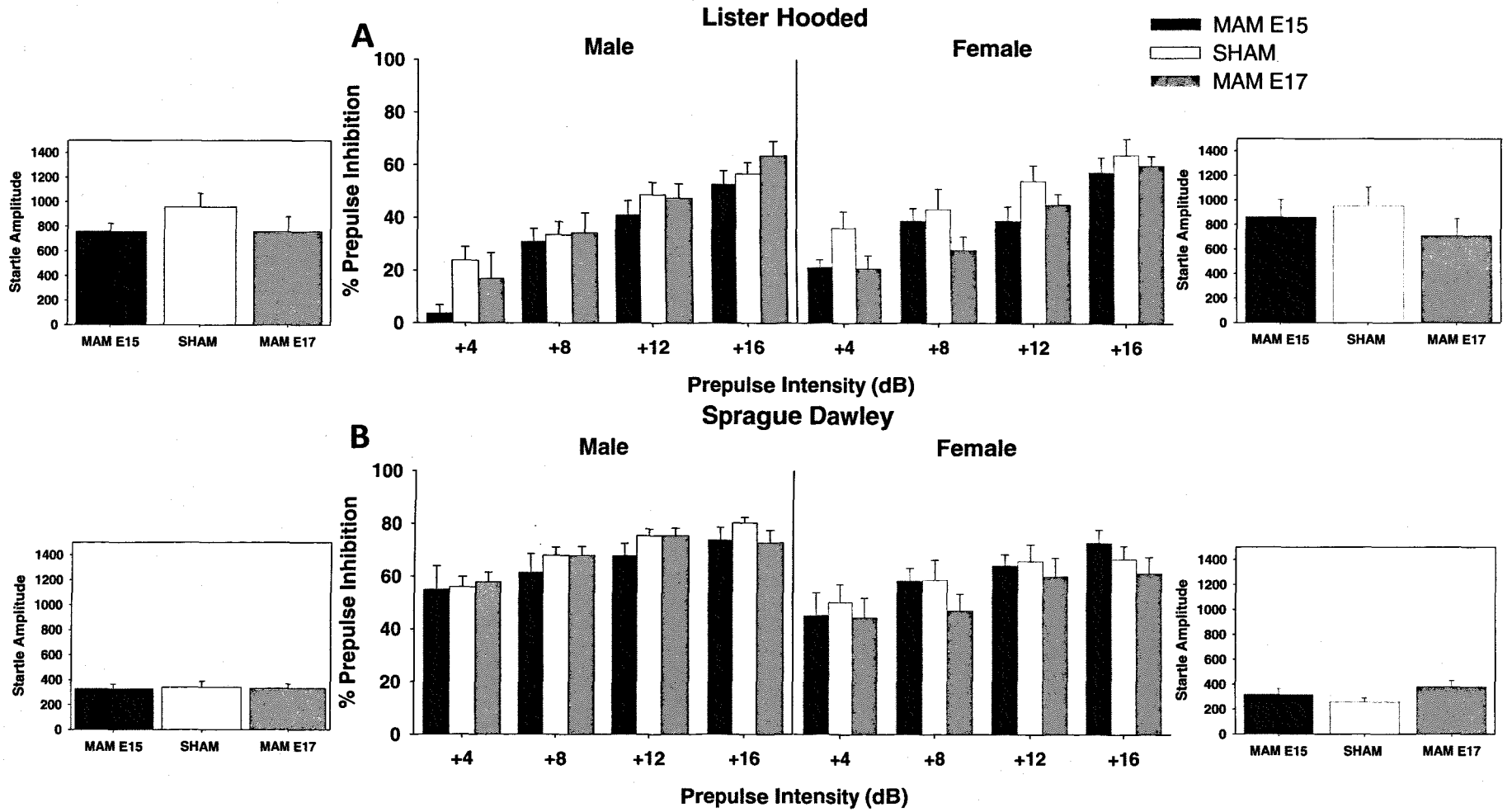


Figure 6: Prepulse inhibition in juvenile (a) Lister Hooded and (b) Charles Derived Sprague Dawley rats treated with MAM on E15 or E17
Effect of MAM treatment on days E15 or E17 in juvenile (a) Lister Hooded rats or (b) Charles River derived Sprague Dawley rats on percentage pre-pulse inhibition. Also presented are the startle amplitudes for each treatment (arbitrary units). Data are presented as mean ± standard error of the mean (n = 8 for all MAM groups and n = 4 for all SHAM groups).

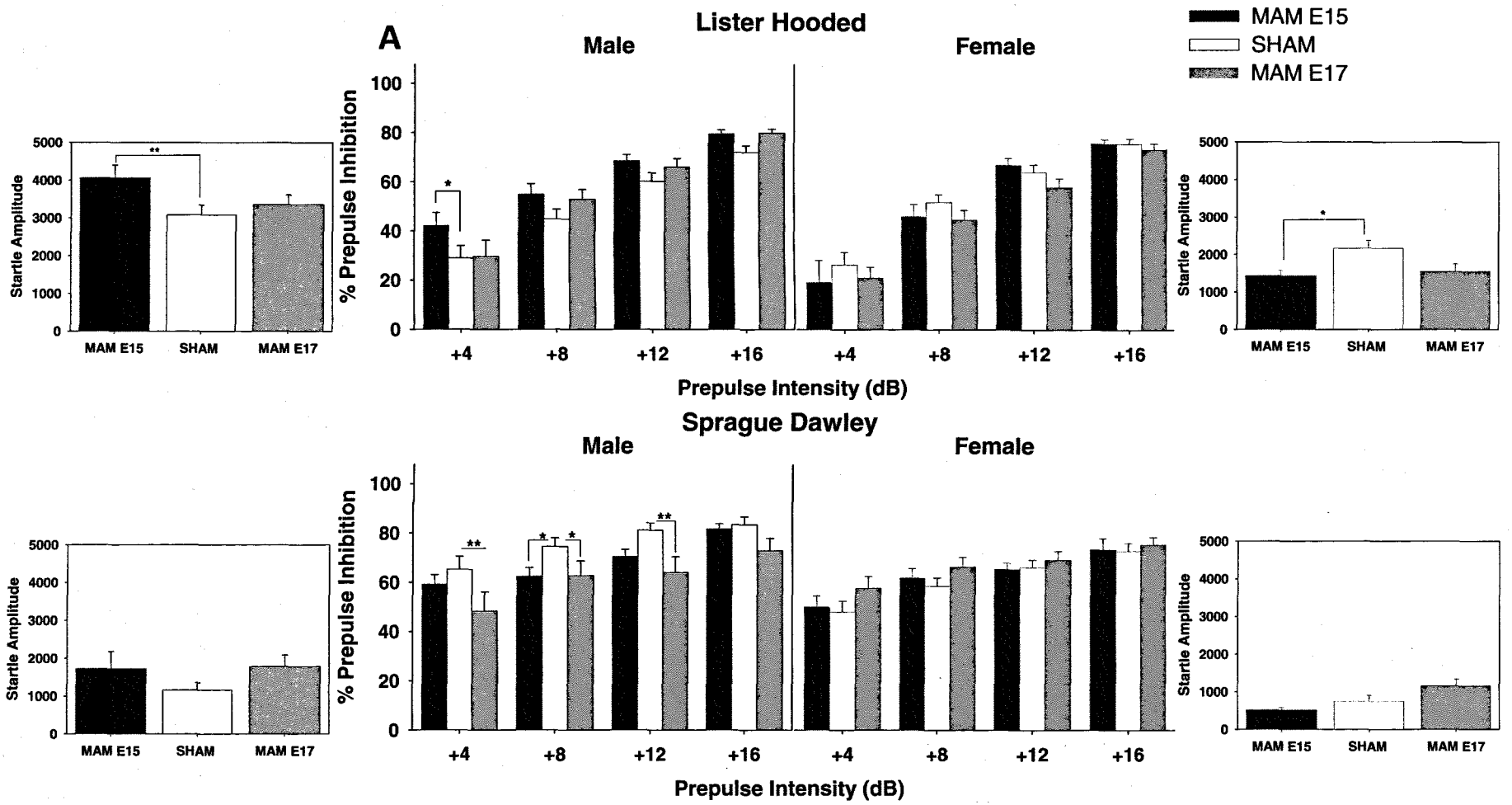


Figure 7: Prepulse inhibition in adult (a) Lister Hooded and (b) Charles Derived Sprague Dawley rats treated with MAM on E15 or E17
Effect of MAM treatment on days E15 or E17 in adult (a) Lister Hooded rats or (b) Charles River derived Sprague Dawley rats on percentage pre-pulse inhibition. Also presented are the startle amplitudes for each treatment (arbitrary units). Data are presented as mean ± standard error of the mean (n = 16 for all MAM groups; n = 12 for all SHAM groups). Significant differences as follows:* P<0.05; ** P<0.01; *** P<0.001.

2.3.4d(ii) Adult PPI

Statistics were also performed on the adult SHAM E15 and E17 groups to see if they could be combined to provide one SHAM group as some of the boxes crashed midway through a session. There was an effect of strain ($F(1,374) = 26.075$, $p<0.0001$) but no effect of treatment group or sex, ($F(1,374) = 0.047$, $p>0.5$) and ($F(1,374) = 1.311$, $p>0.1$) respectively therefore the groups were combined for all further statistical analyses with all data shown in Figure 7B.

There was an effect of sex on prepulse inhibition in adult LH rats ($F(1,424) = 7.540$, $p<0.01$), respectively, but no effect of treatment group ($F(2,424) = 1.776$, $p>0.1$). However there was a significant interaction between sex and treatment group ($F(2,424) = 5.941$, $p<0.01$). Further planned comparisons showed that this significant effect was only in the male E15 rats at prepulse 1 (figure 7A). For the CD Sprague Dawley rats, there was an effect of sex ($F(1,360) = 8.159$, $p<0.01$) but no effect of treatment group ($F(2,360) = 2.259$, $p>0.106$). Again, there was an interaction between treatment group and sex ($F(2,360) = 11.522$, $p<0.0001$). Planned comparisons showed that this effect was due to the male CD Sprague Dawley rats (figure 7B).

2.4 Discussion

The present data show that maternal treatment with methylazoxymethanol acetate (MAM) leads to behavioural, neuroanatomical and pharmacological alterations in the resultant offspring of Charles Derived Sprague Dawley rats. These changes include decreased brain weights, decreased size of the hippocampus, PPI deficits and hypersensitivity to MK-801 measured by changes in locomotor activity; changes that have translational relevance to some of the core symptoms of schizophrenia (Moore *et al.,* 2006). Furthermore, the absence of these deficits before puberty in the same rats indicates that this model may also replicate the post-adolescent onset of psychosis (Wood., 2008).There was no effect of MAM treatment on litter size and all groups gained body weight at the same rate, suggesting that there were no adverse effects of the toxin on gestation or maternal suckling and care. MAM treatment produced a reduction in brain weight in all juvenile Lister Hooded brains, but surprisingly this difference disappeared in the adult males. There was also a reduction in brain weight in MAM E15 and E17 Charles Derived rats very similar to those previously reported in Sprague Dawley (Gourevitch *et al.,* 2004) and Fischer 344 rats (Moore *et al.,* 2006). The structural changes in the brain (reduced size of the prefrontal cortex and dorsal hippocampus and increased ventricular size) are also consistent with the changes reported in the literature in CD-SD (Gourevitch *et al.,* 2004; Le Pen *et al.,* 2006) and Fischer 344 rats (Moore *et al.,* 2006).

Generally, there was a smaller effect of MAM treatment on the behavioural measurements taken in Lister Hooded than in CD-SD rats. MAM had no effect on

locomotor activity in pre-pubertal or adult male LH rats. However, CD-SD MAM treated rats were hypersensitive to MK-801 at adulthood. Hyperlocomotor activity is a plausible correlate of the psychomotor agitation seen in schizophrenia. It is therefore proposed that the hyperlocomotion observed in E17 MAM rats may reflect positive symptoms in schizophrenic patients, in particular, since it has a post-pubertal onset and is exacerbated by NMDA receptor antagonists (Goff and Coyle., 2001). Le Pen *et al.,* (2006) also showed that locomotor activity after MK-801 administration was significantly higher in MAM compared to sham-treated rats in adulthood but not before puberty. Another study found that MAM rats are more responsive to D-amphetamine (Flagstad *et al.,* 2004). Schizophrenic patients are hypersensitive to D-amphetamine both behaviourally and in terms of subcortical DA release (Breier *et al.,* 1997; Angrist *et al.,* 1980). It is also thought that psychological stress may exacerbate psychotic symptoms (Norman and Malla., 1993) and it has also been reported that MAM treated rats are hypersensitive to mild stress (Le Pen *et al.,* 2006).

Abnormalities of sensory-motor gating as measured by pre-pulse inhibition of the acoustic startle response are one of the most robust endophenotypic markers in schizophrenia (Braff *et al.,* 2001). The current data (figure 7B) demonstrate that male Charles Derived E17 MAM rats exhibit a significant deficit in PPI at different prepulse intensities, consistent with previous findings (Le Pen *et al.,* 2006) as well as with other neurodevelopmental models of schizophrenia such as prenatal immune challenge, post-weaning social isolation and neonatal lesions of the ventral

hippocampus (Borrell *et al.,* 2002; Van Den *et al.,* 2003). This study suggests that the changes seen in E17 MAM treated rats are strain-specific since they do not occur in male LH rats under identical experimental conditions.: small differences in brain weight were found in juvenile LH compared to those seen in E15 MAM-treated CD-SD rats and only the female LH rats showed a statistically significant decrease in brain weight in adulthood. Strain differences in rats have been seen in many different assays. For example, in the Isolation Rearing model of schizophrenia, Weiss et al., (2000) showed that LH isolates had significantly higher levels of locomotor activity compared to CD-SD isolates. Both CD-SD and LH rats showed isolation-induced PPI deficits but this was maintained on repeated testing only in the CD-SD isolates. CD-SD rats were also more sensitive to low doses of apomorphine than LH rats.

The differential effects of MAM in the two strains used in this study may be due to a number of factors. Whilst it is possible that the pace and/or pattern of neurodevelopmental processes may differ between the two strains, it seems more likely that the differences are pharmacokinetic in nature. MAM is metabolised to a reactive species that passes through the placenta and methylates purine bases on RNA and DNA (Cannon-Spoor and Freed., 1984; Spatz and Laqueur., 1968). It may be that MAM is less susceptible to metabolism in LH rats or that the drug and/or its metabolites are less easily absorbed or less able to cross the placenta. Surprisingly, the LH rats also seemed to display a 'recovery' as the brain weight deficit seen in

juvenile MAM treated LH rats was lost in adulthood, possibly reflecting enhanced neurogenesis during adolescence.

Female LH and CD-SD rats were more sensitive to the locomotor stimulating effects of MK-801 in comparison to males whereas no deficits were seen in PPI in females. Gender differences in schizophrenia have been reported in the literature ever since Kraepelin first described dementia praecox as predominantly a disorder of young men. Subsequently, it has been one of the most consistent findings in the epidemiology of the disorder. Female schizophrenia patients seem to have a 3–4-year delay of symptom onset and first hospitalization compared with males, and a second peak at the onset of menopause (Hafner *et al.,* 1993).

The acute effects of MAM are dose-related as well as being time-limited, lasting approximately 24h with maximal effects occurring 12h after dosing and only neurons actively dividing during this exposure time are thought to be damaged by the treatment. This is particularly advantageous as the observed abnormalities can be relatively precisely correlated with the different stages of neurodevelopment. We have shown that treatment on E15 results in deficits that are different to those seen on E17. In particular, the reduction in brain weight in both juvenile and adult MAM treated CD-SD rats is much greater after treatment at E15 than at E17. It has also been shown that administration of MAM at E15 severely impairs spatial memory as well as causing a significant reduction in brain weight including a decreased thickness in all cortices and large heterotopias in the hippocampus

(Gourevitch *et al.,* 2004) and a severe reduction in entorhinal cortex, prefrontal cortex and striatum volumes (Jongen-Relo *et al.,* 2004). Other groups examining the behaviour of E15 MAM treated animals have shown impairments of place-navigation in a swim maze (Mohammed at al 1986) and a marked impairment in rule learning as well as in reversal learning and passive avoidance tasks (Moore *et al.,* 2006). However, the consensus is that administration of MAM between E12 and 15 has no validity as a behavioural model for schizophrenia since this leads to marked microencephaly and gross motor impairments, including ataxia and a blunted startle reflex (Jongen-Relo *et al.,* 2004; Moore *et al.,* 2006). Also, despite these morphological and behavioural abnormalities, deficits in prepulse inhibition were only apparent in E17 treated animals, indicating that the timing of MAM administration is very important to outcome.

Timing of injection on E17 could also be important as alluded to by a study by Spatz and Laqueur (1968). They found that one feature of the microencephaly induced by MAM was its uniformity among littermates. They also stated that the differences in the degree of microencephaly between litters were most likely due to the variation in timing of injection. These differences in microencephaly could also result in differences in behaviour between litters whereas the uniformity among littermates could result in litter effects.

In conclusion, the observations from this study and those of other groups indicate that a disturbance in neurodevelopment induced by MAM administration on E17

leads to several behavioural and neuroanatomical changes similar to the core pathophysiological changes characteristic of schizophrenia (Featherstone *et al.,* 2007; Styner *et al.,* 2005). However, our work makes it clear that care must be exercised in the selection of rat strain and gender to be used since male LH rats are essentially resistant to MAM E17 treatment. Further work described in the next chapters will concentrate on the cognitive consequences of MAM E17 treatment.

Chapter 3: Spatial Reversal Learning

3.1 Introduction

Cognitive inflexibility, described as the inability to spontaneously withhold or modify a behaviour in response to changing situational demands, is associated with various psychiatric disorders, most notably schizophrenia, depression, and obsessive-compulsive disorder (Boulougaris., 2008). It is a core component of schizophrenia and in patients it is the single best predictor of long-term outcome of the disease (Holthausen *et al.,* 2006). It is also of particular interest in that unlike many other cognitive deficits in schizophrenia, reversal learning – which is used as an indicator of cognitive (in)flexibility - impairments appear to be unrelated to a general impairment in intelligence and are statistically related to clinical ratings of negative symptoms, including disorganisation and thought disorder (Leeson *et al.,* 2009). The clarification of the underlying mechanisms of cognitive flexibility could therefore be of major importance for the understanding of its etiology and treatment.

Reversal learning defines a fairly broad set of tasks of discrimination learning, where at some point during learning, reinforcement contingencies are swapped

one or more times between choice options. A great benefit of reversal learning paradigms is the operational similarity of such tasks between species, including humans. It has been used as a measure of behavioural flexibility in humans (Rogers *et al.,* 2000; McKirdy *et al.,* 2009, Leeson *et al.,* 2009), nonhuman primates (Owen *et al.,* 1991, Dias *et al.,* 1996a,b; Clarke *et al.,* 2005, 2008), and rats (Birrell and Brown, 2000; Idris *et al.,* 2005; Boulougouris *et al.,* 2007). Rodent tasks of reversal learning usually comprise two distinct components, an initial phase that requires memory of a previously learned reward contingency, followed by a reversal phase, in which the reward contingency is then reversed and the previously correct contingency is now incorrect and vice versa. Animals are required to inhibit a previously rewarded strategy and acquire the new strategy. Thus, effective performance requires animals to demonstrate flexibility, attention and motivation to suppress a previously learned response and implement a new response (Jones *et al.,* 1991). Reversal learning ability can also be assessed using other tasks such as the reversal stages within the attentional set-shifting paradigm (Birrell and Brown, 2000).

Impairments in behavioural flexibility observed in schizophrenia have been attributed to perturbation in frontal lobe functioning. Post-mortem and functional imaging studies of schizophrenic brains have revealed gray matter abnormalities in the prefrontal cortex (PFC), which likely contribute to the impairments seen in executive function tasks such as Wisconsin Card Sorting Test (WCST) and Trail Making Test (TMT) (Bonilha *et al.,* 2008; Rusch *et al.,* 2007). Lesion studies in

rodents and monkeys, electrophysiological studies in rats and mice and studies of brain-damaged patients as well as neuroimaging in healthy volunteers have shown that reversal learning is well-known to engage a specific neural circuitry including the orbitofrontal cortex, amygdala and striatum (Clarke *et al.,* 2008; Dias *et al.,* 1996; Chudasama and Robbins., 2003; McAlonan and Brown., 2003). However, recent findings from both human and animal studies indicate that complex forms of behavioural flexibility mediated by the frontal lobes are also dependent on a number of subcortical systems that interact with the PFC. These include midline thalamic nuclei, dorsal and ventral regions of the striatum, and the mesocorticolimbic dopamine (DA) system. It is of note that abnormalities in each of these systems have also been proposed to contribute to the pathophysiology of schizophrenia (Andreasen *et al.,* 1990; Shenton *et al.,* 2001; Danos *et al.,* 2003; Bonilha *et al.,* 2008).

Converging evidence from a number of studies has specifically implicated the orbitofrontal cortex (OFC) for successful reversal learning ability in rodents. It has been shown that lesions of the orbital prefrontal cortex (OPFC) impair the reversal learning ability within the attentional set-shifting task (McAlonan and Brown., 2003) as well as visual discrimination tasks (Chudasama and Robbins., 2003; Boulougouris *et al.,* 2007). These results are concurrent with those of Bohn and co-workers, showing that lesions of the OFC impair reversal learning in an operant lever pressing-based task (Bohn *et al.,* 2003) while other studies using an olfactory-

guided go/no-go discrimination task, show that rats with OFC lesions are impaired during the second, but not the first, reversal stage (Ferry *et al.,* 2000).

In contrast to the OFC, investigating the involvement of the rat medial prefrontal cortex (mPFC) has produced somewhat ambivalent effects on simple reversal learning. Despite earlier studies having found that lesions in the mPFC produce reversal deficits (Kolb *et al.,* 1974, Nonneman *et al.,* 1974), more recent studies have reported both reversal deficits (Bussey *et al.,* 1996; Ferry *et al.,* 2000; Li and Shao., 1998) as well as unimpaired reversal (Birrell and Brown 2000, Boulougaris *et al.,* 2007; Floresco *et al.,* 2008). The mPFC of rats, however, consists of several sub-areas that are different from each other in both cytoarchitecture and neural connectivity, suggesting a functional dissociation among the mPFC sub-areas. Li and Shao (1998) have shown no difference in acquisition between control and mPFC lesioned rats. However, lesions of either the pre-limibic or the infralimbic mPFC produced a marked deficit in the reversal task. This behavioural deficit was not found in rats with lesions of the anterior cingulate. The results indicate that the mPFC of rats is not essential for discrimination learning, but that each of the two ventral sub-areas of the mPFC, pre-limbic and infra-limbic, play a critical role in reversal learning.

Variants of reversal learning may also engage other brain regions; spatial reversal is often associated with the hippocampus, and probabilistic reversal engages the ventrolateral PFC (Cools *et al.,* 2002), as does serial reversal learning (Rygula *et al.,*

2010). It has been suggested that spatial reversal learning may be more sensitive to hippocampal dysfunction than spatial acquisition (O'Keefe and Nadel., 1978; Whishaw and Jarrard., 1996). This is supported by studies that have investigated the effects of the N-methyl-D-aspartate (NMDA) receptor antagonist D-(-)-amino-5-phosphonopentanoic acid (AP5) on variants of the hidden-platform water maze task. Although infusion of AP5 blocked the induction of hippocampal LTP and impaired acquisition of the spatial reference memory water maze task in naive animals (Bannerman *et al.,* 1995), there was no drug effect on spatial learning if the animals had received spatial water maze training in a separate and distinct testing room, prior to testing with the drug (Bannerman *et al.,* 1995). Schizophrenic patients with a hippocampal lesion have been shown to commit significantly more perseverative errors when performing on the WCST, than subjects with frontal lobe damage (Corcoran and Upton., 1993), confirming that intact hippocampal function is required for reversal learning. In addition, lesion of the hippocampus in rats produces a significant impairment in working memory tasks (Jackson *et al.,* 1998; Sziklas and Petrides., 2002). Therefore, the inability of sub-chronic PCP treated animals to perform adequately in a reversal task (Jentsch and Taylor., 2001; Idris *et al.,* 2005), may be representative of hippocampal as well as frontal cortical dysfunction.

In terms of animal models that may bear relevance to schizophrenia, Kellendonk *et al.,* (2006) have shown that reversal learning was impaired by over-expression of the D2 receptor in mouse striatum. The test is also sensitive to other standard

models of schizophrenia including PCP administration (Gastambide *et al.,* 2012) and social isolation reared rats (Jones *et al.,* 1991). Reversal deficits have also been shown in the MAM-E17 rats by way of the Morris-water maze (Flagsted *et al.,* 2005) and the IDED set shifting task (Gastambide *et al.,* 2012).

Reversal learning can be conducted reliably and fairly quickly in mice, rats and non-human primates, although typical paradigms are only applied once per animal (unless specifically interested in serial reversal effects). As such, reversal learning tests may be useful both in the validation of animal models of cognitive impairment in schizophrenia, and in the subsequent testing of candidate compounds in these models. A recent thrust of drug discovery research has focused on developing novel pro-cognitive compounds to treat the cognitive deficits in schizophrenia, and preclinical animal models are an essential first step in this process. Given that impairments in behavioural flexibility seem to be a core deficit in this disorder, modelling these impairments would be extremely beneficial in testing the potential efficacy of novel cognitive enhancers. Some work has began to address this topic recently (Elsworth *et al.,* 2012; McLean *et al.,* 2009, Gastambide *et al.,* 2013), although more evidence of effects of a broader range of pharmacologies in different animal models would aid convergent validity. With this is mind, the aim of the present study was to investigate a rodent reversal-learning procedure that might be used to evaluate novel antipsychotic drugs. The procedure used was an adapted version of a serial reversal learning procedure used by Boulougaris *et al.,* (2007) and the novel antipsychotic was a metabotropic glutamate 5 (mGlu5)

receptor positive allosteric modulator, LSN2463359, developed by Eli Lilly. As discussed in Chapter 1, the functional interaction of mGlu5 receptors with N-methyl-D-aspartate (NMDA) receptors has prompted speculation that their activation may offer a potential treatment for aspects of schizophrenia.

3.2 Methods

3.2.1 Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Reversal Learning Performance

3.2.1a Subjects

Animals were prepared as previously described in Chapter 2.

30 Male (15 MAM, 15 SHAM), Sprague Dawley (Crl:CD(SD) (SD), Charles River, Kent) aged 12 weeks at the start of the experiment were used. All pup information is detailed in table 1. Rats were housed in groups of 3 or 4 and had *ad libitum* access to water but were food restricted to 85% of their free feeding weight from 8 weeks of age. Food restriction was maintained for the entire duration of the experiment. The rats had environmental enrichment (Jolly BallsTM, (Lillico) plastic tubes and wooden blocks). The animals were maintained on a 12-hour light dark cycle with lights on at 7.00. The experiments were conducted during the same part of the light phase each day and a timeline of studies is shown in figure 1. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, under Home Office project licence 70/6560 and personal licence 71/17650 (N. N. Malik).

Table 1: Detail of pup treatment groups and lever assignment for Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Reversal Learning Performance.

Number	Pup	Tag	Group	Cage	Lever Assignment
1	AA156	2408970	SHAM E17	3	Right
2	D22	2399156			Left
3	II196	2401669			Left
4	A4	2400337			Left
5	Y146	2450846	SHAM E17	6	Right
6	A1	2408525			Right
7	AA153	2449934			Right
8	I62	2409918			Right
9	KK209	2407523	MAM E17	17	Left
10	S119	2403611			Right
11	N90	2449379			Left
12	Q104	2403405			Right
13	M83	2450502	SHAM E17	21	Left
14	GG191	2409229			Right
15	U126	2408868			Left
16					Right
17	H54	2400818	MAM E17	26	Right
18	BB158	2454027			Left
19	DD173	2403289			Left
20	KK208	2408915			Left
21	C20	2408695	MAM E17	32	Left
22	N92	2458906			Right
23	H53	2403325			Right
24					Right
25	Z147	2449066	MAM E17	36	Left
26	C19	2408443			Right
27	CC162	2447768			Left
28	F42	2409199			Left
29	N91	2398865	MAM E17	41	Left
30	E34	2403028			Left
31	F38	2401938			Left
32	CC163	2447816			Left

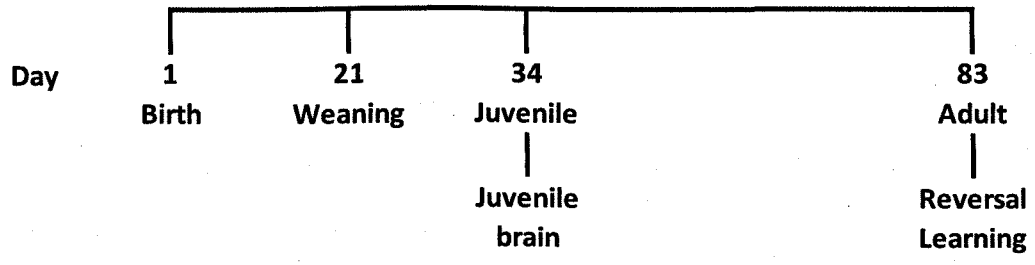


Figure 1: Timeline of events and studies performed for Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Reversal Learning Performance.

3.2.1b Reversal Learning

3.2.2b(i) Apparatus

All rats were tested in one of eight operant chambers. Each chamber (30.5×24×21 cm; Med Associates, Vermont, USA) consisted of Plexiglas walls and ceiling, and a metal grid floor over sawdust. A hinged Plexiglas panel (6×6 cm) provided access to a food hopper containing food pellets (45 mg Noyes pellets, Sandown Scientific, UK). Two retractable levers (4×2 cm) were positioned on either side of the food hopper. A light emitting diode (LED) was positioned centrally above each lever and a house light was located in the ceiling of each chamber. The chambers were placed individually within ventilated sound-attenuating hardboard boxes. A small fan was built into each chamber to provide ventilation and mask external noise. Each animal was tested in the same operant chamber throughout the study. All boxes were controlled by Med-PC software and programmes were written using Medstate notation.

3.2.1b(ii) Reversal Learning Procedure

Training always started on a Thursday to allow the test phases of the procedure to occur in the same week as shown by figure 2. Rats were habituation to the operant chambers during magazine training where they learnt to associate the magazine with receiving a food pellet. Following this, rats were trained to respond for food on a fixed ratio 1 (FR1) schedule of reinforcement with both levers active for 2 days after which they moved onto a fixed ratio 3 (FR3) schedule, again for 2 days. The session began, in both cases, with the house light being illuminated and

presentation of both levers. A single lever press in the case of the FR1 program, and three lever presses for the FR3 program on either lever, resulted in a food pellet being delivered. The session lasted 30 min and the levers were presented throughout. The next stage, biconditional training (BCT), began with illumination of the house light. A head entry within 20 s resulted in both levers being presented but only one lever was active. The active lever was randomly assigned to each rat using an internal random number generator. Assignments are detailed in table 1. A correct response within 10 s on the active lever resulted in delivery of a food pellet. After a correct or incorrect response, the levers were retracted and the house light turned off. The house light was then turned on again and the cycle repeated. If no head entry was made within 20 s or no lever press in 10 s, this was counted as either head omissions or lever omissions, respectively. During each trial, the lever lights were also randomly presented but they gave no indication to which lever was active. This was for the possibility of utilising the program in future for other cognitive tasks such as a set shifting task. Each session lasted either 30 min or 100 trials, whichever came first. Each rat had one training session per day. The BCT stage lasted 3 days, and only rats reaching criterion at the end of the third day, >70% correct responding and <30% omissions, went onto the reversal stage. The reversal program was the same as the BCT program for each rat but the previously inactive lever was now active and the previously active lever became inactive. Each session lasted either 30 min or 100 trials and each rat had one session per day. The reversal stage also lasted 3 days (2 days retention after the reversal day), after

which all rats went onto the final reversal stage. Here, the active lever was the original active lever i.e. the same as in BCT.

								Test Phase						
Day	THURS	FRI	MON	TUE	WED	THU	FRI	MON	TUE	WED	THU	FRI	MON	TUE
Stage	Magazine	FR1		FR3		BCT			Reversal 1	Retention		Reversal 2	Retention	

Figure 2: Reversal learning training procedure for Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Reversal Learning Performance.

3.2.2 Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning

3.2.2a Subjects

Animals were prepared as previously described in Chapter 2.

48 Male, Sprague Dawley (Crl:CD(SD) (SD), Charles River, Kent) aged 12 weeks at the start of the experiment were used and the details are as in 3.2.$_{1A}$. Pup information is shown in table 2 and a timeline of studies is shown in figure 3.

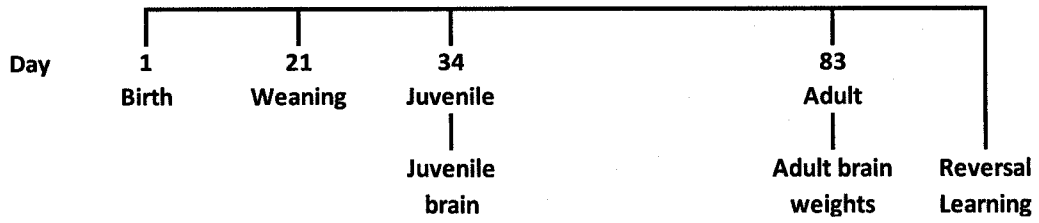


Figure 3: Timeline of events and studies performed for Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning.

3.2.2b Reversal Learning

3.2.2b(i) Apparatus

Apparatus remained the same as in $3.2._{1A}$.

3.2.2b(ii) Reversal Learning Procedure

Reversal Learning remained the same as in 3.2.1b. However, in this case, the subjects were divided into 3 groups, vehicle (MAM n = 10, SHAM n = 8), mGlu5 before Reversal 1 (MAM n = 7, SHAM n = 8), and mGlu5 after Reversal 1 (MAM n = 7, SHAM n = 8). All assignments are shown in table 2. On reversal 1 day, subjects were orally administered with either vehicle or mGlu5 either 30 mins before being put into the operant chamber or immediately after they finished the 30min procedure (figure 4).

3.2.2b(iii) Drugs

LSN2814617 (Lilly, UK), 1 mg/kg, 1 mg/ml accounting for salt weight in 1% CMC, 0.25% tween, 0.05% antifoam administered orally either 30 min before reversal 1 or directly after reversal 1.

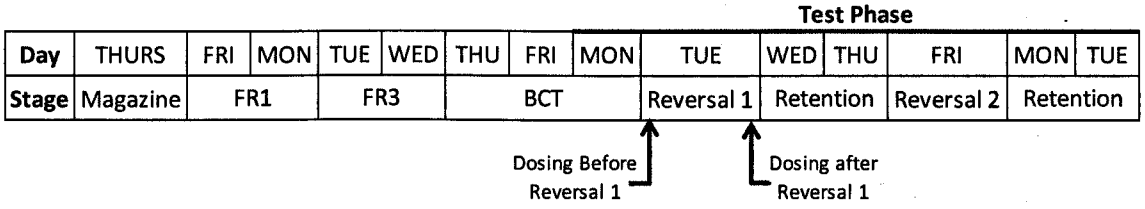


Figure 4: Reversal Learning training and dosing procedure for Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning.

Table 2: Detail of pup treatment groups and lever assignment for Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning.

Number	Pup	Tag	Group	Cage	Active Lever	mGlu5 Treatment
1	NN222	2407985	SHAM E17	1	Right	After Rev1
2	AA155	2450817			Left	Before Rev1
3	EE180	2409630			Left	Before Rev1
4	FF184	2408999			Left	Before Rev1
5	FF187	2407398	SHAM E17	7	Right	After Rev1
6	U129	2410268			Left	Before Rev1
7	A2	2409324			Left	Before Rev1
8	B8	2409600			Left	Before Rev1
9	N86	2448551	MAM E17	9	Left	After Rev1
10	C17	2333806			Right	Vehicle
11	Q105	2402719			Left	Before Rev1
12	KK215	2410162			Left	Vehicle
13	F43	2407848	MAM E17	11	Right	Before Rev1
14	JJ205	2402462			Right	Before Rev1
15	KK212	2408884			Right	Vehicle
16	R113	2409576			Left	After Rev1
17	Y144	2410285	SHAM E17	14	Right	Vehicle
18	B10	2408971			Left	Vehicle
19	LL216	2407645			Left	After Rev1
20	U128	2410204			Right	Vehicle
21	I61	2409358	SHAM E17	15	Right	Vehicle
22	GG189	2399045			Right	After Rev1
23	K77	2451790			Right	After Rev1
24	EE174	2450109			Right	Vehicle
25	NN224	2410335	SHAM E17	18	Right	After Rev1
26	FF185	2449966			Right	Before Rev1
27	M85	2453466			Right	Before Rev1
28	X138	2403963			Left	After Rev1
29	JJ204	2404041	MAM E17	24	Left	Vehicle
30	H55	2399546			Left	Vehicle
31	KK214	2407917			Right	Vehicle
32	E35	2409170			Left	Vehicle
33	CC164	2451702	MAM E17	28	Right	Before Rev1
34	H57	2409336			Right	Before Rev1
35	N87	2452068			Right	After Rev1
36	F39	2407565			Right	Vehicle
37	P99	2399451	MAM E17	31	Left	Before Rev1
38	N88	2451506			Left	After Rev1
39	JJ203	2402546			Left	Vehicle
40	F37	2399078			Left	Vehicle
41	L81	2449219	SHAM E17	33	Left	Vehicle
42	LL218	2403496			Left	Vehicle
43	EE175	2408852			Right	After Rev1
44	G50	2402914			Left	Vehicle
45	Q107	2409815	MAM E17	37	Right	After Rev1
46	E29	2407789			Right	After Rev1
47	T120	2398995			Right	Before Rev1
48	H58	2410132			Left	After Rev1

3.2.3 Experiment 3: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning

3.2.3a Subjects

Animals were prepared as previously described in Chapter 2.

72 Male, Sprague Dawley (Crl:CD(SD) (SD), Charles River, Kent) aged 12 weeks at the start of the experiment were used and the details are as in 3.2.$_{1A}$. Pup information is shown in table 3 and a timeline of studies is shown in figure 5.

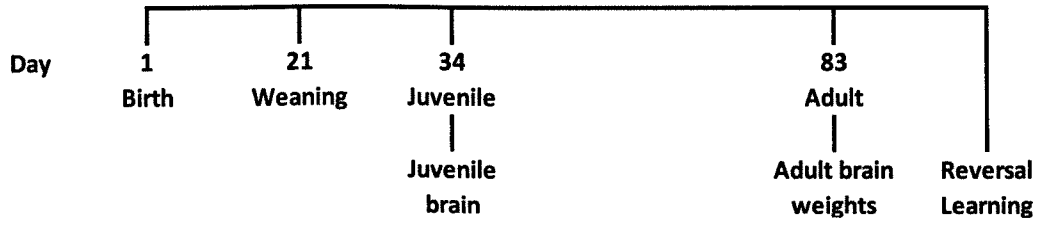


Figure 5: Timeline of events and studies performed for Experiment 3: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning

3.2.3b Reversal Learning

5.2.3b(i) Apparatus

Apparatus remained the same as in 3.2.$_{1A}$.

Table 3: Detail of pup treatment groups and lever assignment for Experiment 3: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning. Those highlighted in yellow were excluded from the final results as they were born a day too late.

Rat	Pup	Tag	Group	Cage	Lever	mGlu5 Treatment	Birth
1	I53	2556711	MAM	3	Right	Before Rev2	28
2	L67	2555620	MAM	3	Right	Before Rev1	28
3	S124	2575301	MAM	3	Left	Vehicle	29
4	JJ206	2575035	MAM	3	Right	Before Rev1	28
5	B12	2553107	MAM	4	Right	Before Rev1	28
6	M75	2553351	MAM	4	Right	Vehicle	28
7	T129	2573910	MAM	4	Right	Before Rev2	28
8	JJ211	2571603	MAM	4	Right	Before Rev2	28
9	C16	2555759	SHAM	5	Right	Before Rev2	28
10	R115	2556998	SHAM	5	Right	Before Rev1	28
11	CC168	2579668	SHAM	5	Left	NT	29
12	N90	2559196	SHAM	5	Left	Before Rev1	28
13	D22	2555696	SHAM	10	Left	Before Rev2	28
14	V138	2574984	SHAM	10	Right	Before Rev1	28
15	CC169	2570311	SHAM	10	Right	Vehicle	29
16	II200	2575687	SHAM	10	Right	Before Rev2	28
17	E26	2555607	SHAM	12	Left	Before Rev1	29
18	R119	2579816	SHAM	12	Left	Before Rev2	28
19	AA164	2555766	SHAM	12	Right	Vehicle	28
20	EE177	2576673	SHAM	12	Left	Vehicle	28
21	H44	2556782	MAM	17	Right	Before Rev2	28
22	O94	2571855	MAM	17	Right	Vehicle	28
23	Q110	2552085	MAM	17	Right	Vehicle	29
24	DD174	2574137	MAM	17	Left	Before Rev2	28
25	G42	2555849	SHAM	21	Right	Vehicle	29
26	N85	2559629	SHAM	21	Left	Before Rev2	28
27	X148	2575439	SHAM	21	Left	Before Rev1	28
28	FF187	2571714	SHAM	21	Left	Before Rev2	29
29	E25	2557254	SHAM	25	Right	Vehicle	29
30	J59	2557017	SHAM	25	Left	Before Rev1	28
31	BB166	2571094	SHAM	25	Right	Vehicle	28
32	II203	2574367	SHAM	25	Left	Before Rev2	28
33	F31	2558972	MAM	26	Right	Before Rev1	28
34	O96	2572130	MAM	26	Right	Vehicle	28
35	P107	2574643	MAM	26	Right	Before Rev1	28
36	DD173	2573144	MAM	26	Left	Vehicle	28
37	C13	2555117	SHAM	29	Left	Before Rev1	28
38	J57	2553016	SHAM	29	Left	Before Rev1	28
39	X149	2574498	SHAM	29	Right	Vehicle	28
40	GG189	2575597	SHAM	29	Left	Before Rev2	28
41	B5	2551583	MAM	32	Left	Before Rev1	28
42	K61	2558280	MAM	32	Left	Before Rev2	28
43	Q109	2558896	MAM	32	Right	Before Rev1	29
44	JJ205	2578688	MAM	32	Left	Before Rev1	28
45	G39	2552670	SHAM	35	Left	Before Rev1	29
46	N89	2555781	SHAM	35	Right	Vehicle	28
47	AA162	2571607	SHAM	35	Right	Before Rev2	28
48	EE180	2563285	SHAM	35	Right	Vehicle	28
49	D21	2556708	SHAM	37	Left	Vehicle	28
50	J56	2553853	SHAM	37	Right	Vehicle	28
51	AA161	2574764	SHAM	37	Left	Before Rev1	28
52	GG188	2552408	SHAM	37	Right	Before Rev2	28
53	F33	2552948	MAM	41	Left	Before Rev2	28
54	K65	2557582	MAM	41	Right	Vehicle	28
55	U134	2575955	MAM	41	Right	Before Rev1	28
56	HH193	2575298	MAM	41	Left	Before Rev2	29
57	B7	2559046	MAM	42	Left	Vehicle	28
58	M83	2557883	MAM	42	Left	Before Rev2	28
59	S125	2574062	MAM	42	Left	Vehicle	29
60	W146	2570960	MAM	42	Right	Before Rev1	29
61	E23	2558367	SHAM	46	Right	Before Rev1	29
62	R120	2579006	SHAM	46	Left	Before Rev2	28
63	V143	2573364	SHAM	46	Right	Before Rev1	28
64	II201	2572363	SHAM	46	Right	Before Rev2	28
65	F36	2554420	MAM	47	Right	Before Rev2	28
66	K64	2555509	MAM	47	Left	Vehicle	28
67	T132	2573061	MAM	47	Right	Before Rev1	28
68	HH196	2576101	MAM	47	Right	Before Rev2	29
69	B6	2556674	MAM	48	Right	Before Rev1	28
70	L72	2555437	MAM	48	Left	Before Rev2	28
71	U133	2573628	MAM	48	Left	Vehicle	28
72	JJ208	2553470	MAM	48	Right	NT	28

3.2.3b(ii) Reversal Learning Procedure

Reversal Learning remained mainly the same as in 3.2.2b but a third reversal was introduced to test of the MAM deficit seen in reversal 2 remained and if treatment with the mGlu5 PAM could attenuate the deficit. Subjects were originally divided into 3 groups, vehicle (MAM n = 11, SHAM n = 11), mGlu5 before Reversal 1 (MAM n = 12, SHAM n = 12), and mGlu5 before Reversal 2 (MAM n = 12, SHAM n = 12) but as it was found that birth date might be an important factor, all the rats that were born a day too late (29th) were excluded. The resulting n numbers were: vehicle (MAM n = 8, SHAM n = 8), mGlu5 before Reversal 1 (MAM n = 10, SHAM n = 9), and mGlu5 before Reversal 2 (MAM n = 10, SHAM n = 11).

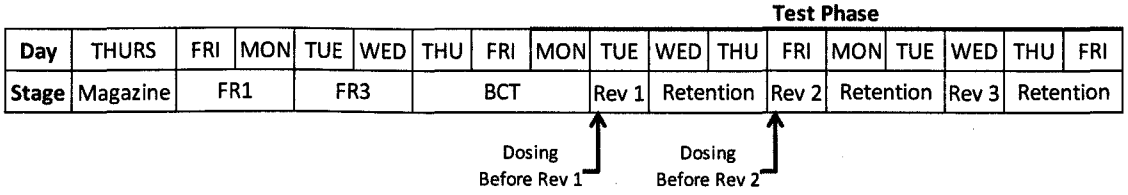


Figure 6: Reversal Learning training and dosing procedure for Experiment 3: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning (repeat)

3.2.3b(iii) Drugs

LSN2814617 (Lilly, UK), 1 mg/kg, 1 mg/ml accounting for salt weight in 1% CMC, 0.25% tween, 0.05% antifoam administered orally either 30 min before reversal 1 or 30 min before reversal 2.

3.2.4 Statistical Analysis

All statistical analysis were calculated using STATISTICA v.7 and all data are expressed as mean ± standard error of the mean (s.e.m). In general, data was analysed using two-way analysis of variance, followed where appropriate by planned comparisons (univariate test of significance) with between subject factors of group (MAM; SHAM) vs. treatment (veh; mGlu5). In the case of significant interactions, these were further submitted to analyses of simple effects and if appropriate to planned comparisons. Brain and body weight was analysed separately for each strain using two-way analyses of variance with between subject factors of group (MAM; SHAM). In all cases, $P<0.05$ indicated significance.

3.3 Results

3.3.1 Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Reversal Learning Performance

3.3.1a Brain and Body Weights.

Repeated measures ANOVA showed no significant difference in the body weight gain (Figure 7a) between MAM and SHAM treated rats ($F_{(3,40)} = 0.997$, $p>0.1$). There was an effect of treatment on brain weights in that MAM brains were significantly smaller than those of SHAM rats ($F_{(1,10)} = 0.098$, $p<0.001$)(Figure 7b).

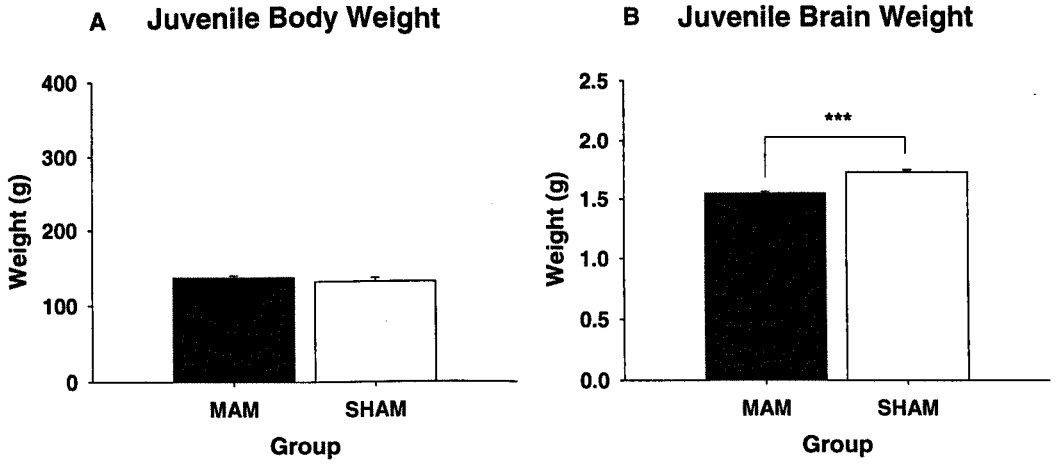


Figure 7: (a) Body weights and (b) brain weights in juvenile rats treated with MAM on E17
Effect of MAM treatment on day E17 on (a) body weights (g) and (b) brain weights (g) in juvenile rats (n = 8 per group). Data are presented as mean ± standard error of the mean. Significant differences are indicated on the figure as follows: *** P<0.001.

3.3.1b Reversal Learning Task

Repeated measures ANOVA showed no significant difference in the acquisition (BCT) phase of the reversal task over the 3 days between MAM and SHAM treated rats ($F_{(1,39)} = 0.001$, $p>0.1$). There was however a deficit in performance in reversal 1 ($F_{(1,39)} = 13.675$, $p<0.001$) and reversal 2 ($F_{(1,39)} = 5.675$, $p<0.05$) where the percentage accuracy was significantly lower in the MAM treated animals compared to the SHAMs (figure 8).

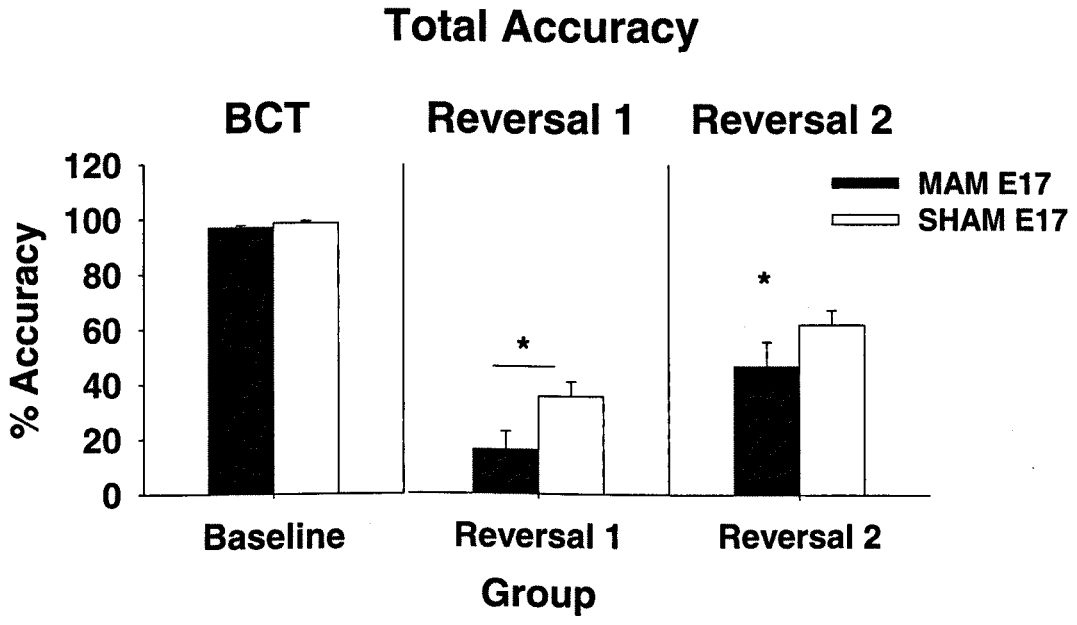


Figure 8: Performance of adult rats treated with MAM on E17 on the reversal learning task
Effect of MAM treatment on E17 in adult rats on percentage accuracy in reversal learning. Data are presented as mean ± standard error of the mean (n = 15 per group). Significant differences are indicated on the figure as follows:* P<0.05.

3.3.2 Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning

3.3.2a Brain and Body Weights.

Repeated measures ANOVA showed no significant difference in the body weight gain (Figure 9a,b) between MAM and SHAM treated rats ($F_{(3,40)} = 0.997$, $p>0.1$). There was an effect of treatment ($F_{(1,28)} = 71.97$, $p<0.001$) and age ($F_{(1,28)} = 414.11$, $p<0.001$) on brain weights (Figure 10a,b).

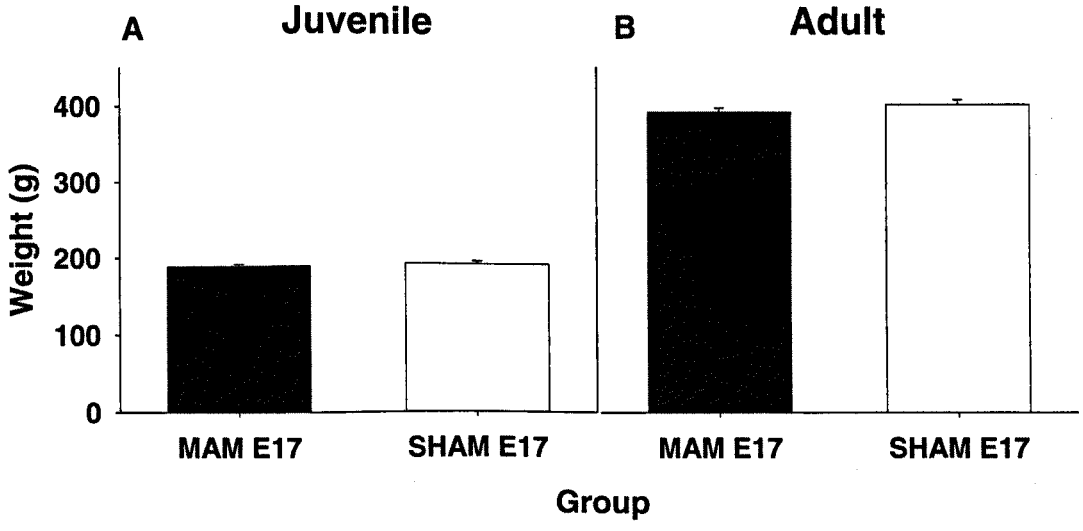


Figure 9: Body weights in (a) juvenile and (b) adult rats treated with MAM on E17 Effect of MAM treatment on days E17 in (a) juvenile and (b) adult rats on body weights (g) (n = 8 per group). Data are presented as mean ± standard error of the mean.

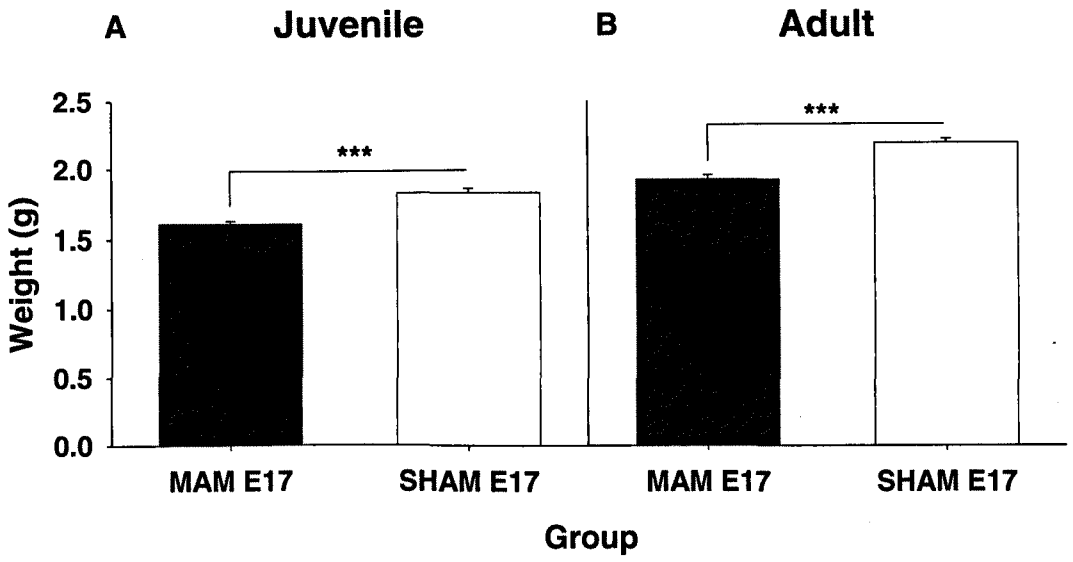


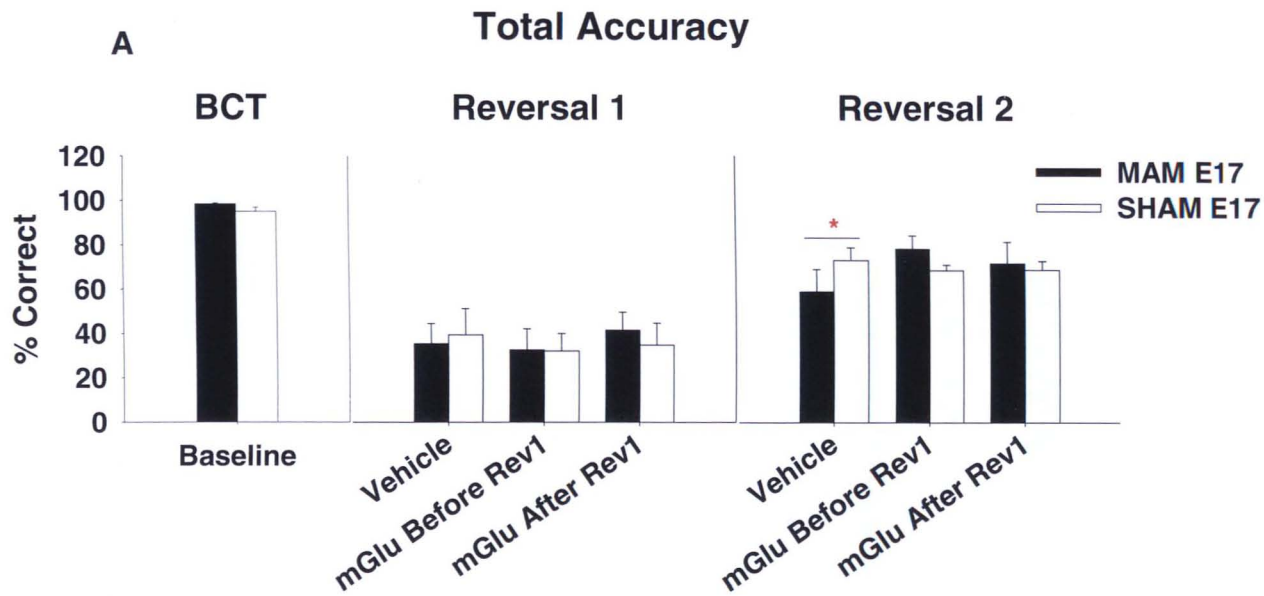
Figure 10: Brain weights in (a) juvenile and (b) adult rats treated with MAM on E17

Effect of MAM treatment on days E17 in (a) juvenile and (b) adult rats on brain weights (g) (n = 8 per group). Data are presented as mean ± standard error of the mean. Significant differences are indicated on the figure as follows: *** P<0.001.

3.3.2b Reversal Learning Task

Repeated measures ANOVA showed no significant difference in the acquisition of the reversal task over the 3 days between MAM and SHAM treated rats ($F_{(2,122)}$ = 1.92, p>0.1). There was also no significant deficit in performance at reversal 1 ($F_{(1,111)}$ = 0.143, p>0.5) in the MAM treated rats, however there was a significant deficit at reversal 2 where the percentage accuracy of MAM treated rats was significantly lower than the SHAM rats ($F_{(1,111)}$ = 5.333, p<0.05)(Figure 1_{1A},b). Treatment with the mGlu5 PAM attenuated this deficit as there was no significant difference the MAM and SHAM rats treated with the PAM either before or after Reversal 1 (Figure 1_{1A}). This is further highlighted by figure 11b which shows a

significant difference between MAM rats treated with vehicle and those treated with the mGlu5 PAM but no significant difference between the same SHAM groups.



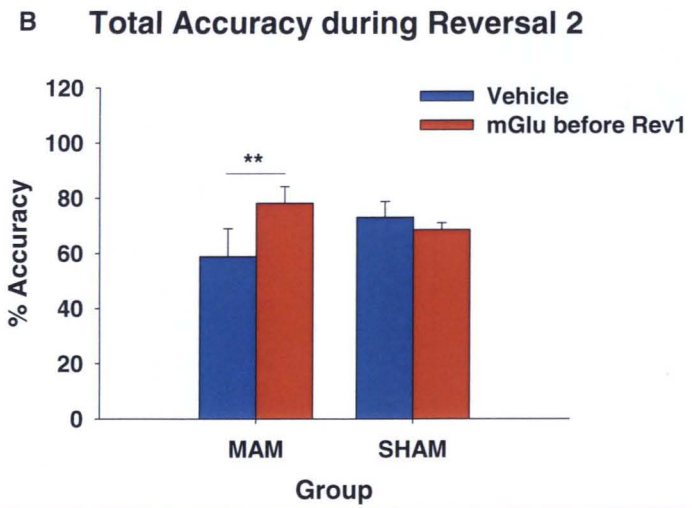


Figure 11: Effect of mGlu5 PAM treatment on performance on the (a) reversal learning task and the (b) reversal 2 phase of the task in adult MAM E17 treated rats. Effect of 1 mg/kg mGlu5 PAM on percentage accuracy in the (a) reversal learning and (b) the reversal 2 phase of the task in MAM E17 treated rats. N numbers are as follows: vehicle (MAM = 10, SHAM = 8), mGlu5 before Reversal 1 (MAM = 7, SHAM = 8), and mGlu5 after Reversal 1 (MAM = 7, SHAM = 8). Data are presented as mean ± standard error of the mean. Significant differences are indicated on the figure as follows: * $P<0.05$; ** $P<0.01$.

3.3.3 Experiment 3: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Reversal Learning (repeat)

3.3.3a Brain and Body Weights.

Repeated measures ANOVA showed no significant difference in the body weight gain (Figure 12a,b) between MAM and SHAM treated rats ($F_{(2,314)} = 0.46$, $p>0.5$). There was an effect of treatment on brain weights ($F_{(1,21)} = 57.78$, $p<0.001$)(Figure 13a,b).

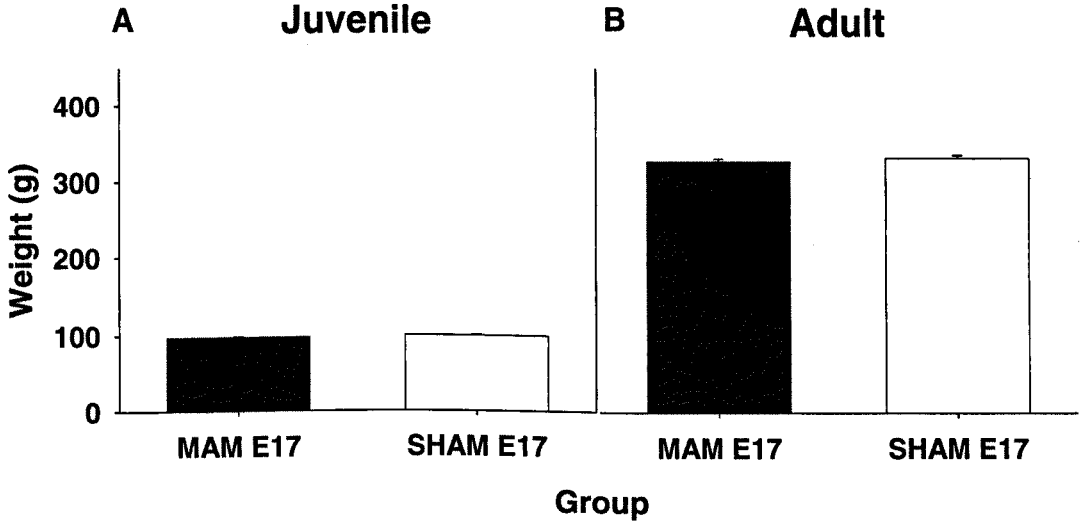


Figure 12: Body weights in (a) juvenile and (b) adult rats treated with MAM on E17

Effect of MAM treatment on days E17 in (a) juvenile and (b) adult rats on body weights (g) (n = 8 per group). Data are presented as mean ± standard error of the mean.

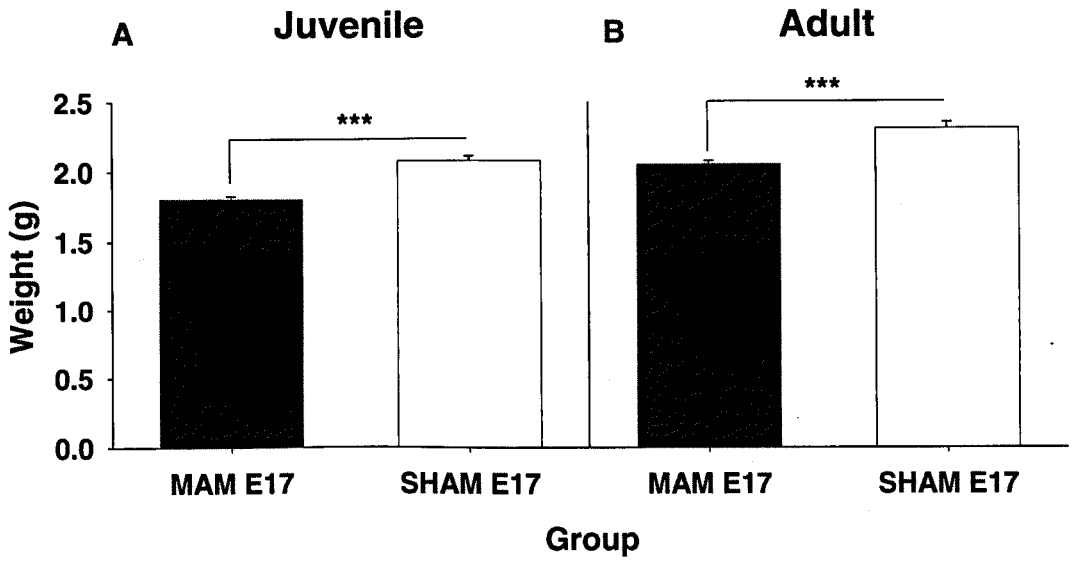


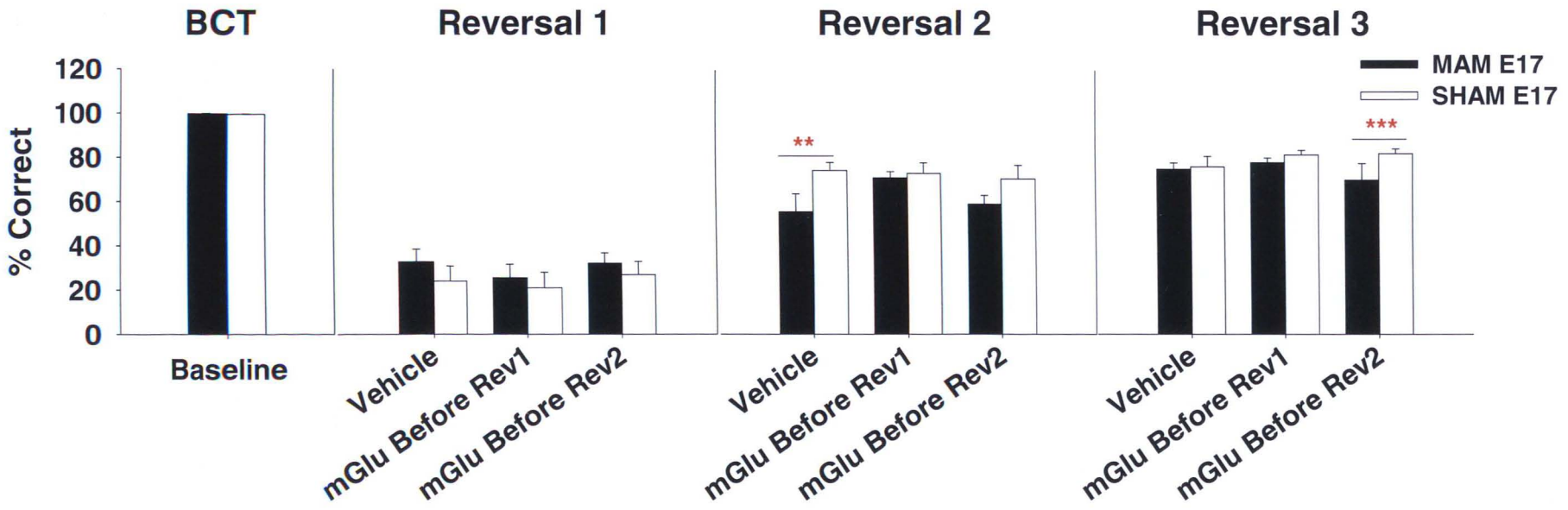
Figure 13: Brain weights in (a) juvenile and (b) adult rats treated with MAM on E17

Effect of MAM treatment on days E17 in (a) juvenile and (b) adult rats on brain weights (g) (n = 8 per group). Data are presented as mean ± standard error of the mean. Significant differences are indicated on the figure as follows: *** P<0.001.

3.3.3b Reversal Learning Task

Repeated measures ANOVA showed no significant difference in the acquisition of the reversal task over the 3 days between MAM and SHAM treated rats ($F_{(1,216)}$ = 1.7, p>0.1). There was also no significant deficit in performance at reversal 1 in any of the treatment groups ($F_{(1,147)}$ = 1.613, p>0.1) in the MAM treated rats, however there was a significant deficit at reversal 2 ($F_{(1,147)}$ = 4.345, p<0.05) where the vehicle treated MAM rats performed significantly lower than the SHAM rats. Treatment with the mGlu5 PAM on the day of reversal 1 significantly attenuated this deficit as shown by Figure 14a and b. The group that received the mGlu5 PAM on the day of reversal 2 instead of reversal 1 showed a trend towards MAMs

performing lower than SHAMs ($p = 0.07$) (Figure 14a; Reversal 2). This same group did not show improved performance on reversal 3 whereas the MAM group that received treatment on reversal 1 were still performing at the higher level of the SHAMs.



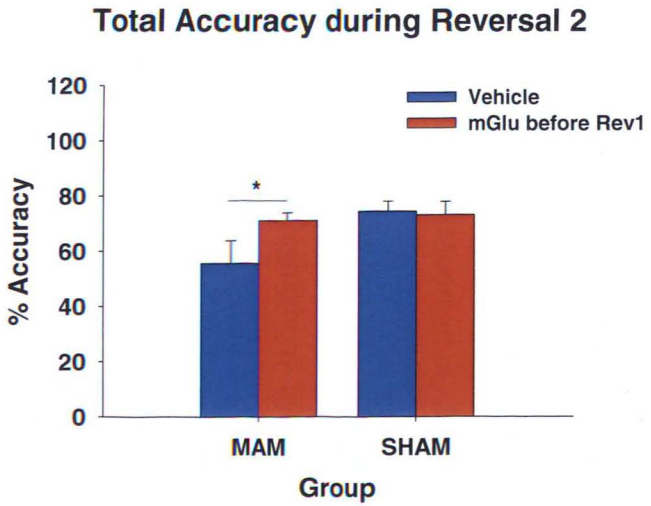


Figure 14: Effect of mGlu5 PAM treatment on performance on the (a) reversal learning task and the (b) reversal 2 phase of the task in adult rats treated with MAM on E17.

Effect of 1 mg/kg mGlu5 PAM on percentage accuracy in the (a) reversal learning and (b) the reversal 2 phase of the task in MAM E17 treated rats. N numbers are as follows: vehicle (MAM = 8, SHAM = 8), mGlu5 before Reversal 1 (MAM = 10, SHAM = 9), and mGlu5 before Reversal 2 (MAM = 10, SHAM = 11)Data are presented as mean ± standard error of the mean. Significant differences are indicated on the figure as follows: * P<0.05; ** P<0.01; *** P<0.001.

3.4 Discussion

This study demonstrates that the novel mGlu5 PAM LSN2814617 attenuates reversal learning deficits in the MAM E17 model of schizophrenia, as measured by the rodent spatial reversal learning task. Overall, these findings are noteworthy for their demonstration of a cognitive enhancing effect of LSN2814617 in a test of fronto-executive function in an animal model of schizophrenia. As shown in previous chapters, there was no effect of MAM treatment on body weight and MAM treatment produced a reduction in brain weight in both juvenile and adult brains, indicating successful MAM treatment.

All three studies within this chapter showed a consistent deficit in reversal learning in the MAM treated rats. Effective performance in the reversal-learning task requires intact cognitive ability; thus animals are required to demonstrate flexibility, attention, motivation, and ability to suppress a previously learned response and implement a new one (Jones *et al.,* 1991). The MAM E17 model has been shown previously to produce a wide variety of cognitive impairments, including impaired reversal learning and attentional set-shifting (Moore *et al.,* 2006; Featherstone *et al.,* 2007) although these were not in an operant reversal learning task such as this one. The reversal-learning task has been highlighted by the MATRICS initiative as a test of reasoning and problem solving i.e. executive function. Similar tests in schizophrenia patients, such as the Wisconsin Card Sorting Test, require intact functioning of the PFC (Deicken *et al.,* 1995; Dias et al., 1997). It

has been shown more specifically that lesions of the orbital prefrontal cortex impair reversal learning (McAlonan and Brown., 2003; Tait and Brown., 2007). Through lesion and electrophysiological studies in rats and monkeys, reversal learning is known to be controlled by a neural network that include several regions of importance in schizophrenia, such as the hippocampus, amygdala, medial striatum, orbitofrontal (OFC) and medial prefrontal cortex (mPFC) (Clarke *et al.,* 2008; Dias *et al.,* 1996; Chudasama and Robbins., 2003; McAlonan and Brown., 2003) as discussed in the introduction. As shown in chapter 2, MAM treated rats have decreased prefrontal cortex thickness compared to SHAM rats which could be an indication that the orbital and/or medial PFC of these rats is altered. As discussed in chapter 1, MAM is a CNS specific mitotoxin and therefore affects cell division. Dosing MAM on E17 coincides with the cell division of the neurones migrating to the hippocampus and prefrontal cortex. Indeed, MAM-treated rats have been shown to exhibit morphological changes in the PFC (Featherstone *et al.,* 2007; Gourevitch *et al.,* 2004). A loss of parvulbumin containing internurones in the PFC has also been shown in MAM treated animals (Lodge *et al.,* 2009) as well as in schizophrenic patients (Lewis and Moghaddam., 2006).

Impaired reversal learning has also recently been highlighted to occur reliably in schizophrenia showing some translational validity of the model. Leeson *et al.,* (2009) found in a large group of first episode schizophrenic patients exhibited small but consistent and highly significant deficits in reversal learning. These deficits in reversal learning, unlike those found in set-shifting, were unrelated to general

intelligence and correlated significantly with the disorganization syndrome, especially of positive formal thought disorder, which presumably interferes with cognitive function and has been shown to be related to reductions in OFC volume (Nakamura *et al.,* 2008). Similarly, reversal learning impairments have been observed in schizophrenics in the context of changes in frontostriatal activation (Murray *et al.,* 2008).

Gastambide *et al.,* (2012) have shown that the reversal learning deficit seen in MAM-treated rats is a specific form, namely in perseverative responding (where the rat continues to choose the previously correct stimulus). Interestingly, perseverative behaviour is widely observed in patients with schizophrenia undertaking tasks such as the Winconsin Card Sorting test (Perry and Braff, 1998; Pantelis *et al.,* 1999; Liddle., 2000) and schizophrenic patients with a hippocampal lesion have also been shown to commit significantly more perseverative errors when performing on the WCST, than subjects with frontal lobe damage (Corcoran and Upton., 1993). Such evidence supports the notion that reversal learning may be a viable translational element of schizophrenic cognitive deficits that can be studied in both rodents and humans.

There were however some discrepancies with regards to the reversal learning deficits. The deficits were seen in reversal 1 and 2 in the first study but only in reversal 2 in the second and third studies. In the reversal paradigm of the Morris Water Maze task, MAM-treated rats performed significantly worse than controls on

days 2 and 3 of testing. On the last day, however, they performed as well as the controls (Flagstad *et al.,* 2005). In set-shifting task that contains three reversals, MAM rats have been shown to have deficits in the first and third reversals in some instances (Featherstone *et al.,* 2007), and in the first and second reversal in others (Gastambide *et al.,* 2012). Orbital frontal lesions, however, routinely cause an impairment in initial reversal learning in an operant reversal learning task which then improves across reversals (Boulougouris *et al.*, 2007).

mGlu5 receptors are expressed in key brain regions for cognition, including hippocampus, amygdala, striatum and prefrontal cortex (Shigemoto *et al.,* 1993; Romano *et al.,* 1995). Modulation of mGlu5 can therefore alter a wide range of cognitive functions in rodents including working memory, attention and cognitive flexibility (Kinney *et al.,* 2003; Homayoun *et al.,* 2004; Manahan-Vaughan and Braunewell., 2005; Semenova and Markou, 2007; Ayala *et al.,* 2009; Xu *et al.,* 2009; Homayoun and Moghaddam, 2010). The findings from the current study demonstrate that administration of the mGlu5 PAM LSN2814617 can improve the performance of MAM treated rats in reversal learning. However, the underlying mechanism of this remains unclear. Gastambide *et al.,* (2012) assessed the regional effects of MAM on the expression of mGlu5 receptors using immunohistochemical techniques, as well as the expression of parvalbumin (PV)-containing GABAergic interneurons known to be implicated in the neuropathology of schizophrenia (Lewis., 2000). They found reductions of PV and mGlu5 expression in the MAM treated rats in both the orbitofrontal and medial prefrontal cortex, which also

mediate reversal learning and extra-dimensional set-shifting. mGlu5 receptors are key postsynaptic signalling partners of NMDA receptors (Marino and Conn., 2002). Activation of mGlu5 may therefore potentiate the function of NMDA receptors within the reversal learning 'network' and as a result, restore plasticity mechanisms which have been reported to be disrupted in the model (Moore *et al.,* 2006; Lodge *et al.,* 2009; Sanderson *et al.,* 2011). While there are no published reports of down-regulation of mGlu5 receptors in schizophrenia to date, such a finding would be consistent with the hypoglutamatergic theory of the disorder.

Overall, the present data suggest that mGlu5 potentiation may have beneficial effects in the treatment of certain cognitive impairments associated with schizophrenia. The efficacy of LSN2814617 in this reversal learning model is therefore of particular importance for demonstrating a role for positive modulation of mGlu5 receptors in alleviating cognitive deficits in schizophrenia. Further studies are needed to test the mGlu5 effects in other memory and executive functions, such as working memory. However, the present findings do show face and construct validity and also indicate the predictive validity of the MAM E17 model for testing new drugs that may reverse the specific cognitive deficits seen in schizophrenia.

Chapter 4: Fear Conditioning

4.1 Introduction

One of the behavioural deficits that has consistently been observed in schizophrenia is impaired emotional learning and memory. It is mediated by several discrete neurological regions and requires the interaction of several systems (McGaugh., 2004; Phelps., 2004). Specifically, data indicate a deficit in the impact of emotion on memory (Hamann *et al.,* 1999; Gur *et al.,* 2002; Kring and Caponigro., 2010). In non-schizophrenic populations, neuroimaging studies have demonstrated that amygdala activation is positively correlated with hippocampal activity and subsequent memory while viewing emotional scenes (Hall *et al.,* 2007, Hamann *et al.,* 1999; Dolcos *et al.,* 2004). Patients with schizophrenia have been shown to display a reduction in amygdala activation when viewing emotional faces and reduced activation in the hippocampus during episodic memory tasks (Hall *et al.,* 2007; Gur *et al.,* 2002; Achim and Lepage., 2005). These findings not only indicate a change in emotional processing, but may also represent an alteration in connectivity between the amygdala and hippocampus.

Pavlovian or classical fear conditioning is recognised as a model system to investigate the neurobiological mechanisms of learning and memory in the mammalian brain and to understand the basis of fear-related disorders in humans.

Fear conditioning is a behavioural task where an association is made between a neutral auditory or visual stimulus (the conditioned stimulus, CS) and an aversive unconditioned stimulus (US) such as a foot shock. Following the CS-US paired training the animal is then presented with the CS alone and since it is associated with the US it evokes a conditioned fear response (CR), characterised by freezing behaviour in rodents, autonomic nervous system responses (increase in blood pressure and heart rate), and neuroendocrine responses (release of hormones from the pituitary and adrenal glands).

In a typical auditory fear conditioning paradigm, there are three distinct phases: the habituation phase where the rat is left in the chamber and no stimuli are presented, the conditioning phase where the tone conditioned stimulus (CS) is paired with the footshock unconditioned stimulus (US). The third phase is the extinction or test phase. Here the CS is presented in the absence of the US and typically, the rat exhibits freezing responses to the CS during this phase. This is known as cued fear conditioning, in which the training protocol produces an association between the CS and the US. The association can be quantified based on the fear response (freezing) to the CS (cued fear) and serves as a measure of how well the animal learned an association. In addition, animals also learn a quantifiable association between the US and the environment in which the training took place, this is known as contextual fear conditioning.

An advantage of this task is that alterations in the training protocol have been demonstrated to recruit differential involvement of specific neurological regions in order to learn the association. In standard delay cue contextual fear procedures, when the CS and US overlap in time and co-terminate, the cued fear component requires amygdala function (Phillips and LeDoux., 1992; LeDoux., 2003) and contextual fear conditioning depends not only on the amygdala but also on the hippocampus. As the amygdala is crucial for the formation of the association between the CS and the US it creates the link between the hippocampal representation of the context and the aversive shock (US) (LeDoux., 2000; Phillips and LeDoux., 1992; Chen at al., 1996; Logue *et al.,* 1997; Holland and Bouton., 1999). A variation known as trace conditioning modifies this task by inserting a temporal gap between the cessation of the CS and the onset of the US (Kinney *et al.,* 2002). The animal therefore has to maintain a memory `trace' of the conditioned stimulus to associate it with the unconditioned stimulus (Solomon and Schaaf *et al.,* 1986). The insertion of the interval between the CS and US has been demonstrated to make the association more difficult, as additional training trials are required for equivalent learning (Kinney *et al.,* 2002; Beylin *et al.,* 2001). Further, data indicate that not only does the task become more difficult, but lesion studies demonstrate the trace cued fear association is also dependent on both the hippocampus and amygdala (Kinney *et al.,* 2002; Sutherland and McDonald., 1990; McEchron *et al.,* 1998). Disruption of protein synthesis in the amygdala immediately following trace conditioning has been shown to impair the consolidation of cue and context memories (Kwapis *et al.,* 2011). Several studies have also demonstrated a

role for a differential role of the dorsal (DH) and ventral (VH) hippocampus (Yoon and Otto., 2007; Czerniawski *et al.,* 2009; Esclassan *et al.,* 2009) in the acquisition of trace fear conditioning. Neurotoxic lesions of the whole hippocampus or reversible inactivation of the ventral hippocampus impaired both contextual and tone freezing in both trace and delay conditioned rats. However, dorsal hippocampal injections impaired contextual freezing and trace conditioning, but not delay conditioning (Esclassan *et al.,* 2009). Yoon and Otto (2007) found that both pre- and post-training lesions of ventral hippocampus impaired the acquisition and expression of auditory trace fear conditioning. However, pre-training lesions of the dorsal hippocampus had no effect on the acquisition of trace fear conditioning, while post-training lesions of the dorsal hippocampus dramatically impaired expression during subsequent testing. The mPFC has also been implicated in trace fear memory processes (Blum *et al.,* 2006; Gilmartin and Helmstetter., 2010) and it has been shown that the prelimbic (PL) area of the mPFC is necessary for trace but not delay fear conditioning (Gilmartin and Helmstetter., 2010). The dependence on mPFC and hippocampus in trace fear conditioning suggests that memory formation may require communication between these two regions. The PL mPFC may be important for helping to maintain the CS across the trace interval (Gilmartin and McEchron., 2005) and Gilmartin *et al.,* (2012) hypothesized that communication between the mPFC and hippocampus is necessary for the association of the CS and US across this interval. They found that unilateral inactivation of the ventral hippocampus or amygdala impairs memory, while bilateral inactivation of the PL is required to produce a deficit. However, deficits after unilateral inactivation of the ventral

hippocampus or amygdala prevented them from determining whether the mPFC functionally interacts with the hippocampus but their findings suggest that the trace fear network is more integrated than previously thought.

Once fear conditioning has been learned, it can be very difficult to reverse. Nevertheless, fear conditioning and its associated fear responses can be inhibited by an active process known as fear extinction. Fear extinction occurs when an animal is repeatedly exposed to the CS in the absence of the US which leads to a decline in the magnitude of the fear-associated response. Like other forms of learning and memory, fear extinction involves encoding, consolidation, retrieval and expression stages, which are mediated by different neural mechanisms (Myers *et al.,* 2011). Unlike fear conditioning, fear extinction is a labile process that tends to reverse over time. For instance, exposure to the CS alone, after the extinction process has been established, leads to the reappearance of the CR, a phenomenon called reinstatement (Myers *et al.,* 2011). Also, fear conditioning can return if the old fear stimuli are presented in a different context from the one in which extinction was learned, a process termed renewal (Myers *et al.,* 2011). Though fear-extinction can suppress the fear-associated response, it does not seem to remove the synaptic changes acquired during fear conditioning. Thus, fear extinction involves new learning and new synaptic changes in the amygdala (Myers *et al.,* 2011). Fear-extinction learning and memory process, as well as its modulation by context, involves three main components: the amygdala, mPFC, and hippocampus (Myers *et al.,* 2011; Quirk and Mueller, 2008).

As discussed in Chapter 1, one of the prevailing models of schizophrenia proposes a reduction in NMDA receptor function on inhibitory interneurons and the resulting disinhibition may give rise to aspects of the disorder. Studies using NMDA receptor antagonists such as PCP and ketamine have induced schizophrenia-like behavioural deficits in animal model systems as well as changes in inhibitory circuits. Bolton *et al.,* (2012) utilized both standard delay and trace cued and contextual fear conditioning paradigms to examine if ketamine produces differential effects when the task is more difficult and relies on connectivity between specific brain regions. Rats administered ketamine displayed no significant deficits in cued or contextual fear following the delay conditioning protocol. However, ketamine did produce a significant impairment in the more difficult trace conditioning protocol.

As fear conditioning is present across animal species, where the same primitive neural circuits may be involved, it has gained much attention as a useful animal model for the investigation of the neurobiological components of learning and stress-related memory (Blair *et al.,* 2001; Rogan *et al.,* 2001), because drugs affecting fear conditioning may have potential utility for the treatment of anxiety-related disorders (Millan and Brocco., 2003). Subtype 5 metabotropic glutamate receptors (mGluR5) are abundant in the basal ganglia, amygdala, septum, hippocampus, peripheral sensory neurones and dorsal horn of the spinal cord. Thus, mGluR5 has been implicated in central processes underlying movement control, emotion, learning, and nociception. In the last two decades, the role of glutamate in fear conditioning and anxiety-related disorders is becoming more

recognized. The first evidence for the role of mGlu5 receptors in fear conditioning stems from work using mGlu5 receptor knockout mice. The knockout mice showed impairments in acquisition of fear and deficits in the ability to extinguish contextual fear (Lu *et al.,* 1997; Xu *et al.,* 2009). Fear conditioning has also been shown to cause an up-regulation of mGlu5 receptor protein levels in the hippocampus (Riedel *et al.,* 2000) and mGlu5 antagonists disrupt contextual fear conditioning but are only effective when applied before conditioning rather than after (Rodrigues *et al.,* 2002; Jacob *et al.,* 2009; Gravius *et al.,* 2008). With this in mind, the aim of the present study was to further investigate the effect of prenatal exposure to MAM on E17 in delay and trace conditioning and to see if any deficits could be reversed by the same mGlu5 positive allosteric modulator that was used in the reversal learning experiments in the previous chapter.

4.2 Methods

4.2.1 Experiment 1: Delay and Trace Fear Conditioning performance in Methylazoxymethanol Acetate (MAM) treated animals

4.2.1a Subjects

Animals were prepared as previously described in Chapter 2.

36 (20 MAM; 16 SHAM) male, Sprague Dawley (Crl:CD(SD) (SD), Charles River, Kent) aged 12 weeks at the start of the experiment were used. Rats were housed in groups of 3 or 4 and had *ad libitum* access to water but were food restricted to 85% of their free feeding weight from 8 weeks of age to stop the rats from increasing in weight too quickly. Food restriction was maintained for the entire duration of the experiment. The rats had environmental enrichment (Jolly BallsTM, (Lillico) plastic tubes and wooden blocks). The animals were maintained on a 12-hour light dark cycle with lights on at 7.00. The experiments were conducted during the same part of the light phase each day and the timeline is shown in figure 1. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, under Home Office project licence 70/6560 and personal licence 71/17650 (N. N. Malik).

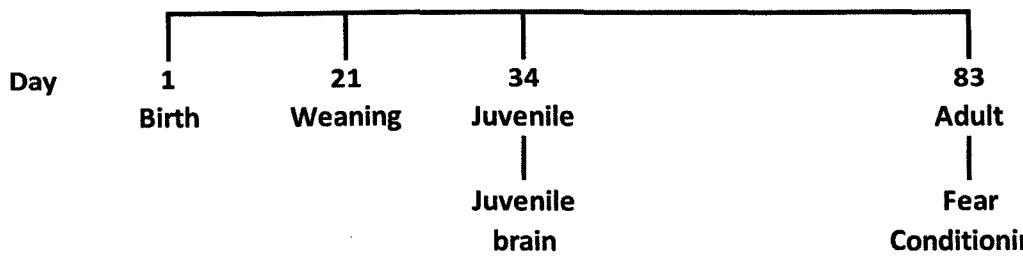


Figure 1: Timeline of events and studies performed for Experiment 1: Delay and Trace Fear Conditioning performance in Methylazoxymethanol Acetate (MAM) treated animals

4.2.1b Fear Conditioning

4.2.1b(i) Apparatus

The operant test chambers (Med Associates; ENV 008) were placed inside sound attenuating cubicles (Med Associates; ENV 022MD). Each chamber had two aluminium walls and two Perspex walls. A house light (100mA, Med Associates; ENV 215M) positioned 1.8 cm from the ceiling on one wall, was lit during all the testing in these chambers. The floor of the chamber consisted of 19 stainless steel bars (diameter 0.48 cm, spaced 1.6 cm apart) through which the shocks were delivered. Shocks were generated and delivered by a Med Associates aversive stimulator/scrambler module (ENV 414). A video camera was attached to the centre of the chamber's ceiling and sent black-and-white images of each experimental session to a PC equipped with a digital video capture card (Geovision Inc., GV-650), and the session videos were recording using Geovision software (Multicam surveillance system, version 8.12). The tone that served as the auditory conditioned stimulus (CS) was 90 dB and 2000 Hz, generated by a programmable

generator package (Med Associates, ANL 926). Presentation of stimuli and shocks was controlled by Med-PC software (version IV, Med Associates). On the test days, the context was modified by covering the two clear Perspex walls with opaque white Perspex panels fixed with Velcro, by covering the metal grid with black Perspex grids, and by introducing a novel odour (vanilla oil) into the waste tray.

Table 1: Detail of pup treatment groups and lever assignment for Experiment 1: Delay and Trace Fear Conditioning performance in Methylazoxymethanol Acetate (MAM) treated animals

Rat	Pup	Tag	Group	Cage	Program
1	N85	2391842	MAM	4	Delay
2	E28	2392234	MAM	4	Trace
3	N82	2392110	MAM	4	Trace
4	L70	2391729	MAM	4	Delay
5	A1	2392809	SHAM	5	Trace
6	B16	2392723	SHAM	5	Trace
7	D26	2394083	MAM	12	Trace
8	L63	2392172	MAM	12	Delay
9	J53	2129402	MAM	12	Trace
10	N83	2391323	MAM	12	Delay
11	L64	2393063	MAM	14	Delay
12	D24	2393257	MAM	14	Trace
13	J62	2128688	MAM	14	Delay
14	N84	2392823	MAM	14	Trace
15	E31	2391277	MAM	16	Trace
16	L65	2392122	MAM	16	Delay
17	J58	2129078	MAM	16	Delay
18	D25	2390773	MAM	16	Trace
19	B15	2392706	SHAM	17	Delay
20	C19	2390867	SHAM	17	Delay
21	O95	2392153	SHAM	19	Trace
22	B13	2392371	SHAM	19	Delay
23	A10	2393736	SHAM	19	Delay
24	F35	2394020	SHAM	19	Trace
25	P98	2394387	MAM	20	Delay
26	E30	2393107	MAM	20	Trace
27	D21	2392807	MAM	20	Trace
28	L66	2394574	MAM	20	Delay
29	A5	2390978	SHAM	21	Trace
30	O94	2392757	SHAM	21	Delay
31	B14	2390745	SHAM	21	Delay
32	H47	2128686	SHAM	21	Trace
33	H42	2392347	SHAM	23	Delay
34	I52	2128751	SHAM	23	Trace
35	C20	2391058	SHAM	23	Trace
36	A8	2394554	SHAM	23	Delay

Delay Fear Conditioning

Subjects were divided into 2 groups, Delay (MAM n = 10, SHAM n = 8) and Trace (MAM n = 10, SHAM n = 8) and all assignments are shown in table 1.

On pre exposure day, animals were placed in the operant chamber (training context) on the first day and were given 10 27s CS (tone) presentations with a random inter-trial interval of between 100-140s (mean of 120s) following a 5 minute habituation period. On conditioning day approximately 24 hours later, animals were again placed into the training context and following a 5min habituation period, they were given 10 CS/shock pairs, in which a 0.5s, 0.5mA foot shock co-terminated with the 27s CS. The next 6 days were extinction sessions which were given in the modified context (white Perspex walls, black Perspex grid floor and vanilla odour in the waste tray) during which rats were presented with the 27s CS 10 times without any shocks.

Trace Fear Conditioning

On pre exposure day, animals were placed in the operant chamber (training context) on the first day and were given 10 10s CS (tone) presentations with a random inter-trial interval of between 100-140s (mean of 120s) following a 5minute habituation period. On conditioning day approximately 24 hours later, animals were again placed into the training context and following a 5min habituation period, they were given 10 CS/shock pairs, in which a 0.5s, 0.5mA foot shock was given after a 16.5s trace interval, i.e. the shock was given 16.5s after the end of the CS. On the next 6 days, extinction sessions were given in the modified context (white Perspex

walls, black Perspex grid floor and vanilla odour in the waste tray) during which rats were presented with the 10s CS 10 times without any shocks.

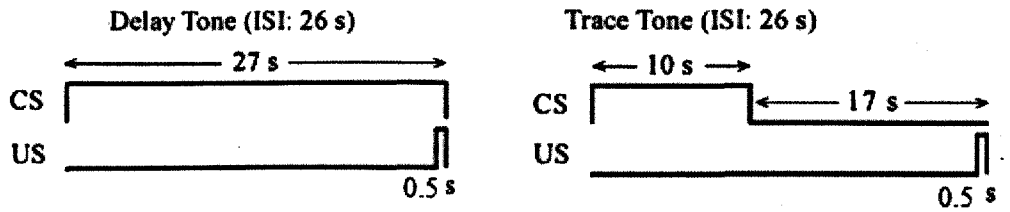


Figure 2: A schematic of the conditioning protocol used

4.2.2 Experiment 2: Effect of mGlu5 treatment on MAM treated rats in Trace and Delay Fear Conditioning

4.2.2a Subjects

Animals were prepared as previously described in Chapter 2.

96 (48 MAM; 48 SHAM) male, Sprague Dawley (Crl:CD(SD) (SD), Charles River, Kent) aged 12 weeks at the start of the experiment were used (details as in 4.2.$_{1A}$). The timeline of experiments and assignments are shown in figure 3 and table 2, respectively.

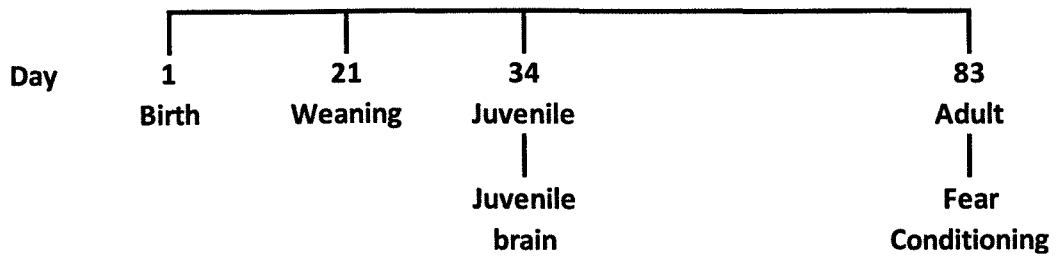


Figure 3: Timeline of events and studies performed for Experiment 2: Effect of mGlu5 treatment on MAM treated rats in Trace and Delay Fear Conditioning

4.2.2b Fear Conditioning

4.2.2b(i) Apparatus

Apparatus remained the same as in $4.2._{1A}$.

4.2.2b(ii) Fear Conditioning Procedure

The procedure remained the same as in 4.2.1b but the rats received a context test day following conditioning and then 3 days of extinction. Subjects were divided into 4 groups, delay vehicle (MAM n = 12, SHAM n = 12), trace vehicle (MAM n = 12, SHAM n = 12), delay mGlu5 (MAM n = 12, SHAM n = 12), and trace mGlu5 (MAM n = 12, SHAM n = 12). All assignments are shown in table2. On the day of conditioning, subjects were orally administered with either vehicle or mGlu5 30 mins before being put into the operant chamber.

4.2.2b(iii) Drugs

LSN2814617 (Lilly, UK), 1 mg/kg, 1 mg/ml accounting for salt weight in 1% CMC, 0.25% tween, 0.05% antifoam administered orally.

Table 2: Detail of pup treatment groups and lever assignment for Experiment 2: Effect of mGlu5 treatment on MAM treated rats in Trace and Delay Fear Conditioning

Rat	Pup	Tag	Group	Cage	Program	Treatment
1	AK227	2393758	SHAM	1	Trace	mGlu5
2	AL235	2391038	SHAM	1	Delay	mGlu5
3	D18	2393045	SHAM	1	Delay	VEH
4	AB167	2393232	SHAM	1	Trace	VEH
5	S122	2393051	MAM	2	Trace	mGlu5
6	K73	2393016	MAM	2	Delay	VEH
7	A2	2391318	MAM	2	Delay	mGlu5
8	C15	2390830	MAM	2	Delay	mGlu5
9	O102	2391440	SHAM	5	Trace	VEH
10	AK225	2393167	SHAM	5	Delay	VEH
11	N92	2391036	SHAM	5	Trace	VEH
12	AE186	2394190	SHAM	5	Delay	mGlu5
13	AC176	2390932	MAM	6	Trace	mGlu5
14	C11	2392585	MAM	6	Trace	mGlu5
15	AD179	2392838	MAM	6	Trace	VEH
16	AH208	2392329	MAM	6	Delay	VEH
17	AB163	2393703	SHAM	7	Trace	VEH
18	Q110	2391890	SHAM	7	Delay	mGlu5
19	X230	2391482	SHAM	7	Trace	mGlu5
20	F33	2394912	SHAM	7	Delay	mGlu5
21	M87	2391603	MAM	8	Trace	mGlu5
22	A9	2391463	MAM	8	Delay	mGlu5
23	AM237	2390722	MAM	8	Trace	VEH
24	K74	2393163	MAM	8	Trace	mGlu5
25	X140	2394717	SHAM	9	Delay	VEH
26	F36	2394087	SHAM	9	Trace	mGlu5
27	G41	2390818	SHAM	9	Trace	VEH
28	N91	2390885	SHAM	9	Delay	VEH
29	C13	2391963	MAM	10	Trace	VEH
30	R116	2391798	MAM	10	Delay	mGlu5
31	AC177	2353595	MAM	10	Delay	mGlu5
32	AH207	2391190	MAM	10	Trace	mGlu5
33	AF191	2393075	SHAM	11	Trace	VEH
34	D21	2393465	SHAM	11	Delay	mGlu5
35	AK229	2391713	SHAM	11	Delay	mGlu5
36	I57	2392119	SHAM	11	Delay	VEH
37	H47	2392410	SHAM	13	Delay	mGlu5
38	E30	2393441	SHAM	13	Trace	VEH
39	AL233	2391835	SHAM	13	Delay	mGlu5
40	F34	2393224	SHAM	13	Delay	VEH
41	AG202	2391978	MAM	14	Trace	VEH
42	R117	2394748	MAM	14	Delay	VEH
43	J61	2393249	MAM	14	Trace	mGlu5
44	AC172	2392111	MAM	14	Delay	VEH
45	AB161	2394710	SHAM	15	Delay	VEH
46	D20	2390655	SHAM	15	Trace	VEH
47	G44	2395067	SHAM	15	Delay	mGlu5
48	I56	2394412	SHAM	15	Trace	mGlu5
49	P105	2393155	MAM	22	Delay	VEH
50	AG199	2393696	MAM	22	Delay	mGlu5
51	AC170	2391725	MAM	22	Delay	mGlu5
52	R114	2392162	MAM	22	Trace	VEH
53	AB164	2393185	SHAM	23	Trace	mGlu5
54	AE188	2392194	SHAM	23	Delay	VEH
55	AI211	2394651	SHAM	23	Trace	mGlu5
56	Y148	2393495	SHAM	23	Delay	VEH
57	AJ221	2390649	MAM	24	Delay	VEH
58	R112	2393032	MAM	24	Delay	VEH
59	K70	2354455	MAM	24	Trace	VEH
60	AD178	2390938	MAM	24	Trace	mGlu5
61	E26	2392382	SHAM	27	Delay	mGlu5
62	Y146	2392595	SHAM	27	Trace	mGlu5
63	I53	2392645	SHAM	27	Trace	VEH
64	AK223	2391429	SHAM	27	Trace	VEH
65	AH209	2395210	MAM	28	Delay	mGlu5
66	J62	2345914	MAM	28	Trace	VEH
67	A5	2394004	MAM	28	Trace	mGlu5
68	AD182	2391389	MAM	28	Delay	VEH
69	U128	2394069	MAM	32	Trace	mGlu5
70	M83	2395159	MAM	32	Delay	VEH
71	V131	2394525	MAM	32	Delay	VEH
72	AC169	2395065	MAM	32	Trace	VEH
73	W137	2393276	SHAM	33	Trace	VEH
74	AN241	2394690	SHAM	33	Delay	VEH
75	Z151	2393134	SHAM	33	Trace	mGlu5
76	AF196	2393872	SHAM	33	Trace	mGlu5
77	AJ220	2395238	MAM	34	Trace	VEH
78	K75	2392669	MAM	34	Delay	mGlu5
79	AH205	2393629	MAM	34	Trace	VEH
80	P104	2395164	MAM	34	Delay	mGlu5
81	AE189	2390972	SHAM	35	Trace	VEH
82	Q108	2392065	SHAM	35	Delay	VEH
83	W135	2391551	SHAM	35	Trace	mGlu5
84	AI210	2394763	SHAM	35	Delay	mGlu5
85	AJ215	2392791	MAM	36	Delay	mGlu5
86	P103	2394894	MAM	36	Trace	mGlu5
87	R115	2394343	MAM	36	Trace	mGlu5
88	A1	2392988	MAM	36	Delay	VEH
89	V129	2394047	MAM	44	Trace	VEH
90	A3	2394084	MAM	44	Trace	VEH
91	AG198	2348081	MAM	44	Delay	VEH
92	M84	2353025	MAM	44	Delay	mGlu5
93	O99	2391022	SHAM	45	Trace	mGlu5
94	AB168	2393888	SHAM	45	Delay	mGlu5
95	N90	2393346	SHAM	45	Trace	mGlu5
96	W136	2393071	SHAM	45	Delay	VEH

4.2.3 Analysis

4.2.3a Freezing Scoring

Videos were recorded for all sessions to be analysed for freezing behaviour in Freezescan software (CleverSys Inc.). The recorded videos were run through the software off-line and the measures recorded were duration of freezing, percent freezing, total motion, and average motion (pixels/second). These data were exported in 30s time bins for plotting the % freezing over the whole session (including the 5min habituation before the session) on each day. The data was also exported in 1s time bins and the timestamps of when the CSs came on (from MedPC data) were found in the motion and freezing data. The average % freezing over 10-17s (the last 17s of the CS in the delay program, and the 17s trace interval in the trace program) of all 10 CSs on Cond, Ext1, Ext2 and Ext3 were calculated and plotted for each group.

4.2.3b Statistical Analysis

All statistical analysis were calculated using STATISTICA v.9 and all data are expressed as mean ± standard error of the mean (s.e.m) In general, data was analysed using two-way analysis of variance, followed where appropriate by planned comparisons (univariate test of significance) with between subject factors of group (MAM; SHAM) vs. treatment (veh;mGlu5). In the case of significant interactions, these were further submitted to analyses of simple effects and if

appropriate to planned comparisons. Brain and body weight was analysed separately for each strain using two-way analyses of variance with between subject factors of group (MAM; SHAM). In all cases, $P<0.05$ indicated significance.

4.3 Results

4.3.1 Experiment 1: Effect of Methylazoxymethanol Acetate (MAM) treatment on Fear Conditioning Performance

4.3.1a Brain and Body Weights

Repeated measures ANOVA showed no significant difference in the body weight (Figure 4a) between MAM and SHAM treated rats ($F_{(1,10)} = 0.681$, $p>0.1$). There was, as expected, a significant effect of MAM treatment on brain weights ($F_{(1,10)} = 19.170$, $p<0.01$)(Figure 4b).

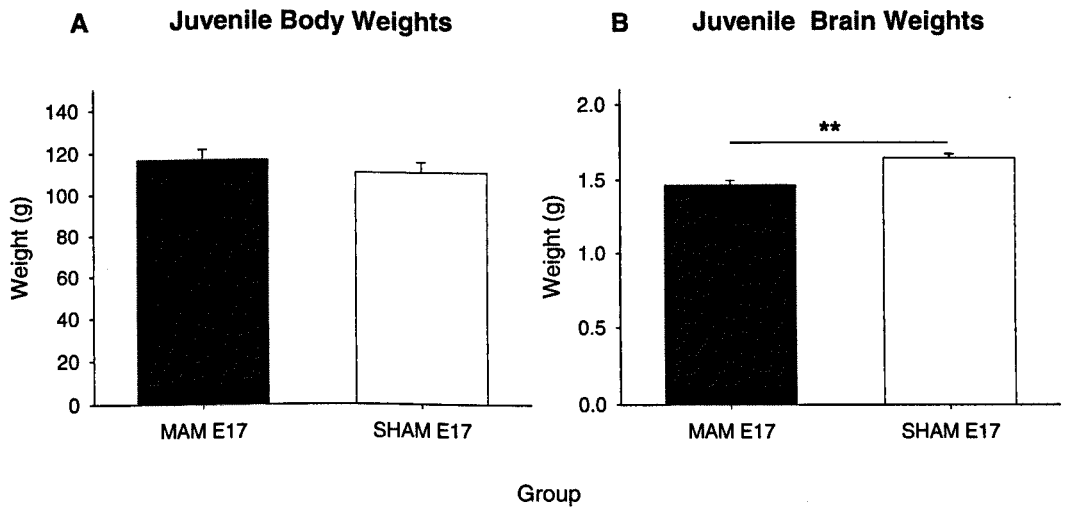


Figure 4: (a) Body weights and (b) brain weights in juvenile rats treated with MAM on E17
Effect of MAM treatment on day E17 on (a) body weights (g) and (b) brain weights (g) in juvenile rats. Data are presented as mean ± standard error of the mean. (n = 8 per group). Significant differences are indicated on the figure as follows: ** P<0.01.

4.3.1b Fear Conditioning Task

4.3.1b(i) Conditioning

There was an effect of treatment and task type on acquisition of during the conditioning day, $F(1,320) = 5.632$, $p<0.05$) and ($F(1,320) = 11.505$, $p<0.001$), respectively. However there was no interaction between the two ($F(1,320) = 0.836$, $p>0.1$. Further planned comparisons on individual CSs showed there was no significant difference between the MAM and SHAM groups in Delay conditioning. There was however a significant effect at CS6 in Trace conditioning in that MAM treated rats froze significantly less than SHAM rats with trend effects at CS5, CS7 and CS8 (Figure 5b).

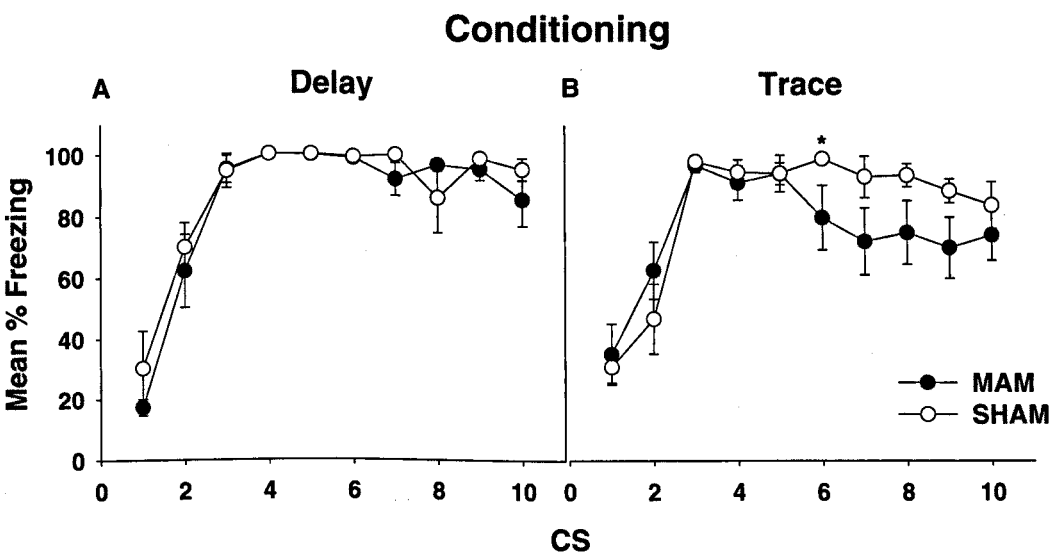


Figure 5: Acquisition of freezing behaviour during (a) Delay (b) Trace conditioning in adult rats treated with MAM on E17.
Effect of MAM treatment on day E17 on (a) Delay and (b) Trace conditioning in adult rats. Data are presented as mean ± standard error of the mean. (N numbers are as follows: delay (MAM = 10, SHAM = 8), trace (MAM = 10, SHAM = 8). Significant differences are indicated on the figure as follows:* $P<0.05$.

4.3.1b(ii) Extinction

Average freezing on Extinction days were analysed in 1s time bins and the mean % freezing from CS1-4 was calculated. This was because rats normally start to extinguish to the cue after this point as can be seen in figure 6.

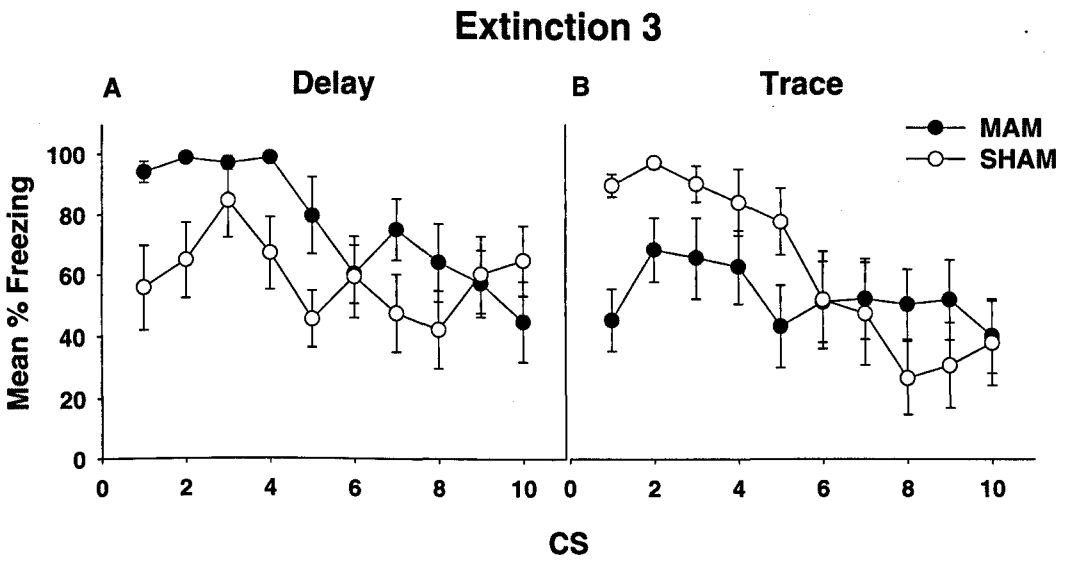


Figure 6: An example of freezing behaviour during (a) Delay (b) Trace Extinction Day 3 in adult rats treated with MAM on E17.
Effect of MAM treatment on day E17 on (a) Delay and (b) Trace extinction in adult rats. Data are presented as mean ± standard error of the mean. (N numbers are as follows: delay (MAM = 10, SHAM = 8), trace (MAM = 10, SHAM = 8).

MAM treatment had no significant effect on freezing on extinction days 1, 4, 5 and 6, $F(1,32) = 0.176$, $p>0.5$) , $F(1,32) = 1.198$, $p>0.1$) , $F(1,32) = 0.324$, $p>0.5$) and ($F(1,32) = 0.384$, $p>0.5$) respectively and there was also no interaction between treatment*program, $F(1,32) = 2.8721$, $p>0.05$) , $F(1,32) = 0.657$, $p>0.1$) , $F(1,32) = 0.369$, $p>0.5$) and ($F(1,32) = 1.032$, $p>0.1$).

There was also no significant effect of treatment on days 2 and 3 ($F(1,32) = 0.094$, $p>0.5$) and $F(1,32) = 0.002$, $p>0.5$)) but there was an interaction between

treatment and program (fear conditioning type) on both of these days, $F(1,32) = 7.534$, $p<0.01$) and $F(1,32) = 15.345$, $p<0.001$) respectively.

Further planned comparisons on individual CSs showed significant differences between the MAM and SHAM groups in Delay conditioning on extinction day 3 where MAM animals were freezing significantly more than SHAM rats. Rats ran in Trace conditioning had the opposite effect where the MAM treated rats froze considerably less than the SHAM rats on extinction days 2 and 3 (Figure 7).

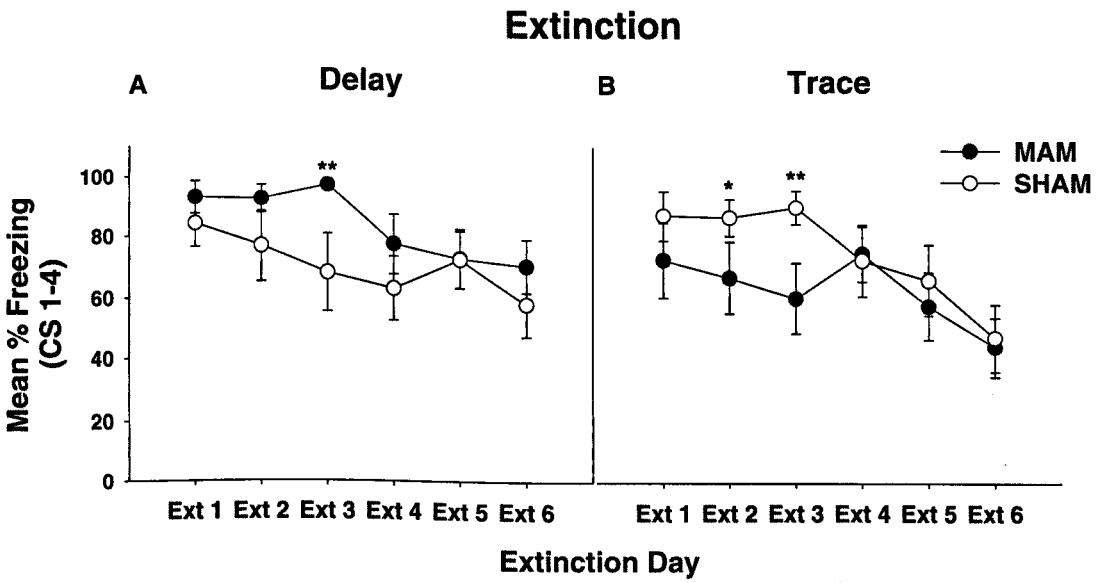


Figure 7: Delay and Trace Extinction days 1-6 in adult rats treated with MAM on E17.

Effect of E17 MAM treatment on extinction in Delay and Trace fear conditioning in adult rats. Data are presented as mean (CS 1-4) ± standard error of the mean. (N numbers are as follows: delay (MAM = 10, SHAM = 8), trace (MAM = 10, SHAM = 8). Significant differences are indicated on the figure as follows:* $P<0.05$; ** $P<0.01$.

4.3.2 Experiment 2: Effect of mGlu5 PAM on Methylazoxymethanol Acetate (MAM) treated rats in Fear Conditioning

4.3.2a Brain and Body Weights

There was no significant difference in the body weight (Figure 8a) between MAM and SHAM treated rats ($F_{(1,8)} = 2.549$, $p>0.1$). There was an effect of treatment ($F_{(1,8)} = 32.629$, $p<0.001$) on brain weight (Figure 8b).

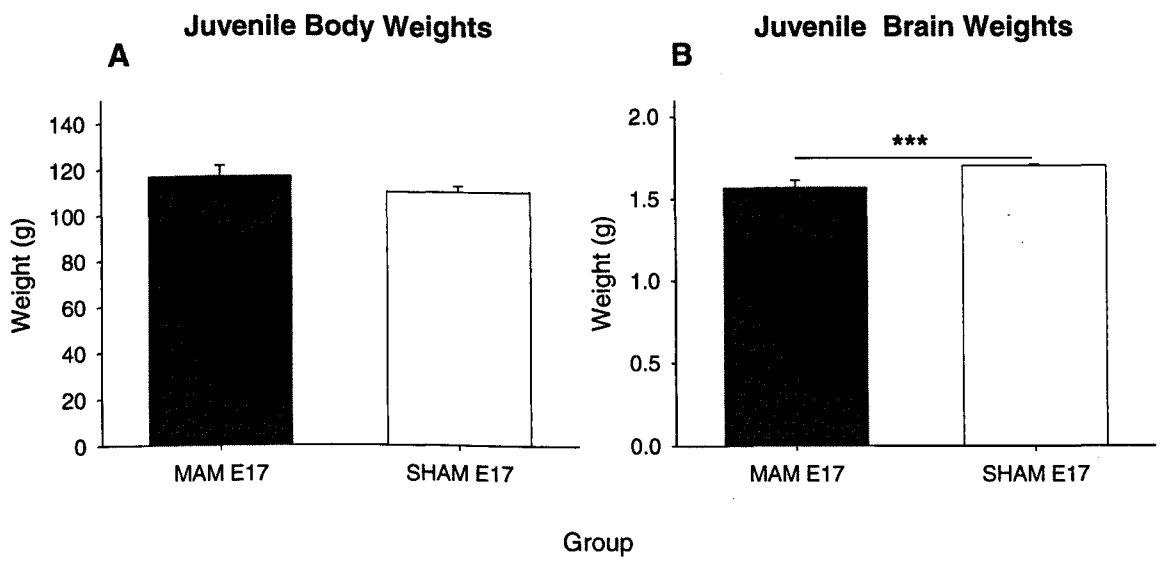


Figure 8: (a) Body weights and (b) brain weights in juvenile rats treated with MAM on E17
Effect of MAM treatment on day E17 on (a) body weights (g) and (b) brain weights (g) in juvenile rats. Data are presented as mean ± standard error of the mean (n = 8 for all groups). Significant differences are indicated on the figure as follows: *** P<0.001.

4.3.2b Fear Conditioning Task

4.3.2b(i) Conditioning

There was an effect of treatment and task type on acquisition of during the conditioning day, $F(3,888) = 4.556$, $p<0.01$) and ($F(1,888) = 21.148$, $p<0.0001$) respectively. Further planned comparisons on individual CSs showed there was no significant difference between the MAM and SHAM groups in Delay conditioning. There was however a significant effect at CS2 in Trace conditioning in that MAM treated rats froze significantly less than SHAM rats with and that treatment with mGlu5 seemed to increase the freezing back to that of the SHAM groups.

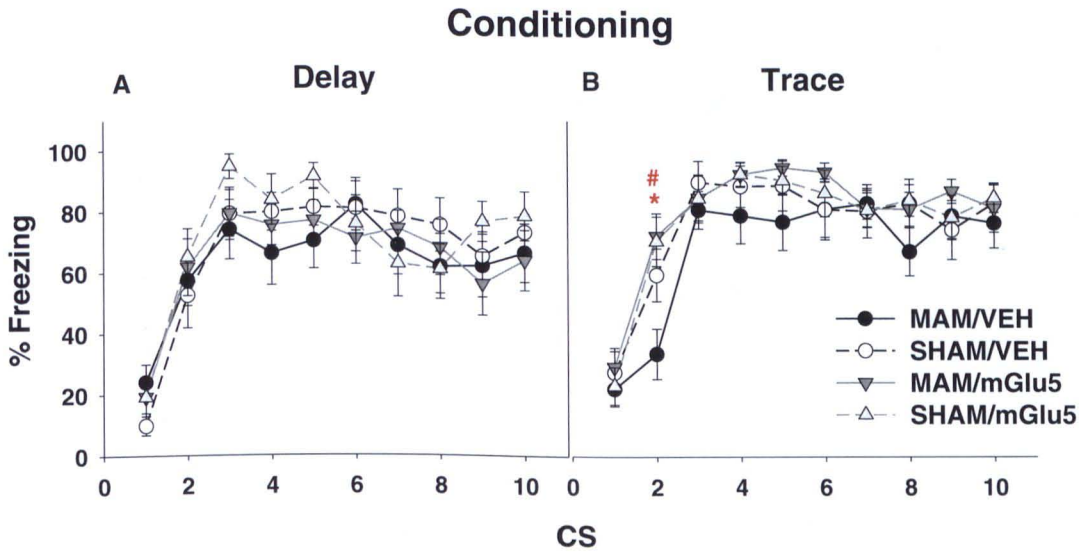
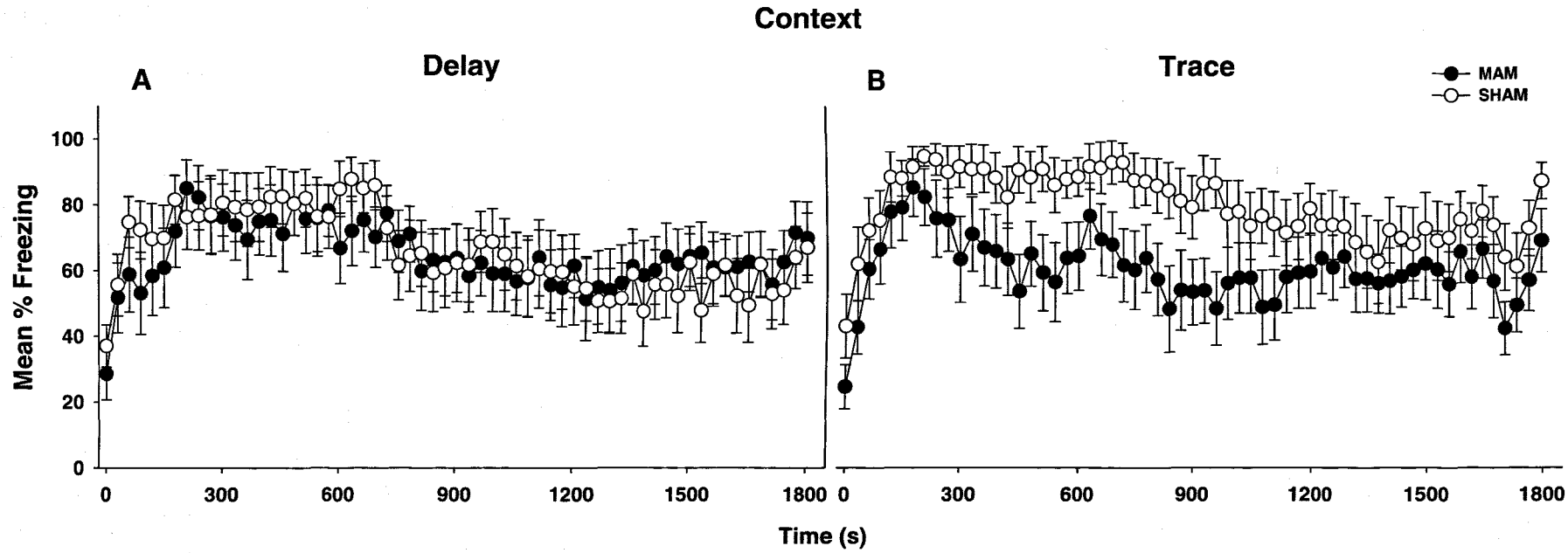


Figure 9: Effect of mGlu5 PAM treatment on performance on (a) Delay and (b) Trace fear conditioning in adult rats treated with MAM on E17.
Effect of 1 mg/kg mGlu5 PAM on percentage freezing in the (a) Delay and (b) Trace fear conditioning in MAM E17 treated rats. Data are presented as mean ± standard error of the mean (n = 12 for all groups). Significant differences are indicated on the figure as follows: vs. vehicle * $P<0.05$; vs. mGlu5 # $P<0.05$.

4.3.2b(ii) Context

MAM treatment had a significant effect on freezing on context day, $F(3,88) = 5.5791$, $p<0.01$) but there was no effect of program type or an interaction between the two, $F(1,88) = 2.383$, $p>0.05$) , $F(3,88) = 0.739$, $p>0.5$) respectively.

Further analysis showed that this treatment effect was specifically on Trace conditioning where MAM treated rats froze significantly less that SHAM treated rats (Figure 10b) but treatment with the mGlu5 PAM did not ameliorate this deficit (Figure 10c).



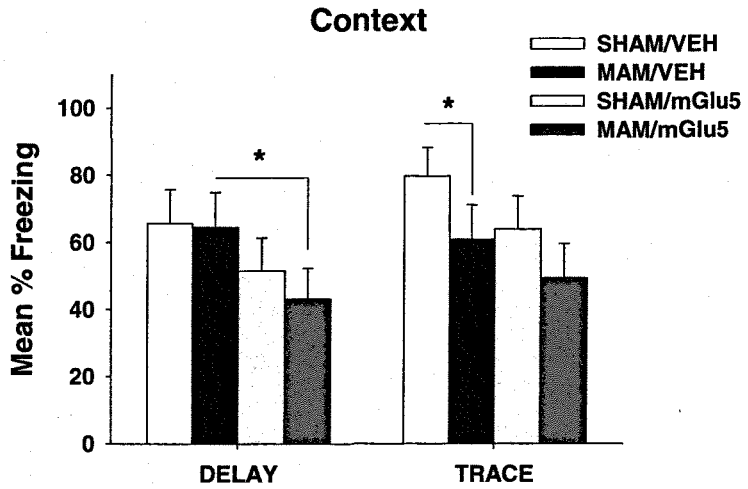


Figure 10: % freezing on Context day in (a) Delay and (b) Trace fear conditioning in adult rats treated with MAM on E17. (c) Mean % freezing in response to mGlu5 PAM.

Effect of MAM treatment on percentage freezing in the (a) Delay and (b) Trace fear conditioning in MAM E17 treated rats. (c) Effect of 1 mg/kg mGlu5 PAM on percentage freezing in the Delay and Trace fear conditioning in MAM E17 treated rats. Data are presented as mean ± standard error of the mean (n = 12 for all groups). Significant differences are indicated on the figure as follows:* $P<0.05$.

4.3.2b(ii) Extinction

MAM treatment had no significant effect on freezing on any of the extinction days, $F(1,88) = 1.083$, $p>0.1$) , $F(1,88) = 0.016$, $p>0.5$) , $F(1,88) = 0.681$, $p>0.5$) and ($F(1,88) = 0.110$, $p>0.5$) respectively and there was also no interaction between treatment*program, $F(3,88) = 0.552$, $p>0.05$) , $F(3,88) = 0.982$, $p>0.1$) , $F(3,88) = 0.414$, $p>0.1$) and ($F(3,88) = 1.152$, $p>0.1$).

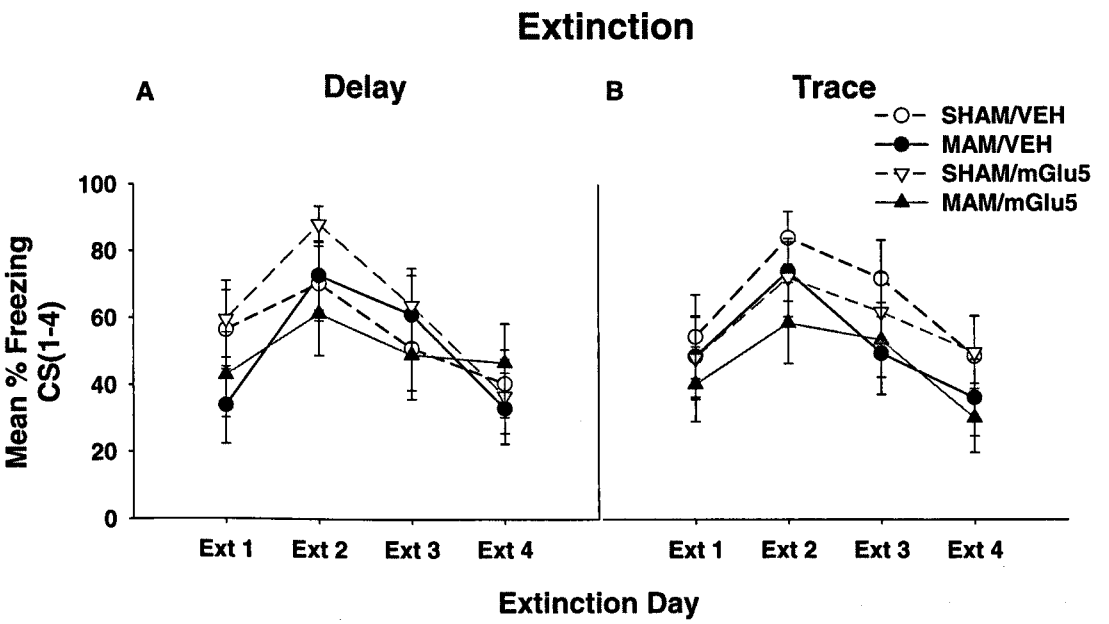


Figure 11: Effect of mGlu5 PAM treatment on % Freezing in (a) Delay and (b) Trace Extinction in adult rats treated with MAM on E17.
Effect of 1 mg/kg mGlu5 PAM on percentage freezing in (a) delay and (b) trace fear extinction in MAM E17 treated rats. Data are presented as mean ± standard error of the mean (n = 12 for all groups).

4.4 Discussion

In the above studies we have observed that maternal treatment with methylazoxymethanol acetate (MAM) results in emotional learning and memory deficits as shown by impairments in delay and trace fear conditioning but to varying degrees and variability. MAM treated rats in study 1 had impairments in trace conditioning and delay and trace extinction but study 2 only showed deficits in trace contextual freezing and treatment with the mGlu5 positive allosteric modulator did not ameliorate these deficits. There was no effect of MAM treatment on body weight and MAM treatment produced a reduction in juvenile brain weight.

Esclassan *et al.,* (2009) have previously shown that neurotoxic lesions of the whole hippocampus impair both contextual and tone freezing in both trace and delay conditioned rats. They went on to assess the dorsal and ventral hippocampal contributions to the deficit. Ventral and dorsal inactivation abolished contextual freezing in the trace group but had no effect in the delay conditioned group. With regards to extinction, dorsal hippocampus inactivation impaired trace fear only whereas ventral inactivation impaired both delay and trace fear. They suggest that the ventral and dorsal parts of the hippocampus compute different aspects of trace conditioning with the ventral being involved in fear and anxiety processes and the dorsal involved more with the temporal and contextual aspects. As the MAM-treated rats in study 1 showed impairments in trace conditioning and both delay and trace fear extinction, and those in study 2 showed impairments in trace

contextual freezing, this supports a hippocampal deficit in the model, be it variable. This is further supported by data that show that there is reduced synaptic transmission and innervations in the dorsal but not the ventral hippocampus in MAM treated rats (Sanderson *et al.,* 2012) whereas Lodge and Grace (2007) demonstrate that adult MAM rats display a significantly greater number of spontaneously firing ventral tegmental DA neurons. They postulate this is due to a consequence of excessive ventral hippocampal activity as inactivation of this area completely reversed the elevated DA neuron population activity.

The MAM E17 model has also previously been shown to produce deficits in various versions of fear conditioning. Shors *et al.,* (2002) injected adult rats with MAM for 7 days to reduce hippocampal neurogenesis by approximately 80% (Shors *et al.,* 2001; Shors *et al.,* 2002). Consequently, they reported deficits in two hippocampus-dependent tasks: trace-eyeblink and trace-fear conditioning, impairments that were reduced upon recovery of neurogenesis. Another group used the MAM E17 model in a classic 1 trial delay fear conditioning task across successive days. They found increased asymptotic freezing to context and cue in the MAM-treated animals compared to controls. They suggest the treatment on E17 may result in a relative increase in the efficacy of the amygdala due to compensatory mechanisms that could lead to the observed increase in fear conditioning (Tinsley *et al.,* 2003). This is further supported by our delay data. The same group also subjected the MAM-treated rats to delay or trace variants of fear conditioning. Contextual- and tone-elicited freezing were comparably high for all animals 24 hr after training. However,

they found that MAM rats extinguished more rapidly to the context than the controls in the delay paradigm whereas we found this was the case for trace not delay conditioning. Both data suggest that the context-fear association is weaker in MAM rats. Furthermore, 24 hrs after extinction, they found MAM rats showed evidence of impaired retention of extinction, which is consistent with new-rule-learning deficits observed in schizophrenia patients and prefrontal cortex lesioned subjects (Davoli *et al.,* 2003). These findings further suggest that behavioural regulation by the hippocampus and prefrontal cortex is abnormal in MAM rats, which results in contextual stimuli being rendered less salient and new-rule learning impairment. This is further supported by our findings in Chapter 2 showing that treating rats prenatally with MAM disrupts limbic and cortical development resulting in an adult phenotype reminiscent of schizophrenia.

Holt *et al.,* (2009) examined fear and extinction learning and memory in patients with schizophrenia by measuring skin conductance responses (SCRs) using a 2 day Pavlovian fear conditioning and extinction paradigm (Milad *et al., 2005*). They found that both healthy control subjects and schizophrenic patients were able to successfully acquire and extinguish conditioned fear responses. Twenty-four hours following successful extinction, healthy control subjects exhibited lower SCRs compared to the schizophrenic patients who showed an excessive fear response (high SCRs), thus failing to demonstrate appropriate context gating of extinction memory retrieval. The same group also found that the extinction memory impairment found was associated with ventral medial PFC dysfunction (Holt *et al.,*

2012) by measuring fear and extinction learning and memory while simultaneously collecting functional magnetic resonance imaging (fMRI) data. Interestingly, a substantial proportion of the patient group (43%) were also excluded from the study as they were deemed non-responders. Other studies of aversive conditioning in schizophrenia have also produced mixed results, (Sears *et al.,* 2000; Jensen *et al.,* 2008; Kosmidis *et al.,* 1999; Hofer *et al.,* 2001; Howe *et al.,* 1958). These inconsistencies might be due in part heterogeneity within patient samples. Some studies have found that one-third to one-half of the patients exhibited no learning at all, whereas the remaining patients showed normal acquisition of aversive conditioning (Kosmidis *et al.,* 1999; Howe *et al.,* 1958). These findings are consistent with evidence for the existence of a subpopulation of patients with schizophrenia with abnormally low or absent autonomic responses to salient stimuli (Ohman *et al.,* 1981). This could also account for the variability seen in our current data where maybe not all offspring were equally affected by MAM treatment resulting in the inconsistencies between the studies.

The ultimate aim of the present study was to further evaluate the validity of the prenatal MAM treatment as a neurodevelopmental model for the emotional learning and memory behavioural changes observed in schizophrenia. Although we did detect deficits in both delay and trace fear conditioning paradigms, these were highly inconsistent despite the consistent microencephaly observed in the MAM 17 animals. This lack of consistency observed in the present investigation leads us to the general conclusion that the prenatal MAM treatment may have validity as a

behavioural model for deficits related to hippocampal abnormalities seen in schizophrenia but an alternative hippocampal dependent assay may be more appropriate.

Chapter 5: General Discussion

5.1 Introduction

The main aim of this thesis was to perform a behavioural characterisation of the Methylazoxymethanol Acetate E17 neurodevelopmental model of schizophrenia in rats. One of the major challenges in schizophrenia-related drug discovery is to find an appropriate animal model of the illness so that novel hypotheses can be tested at the basic science level. As more knowledge of the pathophysiology of schizophrenia accrues, it is essential that appropriate animal models of the illness be developed that have better translational value. Secondly, the model was utilised to elucidate the pro-cognitive effects of an mGlu5 positive allosteric modulator in assays of cognitive flexibility and learning and memory to determine the use of the model as a tool for drug discovery. The purpose of this chapter is to review the results of the previous chapters and discuss the implications of the combined findings with regards to the aims of the thesis.

Chapter 1 provides a general overview of schizophrenia, hypotheses, treatment and how the disorder can be modelled in animals. In its simplest form the neurodevelopmental hypothesis posits that schizophrenia is the behavioural outcome of an aberration in neurodevelopmental processes that begins long before the onset of clinical symptoms and is caused by a combination of environmental and genetic factors (Rapoport *et al.,* 2005). There are several potential difficulties

associated with modelling schizophrenia in animals, including the standard caveat of reproducing what is generally perceived to be a cognitive disorder in less cognitively developed animals. More specifically, heterogeneity in clinical symptoms, course of the disorder and potential causative factors represent significant obstacles to model building. Patients typically experience a combination of symptoms, often divided into positive (e.g., hallucinations, delusions, thought disorganizations), negative (e.g., loss of motivation, affective blunting, alogia, social withdrawal) and cognitive (e.g., deficits in attention, memory and executive functions) (Andreasen 1995). Accordingly, current animal models of schizophrenia are not intended to serve as the complete animal equivalent of the human disorder. Rather, they are often designed to test specific causative or mechanistic hypotheses regarding schizophrenia. The models can be validated on the basis of how well their performance in a given test predicts the performance of humans with schizophrenia and/or on whether the model provides a sound theoretical rationale.

5.2 Summary of findings

The first part of this thesis (Chapter 2) investigated the basic behavioural profile of the MAM E17 model in different strains, sexes and ages. The data show that maternal treatment with MAM on E17 leads to behavioural, neuroanatomical and pharmacological alterations in the resultant male offspring of Sprague Dawley CD rats. These changes include decreased brain weights, PPI deficits and hypersensitivity to MK-801 measured by changes in locomotor activity that have

translational relevance to some of the core symptoms seen in schizophrenia. Furthermore, the absence of these deficits pre-puberty in the same rats indicates that this model may also replicate the adolescent onset of psychosis seen in this human disorder. However this study suggests that these changes are strain and sex specific, suggesting a genetic component could be involved, since they do not occur in male LH or female CD rats under identical experimental conditions.

To investigate any potential cognitive deficits observed in the model, the second and third part of the thesis involved looking at the performance of MAM E17 treated rats in assays of behavioural flexibility and learning and memory. The utility of the model to investigate the effects of a potential pro-cognitive compound for drug discovery in these tasks were also determined. The studies in chapter 3 consistently demonstrated that maternal treatment with MAM on E17 results in deficits in cognitive flexibility as measured by the rodent spatial reversal learning task. The studies also show that the novel mGlu5 PAM LSN2814617 reverses these deficits. In the fear conditioning chapter, we have observed that maternal treatment with methylazoxymethanol acetate (MAM) results in emotional learning and memory deficits as shown by impairments in delay and trace fear conditioning but to varying degrees and variability. In the first study we saw impairments in trace conditioning and in delay and trace extinction but the second only showed deficits in trace contextual freezing with no change in extinction. Treatment with the mGlu5 PAM in this case had no effect on the deficits observed.

Overall, the observations from this study and those of other groups indicate that a disturbance in neurodevelopment induced by MAM administration on E17 leads to several behavioural and neuroanatomical changes similar to the core pathophysiological changes characteristic of schizophrenia (Featherstone *et al.,* 2007; Rizos et al. 2007; Styner *et al.,* 2005). These include reduction in tissue volume, increased neuronal density and ventricle size, and disorganisation of the hippocampus. Deficits are also seen in several behavioural measures that emerge after puberty. These include deficits in PPI, hypersensitivity to NMDA receptor antagonists in locomotor activity and deficits in cognitive flexibility. We also confirm that positive modulation of mGlu5 receptors may have beneficial effects in the treatment of certain cognitive impairments associated with schizophrenia. Further studies are needed to test the mGlu5 effects in other memory and executive functions, such as working memory. The present findings also show face and construct validity and but also present an opportunity for the predictive validity of the MAM E17 model for remediating specific cognitive deficits in schizophrenia to be tested. However, our work makes it clear that care must be exercised in the selection of rat strain and gender to be used since male LH rats are essentially resistant to MAM E17 treatment.

5.3 Variability

Although the summary of the findings presented in this thesis paints a very positive picture with regards to the model, this is not always the case. Table 1 displays all the different MAM batches that have been generated at Lilly since I started my PhD and the different assays used to test them. A yes indicates whether a deficit is seen in a particular assay. The table shows, that a deficit in an assay in one particular batch may not mean that the same deficit will be present in another batch.

Deficit Present?	Brain Weights	Neuropath	Sleep	PPI	LMA	Lever Reversals	Digging Reversals	Touchscreen Reversals	Fear Conditioning	Watermaze & other spatial tasks
NM_MAM_01	YES	YES		YES	YES					
NM_MAM_02	YES			YES	NO					
NM_MAM_03	YES	YES				YES				
NM_MAM_04	YES	YES				YES				
NM_MAM_05	YES				NO	NO				
NM_MAM_06	YES	YES	YES	YES		NO			YES	
NM_MAM_07	YES					NO				
NM_MAM_08	YES					NO				
NM_MAM_09	YES			NO	NO	NO				
KP_MAM_01	YES		YES							
KP_MAM_02	YES		YES							
KP_MAM_03	YES		YES							
FG_MAM_01	YES						YES			
FG_MAM_02	YES						YES			
TS_MAM_01	YES					YES	YES		YES	
NM_MAM_11	YES					NO	NO			
FG_MAM_04	NO						NO			
NM_MAM_12	YES					NO				
NM_MAM_13	YES						NO			YES
NM_MAM_14	YES	YES	YES	YES		NO	YES		NO	
NM_MAM_15	YES			NO	YES			NO		YES
NM_MAM_17	YES						NO			
TOTAL	22/23	5/5	5/5	4/6	2/5	3/11	4/8	0/1	2/3	2/2

Figure 1: Table displaying the different MAM batches generated at Lilly and the different assays used to test them. A yes indicates a deficit.

This variability could actually be that the model actually 'models' the heterogeneous nature of schizophrenia very well. Taking our results in fear conditioning as an example where we found deficits in extinction in the first study

but not the second. Studies of aversive conditioning in schizophrenia patients have also produced mixed results where some have found deficits (Jensen *et al.,* 2008, Kosmidis *et al.,* 1999, Hofer *et al.,* 2001) and others no change compared to control subjects (Kosmidis *et al.,* 1999, Howe *et al.,* 1958). However, variability within a preclinical model is not conducive to drug discovery.

Another reason for the variability seen in the MAM E17 model may be because specific litters are affected more by MAM treatment than others. Unfortunately this cannot be directly controlled as it is the DAM that is treated by MAM and not the offspring. However, this could be accounted for any future experiments in the experimental design and statistical analysis. As it is the DAM that is the actual experimental unit and the pups are the observational units, we need to take into account the nesting structure – this is because the average correlation between variables measured on pups from the same litter could be higher than the average correlation between variables measured on pups from different litters. Spatz and Laquer., (1986) have stated that a striking feature of chemically induced microencephaly (by MAM) was its uniformity among littermates. Standard statistical tests rely heavily on the assumption of independence of the observations. If this assumption is violated (as is usually the case in nested data) the estimates of the standard errors of conventional statistical tests are much too small and this can result in false positives.

Variability in preclinical experiments is not specific to this model. A commentary by Peers *et al.,* (2012) highlight that a major factor in the high clinical failure rates in pharmaceutical research and development is due to insufficient robustness in preclinical studies. Although not specific to schizophrenia or even psychiatry, one example they cite is from Begley and Ellis., (2012) who reported that only 6 out of 53 landmark studies in oncology were reproducible. They also cite an investigation at Bayer (Prinz *et al.,* 2011) where they found that around two thirds of studies were stopped due to lack of reproducibility seen in preclinical research. Indeed, one the factors suggested by Peers *et al.,* (2012) to contribute to the lack of reproducibility is the inadequate use of statistics. They state that statistical issues such as variability and bias and their impact their impact on design, analysis and interpretation of scientific experiments are often misunderstood.

Given the high variability seen in our findings, we contacted other laboratories who either work on the model or have done previously, and found that all found the same level of variability in their data and many cited this as a reason for not taking the model further. However if you search the current MAM E17 literature you will find no mention of this variability. Begley and Ellis., (2012) hypothesise that "to obtain funding, a job, promotion or tenure, researchers need a strong publication record, often including a first-authored high-impact journal. Journal editors, reviewers and grant-review committees often look for a scientific finding that is simple and clear and complete – a 'perfect' story. It is therefore tempting for investigators to submit selected data sets for publication, or even to massage data

to fit the underlying hypothesis." Drug development relies heavily on the literature, especially with regards to new targets, biology and assays and as Begley and Ellis rightly state, there are no perfect stories in biology. In fact, gaps in stories can provide opportunities for further research and if this is not the case, not disclosing this so called 'negative data' often results in another research group pursuing a similar, wasting time and money in uninformed redundancy. I think this is an issue that we have generally have in the science community and we need to be working more on sharing all types of data, not just those data that are deemed positive.

5.4 Future Work

As mentioned previously, accounting for litter effects in the experimental design and analysis will be an important aspect of any future work but could we use the litter effects to our advantage. Could we use it to assess which litters would have the largest deficits and so only use those litters in experiments thereby potentially erasing high variability of findings? We have just undertaken a study where MAM E17 rats are assessed, consecutively, in several assays, including PPI, reversal learning, fear conditioning, electrophysiology and then the brains extracted for neuropathology. Assessing the same animals through several assays will allow performance of a correlation analysis to assess if a particular rat or litter has a deficit in one assay, for example PPI, would that mean it will also have a deficit in other assays. Running a large number of animals through each assay will also allow assessment of any within litter correlations. If any correlations arise, then

potentially, one particular assay could be utilised as a 'biomarker' to allow incorporation of only those animals with a MAM deficit in any further experiments.

NEWMEDS (Novel Methods leading to New Medications in Depression and Schizophrenia) is an international consortium of scientists that has launched one of the largest ever research academic-industry collaboration projects to find new methods for the development of drugs for schizophrenia and depression. One of the aims of the initiative is to assess pharmacological, neurodevelopmental and genetic models of schizophrenia, and the MAM E17 model has been chosen to be one of these. MAM treated animals will be generated at two sites, Charles River in the UK and Charles River in the US and then assessed in a wide range of assays by many of the different sites to assess reproducibility in the model as well as in the assays being tested.

5.6 Conclusion

To conclude, our data suggests that maternal treatment with MAM on embryonic day 17 leads to persistent alterations in the adult offspring of CD rats that are relevant for modelling aspects of schizophrenia and could be important for discovery of novel therapeutic drugs for the treatment of schizophrenia. However, revised methods for study design and statistical analysis are crucial to avoid misinterpretation of findings. "Negative" observations and conclusions, based on rigorous experimentation and thorough documentation, should be published in

order for them to be discussed, confirmed or refuted by others. This may also reveal fundamental flaws and obstacles in commonly used methods, ultimately leading to improvements in experimental designs.

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