

**The Impact of Social Isolation on  
Rat Behaviour**

Malini Veronica Porkess

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## Abstract

Schizophrenia is a psychiatric disorder with symptoms including delusions, social withdrawal and cognitive deficits. The cognitive symptoms respond poorly to current antipsychotic medication and in order to develop new treatments it is necessary to model these deficits in animals. Rearing rats in isolation from weaning causes behavioural, cognitive and neurochemical alterations, some of which have relevance to the symptoms of schizophrenia. The work described in this thesis aimed to further characterise the behavioural and cognitive changes found isolation reared rats.

After five weeks of isolation rats demonstrated increased locomotor activity in a novel environment and a gender specific impairment in recognition memory. After six weeks of isolation rats developed attenuated prepulse inhibition of acoustic startle. Isolation reared rats did not develop impairments in the attentional set shifting test of behavioural flexibility. However, in a further study isolated rats did show deficits in reversal learning (but not acquisition) in the water maze, which were reversed by the pro-cognitive 5-HT<sub>6</sub> antagonist Ro 04-6790. Sub-chronic treatment with aniracetam, a modulator of the AMPA receptor had no effect on fear-related memory impairments seen in passive avoidance but aniracetam-treated isolation reared rats were able to discriminate the novel object.

Finally, following controversial reports linking heavy cannabis use with increased risk of schizophrenia, weanling rats were dosed with a component of cannabis,  $\Delta^9$ -tetrahydrocannabinol (THC). The interactions between isolation rearing and two regimens of THC treatment (low: 4x 2mg/kg and high: 8x 5mg/kg) were observed in adulthood. The low dose of THC had no effect on any behavioural test used. The high dose of THC led to impairments in recognition memory but had no effect on attentional set shifting or prepulse inhibition. High-THC and isolation rearing interacted to improve passive avoidance performance in isolates, but impair social rats.

In conclusion, isolation rearing induces varied cognitive deficits which are responsive to nootropic compounds and as such is an important tool in the development of cognition enhancing and antipsychotic drugs.

## Publications

### **Papers:**

Fone, K.C.F & Porkess, M.V. 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents – relevance to developmental neuropsychiatric disorders. *Neuroscience and Biobehavioral Reviews*: doi: 10.1016/j.neubiorev.2008.03.003

### **Abstracts:**

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Porkess, M.V., Baxter, A., Cui, H., Graylen, S., Mok, K., Watson, D.J.G., Topham, I.A., Marsden, C.A. & Fone, K.C.F. Investigation of the interaction between low dose sub-chronic  $\Delta^9$ -tetrahydrocannabinol at weaning and isolation rearing in rats. *Journal of Psychopharmacology* 2007 21(7 Suppl.): A34. Presented at British Association of Psychopharmacology 2007 conference.

Porkess, M.V., Dhanji, H., Huan, S., Watson, D.J.G., Topham, I.A., Marsden, C.A. & Fone, K.C.F. Aniracetam has no effect on isolation rearing induced hyperactivity, PPI deficits or passive avoidance impairment in rats, but does improve novel object recognition. *Journal of Psychopharmacology* 2006 20(5 Suppl.): A46. Presented at British Association of Psychopharmacology 2006 conference.

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## List of Abbreviations Used

2-AG	2-arachidonyl glycerol
5-CSRTT	Five choice serial reaction time task
5-HT	5-hydroxytryptamine
ACh	Acetylcholine
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc	Activity related cytoskeletal protein
BDNF	Brain-derived neurotrophic factor
BMSU	Biomedical services unit
CaMKII	Calcium/calmodulin dependent protein kinase II
CANTAB	Cambridge Automated Neuropsychological Testing Battery
CB	Cannabinoid
CNS	Central Nervous System
COMT	Catechol-O-methyl-transferase
CRUK	Charles River United Kingdom
CSF	Cerebrospinal fluid
DA	Dopamine
DAAO	D-amino acid oxidase
DISC1	Disrupted in schizophrenia 1
dIPFC	Dorsolateral prefrontal cortex
DR	Discrimination ratio
ECL	Enhanced chemi-luminescence
ED	Extra-dimensional
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid
EPS	Extra pyramidal side effects
FDA	Food & Drug Administration
fMRI	Functional magnetic resonance imaging
GABA	$\gamma$ -aminobutyric acid
GAP-43	Growth associated protein 43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu-Tub	Detyrosinated $\alpha$ -tubulin
GlyT1	Glycine transporter 1
HPA	Hypothalamo-pituitary-adrenal
HRP	Horse-radish peroxidase
ID	Intra-dimensional
i.p.	Intraperitoneal
ISI	Inter-stimulus interval
ITI	Inter-trial interval
LH	Lister hooded
LI	Latent inhibition
LMA	Locomotor activity
MAM	Methylazoxymethanol
MATRICES	Measurement and Treatment Research to Improve Cognition in Schizophrenia
mCPP	m-chlorophenylpiperazine

mGluR	Metabotropic glutamate receptor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NAA	N-acetyl aspartate
NAcc	Nucleus accumbens
NIMH	National Institute of Mental Health
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
NOD	Novel object discrimination
NVHL	Neonatal ventral hippocampal lesion
OD	Optical density
PCP	Phencyclidine
PFC	Prefrontal cortex
PLC	Phospholipase C
PND	Post natal day
PPI	Prepulse inhibition
PRODH	Proline dehydrogenase
PSD	Post-synaptic density
RGS4	Regulator of G-protein signalling 4
RM ANOVA	Repeated measures analysis of variance
s.c.	Subcutaneous
SDS	sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
T1	Trial 1
T2	Trial 2
THC	$\Delta^9$ -tetrahydrocannabinol
Tris	Tris(hydroxymethyl) amidomethane
Tyr-Tub	Tyrosinated $\alpha$ -tubulin
VGLUT	Vesicular glutamate transporter
WCST	Wisconsin card sort test
WM	Working memory

# 1 General Introduction

## 1.1 Schizophrenia

In 1893 Emil Kraepelin made the first detailed description of a psychiatric disorder which he called dementia praecox (Ion and Beer, 2002). Kraepelin described in great detail the symptoms, diagnosis, clinical subtypes and possible causes of the disease as was known at that time (Kraepelin, 1968 (1904)). In 1908 Eugen Bleuler was the first refer to dementia praecox as schizophrenia (Kuhn, 2004), meaning “split mind”.

### 1.1.1 Symptoms

The foundation work of Kraepelin and Bleuler, along with many others over the years, led to the development of diagnostic criteria for schizophrenia, such as the Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association, 2000). Schizophrenia is a heterogeneous disease characterised by 3 groups of symptoms: positive, negative and cognitive (see below). For a diagnosis of schizophrenia to be made the patient must exhibit two or more core symptoms for a significant portion of one month and show some signs of symptoms for approximately 6 months. The core symptoms include delusions, hallucinations, disorganised speech and/or behaviour and negative symptoms (American Psychiatric Association, 2000). Possible other causes of the symptoms, such as substance abuse, must be excluded.

#### *Positive Symptoms*

The positive symptoms are behaviours which are not normally found in unaffected individuals. These include delusions, which are often paranoid and hallucinations. Another common positive symptom is hearing voices that may be talking about the patient (Schultz et al., 2007).



### *Negative Symptoms*

Conversely, negative symptoms are characteristics that unaffected individuals usually display, but which are absent in schizophrenia e.g. social interactions are impaired in schizophrenia, leading to withdrawal from society. Other negative symptoms include anhedonia (loss of feelings of pleasure), apathy, and flattened affect (Tamminga and Holcomb, 2005, Montgomery and van Zwieten-Boot, 2007).

### *Cognitive Symptoms*

In addition, schizophrenics also suffer cognitive deficits (Tyson et al., 2004, Bozikas et al., 2006), the severity of which is a major predictor of successful re-integration into society (Green et al., 2004, Alptekin et al., 2005, Hofer et al., 2005). These cognitive impairments are core features of schizophrenia itself, not side effects of psychosis (Gold, 2004) or negative symptoms (Bell and Mishara, 2006). The cognitive impairments are poorly treated by currently available antipsychotic drugs and as such represent a large unmet treatment area (Green et al., 2002). In 2003 the National Institute of Mental Health (NIMH), with the support of the US Food and Drug Administration (FDA), set up the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Marder and Fenton, 2004). This group aimed to identify the main cognitive domains which are affected in schizophrenia, the behavioural tests which give the best indicators of functional ability in these domains and the corresponding pre-clinical tests. This will provide a battery of tests, covering the main cognitive aspects of schizophrenia for use in the development of new cognition-enhancing drugs at both the preclinical and clinical level. The seven main cognitive domains affected in schizophrenia identified by MATRICS and targeted for drug development are shown and briefly described in Table 1.1 (Nuechterlein et al., 2004).

Table 1.1: The cognitive domains identified by MATRICS (adapted from (Kee et al., 2003, Geyer, 2005, Reichenberg and Harvey, 2007).

Cognitive Domain	Description	Example Test Measures	Rodent test
Speed of processing	The speed with which cognitive processes are executed.	Naming words beginning with a certain letter as quickly as possible	5-Choice Serial Reaction Time Simple reaction time tasks
Attention/vigilance	The ability to maintain a readiness to respond to a signal over a period of time.	Continuous Performance Tests	5-Choice Serial Reaction Time PPI/ auditory gating
Working Memory (WM)	Short-term memory holding a small amount of information for immediate use, e.g. remembering a telephone number	Recall of numbers of digits over a short time period-digit span tests.	T-maze, Delayed non match to sample, Radial Arm maze
Verbal learning and memory	Memory of words and language.	Recall of word lists (longer than WM) and paragraph-long stories	No animal correlate
Visual learning and memory	Memory of visual stimuli.	Facial recognition Reproduction of line drawings	Novel Object Recognition
Reasoning and problem solving	Also referred to as 'executive function'. High level decision-making and strategic planning.	Wisconsin Card Sort Test (WCST), card category sorting.	Attentional set shifting Maze tasks
Social Cognition	Interpretation of facial and vocal emotions.	Recognition of emotion on faces.	Social Interactions Social Recognition

### **1.1.2 Sub-types of Schizophrenia**

There are five types of schizophrenia: paranoid, disorganised, catatonic, undifferentiated and residual (American Psychiatric Association, 2000). Paranoid schizophrenia is typified by mainly delusions and hallucinatory symptoms, with fewer negative symptoms or disordered thought. Catatonic schizophrenics exhibit catalepsy, stupor or meaningless excessive motor activity, bizarre posturing or prominent grimacing and extreme negativism including a resistance to all instruction and mutism. A diagnosis of disorganised schizophrenia is made when disordered speech or behaviour and flattened affect are present, but the symptoms do not meet criteria for the catatonic subtype. Residual schizophrenics usually do not suffer from prominent positive symptoms or catatonic behaviour, but exhibit negative symptoms and some core features of schizophrenia in a less severe form, such as having eccentric ideas and mildly disorganised speech. Undifferentiated schizophrenia is diagnosed if patients have some core symptoms, but do not meet the criteria for the paranoid, catatonic or disorganised sub-types (American Psychiatric Association, 2000).

### **1.1.3 Treatments**

There are two classes of drugs used to treat schizophrenia, typical antipsychotics and atypical antipsychotics. The first typical antipsychotic, chlorpromazine, was discovered serendipitously in the early 1950s (Kurland, 1955) and this class of drugs is typified by haloperidol (Kapur and Mamo, 2003). The antipsychotic efficacy of typical antipsychotics correlates well with their dopamine (DA) D<sub>2</sub> receptor affinity where they act as antagonists (Seeman et al., 1976). The typical antipsychotics are effective at controlling the positive symptoms of schizophrenia, but have little effect on the negative symptoms or cognitive impairments (Murphy et al., 2006). They are also ineffective in up to 30% of patients (Wong and Van Tol, 2003).

Typical antipsychotics also cause a number of unpleasant side effects, especially extra pyramidal side effects (EPS) which include tremors and rigidity similar to that seen in Parkinson's Disease. Furthermore EPS can develop into uncontrollable movements, called tardive dyskinesia, which can be irreversible (Wong and Van Tol, 2003).

In 1958 the first atypical antipsychotic, clozapine, was synthesised (Kapur and Mamo, 2003). Clozapine has affinity for many receptors including serotonergic, dopaminergic, adrenergic and muscarinic (Wong and Van Tol, 2003). This drug, and the others that have followed, show efficacy at treating some of the positive and negative symptoms of the disease, in some patients, as well as having a reduced propensity towards development of EPS (Lublin et al., 2005). Clozapine has also proved effective in many patients resistant to treatment with typical antipsychotics. However, atypical antipsychotic treatment does not appear to improve the cognitive deficits seen in schizophrenia (Green et al., 2002, Fenton et al., 2003), which is important to enable the patient to successfully reintegrate into society (Alptekin et al., 2005). It is therefore necessary to find either a new type of antipsychotic or an add-on therapy to treat these cognitive deficits.

#### **1.1.4 Aetiology**

Although schizophrenia affects 1% of the population, the cause of the disease is not known. The risk factors for developing schizophrenia are both genetic and environmental, with monozygotic siblings of affected individuals showing 50-80% risk of developing the disease (Lewis and Lieberman, 2000) (Sullivan et al., 2003). Various theories of the underlying neurochemistry of schizophrenia have been proposed. Initially

the neurotransmitter dopamine was implicated, but more recently a glutamatergic role has been suggested.

### *Dopamine Hypothesis*

The dopamine hypothesis proposed that schizophrenia is caused by an over activity of the mesolimbic dopamine system. This theory was based on two observations (Meltzer and Stahl, 1976): firstly, all the typical antipsychotics are potent dopamine D<sub>2</sub> receptor antagonists and the efficacy of these drugs at treating schizophrenia is strongly correlated with their D<sub>2</sub> receptor affinity (Seeman et al., 1976, Miyamoto et al., 2005). Secondly, it was noted that treatment with amphetamine, an indirect dopamine agonist, induced psychoses in humans that are very similar to the positive symptoms of schizophrenia (Johnson and Milner, 1966). According to this hypothesis a hyperactive subcortical/ mesolimbic dopamine system causes the positive symptoms of schizophrenia, whereas the negative symptoms arise from hypoactivity of mesocortical dopamine, which may contribute to the reduced activation of the frontal cortex seen in schizophrenia, so-called hypofrontality (Goldman-Rakic et al., 2000).

However, the typical antipsychotics do not treat all the symptoms of schizophrenia, which indicates there is more to the disease than simple changes in DA. The discovery of the atypical antipsychotics, specifically clozapine which has comparatively weak affinity for the D<sub>2</sub> receptor, suggested that this was not the only desirable pharmacological property for an effective antipsychotic. Clozapine has been shown to have moderate affinity at many receptors including D<sub>1</sub>, D<sub>2</sub>, D<sub>4</sub>, 5-hydroxytryptamine (5-HT) 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, muscarinic acetylcholine (ACh) M<sub>1</sub> receptor, histamine H<sub>1</sub> and  $\alpha_1$  adrenoceptors (Ashby and Wang, 1996). Importantly, whereas D<sub>2</sub> receptor antagonists seem to be efficacious against only the positive symptoms of schizophrenia, clozapine and the other atypical antipsychotics can also improve the negative symptoms in some, but not all, patients.

### *Glutamate Hypothesis*

Glutamate is the primary excitatory neurotransmitter in the brain and has both ion-channel (ionotropic) and G-protein coupled (metabotropic, mGluR) receptors (Konradi and Heckers, 2003). There are 3 types of ionotropic glutamate receptor, the *N*-methyl-*D*-aspartate (NMDA) receptor, the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the kainate receptor, based on their ligand affinity.

The role of glutamate in schizophrenia began to be investigated more closely when it was noticed that the NMDA receptor antagonists phencyclidine (PCP) and ketamine induced psychoses in normal individuals which were almost indistinguishable from those seen in schizophrenia (Allen and Young, 1978). Furthermore schizophrenics taking PCP experienced very similar symptoms to their individual schizophrenia related psychoses. As PCP is an NMDA receptor antagonist it was hypothesised that schizophrenia is caused by NMDA receptor or glutamatergic hypofunction (Olney and Farber, 1995). Consistent with this hypothesis the density of AMPA, kainate and NMDA receptors have all been reported to be altered in the post mortem brain of schizophrenics (Eastwood et al., 1997, Nudmamud and Reynolds, 2001).

### **Modulating Glutamatergic Neurotransmission**

If schizophrenia is caused by reduced NMDA receptor activation then increased activation of this receptor could be an effective treatment of the disease. However, excessive activation of the NMDA receptor (e.g. with an NMDA receptor agonist) may lead to excitotoxicity (cell death), so current research efforts are focussing on indirect modulation of NMDA receptors.

NMDA receptor activation requires the presence of a co-agonist, glycine, as well as glutamate. Increasing glycine availability by either giving large doses of glycine or using agonists (D-serine) and partial agonists (D-cycloserine) (Goff and Coyle, 2001), activates the NMDA receptor. Such compounds improve some of the positive, negative and cognitive symptoms (although the partial glycine agonist D-cycloserine actually exacerbates symptoms at high doses when it acts as an antagonist (Heresco-Levy, 2003)). This data further implicates NMDA receptor hypofunction in schizophrenia. NMDA receptor activation has also been potentiated by increasing glycine availability at the synapse by inhibiting the glycine transporter 1 (GlyT1) (Chen et al., 2003, Sur and Kinney, 2004).

As well as requiring the presence of glycine, NMDA receptor activation is also modulated by a number of other receptors. It is possible that altering the activity of these other receptors may offer a mechanism by which NMDA receptor activation can be increased, without excitotoxic effects. At the normal resting membrane potential  $Mg^{2+}$  blocks the NMDA receptor ion channel. Depolarisation of the AMPA or kainate receptors removes this  $Mg^{2+}$  blockade, allowing influx of  $Na^+$ ,  $K^+$  or  $Ca^{2+}$  through the NMDA receptor channel. Therefore modulation of AMPA or kainate receptors may offer a possible new treatment strategy. Initial preclinical studies with positive AMPA receptor modulators looked promising, with improved performance in delayed match to sample (Black, 2005), although preliminary clinical trials did not show any antipsychotic or precognitive effects either alone (Marenco et al., 2002) or in combination with antipsychotics (Tuominen et al., 2005).

As well as being modulated by AMPA and kainate receptors, NMDA receptors are also modulated by some mGluRs. Eight mGluRs have been cloned and these have been divided into 3 groups, based on their pharmacology, sequence and signal transduction mechanisms (Swanson et al., 2005). Group 1 consists of mGluR 1 and 5. They are positively

coupled to phospholipase C (PLC). Group 2 consists of mGluR 2 and 3 and Group 3 consists of mGluR 4, 6, 7, and 8, which all inhibit adenylate cyclase. Of these receptors mGluR 2, and 5 are of particular interest as targets to treat schizophrenia (Moghaddam, 2004). mGluR<sub>2</sub> is mostly expressed pre-synaptically at glutamatergic synapses where it suppresses glutamate release (Moghaddam, 2003, Swanson et al., 2005), so antagonists could increase glutamatergic function. mGluR<sub>5</sub> is found post-synaptically at glutamatergic synapses where the mGluR<sub>5</sub> is positively coupled to the NMDA receptor. Activation of group I mGluRs has been shown to potentiate NMDA function (Kinney et al., 2003) and it has been seen that mGluR<sub>5</sub> knock-out mice have a deficit in NMDA mediated long term potentiation (Jia et al., 1998). Therefore stimulation of mGluR<sub>5</sub> may be beneficial in the treatment of schizophrenia by increasing NMDA receptor activation.

### *Genetic Risk Factors*

Schizophrenia is a polygenetic disorder, i.e. rather than one or two large gene effects schizophrenia is controlled by the cumulative effects of many risk genes. Several genome wide scans have searched for genes linked to schizophrenia and between them have associated much of the genome with the disease. However, the most of the regions implicated in any one study have not been replicated in others (Harrison and Weinberger, 2005). Given that schizophrenia is a heterogeneous disease with patients exhibiting a wide range of symptoms, it is not surprising that each patient carries a different set of risk genes. In a recent review Harrison and Weinberger considered the strength of evidence of several putative schizophrenia risk genes (Harrison and Weinberger, 2005). Harrison previously observed that most of the susceptibility genes are involved in synaptic function (Harrison and Owen, 2003) and the additional genes reviewed more recently strengthen this hypothesis (Harrison and Weinberger, 2005), with glutamatergic synapses being particularly affected (Eastwood and Harrison, 2005). Some of the genes with the most compelling evidence for



an association with schizophrenia, based on genome scans, are discussed briefly in Table 1.2 below. However it must be reiterated that no risk gene has been found to be associated with schizophrenia in all studies.

Table 1.2: Function of protein products of schizophrenia risk genes

Protein	Function
Catechol-O-methyl-Transferase (COMT)	Metabolises catechol amines, including dopamine Expressed in neurons, mainly in the prefrontal cortex and hippocampus. The val158met polymorphism in the COMT gene leads to decreased activity in the methionine containing enzyme. Val-COMT has been associated with schizophrenia and poor pre-frontal cortex function due to hypofrontality caused by increased metabolism of dopamine in frontal cortex (Harrison and Weinberger, 2005, Tunbridge et al., 2006).
Dysbindin	Dysbindin is involved in functioning of the post-synaptic density (PSD), including receptor and signal transduction protein trafficking. (Harrison and Weinberger, 2005)
Neuregulin 1	Neuregulin 1 protein has several isoforms. All the isoforms are involved in cell-cell signalling, via various mechanisms. (Harrison and Weinberger, 2005) The functional effects of neuregulin signalling include neuronal migration, synaptogenesis, neurotransmission and synaptic plasticity (Stefansson et al., 2004).
Regulator of G-protein signalling 4 (RGS4)	RGS4 negatively modulates G-protein mediated signalling at some dopamine, mGluR and muscarinic receptors and is regulated by dopamine (Harrison and Weinberger, 2005).
Disrupted in Schizophrenia 1 (DISC1)	Role of DISC1 protein is not fully understood (Harrison and Weinberger, 2005). DISC1 associates with cytoskeletal proteins involved in cell migration and trafficking of receptors.
Metabotropic glutamate receptor 3 (mGluR <sub>3</sub> )	A SNP is associated with abnormal prefrontal activation, poor episodic memory and attention (Harrison and Weinberger, 2005). Modulates 5-HT and dopaminergic transmission (Cartmell and Schoepp, 2000, Spooren et al., 2003). Polymorphisms predict response to olanzapine (Bishop et al., 2005).
G72 and D-amino acid oxidase (DAAO)	G72 activates DAAO. DAAO metabolises D-serine, an agonist at modulatory glycine site on the NMDA receptor. (Harrison and Weinberger, 2005, Kapoor et al., 2006, Boks et al., 2007).

Calcineurin	Involved in pre and post-synaptic plasticity, especially in glutamate-dopamine interactions (Harrison and Weinberger, 2005)
Nicotinic $\alpha 7$ receptor	Involved in modulating dopamine and glutamate neurotransmission (Harrison and Weinberger, 2005).
Proline Dehydrogenase (PRODH2)	PRODH knock-out mice exhibit PPI deficits and decreased glutamate levels (Harrison and Weinberger, 2005).

### *Environmental Risk Factors*

As with genes, there are several environmental factors which appear to increase risk of developing schizophrenia, although no one factor is present in all cases. These include maternal infection or stress during gestation, obstetric complications and winter birth (Lewis and Lieberman, 2000, Cannon and Clarke, 2005). It is currently believed that exposure to a combination of risk genes and environmental factors early in life can, in the future, lead to the development of schizophrenia. Therefore schizophrenia is now thought of as a neurodevelopmental disorder. Home video footage of schizophrenics as children, taken long before the disease symptoms appeared, show evidence of motor abnormalities (Lewis and Lieberman, 2000, Rapoport et al., 2005). Social and cognitive deficits have also been seen in teenagers, before they fully develop the disease, suggesting that there are underlying problems in existence before the disease manifests completely (Rapoport et al., 2005). There is also considerable evidence that gender plays a role in the development of schizophrenia, with men generally having an earlier age of onset than women (Castle et al., 1993). This gender effect is discussed in more detail in Chapter 2. The role of cannabis abuse in the development of schizophrenia has also attracted much attention in recent years, following controversial studies suggesting that heavy cannabis use during adolescence can increase risk of developing schizophrenia later in life (Zammit et al., 2002). The link between cannabis and schizophrenia is discussed further in Chapter 6.

If schizophrenia is caused by a combination of genetic and early life (pre-natal or early post-natal) events, why do the main symptoms of the disease not develop until young adulthood? Brain development is not complete until after adolescence when the brain undergoes various maturational processes, such as apoptosis, synaptic pruning and myelination (Lewis and Lieberman, 2000, Woo and Crowell, 2005). If the genetic differences and environmental insults suffered by schizophrenics have led to impaired brain development, it is possible that the defect may not show up until the brain has undergone this re-organisation. Young adulthood is also a period of change in a person's life, when they first start work or leave home. This stressful period may put added pressure on the brain, leading to the development of schizophrenia.

This developmental hypothesis is supported by evidence of reduced pyramidal cell size and fewer dendritic spines in the hippocampus of schizophrenics, as well as decreased pre-synaptic markers such as synaptophysin and growth associated protein-43 (GAP43) (Eastwood et al., 1995, Wong and Van Tol, 2003, Honer and Young, 2004, Chambers et al., 2005, Harrison and Weinberger, 2005). It has also been found that brain organisation is impaired in schizophrenia, with evidence suggesting impaired neuronal migration, leading to misplaced cells in the dorsolateral prefrontal cortex (PFC) and entorhinal cortex (Harrison and Weinberger, 2005, Tamminga and Holcomb, 2005). As this neuronal migration occurs during brain development, this is strong evidence for a developmental component to schizophrenia.

### **Glutamate and Brain Development**

During brain development the presence of glutamate allows young neurons to form excitatory synapses containing NMDA receptors (Bolton et al., 2000). Once these synapses have been made the pre- and post synaptic membranes must synchronise to form a functional, stable synapse, which requires NMDA receptor activation. The new synapse must release glutamate for the NMDA receptors, which are present on the postsynaptic membrane, at the same time as a depolarisation dislodges the  $Mg^{2+}$  blockade (Konradi and Heckers, 2003). Once this has occurred AMPA receptors are recruited to the synapse to allow further depolarisation and functional development. This process requires the presence of  $Ca^{2+}$ /calmodulin dependent kinase II (CaMKII), the expression of which has been found to be decreased in schizophrenia (Cochran et al., 2002). It is clear that if synapses require glutamate and functioning NMDA receptors to form correctly, a deficit in either could have severe consequences for the development of brain circuitry.

Post mortem studies of schizophrenic brains have found a reduction in the expression of the calcium binding protein parvalbumin in the prefrontal cortex (PFC) and hippocampus (Beasley et al., 2002, Hashimoto et al., 2003, Morris et al., 2005). Parvalbumin is found in a subpopulation of inhibitory  $\gamma$ -aminobutyric acid (GABA)-ergic interneurons known as chandelier cells and basket cells. NMDA receptors are involved in the basal activity levels of chandelier cells. The activity of PFC pyramidal neurones is normally suppressed by chandelier cells. If NMDA receptor activity on the interneurons is reduced, PFC pyramidal neurones will be disinhibited, leading to excessive firing. PFC pyramidal neurones are part of a cortico-limbic circuit thought to be involved in schizophrenia, running from the PFC to the ventral striatum to the ventral pallidum to the mediodorsal thalamic nucleus and back to the PFC. Interestingly, the GABAergic interneurons continue development in the post-natal period, making them vulnerable to early life events. In rhesus monkeys the distribution and density of these neurones continues to change into

adolescence, which means an impairment in their function may not manifest until after that time, consistent with the age of onset of schizophrenia (Cruz et al., 2003).

## **1.2 Animal Modelling of Schizophrenia**

When modelling psychiatric diseases in animals one is faced with two problems. Firstly, how to induce the symptoms of a psychiatric disease in a rat and secondly, how to detect them? Numerous behavioural tests have been designed to assess some of the core symptoms of schizophrenia in rodents, as described below.

### **1.2.1 Rodent Correlates of Aspects of Schizophrenia**

#### *Positive Symptoms*

While it is not possible to know whether rats are experiencing hallucinations or delusions, there are behavioural tests which do show face, construct and predictive validity for some of the core symptoms of the disease.

Sensorimotor gating is an inhibitory response thought to allow the brain to filter out incoming sensory stimuli to allow prior or weaker stimuli to be processed without being drowned out (Swerdlow et al., 2000). Sensorimotor gating is well conserved across species and can be assessed in both animals and humans by measuring the ability of a subthreshold stimulus to reduce startle response to an intense stimulus, such as a loud noise (a pulse). This phenomenon is known as prepulse inhibition (PPI) of acoustic startle and is thought to be a measure of sensorimotor gating, which is attenuated in schizophrenia. However it is important to note that schizophrenia is not the only psychiatric disease to exhibit PPI deficits, obsessive compulsive disorder, Huntington's disease and Tourette's syndrome also share this symptom (Geyer et al., 2001b).

The DA releasing agent amphetamine causes hyperactivity in animals, as well as inducing psychoses in humans (Johnson and Milner, 1966, Sahakian et al., 1975). This hyperactivity is believed to arise from excessive DA activation in the mesolimbic system, especially the nucleus accumbens (NAcc) (Geyer and Ellenbroek, 2003). Reversal of amphetamine induced locomotor hyperactivity has been used as a screen for antipsychotic drugs for many years, although not it is not always predictive of clinical success (Moser et al., 1996).

### *Negative Symptoms*

The negative symptoms of schizophrenia include social withdrawal, which is measured in animals by watching the interactions between them. The ability to measure anhedonia in animals is controversial, but has been proposed to be achieved by measuring the amount an animal is prepared to work for a reward. Ellenbroek suggests using a breaking-point schedule in which rats are trained to press a lever to receive a reward (Ellenbroek and Cools, 2000). Once trained, the number of lever presses required to obtain the reward is increased. Eventually the breaking-point is reached at which the reward is no longer worth the effort required. In this paradigm an anhedonic rat will reach breaking-point earlier as the perceived reward is less.

### *Cognitive Tests*

Many cognitive tests have been developed for rodents to assess different types of memory.

#### **Novel Object Discrimination (NOD)**

NOD tests recognition memory by taking advantage of the natural preference of rats for novelty (Ennaceur and Delacour, 1988). Rats placed in a familiar chamber with a two unseen identical objects will explore them. The rat is removed from the chamber and one of the objects is

replaced with a novel object. Recognition of the original, familiar, object will lead to more interest in and exploration of the novel object. If the original object has been forgotten equal time will be spent exploring both objects. Visual learning and memory and recognition memory are, at least partly, dependant on the entorhinal cortex, perirhinal cortex and parahippocampal cortex (Bear et al., 2007).

### **Attentional Set-shifting**

Set-shifting measures behavioural flexibility, testing the ability to learn rules and then adapt to a change in rules. In humans the Wisconsin Card Sort Test is used to measure behavioural flexibility. Subjects are given a stack of cards with different numbers of coloured symbols. The subject must sort the cards, using feedback from the administrator to ascertain the sorting criteria (e.g. colour of the symbols). Schizophrenics have been shown to be particularly impaired at the most difficult type of rule change (Haut et al., 1996). A rodent version of this task has been developed by Birrell and Brown (Birrell and Brown, 2000). In this task food-deprived rats dig in scented bowls for food rewards hidden in different substances. The rats must learn to follow one of the cues (e.g. scent) to find the reward. The rats then undergo a series of discriminations, in which they always follow the same type of cue (e.g. a new scent), known as intra-dimensional (ID) or affective shifts. However, in the extra-dimensional (ED) or attentional shift the rat must now learn to follow the other cue (e.g. digging medium), which is the most difficult new rule to acquire.

Attentional set shifting is mediated by the PFC, with lesions of the lateral PFC specifically impairing ED/ ID shifts in monkeys (Dias et al., 1996). Schizophrenia causes a decrease in metabolic function in the PFC, known as hypofrontality, which may explain the inability of schizophrenics to perform this cognitive test. As schizophrenics are so impaired at this ED shifting it is felt that the rodent version of the task most accurately models the specific cognitive deficits seen in schizophrenia. Attentional set shifting is described in more detail in Chapter 3.

**Water Maze**

The Morris water maze was developed to investigate spatial memory (Morris, 1984) and consists of a round pool of opaque water, in which a submerged platform is hidden. Although rats are competent swimmers they will search for the platform to escape from the water. Having been shown the platform position after a trial, rats will use extra-maze cues (shapes on the walls etc) to learn its location, which enables the rats to find the platform quickly on the subsequent first trial of each day (Baldi et al., 2005).

**Passive Avoidance**

Passive avoidance is a test which measures memory of a fearful event, such as receiving a footshock. The test box consists of two chambers, one with white and one with black walls. Animals are placed into the white chamber and move into the black chamber in their own time. Once in the black chamber rats receive an aversive footshock before being returned to their home cage. The following day the rats are returned to the white chamber and the time taken to move into the black side is recorded. This fear-related memory is mediated by the amygdala (Davis, 1992) and discussed in greater detail in Chapter 5.

**1.2.2 Inducing the Symptoms of Schizophrenia in Animals**

Several groups have used exposure of rats to early environmental or pharmacological interventions to attempt to reproduce some of the core behavioural symptoms seen in schizophrenia. When modelling any disease in animals three criteria are frequently used to assess the model. These criteria are known as face, construct and predictive validity. For a model to have face validity it must reflect the clinical situation. In the case of schizophrenia this would ideally mean inducing behavioural changes which are analogous to positive, negative and cognitive symptoms.



Construct validity refers to replicating the underlying pathophysiology of the disease in as many ways as possible, such as neuroanatomical changes, physiological changes and neurochemical markers. Finally, predictive validity is the ability of the model to successfully respond to a treatment known to be efficacious in the clinical situation. Without predictive validity the efficacy of the model in detecting novel treatments for the disease must be questioned. While a comprehensive review of all the animal models of schizophrenia is beyond the scope of this report, a brief overview of some models is given below.

#### *NMDA Antagonists*

The observation that PCP induces psychosis in humans led to the idea that treatment with PCP and other NMDA antagonists such as ketamine and MK801 could be used as animal models of schizophrenia. PCP binds the ion channel of the NMDA receptor, but can only bind when the channel is open, which means PCP causes a use-dependent blockade of the receptor (Morris et al., 2005). Several groups have used NMDA antagonists to model schizophrenia, using various treatment regimens. The acute, chronic and perinatal effects of PCP are reviewed by (Mouri et al., 2007).

#### **Acute NMDA Antagonists**

Acute PCP treatment of primates and rodents has shown promise to model many of the behavioural features of schizophrenia, such as cognitive impairment, PPI deficits and impaired social interactions (Geyer et al., 2001b, Morris et al., 2005). MK801 also robustly impairs PPI in rats (Varty and Higgins, 1995, Bubenikova et al., 2005, Levin et al., 2005). Furthermore, these PPI deficits can be reversed by the antipsychotics clozapine and olanzapine (Geyer et al., 2001b, Bubenikova et al., 2005). Systemic MK801 impaired rule acquisition and the attentional (ED) shift in rats, in a set-shifting paradigm (Stefani and Moghaddam, 2005), which is considered to be particularly relevant to the cognitive deficits seen in schizophrenia.

Acute PCP also models some neurochemical alterations seen in schizophrenia such as reduced expression of parvalbumin in the PFC (Cochran et al., 2002) (Morris et al., 2005). PCP increases expression of activity related cytoskeletal protein (Arc) (an immediate-early gene which is rapidly induced in neurones by synaptic activity) in the PFC, NAcc and posterior cingulate cortex and this can be reversed in some areas by the antipsychotics clozapine, olanzapine and risperidone (Nakahara et al., 2000). However, low doses of PCP also lead to vacuolarisation, with high doses causing cell death and necrosis in many brain regions (Sams-Dodd, 2004). MK-801 also causes a reduction in brain derived neurotrophic factor (BDNF) expression in the hippocampus (Fumagalli et al., 2003), which is involved in regulating neuronal differentiation and survival during development. Low doses of PCP cause initial increases in Arc expression, indicating excitement in the PFC, followed by a depression of activity. As schizophrenics have been found to have reduced activity in the PFC the longer-term effects of PCP are more relevant to the disease leading to investigation of the effects of chronic NMDA antagonist treatment (Morris et al., 2005).

#### **Chronic NMDA Antagonists**

Chronic PCP abuse is more likely to induce psychosis in humans than a single dose (Morris et al., 2005), so the effects of chronic dosing were explored in animals. Cochran et al used a 5 day low dose PCP (2.58mg/kg/day) protocol to induce a reduction in activity in the PFC (measured by glucose utilisation) (Cochran et al., 2003) accompanied by decreased parvalbumin expression in rats (Cochran et al., 2002). They found that treatment with further doses, 3 x per week (chronic intermittent dosing regime) maintained these changes. Chronic treatment with antipsychotics reversed some of the metabolic changes seen, but not those in the PFC (Cochran et al., 2003). Furthermore, while clozapine reversed the PCP-induced decrease in PFC parvalbumin, the typical antipsychotic haloperidol had no effect. The same dosing regime also reduces

parvalbumin expression in the reticular nucleus of the thalamus, which is comprised of GABA-ergic neurones (although in this study PCP treatment had no effect on PFC parvalbumin expression) (Cochran et al., 2002).

The chronic intermittent dosing protocol also causes a reduction in N-acetyl aspartate (NAA) in the rat temporal cortex (Reynolds et al., 2005). NAA is thought to be a marker of neuronal function and integrity and is significantly reduced in the temporal cortex of schizophrenics, providing further evidence that chronic intermittent PCP closely replicates some neurochemical features seen in schizophrenia. The effects of chronic intermittent PCP in behavioural tasks are less well reported, although some evidence suggests the dosing regime may not have sustained effects on cognition (Watson et al., 2005).

A sub-chronic dosing regime, of twice daily PCP dosing for one week has been found to have sustained effects in some cognitive tasks including operant reversal learning (Abdul-Monim et al., 2006), discrimination learning (Dunn and Killcross, 2006), recognition memory (Grayson et al., 2007) and attentional set-shifting (Rodefer et al., 2005) after at least a week drug-free. Sub-chronic PCP has also been found to induce neurochemical changes with relevance to schizophrenia, such as increased BDNF (Harte et al., 2007a) and reduced parvalbumin expression (Abdul-Monim et al., 2007).

### *Developmental Models*

As schizophrenia is believed to be a neurodevelopmental disorder, several groups have attempted to produce a developmental animal model of the disease, hoping this will more accurately replicate the clinical disease. Such paradigms involve an insult early in life to cause schizophrenic-like symptoms to develop later in life. A wealth of information shows that exposure of mammals to early-life adversity negatively affects brain development and adult behaviour (Harlow et al., 1965, Heim et al., 2004,

Rapoport et al., 2005). Although the molecular mechanism involved in producing these developmental adaptations is unclear, similar environmental interventions during early-life in humans may contribute to the development of common psychiatric disorders, such as depression and schizophrenia in genetically predisposed individuals. The availability of a non-pharmacological animal paradigm which elicits robust, reproducible, developmental alterations during critical brain development periods may provide an ideal scenario to gain a better understanding of the genetic and molecular associations and the neurobiological aetiology of developmental psychiatric disorders and enable discovery and evaluation of novel therapeutic agents.

#### **Perinatal PCP**

The effect of NMDA receptor antagonism on the developing brain has been investigated using varying protocols which all involve a challenge with an NMDA receptor antagonist within the first 2 weeks after birth. PCP on post natal days (PND) 7, 9 & 11 (10mg/kg) increases PCP-induced locomotor activity (reflecting sensitisation) and attenuates PPI in resultant adult rats, which can be prevented by pre-treatment with olanzapine (Wang et al., 2001). Perinatal PCP has been shown to cause apoptosis of cortical neurones, which is believed to lead to reduced synaptogenesis in the striatum (Wang et al., 2004), which again resembles the pathology of schizophrenia. Perinatal PCP treatment also induces cognitive deficits in adulthood, with male (but not female rats) showing impaired spatial memory acquisition in the water maze, which is reversed by the glycine agonist D-serine (Anderson and Pouzet, 2004).

#### **Neonatal Ventral Hippocampal Lesions (NVHL)**

The NVHL model uses an infusion of the excitotoxic compound ibotenic acid directly into the rat ventral hippocampus (bilaterally) on PND 7. This leads to the post-pubertal development of a number of behaviours akin to those seen in schizophrenia (Lipska, 2004), such as sensitivity to MK-801

and apomorphine (Schroeder et al., 1999), down-regulation of striatal dopamine D<sub>2</sub> receptors (Schroeder et al., 1999), disrupted dopamine-glutamate interactions in the PFC (Tseng et al., 2007), decreased dendritic branching (Flores et al., 2005) deficits in social behaviour and cognitive impairment (Le Pen and Moreau, 2002). NVH lesions also impair PPI, which was reversed by clozapine and risperidone but not by haloperidol (Le Pen and Moreau, 2002). Chronic clozapine and risperidone treatment also reverse locomotor hyperactivity and PPI deficits, but not social interaction deficits seen in this model (Rueter et al., 2004).

#### **Pre-natal Methylazoxymethanol (MAM)**

The DNA-methylator MAM is an inhibitor of mitosis. Treatment with MAM at specific time points during the gestational period can induce region targeted disruption of brain development. MAM treatment on or earlier than day 15 of gestation leads to development of increased striatal dopamine and deficits in sensorimotor gating and cognitive processes (ref Moore 2006) and (Leng et al., 2005). However, MAM treatment at this stage in development also causes microcephaly (Leng et al., 2005) which is not seen in schizophrenia. MAM treatment on embryonic (E) day 17 has a more specific effect on cerebral cortex, especially the frontal cortex, which develop later in gestation. When the MAM-E17 treated rats reach adulthood they demonstrate impairments in reversal learning, object recognition, latent inhibition (Flagstad et al., 2005) and sensorimotor gating (Moore et al., 2006). As well as behavioural deficits these rats also show region specific volume reductions (including in the hippocampus and prefrontal cortex), a decrease in hippocampal parvalbumin-expressing neurons (Penschuck et al., 2006) and altered response to amphetamine (Flagstad et al., 2004). This the MAM-E17 paradigm can induce behavioural and neurochemical changes with relevance to schizophrenia (Flagstad et al., 2004).

### 1.3 Isolation Rearing

As this work described in this thesis focuses on the effects of social isolation of rats from weaning, this model will now be discussed in greater detail.

Rearing rodents in persistent social isolation from weaning, to deprive them of social play, produces a large array of consistent long-lasting behavioural alterations compared with group housed controls (Valzelli, 1973, Eison and Morgan, 1977, Heidbreder et al., 2000, Lapiz et al., 2003) without causing any consistent alteration in body weight from age-matched controls. Collectively the behavioural changes observed are consistent with the proposal that rats reared in social isolation are unable to appropriately process environmental stimuli. Even from early experiments performed in the 1960's and 1970's rats reared in social isolation were reported to be excessively reactive to handling, anxiogenic, and overly emotional (Koch and Arnold, 1972, Morgan, 1973, Sahakian et al., 1977) which lead to the description of the 'isolation-induced stress syndrome' (Valzelli, 1973, Holson et al., 1991).

Some of the behavioural and neurochemical alterations seen in isolation reared rats have translational relevance to developmental neuropsychological disorders, in particular to several core symptoms of schizophrenia but also to changes seen in depression, which has lead to isolation rearing being proposed as an animal model of these disorders. The majority of such studies have been performed with male rats but where another species or gender has been examined this will be stated.

### 1.3.1 Isolation Procedure

The isolation rearing procedure adopted by most laboratories involves housing rat (or mouse) pups in individual cages from the first day of weaning from the dam, normally on PND21 to PND28 for rats. From that point isolation housed rats are not handled more than once a week (to change bedding material). Isolation reared rats thus have visual, auditory and olfactory contact with other isolation reared and group housed rats kept in the same husbandry conditions. They are however unable to have any form of social interaction with littermates. Noise in the housing facility should be carefully controlled and minimised. To avoid any confounding affect of litter, pups from the same dam should be divided equally into isolation and group housed conditions and litters should be selected for equal size.

The full behavioural change associated with social isolation of rodents from weaning (discussed by topic later) is only observed if this intervention is commenced (from PND20 to PND30) in a critical period around the time of puberty (Einon and Morgan, 1977). For instance, the reduction in; social interaction (Ferdman et al., 2007) and prepulse inhibition of acoustic startle (Wilkinson et al., 1994), or the increase in; self-administration of ethanol (Schenk et al., 1990) and defensive shock probe burying (Arakawa, 2007), only occur when rat pups are isolated and not when the same procedure is applied to adults. Although the actual style of the cage appears to be unimportant (Einon and Morgan, 1977), the housing environment may well affect the precise nature of the outcome (Weiss et al., 1999) and no environmental enrichment should be provided. For instance, basal corticosterone levels may only be elevated by more austere housing conditions, such as wire floored cages where no handling is performed during the isolation period (Holson et al., 1991, Heidbreder et al., 2000) or housing in sound proof cages which also prevent auditory and visual contact with littermates (Greco et al., 1989). Furthermore, it is clear that any form of contact with a conspecific, or excessive handling by the

experimenter (such as daily drug injection), will readily negate any long-term changes and strict adherence to the protocol is required if robust changes are to be reproduced in the laboratory (as discussed later). As isolation rearing leads to an array of behavioural changes it is important that when these animals are being investigated, or used as a research tool (e.g. for antipsychotic drug discovery), more than one behavioural phenotype with strong translational relevance to the core domain defects seen in the disease being modelled should be examined (Powell and Miyakawa, 2006).

### **1.3.2 Behavioural Effects of Isolation Rearing**

The behavioural effects of isolation rearing are summarised in Table 1.3, found at the end of this chapter.

#### *Hyper-reactivity to a Novel Environment*

One of the earliest onset and probably the most robust observations reported in isolation reared rats is a lack of the normal habituation following placement in a novel arena, characterised by motor hyperactivity (horizontal ambulation and rearing) compared with group housed controls (Hughes and Syme, 1972, Syme, 1973, Sahakian and Robbins, 1977, Sahakian et al., 1977, Gentsch et al., 1981b, Gentsch et al., 1982a, Gentsch et al., 1982b, Gentsch et al., 1983, Phillips et al., 1994b, Domeney and Feldon, 1998, Heidbreder et al., 2000, Silva-Gomez et al., 2003) due to less time spent resting. Careful analysis of this behaviour with photocells or computerised tracking shows that the response is strain dependent. For instance the hyperactive locomotor response appears to be much more marked in Lister than in Sprague-Dawley rats which have undergone an identical isolation protocol (Geyer et al., 1993, Weiss et al., 2000) in both male and female rats (Weiss et al., 2001a, Powell et al., 2002). In addition, while the initial activity level is comparable to group housed controls in Lister hooded (Fone et al., 1996) and Long-Evans (Powell et al., 2002) rats the former strain show a slower rate of decline such that



activity levels are typically elevated after 15 minutes of exploration. In contrast, the basal level of activity may be elevated from group housed controls from the outset of placing isolated Wistar rats in a novel arena (Domeney and Feldon, 1998, Heidbreder et al., 2000). This also demonstrates the need to record the response to novelty over a sufficient time period to enable the temporal profile to be recorded and could explain the few reports where the response to a novel arena has appeared unaltered (Rosa et al., 2005) or even reduced during the first five minutes due to initial freezing (Holson et al., 1991). Furthermore, even brief periods of handling twice a week are sufficient to prevent this inappropriate habituation to a novel environment (Holson et al., 1991). This hyperactive response of isolation reared rats is also reduced in bright light (Hall et al., 1998b) and absent when the environment becomes familiar (Phillips et al., 1994b).

Regardless of strain this decreased habituation to novelty is comprised of elevated horizontal locomotion and vertical rears (Gentsch et al., 1981a, Gentsch et al., 1982b) suggestive of an increased propensity to escape and consistent with neophobia (Fone et al., 1996). One recent study using microarray analysis found abnormal expression of immediate early genes and genes that regulate apoptotic genes and cell differentiation in the medial prefrontal cortex which correlated with the extent of hyperactivity in a novel arena after 26 days isolation in a small group of six Sprague Dawley rats (Levine et al., 2007). Thus abnormal prefrontal cortex activity may be related to this behavioural alteration and such behaviour is potentially relevant to the positive symptoms seen in schizophrenia or 'anxiety' accompanying depression. In an analogous fashion isolation reared rats are slower to emerge from a confined space to a larger novel environment (Einin and Morgan, 1977, Arakawa, 2005). Interestingly isolation reared rats appear to show a preference for a novel environment compared with group housed rats (Sahakian et al., 1977), although another study only found this to be the case under red-light conditions (Hall et al., 1997b).

The consistency of the exaggerated response to a novel arena and the ease with which this can be measured makes this a suitable marker with which to confirm the development of the ‘isolation syndrome’ prior to performing more complex behavioural or neurochemical analyses. Few groups have systematically examined the time course of the development of the hyper-reactivity to a novel arena but it is clear that this is also one of the earliest behavioural features to appear, being evident even after two weeks of isolation (Einon and Morgan, 1977, Bakshi and Geyer, 1999). In contrast, other behavioural changes such as the reduction in prepulse inhibition of startle (discussed later) are only apparent after at least four weeks isolation in Sprague-Dawley rats (Varty et al., 1999a) and even longer periods of isolation in Lister hooded rats (Bakshi and Geyer, 1999).

#### *Prepulse Inhibition of Startle*

Prepulse inhibition (PPI) refers to the inhibitory influence of a weak sensory stimulus (the prepulse) on the reaction to a subsequent startle-eliciting stimulus (the pulse). In animals, PPI is typically measured as the inhibition of a motor startle response to a loud (approximately 120 dB) acoustic startle eliciting stimulus by a preceding weaker (typically 70-85 dB) prepulse delivered over a background white noise (usually 65-68dB). PPI is a useful operational index of pre-attentive sensorimotor gating mechanisms essential for the integration of cognitive and sensory information (Geyer et al., 2001b) and shows a similar neurobiology and neuropharmacology in rat and man. Although it is not a unique nor diagnostic feature of the disorder, impairments in PPI are often reported in patients with schizophrenia (Braff et al., 1992, Braff et al., 2001) as well as other psychiatric disorders such as depression, and it may reflect stimulus overload-induced cognitive fragmentation.

Isolation reared mice (Sakaue et al., 2003, Varty et al., 2006) and rats show a robust reduction in PPI of acoustic startle (Geyer et al., 1993).

This finding has been replicated in a large variety of laboratories in both male and female Lister hooded (Varty et al., 1999b, Cilia et al., 2001, Cilia et al., 2005b, Day-Wilson et al., 2006), Long-Evans (Binder et al., 2001, Powell et al., 2002, Powell et al., 2003) and Sprague Dawley rats (Geyer et al., 1993, Varty et al., 1999a), with less consistent and smaller effects seen in Wistar (Domeney and Feldon, 1998, Weiss et al., 1999, Weiss et al., 2000, Weiss and Feldon, 2001, Rosa et al., 2005) and Lewis strains but it may not occur in isolated Fischer (Varty and Geyer, 1998) rats. As previously discussed, the development of PPI may also be dependent on the housing environment and is not seen in Wistar rats raised on a grid rather than a sawdust floor (Weiss et al., 1999). By using comparable isolation procedures (8 weeks of isolation from PND 28) Cilia et al (Cilia et al., 2001, Cilia et al., 2005b) showed that 23 of 27 cohorts of isolated male Lister hooded rats exhibited 30 to 50% reductions in the PPI produced by a single prepulse either 5 or 10 dB above baseline, demonstrating the robust, reproducible nature of this behavioural alteration which is independent of any change in basal startle (Domeney and Feldon, 1998). Once established the deficit in PPI persists consistently with weekly testing (Cilia et al., 2001) is not reversed by re-socialisation of Lister hooded rats for 8 weeks (Cilia et al., 2001) or by restricting water access to 30 min per day early in the isolation period (used as an additional early-life adverse effect) on PND 24-26 (Binder et al., 2001, Powell et al., 2002). Weiss and Feldon (Weiss and Feldon, 2001) performed a similar analysis of the effect of social isolation measured on repeated occasions in multiple cohorts of male Sprague-Dawley rats following 12 to 24 weeks of continuous isolation and found that for each cohort the extent of any impairment was consistent across the isolation period. However, the PPI deficit is developmentally specific and does not occur when adult rats are isolated for an eight week period (Wilkinson et al., 1994). Other groups have also found that the PPI deficit can be lost by assessment of locomotor activity less than one week prior to PPI (Domeney and Feldon, 1998) or handling during the isolation period (Krebs-Thomson et al., 2001) even briefly (grasping the tail three times a week) will prevent the development

of PPI deficits (Rosa et al., 2005). So an adequate recovery period of at least 7 days should be left between test procedures or repeated PPI testing if this is to be performed (Varty and Higgins, 1995, Domeney and Feldon, 1998).

Early experiments showed that the deficit in PPI produced by social isolation could be partially reversed by acute treatment with the dopamine D<sub>2</sub> receptor antagonist, raclopride (Geyer et al., 1993), suggesting that it may involve enhanced dopaminergic neuronal activity. Subsequent studies found that bilateral injections of 6-hydroxydopamine into the nucleus accumbens (NAcc), depleting dopamine levels by 83%, reduced the deficit in PPI seen in female Long-Evans rats produced by 8 weeks isolation rearing (Powell et al., 2003). This supports the view that dopamine hyperfunction in the NAcc may account for the deficit in PPI seen in isolates, probably by enhancing GABAergic output to the ventral pallidum and subsequently the pedunculo pontine pathway (Figure 1.1). Recently it has been shown that the isolation-induced PPI deficit is reversed by pre-treatment with an  $\alpha 7$  nicotinic receptor agonist (Cilia et al., 2005a). However, the same authors showed that isolation rearing did not alter expression of  $\alpha 7$  receptor RNA and protein in the hippocampus or frontal cortex, suggesting that alteration of this receptor is not associated with the phenotypic changes resulting from isolation rearing. Treatment with  $\Delta^9$ -tetrahydrocannabinol (THC) exacerbates the isolation-induced PPI impairment, but has no effect on PPI in socially housed animals. This THC effect is blocked by SR 141716 (an antagonist at the CB<sub>1</sub> receptor), although SR141716 has no effect on PPI when given alone (Malone and Taylor, 2006). This is of particular interest given the reported links between adolescent cannabis exposure and schizophrenia (Zammit et al., 2002, D'Souza et al., 2005) supported by a recent meta-analysis which found that cannabis use increased the risk of psychotic outcome by a factor of 1.4 fold (Moore et al., 2007), This will be discussed further in Chapter 6.

The impairment of PPI seen in isolation reared rats is reversed by atypical antipsychotics, such as quetiapine, olanzapine, clozapine and risperidone, provided that a variable inter-stimulus interval is used (Wilkinson et al., 1994, Varty and Higgins, 1995, Bakshi et al., 1998, Cilia et al., 2001). However, a recent study found that the novel atypical antipsychotic, iloperidone, failed to reverse the PPI deficit in isolation reared Sprague-Dawley rats (Barr et al., 2006), so negative results should be interpreted with care. Furthermore, even acute administration of an antipsychotic is sufficient to reverse the isolation-induced PPI deficit while the clinical benefit of antipsychotic medication takes several weeks to manifest, so the predictive validity to the chronic effect of treatment in schizophrenia is unclear. Although it is not unique to schizophrenia, PPI impairments are believed to reflect the loss of sensory inhibition resulting in cognitive fragmentation (Perry and Braff, 1994). The strong similarity between the neurobiology of PPI in rodents and man (Swerdlow and Geyer, 1998) together with its reversal by most antipsychotic drugs has resulted in several groups using this as a screen for novel antipsychotics (Geyer et al., 1993, Varty and Higgins, 1995, Geyer et al., 1999, Cilia et al., 2005a). For instance the selective 5-HT<sub>2A</sub> receptor antagonist, M100907, partially reversed the PPI deficit produced by 8 weeks of isolation rearing of Sprague-Dawley rats (Geyer et al., 1999), but clinical trials have failed to replicate any similar substantial benefit from the use of this drug in schizophrenia (Gray and Roth, 2007). In addition, acute administration of the 5-HT<sub>1A</sub> agonist, MKC-242 (ozemototan), reversed the PPI deficit produced by 6 weeks isolation of male ddY mice without affecting PPI in group-housed controls (Sakaue et al., 2003) but this has yet to be studied in rats. However, it is obvious that no single phenotypic component of the isolation-induced syndrome will be sufficient to predict antipsychotic potential nor indeed that a single animal paradigm could be used as a safe mechanism for predicting therapeutic potential in such a complex human disorder.

### *Latent Inhibition*

Latent inhibition refers to the constraining effects of prior stimulus exposure on sequential stimulus-response learning (Feldon and Weiner, 1992) and reflects the ability to ignore or suppress irrelevant stimuli to focus on biologically salient input. Latent inhibition represents the interaction of associative and non-associative learning for a specific stimulus, modelling attentional processes that are disrupted in schizophrenia. Several studies have shown that latent inhibition is reduced in schizophrenic patients (Baruch et al., 1988, Gray et al., 1995). Baruch et al. demonstrated that latent inhibition was completely absent in recently diagnosed schizophrenic patients, while chronically ill schizophrenic patients showed distinct latent inhibition (Gray et al., 1995). This led to the hypothesis that latent inhibition is restored by treatment with antipsychotics and therefore latent inhibition rodent paradigms may represent a relevant translational model of the positive symptoms seen in acute schizophrenics (Baruch et al., 1988). Latent inhibition tasks, therefore, fulfil the criteria for face and construct validity as an animal behavioural model of the specific attentional impairments associated with schizophrenia. However, it has also been postulated that disrupted latent inhibition seen in schizophrenia may result from the effects of antipsychotic medication (Williams et al., 1998). In the few studies performed, isolation rearing of Sprague Dawley rats failed to produce any effect on latent inhibition (Wilkinson et al., 1994, Weiss et al., 2001b).

### *Response to Rewarding Stimuli*

Isolation reared rats tend to consume more food than group housed controls and may weigh slightly more than aged matched controls (Fiala et al., 1977). Yet other groups have reported isolation reared rats consume an equal amount of food to socially housed controls under both normal conditions and when food-deprived (Hellemans et al., 2004) but may show an altered preference for different types of food (Hall et al., 1997a). Nonetheless, isolates show a higher response rate in food-reward

motivated procedures consistent with them having enhanced incentive motivation. When given access to increasing concentrations of sucrose solution, isolation reared Lister hooded rats show increased fluid consumption consistent with increased motivation (Hall et al., 1997c). Similarly rearing in isolation enhances the rate of acquisition of a discriminative approach task, which measures association of a stimulus with a sucrose reward (Harmer and Phillips, 1998). Isolates also show an increased response to sucrose reward in a conditioned reward paradigm; an effect further enhanced by intra-accumbens amphetamine, consistent with alteration in the mesolimbic dopamine system contributing to this change (Jones et al., 1990). In contrast, no difference was found in conditioned taste avoidance (Hellemans et al., 2004) in isolation reared rats. However, Morgan (Morgan, 1973) and Jones (Jones et al., 1991) both found that isolation reared rats will continue to perform in food motivated learning tasks, even after satiation.

Several groups have investigated the effect that rearing rats in isolation has on the response to psychostimulant drugs but the results appear to be inconsistent. Isolation rearing has been found to increase both the locomotor (Jones et al., 1990, Jones et al., 1992) and/or the stereotype (Sahakian et al., 1975, Eison and Sahakian, 1979) behavioural response elicited by amphetamine or apomorphine or to systemic cocaine administration (Phillips et al., 1994b). While other groups found no change in either the locomotor or stereotype response to acute amphetamine administration (Weiss et al., 2001a). Furthermore both a complete absence of locomotor sensitisation to repeated amphetamine administration (Weiss et al., 2001a) and a marked increase compared to controls (Bardo et al., 1995) have both been recorded in the same strain (Sprague–Dawley) of rats; although in the latter study group-housed rats were raised in a highly enriched condition which could account for the difference.

Isolation reared rats have been shown to display an increased propensity to self-administer cocaine (Schenk et al., 1987b, Howes et al., 2000) or morphine (Alexander et al., 1978, Hadaway et al., 1979), no enhancement following d-amphetamine (Schenk et al., 1988) or cocaine (Bozarth et al., 1989) or impaired reinforcement to intravenous cocaine (Phillips et al., 1994a, Phillips et al., 1994b) or intra accumbens amphetamine (Phillips et al., 1994a). Unlike other studies cited the isolation procedure utilised by Bozarth et al. (Bozarth et al., 1989) did not begin until PND 61 (i.e. at adulthood) which may explain the apparent discrepancy of this particular work, but why such inconsistency in other studies? Overall, most studies which have reported enhanced responding to stimulant drugs have begun with a series of non-contingent priming infusions (unlike the studies by Phillips et al. (Phillips et al., 1994a, Phillips et al., 1994b)) which could favour detection of 'drug driven' rather than 'drug seeking' behaviour. A recent comprehensive review concluded that isolation rearing causes a modest increase in the initiation of psychostimulant self-administration, particularly following low doses of stimulant, but that there is little change in the maintenance of the behaviour once it is established (Lu et al., 2003b). Such early changes in response to psychostimulants could be the consequence of alteration in the activity of mesolimbic dopaminergic neurones or dopamine receptor function in their terminal areas, as discussed later in the section on neurochemistry.

Similar discrepancies occur in the literature using the conditioned place preference paradigm. Isolation in Lister hooded rats prevents the development of a conditioned place preference to either amphetamine (Wongwitdecha and Marsden, 1995) or morphine (Wongwitdecha and Marsden, 1996a). In Long Evans rats isolated immediately post-weaning (Schenk et al., 1983, Schenk et al., 1985) heroin conditioning was impaired when a biased training procedure which initially paired drug treatment with the non-preferred compartment was used (similar to that used by (Wongwitdecha and Marsden, 1996a)). Notably this impairment was only observed when rats were isolated immediately post-weaning and



not at three months of age, confirming the importance of ‘early-life’ intervention on this behaviour. Interestingly isolation rearing has been shown to alter opioid receptor binding. While seven days isolation increases prefrontal cortex [<sup>3</sup>H]diprenorphine binding measured by autoradiography (Vanderschuren et al., 1995), a marked decrease in whole brain naloxone binding occurred following 43 days isolation (Schenk et al., 1982) more comparable with the duration used in most conditioning paradigms above, which could account for the decreased behaviours reported. Consistent with this proposal isolation rearing has also been found to attenuate both the locomotor and conditioned taste aversion (Schenk et al., 1987a) response to morphine.

Ethanol preference was found to be increased following six weeks isolation of male C57BL/6J mice (Advani et al., 2006). Similarly voluntary ethanol consumption was increased following eight weeks isolation of Wistar rats (Wolffgramm, 1990, Hall et al., 1998c) and operant responding to obtain ethanol was increased following 90 days of isolation of male Long Evans rats (Deehan et al., 2007). Like most other phenotype changes discussed, this increase preference for ethanol does not occur when rats are isolated for 12 weeks at adulthood (Schenk et al., 1990) and this may explain why some early studies which did not state the age at the time of isolation housing failed to show this effect (Deatherage, 1972). Thus early-life isolation in the rodent at most produces a modest increase in the susceptibility to the reinforcing properties of a variety of drugs and a very inconsistent effect in paradigms involving motivation and reward, which is at odds the general picture of anhedonia usually experienced by schizophrenic or depressed patients.

*Social Interaction and Aggressive Behaviour*

Social withdrawal is one of the inherent negative symptoms of schizophrenia, and several researchers have postulated that it is also one of the first symptoms to manifest (Strous et al., 2004). An increase in social interaction occurs in male but not female Wistar rats following isolation rearing which is more evident when isolation begins on PND21 than PND30 (Ferdman et al., 2007). This increase in social interaction is particularly evident under conditions of bright light and may be largely due to an increase in aggressive behaviours (Wongwitdecha and Marsden, 1996c, Vale and Montgomery, 1997). A similar increase in aggressive behaviour is a prominent feature in mice reared in isolation (Figure 1.1) where it was one of the first phenotypical effects of isolation rearing to be identified (Valzelli, 1973). An increase in defensive burying of a shock probe occurs in both adult male and female Wistar rats following as little as two weeks of social isolation (PND 26 – 40), a behaviour which is highly correlated with individual aggressive behaviour and social dominance (Arakawa, 2007).

Furthermore, isolation of male, but not female, Wistar rats causes the development of one of a spectrum of three different behavioural responses to a mouse; indifferent, friendly (attempting to play with it) or muricide (breaking its neck). The proportion of rats displaying muricidal behaviour increases with the duration of isolation (Valzelli and Garattini, 1972). This muricidal activity is not reduced by treatment with benzodiazepines but is abolished by tricyclic antidepressants (Valzelli and Bernasconi, 1976) but no studies have attempted to reverse this behaviour with antipsychotics.

*Pain Sensitivity*

Social isolation appeared to increase the oral response to a mild tail pinch, a behaviour which has been linked to striatal dopaminergic activity (Sahakian and Robbins, 1977), without causing any difference in sensitivity in response to formalin injection. Conflicting results have also been found in the tail flick test of heat sensitivity, with either no difference (Hellemans et al., 2004) or a hypoalgesic effect being found (Gentsch et al., 1988) in isolates. Recent evidence suggests that schizophrenics show a reduced sensitivity to warmth perception and have a higher onset of thermal pain sensation (Jochum et al., 2006), so further translational studies in isolation reared rats are required. The limited data available thus suggest that social isolation does not cause any major change in nociception. This is important since a major change in pain sensitivity would severely confound interpretation of the impact of this early-life intervention on many of the paradigms utilised to assess reward-related or conditioned behavioural responses.

### 1.3.3 Cognitive Deficits in Isolation Reared Rats

#### *Conditioned Learning*

Early cognitive analysis in the isolation reared mouse showed a deficit in avoidance conditioning in the passive avoidance test (Valzelli, 1973) and a similar deficit in retention has been demonstrated after eight weeks isolation of Sprague-Dawley rats (Del Arco et al., 2004). Reductions in contextual fear conditioning have also been found in isolation reared Sprague-Dawley rats irrespective of gender (Weiss et al., 2004) consistent with impairment in associative learning.

#### *Novel Object Discrimination*

Isolation reared rats show impaired recognition memory when an inter-trial time delay is used in the novel object discrimination paradigm (Bianchi et al., 2006). This deficit is probably due to cognitive impairment rather than hyperactivity as the time spent exploring both objects in the first trial is unaltered by isolation. In agreement with this proposal and in an acute object contact task isolation reared rats explored the same number of novel objects as social reared animals (Einson and Morgan, 1977, File, 1978). The apparent reduction in object recognition could also reflect cognitive inflexibility preventing attention of the new stimulus. However, no selective impairment in novel object discrimination occurs after isolation when short inter-trial intervals (such as 1 min) are used (Lapiz et al., 2000). Although this is not surprising as rats will be very proficient at the task using this protocol it is consistent with isolation impairing cognitive rather than attention processes. Deficits in novel object recognition may have construct validity to the visual recognition defects seen in schizophrenia (McClure et al., 2007, Nestor et al., 2007), suggesting that this behavioural deficit may be a valuable tool with which to examine novel therapeutic treatments for cognitive impairments relevant to those seen in schizophrenia.

### *Water Maze*

Several groups have examined the impact of isolation on acquisition and retention of spatial learning in the water maze but results are inconsistent. The majority of groups find no alteration in acquisition using a fixed platform position in the task (Lapiz et al., 2003, Schrijver et al., 2004), although some groups have reported modest improvements (Wongwitdecha and Marsden, 1996b) or impairments in learning (Hellemans et al., 2004) accompanied by reduction in long-term potentiation in the CA1 area of rat hippocampus (Lu et al., 2003a). However, the apparent impairment in acquisition compared to group housed controls may be due to the well documented effect of environmental enrichment in the group housed cage rather than any effect of isolation per se (Schrijver et al., 2002). One group has shown that isolation rearing enhanced reversal performance in the water maze over social housed controls (Wongwitdecha and Marsden, 1996b). However, isolation rearing has been shown to improve retention (Lapiz et al., 2001) and impair reversal learning (Krech et al., 1962, Schrijver et al., 2004) consistent with the induction of behavioural rigidity or impaired learning of a new rule, as discussed in more detail for other tasks below. Taken together these results suggest that isolation rearing may primarily affect prefrontal cortico-striatal pathways involved in reversal learning rather than hippocampal pathways involved in spatial learning.

Schizophrenics show impaired performance in tests of visuo-spatial ability (Bozikas et al., 2006). Recently 22 schizophrenic patients were found to be impaired in both acquisition and retention of a hippocampal-dependent version of computerised virtual water maze task compared to healthy controls but normal in a visible platform and non-hippocampal dependent version of the task (Hanlon et al., 2006). Importantly this study confirms the translational relevance of performing rodent water maze tasks to examining human cognitive deficits.

*Rule Learning, Extra-dimensional Set Shifting & Behavioural Flexibility*

Isolation rearing appears to cause impairment in many rule learning tasks, including reversal learning (Krech et al., 1962). In 1973 Morgan reared rats in isolation with no environmental enrichment and tested them in food motivated learning tasks (Morgan, 1973). Isolates showed no impairment in acquisition of the task but were impaired when required to adopt a different strategy (Morgan, 1973) and continued to perform under conditions of satiation and extinction which was interpreted as a tendency to continue with a previously rewarded behaviour in the absence of an alternative (Morgan et al., 1977).

Jones et al (Jones et al., 1991) also demonstrated altered rule learning in isolates. In a conditional visual discrimination task isolation reared rats achieved the same level of accuracy as group housed controls. However, when distracting stimuli were introduced the accuracy of socially reared animals was reduced while that of isolates was unaffected. Isolates also continued to perform the task when satiated. In a second experiment to test serial reversal learning in a simple discrimination task, isolation reared rats did not improve their performance after several reversals (Jones et al., 1991). Similarly Sprague-Dawley rats isolated for 8 weeks then trained to retrieve a food pellet reward by a light cue in a rotating T-maze in dim light showed no impairment of acquisition (Li et al., 2007). However, isolates were impaired upon reversal of the visual discrimination stimulus-reward contingency (i.e. from light = food to no light = food) and this effect was attenuated by chronic clozapine treatment.

Attentional set-shifting measures behavioural flexibility, testing the ability to learn rules and then adapt to a change. The Wisconsin Card Sort Test measures this in humans, and schizophrenics have been shown to be particularly impaired in this paradigm (Elliott et al., 1995, Haut et al., 1996, Bozikas et al., 2006, Ruiz et al., 2007). A rodent version of this task has been developed (Birrell and Brown, 2000) in which food deprived rats dig in scented bowls for food rewards hidden in different digging

mediums. The rats must learn to follow one of the cues (either scent or digging medium) to find the reward. The rats then undergo a series of discriminations, in which they always follow the same type of cue (e.g. a new scent), known as intra-dimensional (ID) or affective shifts. However, in the extra-dimensional (ED) or attentional shift the rat must now learn to follow the other cue (e.g. digging medium), this transition can be selectively impaired by lesions of the medial prefrontal cortex in monkeys (Dias et al., 1996) and rats (Birrell and Brown, 2000). Schizophrenics find this ED shift particularly difficult (Elliott et al., 1995), as discussed in Section 1.1.1.

Recently one group has found reversal learning to be impaired in isolation reared rats (Table 1.3) using a bowl digging paradigm (Schrijver et al., 2004). Furthermore, although isolation reared rats were able to perform ID shifts and reversals, they were impaired in ED shifts when tested in a radial arm maze ED/ID paradigm, which involves switching between spatial and non-spatial cues (Schrijver and Wurbel, 2001).

While spatial learning and acquisition (which appear to be largely unaltered in isolates) is dependent on hippocampal-neocortical pathways, reversal learning depends primarily on prefrontal cortico-striatal pathways which would appear to be preferentially affected by social isolation from weaning. This would account for the observation that isolation rearing particularly disrupts spatial or discrimination learning in paradigms which require reversal learning (Jones et al., 1991, Schrijver et al., 2004, Li et al., 2007), an alteration in the modality of the response rule (Morgan, 1973, Schrijver and Wurbel, 2001), or tasks based on extinction (Jones et al., 1991) rather than impairing simple learning of a new rule.

### *Visuospatial Attention*

The rat 5-choice serial reaction time task (5-CSRTT) measures sustained visuospatial attention and aspects of executive function, including perseverance and impulsivity (Chudasama and Robbins, 2004). Rats placed into a chamber with five operant response holes in the wall and are trained by food reward to make appropriate operant noses pokes in response to brief visual stimuli. Once training has achieved stable criteria premature, perseverative and omission responses are recorded to probe attentional defects. Apart from being slower to collect food rewards and making more perseverative responses to an auditory distractor, isolation reared rats were not found to be impaired in accuracy, impulsivity or correct latency in the 5-CSRTT (Dalley et al., 2004). This is consistent with the previous suggestion that isolation-induced impairments in cognitive tasks is not solely the result of impaired attention.

### *Anxiety-like Behaviour*

Some groups have reported modest increases in anxiety-related behaviours on the elevated plus maze in isolation reared rats (Parker and Morinan, 1986, Wright et al., 1991b, Bickerdike et al., 1993, Hellemans et al., 2004) while others have found no effect using a similar protocol and strain of rats (Fone et al., 1996). Similarly following 90 days isolation of Long-Evans rats there was no effect on the behavioural profile in the black/white box (Hellemans et al., 2004) and although these workers reported an anxiogenic-like profile in the elevated plus maze test an error in data collection meant that some control groups were not reported in their study. Isolation reared rats also show reduced consumption of new food and a reduced latency to escape from a novel open top cage (Parker and Morinan, 1986), both of which are behaviours consistent with a small increase in anxiety in tasks involving mild aversion. Similarly male ddY (outbred) mice reared in isolation for six weeks show a small but significant increase in the number of steps climbed in the staircase test,



similar to the pattern of behaviour reported following anxiogenic drugs such as benzodiazepine inverse agonists (Ago et al., 2007). Interestingly both mice and rats reared in social isolation for two months showed a reduction in sodium pentobarbital-induced sleep time compared to group reared controls (Watanabe et al., 1992). However, this was attributed to an increase in hepatic metabolism of pentobarbital in isolation reared rats as the urinary concentration of the major metabolite of pentobarbital was increased in isolation reared rats. This potential increase in hepatic metabolism by isolation reared rats has not been investigated further but could confound any isolation rearing studies involving compounds which are metabolised by the same enzymes.

Loss of social contact and behavioural withdrawal are associated with the aetiology of depression in man. Therefore some groups have examined whether rearing rodents in isolation may result in the development of behavioural despair, such as the immobility adopted by rats placed in a small chamber containing water, known as the Porsolt Forced swim test (Porsolt et al., 1977, Porsolt et al., 1978). In mice although short periods of social isolation (for between 24h to 5 days) near the age of weaning (Hilakivi et al., 1989) may reduce immobility time in the forced swim test (although see (Yates et al., 1991)) longer periods of isolation for 10 to 20 days saw this behaviour return to that seen in group housed controls. Similarly in a modified version of the Porsolt forced swim test, twelve weeks isolation rearing from PND 21 failed to alter either immobility or escape behaviour in either Wistar or Fawn-hooded rats strains (Hall et al., 1998a, Hall et al., 2001). This suggests that prolonged social isolation does not affect the ability of the rodent to cope with a short period of severe stress. Furthermore, taken together, these data do not support the early view that isolation reared rats may have a 'depressed-like' phenotype that could be useful to investigate the neurobiology of depressive disorders.

### 1.3.4 Neurochemical Effects of Isolation Rearing

The neurochemical changes induced by isolation rearing are summarised in Table 1.4, found at the end of this chapter.

#### *Neurotransmitter Alterations*

##### **Dopamine**

Overall, post-mortem analysis has found few consistent alterations in tissue levels of dopamine, DOPAC (Jones et al., 1992, Leng et al., 2004), 5-HT or 5-HIAA or acetylcholine (Leng et al., 2004) in the NAcc or striatum of isolation compared with group housed rats, although basal prefrontal cortex dopamine levels may be elevated (Jones et al., 1992). While isolation rearing does cause a variety of changes to brain neurochemistry, evidence is often contradictory as will be discussed in detail below (Figure 1.1 and Table 1.4). Some groups have reported that isolation rearing increases basal DA turnover in amygdala (Heidbreder et al., 2000) and NAcc (Hall et al., 1998d). Isolation reared rats have an enhanced locomotor response to amphetamine and show a potentiation of amphetamine-induced DA release in the NAcc (Hall et al., 1998d), suggesting a possible sensitisation of the mesoaccumbens DA projection (Jones et al., 1990). Isolation reared rats are impaired in the development of schedule-induced polydipsia, which has been shown to be dependent on optimal mesolimbic dopamine activity, specifically in the NAcc (Jones et al., 1989). Isolation rearing also decreases dopamine turnover in the medial PFC (Heidbreder et al., 2000). In a conditioned emotional response (CER) paradigm foot shock increased DA levels in the shell of the NAcc to a greater magnitude and for a more prolonged period in isolation reared rats (Fulford and Marsden, 1998b) consistent with enhanced dopaminergic activity in the NAcc contributing to components of the isolation behavioural syndrome.

Hall et al showed that the inhibitory effect of the DA D<sub>2</sub> receptor on DA D<sub>1</sub> receptor function in the striatum was also attenuated in isolates,

suggesting functional down-regulation of DA D<sub>2</sub> receptors (Hall et al., 1998d). However, other groups have found no change in either the density or affinity of either DA D<sub>1</sub> or D<sub>2</sub> receptors using autoradiography (and the radioligands [<sup>3</sup>H]SCH 23390 and [<sup>3</sup>H]spiroperidol, respectively) in the mesolimbic or nigrostriatal systems (Bardo and Hammer, 1991) or striatum (Del Arco et al., 2004) following thirty days isolation of Sprague-Dawley rats. In contrast, some groups (Djouma et al., 2006) have found a selective elevation in DA D<sub>2</sub> but not D<sub>1</sub> receptor binding measured using autoradiography with [<sup>125</sup>I]-NCQ 298 in the NAcc and amygdala of isolation reared Fawn hooded rats and elevation in striatal [<sup>3</sup>H]spiroperidone DA D<sub>2</sub> receptor binding in Wistar rats. Part of this discrepancy may be due to the inability of D<sub>2</sub> ligands to differentiate between presynaptic autoreceptors and postsynaptic D<sub>2</sub> receptors which may be differentially regulated by isolation. In addition there may be an increase in the ratio of high:low affinity states of the DA D<sub>2</sub> receptors in isolation rearing (which maybe a common feature of psychosis (Seeman et al., 2006)) but this has not yet been fully investigated.

The level of immunoreactivity of the presynaptic protein CDCrel-1 was reduced in the striatum and increased in the hippocampus of isolated rats (Barr et al., 2004). This protein modulates DA neurotransmission and is normally co-localised with syntaxin, but this relationship appears to be lost in isolation reared rats (Barr et al., 2004). The overall picture from these studies suggests an enhanced dopaminergic activity in the NAcc and ventral striatum but reduced dopamine function in the prefrontal cortex of isolation reared rats, but the exact receptor alterations are not yet clear (Figure 1.1).

### **5-Hydroxytryptamine**

A similar confusion is found in studies of 5-hydroxytryptamine (5-HT) function which appears to be affected in a brain region specific manner (Table 1.4). Isolation rearing has been shown to decrease basal 5-HT turnover in NAcc (Heidbreder et al., 2000), but not in the prefrontal cortex or caudate putamen (Jones et al., 1992). Furthermore, exposure to inescapable footshock induced an increase in 5-HT release from the medial NAcc in isolation but not social reared rats measured by microdialysis (Fulford and Marsden, 1998a, Fulford and Marsden, 2007). Repeated exposure to the contextual stimulus also caused 5-HT release in the isolated and not in social housed rats. Thus isolation rearing increases presynaptic serotonergic function in the NAcc which may represent an adaptation to preceding early-life stress or be secondary to changes in the function of other neurotransmitters such as dopamine. In a recent follow-up to earlier experiments this group (Fulford and Marsden, 2007) has also shown that on re-exposure to the context where the shock was received the significant elevation in n. accumbens 5-HT and DA release seen only in isolation and not group reared rats, are both blocked by depletion of DA with  $\alpha$ -methyl-p-tyrosine suggesting that the enhanced presynaptic dopaminergic input may contribute to the augmented serotonergic neuronal function in this brain region.

In contrast, Lister hooded rats reared in social isolation from weaning for 6 weeks show no change in hippocampal post-synaptic 5-HT<sub>1A</sub> receptor function *in vivo* using electrophysiology but a decrease in 5-HT<sub>1B</sub> mediated inhibition of endogenous 5-HT release, consistent with impaired presynaptic 5-HT function in this area (Muchimapura et al., 2002, Muchimapura et al., 2003). Isolation rearing significantly increased 5-HT<sub>2A</sub> receptor binding in the prelimbic, motor and cingulate cortex, whereas 5-HT<sub>1A</sub> receptor binding was significantly reduced in the prelimbic cortex and increased in the motor cortex and hippocampus (Preece et al., 2004). Consistent with this suggestion 30 days isolation

rearing in Lister hooded rats caused an elevation in wet-dog shakes and back muscle contractions that were elicited by systemic administration of a 5-HT<sub>2A</sub> receptor agonist and enhanced 5-HT<sub>1A</sub> receptor mediated flat body posture and reciprocal forepaw treading (Wright et al., 1991a). A possible cause of this apparent post-synaptic 5-HT receptor supersensitivity could be reduced 5-HT release. Indeed, Bickerdike (Bickerdike et al., 1993) found evidence of attenuated 5-HT release in cortical and hippocampal microdialysates evoked by potassium or elevated plus maze exposure respectively in isolation compared with social housed Lister hooded rats. Similarly, although basal 5-HT release in the frontal cortex measured using *in vivo* microdialysis in the conscious Lister hooded rat was unaltered, the normal elevation in 5-HT release induced by systemic amphetamine was attenuated in isolation compared with group reared rats (Dalley et al., 2002). Furthermore, 5-HT<sub>1A</sub> function (measured by [<sup>35</sup>S]GTPγS binding) was increased in the dorsal raphe nucleus of isolation reared CB57BL/6J mice (Advani et al., 2006), consistent with heightened presynaptic autoreceptor inhibition of serotonergic neuronal function. Isolation rearing caused an increased anxiogenic response to the 5-HT agonist mCPP on the elevated plus maze, compared to socially reared animals, which has been attributed to increased 5-HT<sub>2C</sub> receptor responsiveness, possibly in the hippocampus (Fone et al., 1996). Overall the effect of isolation rearing on 5-HT function in the NAcc remains unclear, but 5-HT release in the PFC appears to be reduced. However much further work is required to elucidate the exact mechanisms behind the changes.

Although there have been fewer studies on noradrenergic function in isolation-reared rats, early studies found an increase in noradrenaline turnover in the hippocampus, cerebellum and cortex of Wistar rats (Miachon et al., 1993). Consistent with this observation isolation reared rats also appear to have enhanced presynaptic α<sub>2</sub>-adrenoceptor autoreceptor function in the hippocampus (Fulford and Marsden, 1997b, Fulford and Marsden, 1997a).

**Glutamate**

Very few studies have examined the effect of isolation on glutamatergic neurotransmission in the brain which is clearly of great relevance to clarify the translational relevance of the neurobiological basis to that seen in schizophrenia. However, studies have found a reduction in NMDAR1A mRNA expression by in situ hybridization in Fawn hooded rats compared to Wistars, which is potentiated by isolation rearing in some areas of the striatum and prefrontal cortex (Hall et al., 2002), and could reflect the observed enhanced dopamine release in the NAcc. Indeed sub-chronic PCP administration appears to enhance the isolation induced locomotor hyperactivity (Lapiz et al., 2003) supporting the proposal that a deficit in glutamatergic innervation of the NAcc may contribute to the enhanced presynaptic dopamine release seen in this brain area.

One recent study using microarray analysis reported an increase in mGluR<sub>6</sub> and AMPA3 ionotropic glutamate receptor subunits following 26 days isolation of Sprague-Dawley rats (Levine et al., 2007). However these findings require replication as only six rats were used in each rearing condition. Sprague Dawley rats reared in isolation from PND21 on wire grids without handling resulted in a specific decrease in mGluR<sub>1</sub> and mGluR<sub>5</sub> in the dorsal PFC but not in the ventral PFC or striatum (Melendez et al., 2004). In addition, while there was no change in basal glutamate release in the PFC of isolates, measured by microdialysis, there was a blunted elevation in release associated with mGluR<sub>1</sub> agonist (DHPG) or mGluR<sub>2</sub> antagonist (LY341495) administration (Melendez et al., 2004). This reduction in PFC glutamate was accompanied by impaired performance of isolation reared rats in the T-maze delayed alternation task, showing a deficit in working memory performance. Although this evidence is at a preliminary stage it would appear that isolation rearing is associated with glutamatergic hypofunction in the PFC, which could lead to reduced PFC activity analogous to the hypofrontality observed in schizophrenia.

*Other Neurochemical and Histological Changes*

Since synaptic connectivity and the neurochemical phenotype of neurones in the rodent brain is incomplete until well after weaning it is not surprising that early post-natal social deprivation alters brain development. For instance, most of the monoamine neurotransmitters and their associated receptor complement continue to change, only reaching the adult composition between PND 30-50 of age in the rat.

The hippocampus and PFC show a number of alterations indicative of change in plasticity as a consequence of social isolation at weaning (Figure 1.1). Comery et al found reduced spine density and dendritic branching measured by Golgi-Cox staining on medium sized spiny neurones in the corpus striatum and Silva-Gomez reported a similar reduction in pyramidal neurones of the PFC and hippocampus (Comery et al., 1995, Comery et al., 1996, Silva-Gomez et al., 2003). These changes are indicative of persistent morphological alterations following isolation rearing of Long Evans and Sprague-Dawley rats, respectively (Comery et al., 1995, Comery et al., 1996, Silva-Gomez et al., 2003). Just like the isolation behavioural syndrome these reductions in dendritic branching in the prefrontal cortex (Pascual et al., 2007) are not reversed by 30 days of resocialisation in Sprague Dawley rats (Pascual et al., 2006), but appear to partially recover following chronic administration with deprenyl (Pascual and Zamora-Leon, 2007).

Consistent with this rearing-induced loss of neuropil, Lister hooded rats reared in isolation for 8 weeks show a 7% decrease in medial PFC volume without any change in neuronal number measured by stereology, accompanied by deficits in PPI (Day-Wilson et al., 2006). Our group has also recently reported a small but significant decrease in prefrontal cortical volume measure by MRI in isolation reared Lister hooded rats accompanied by the characteristic isolation behavioural syndrome (Porkess et al., 2007). Furthermore, a reduction in cortical thickness has also been found in isolation reared rats (Hellemans et al., 2004).

Numerous markers of synaptic function appear to be altered by isolation rearing reminiscent of alteration in synaptic plasticity (Table 1.4). Synaptophysin, a synapse specific protein associated with presynaptic release of neurotransmitters, was reduced in the molecular layer of the dentate gyrus after eight weeks of isolation in Lister hooded rats (Varty et al., 1999b). Although this technique does not permit analysis of the neurotransmitter phenotype of affected neurones, it suggests that isolation also reduces the gross synaptic density in this hippocampal subfield, which receives a dense innervation from the entorhinal cortex. Consistent with this suggestion, isolation rearing appears to induce dynamic instability of microtubules in the hippocampus, as suggested by a decrease in hippocampal tyrosinated  $\alpha$ -tubulin (Tyr-Tub) and a parallel increase of detyrosinated  $\alpha$ -tubulin (Glu-Tub) together with a decrease in the Tyr/Glu-Tub ratio without any gross change in total  $\alpha$ -tubulin content (Bianchi et al., 2006). Furthermore, this was accompanied by a decrease in the neuronal-specific dendritic marker, microtubule associated protein-2 (Bianchi et al., 2006), suggesting impaired dendritic development in agreement with previous studies which could account for changes in cognitive flexibility shown in isolation reared rats.

Following 3 months of isolation of Wistar rats Miachon et al (Miachon et al., 1990) reported a 70 % increase in [ $^3$ H]flunitrazepam binding affinity in hippocampal tissue which they speculated might be due to alteration in endogenous levels of allosteric modulators of the GABA receptor and similar isolation-induced changes in hippocampal benzodiazepine binding were reported by Petkov and Yanev (Petkov and Yanev, 1982). More recently eleven weeks of isolation rearing in the female Sprague Dawley rat has been found to produce reduced numbers of the parvalbumin and calbindin (but not calretinin) positive subsets of GABAergic interneurons within the hippocampus (Harte et al., 2007b) consistent with a developmental alteration in local hippocampal circuits. These hippocampal changes in isolation reared rats agree well with substantial indirect



evidence of functional impairment of inhibitory GABAergic interneurons in the hippocampus seen in post-mortem schizophrenic brain tissue.

Eight weeks isolation of two month old Sprague Dawley rats also results in a selective elevation of hippocampal BDNF measured by ELISA with no concomitant alteration in the PFC or striatum (Scaccianoce et al., 2006). In contrast, in Fawn hooded rats, BDNF mRNA is decreased in the dentate gyrus but increased in the retrosplenial cortex (Djouma et al., 2006). Thus changes in hippocampal neuronal morphology may result from accompanying changes in neurotrophic factors induced by the isolation procedure. Consistent with these findings, neurogenesis appears to be significantly reduced in isolation reared rats, as was LTP in CA1 region of the hippocampus (Lu et al., 2003a), and as predicted these rats were impaired at spatial learning in the water maze, even though this conflicts with results from other groups (Wongwitdecha and Marsden, 1996b).

The level of N-acetyl aspartic acid (NAA), a marker of neuronal integrity, is reduced in the temporal cortex of isolated rats, indicating possible neuronal loss or dysfunction, while no similar change has been reported in the hippocampus, striatum or frontal cortex (Harte et al., 2004). This closely mimics the change seen in schizophrenia where post mortem NAA reductions occur in the temporal but not frontal cortex.

Finally, the electrophysiological properties of some neurones are altered in isolation reared rats, with pyramidal neurones showing reduced action potential height and increased action potential threshold, with no changes in resting membrane potential (Greene et al., 2001). Altered long term potentiation has also been seen in the CA1 to subiculum pathway of isolation reared rats (Roberts and Greene, 2003).

Basal corticosterone levels are not altered by social isolation, if the animals are housed in standard cages (Schrijver et al., 2002) but are only elevated by more aversive isolation in wire floor cages (Heidbreder et al.,

2000). Neither is there any effect of isolation on hippocampal glucocorticoid or mineralocorticoid receptor mRNA or adrenal gland weight but levels of ACTH may be elevated (Miachon et al., 1993, Weiss et al., 2004). After a 10 min open-field test isolation reared rats have lower plasma corticosterone than socially housed rats (Gentsch et al., 1981a). In contrast, the ACTH and corticosterone response to an acoustic startle stress was enhanced in both male and female Sprague-Dawley rats, consistent with a hyper-responsivity to stress accompanied by a mild hyperfunction of the HPA-axis, which again may depend on strain, duration of isolation and severity of the stress experienced.

### **1.3.5 Social Isolation as a Model of Core Symptoms of Developmental Neuropsychological Disorders**

As extensively reviewed elsewhere, no single animal model can possibly replicate all the myriad of symptoms associated with schizophrenia, which in itself is a heterogeneous, polygenetic, disorder influenced by early-life adverse environment (Lipska and Weinberger, 2000, Gainetdinov et al., 2001). Furthermore, no single behavioural paradigm is uniquely pertinent to schizophrenia. Therefore it is clearly inappropriate to describe isolation reared rats as a model for schizophrenia. Nonetheless, isolation rearing of rats from weaning produces reproducible, long-term changes in behaviour including; locomotor hyperactivity, impaired sensorimotor gating, social withdrawal and cognitive inflexibility which cover the three domains affected in schizophrenia and is a desired feature of a suitable rodent paradigm to investigate the neurobiology of the disorder (Powell and Miyakawa, 2006). In addition the isolation-induced behavioural syndrome is accompanied by reductions in prefrontal cortex volume and hippocampal synaptic plasticity, hyperfunction of mesolimbic dopaminergic systems and hypofunction of mesocortical dopamine which also resemble abnormalities seen in schizophrenia (Figure 1.1). This

favours the characterisation of a battery of behavioural, neuroanatomical and neurochemical measures with robust translational relevance to core defects in schizophrenia (positive, negative and cognitive impairments) in rats reared in social isolation to investigate the neurodevelopment aetiology of this disorder to identify longitudinal biomarkers of dysfunction and serve as a predictive screen for novel compounds with potential antipsychotic efficacy.

Figure 1.1: Behavioural and neurochemical consequences of isolation rearing

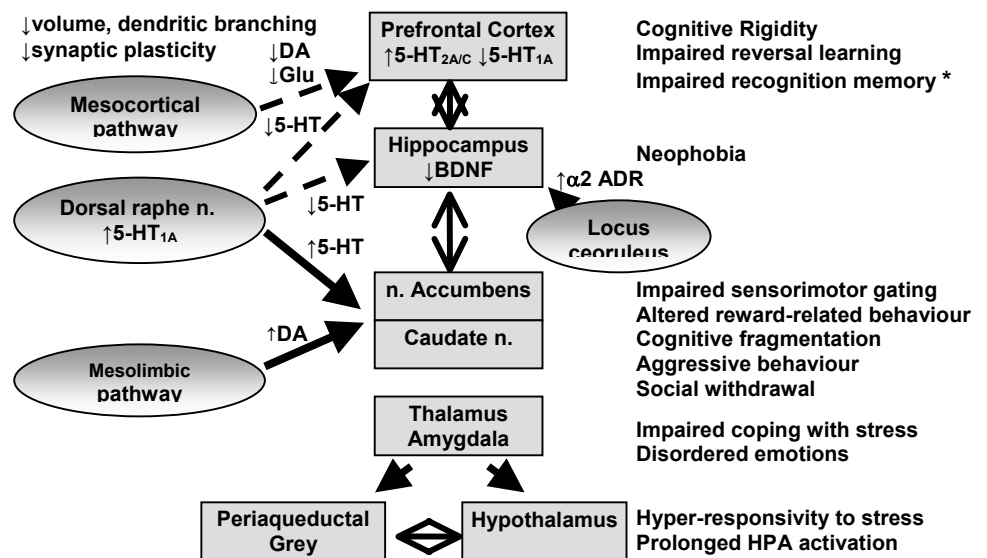


Figure 1. Schematic overview of the most consistent behavioural and neurochemical observations reported following at least six weeks social isolation from weaning of rat pups compared with control responses in group housed conspecifics. Single headed lines reflect a decrease (dotted lines) or increase (solid bold) in the neurotransmitter function identified. DA = dopamine, 5-HT = 5-hydroxytryptamine, Glu = glutamate, BDNF = brain derived neurotrophic factor, α<sub>2</sub> ADR = α<sub>2</sub> adrenoreceptor, HPA = hypothalamic pituitary adrenal axis and 5-HT receptors are named according to the IUPHAR nomenclature. \* Impairment in recognition memory may involve deficits in the entorhinal and perirhinal cortex and hippocampus not shown on this figure.

## 1.4 Aims of this Project

The first aim of this project was to further characterise the behavioural changes seen in isolation reared rats, with particular emphasis on cognitive impairments. This initially involved establishing the isolation rearing protocol within our institution. As it is preferable to have a battery of tests measuring different aspects of behaviour a set of three core behaviours were assessed in all cohorts of isolation reared rats: locomotor activity, novel object recognition and prepulse inhibition of acoustic startle. The project then moved forward to examine the effects of isolation rearing on three further cognitive assays: attentional set shifting, reversal learning in the water maze and passive avoidance.

The reversibility of isolation rearing induced cognitive changes was assessed using two putative cognition enhancing compounds that act via different pharmacological mechanisms.

Finally, after reports of heavy cannabis use increasing the risk of schizophrenia developing in some individuals, the effect of an active component of cannabis,  $\Delta^9$ -tetrahydrocannabinol, was investigated in isolation reared rats.

Table 1.3: Summary of the long-term behavioural phenotype changes associated with isolation rearing of rat pups from weaning.

<b>Behaviour</b>	<b>Consequence of isolation rearing</b>
Body weight	Males may be slightly heavier (Fiala et al., 1977) but mostly no change No change in food consumption (Hellemans et al., 2004) Increased food hoarding behaviour (Heidbreder et al., 2000)
Exploratory behaviour	Hyper-reactivity to a novel environment (Sahakian et al., 1977, Gentsch et al., 1982a, Gentsch et al., 1988, Varty et al., 2000) Decreased in mice (Valzelli et al., 1974)
Prepulse inhibition of acoustic startle	Impairment in PPI without change in control startle amplitude, (Bakshi et al., 1998, Varty et al., 2000, Cilia et al., 2001, Barr et al., 2006, Day-Wilson et al., 2006)
Latent inhibition	No effects (Wilkinson et al., 1994, Weiss et al., 2001b)
Anhedonia	Increased sucrose consumption (increased motivation) (Hall et al., 1997c) Hyperphagia (Fiala et al., 1977) Increased reward sensitivity (Jones et al., 1990) Increased anticipatory behaviour (Morgan and Einon, 1975) Increased self-administration with low but impaired acquisition with high doses of cocaine (Howes et al., 2000) Increased ethanol preference in mice (Advani et al., 2006) and Long Evans rats (Deehan et al., 2007)
Social interaction	Increased interactions and aggression (Wongwitdecha and Marsden, 1996c) Progressive increase in muricidal behaviour in isolation reared rats (Valzelli and Garattini, 1972) Increase in defensive burying of a shock probe (Arakawa, 2007) Mice- increased aggression (Valzelli, 1973)
Pain	Hypoalgesia (Gentsch et al., 1988) No changes (Hellemans et al., 2004) Increased oral response to tail pinch (Sahakian and Robbins, 1977)

Passive avoidance	Mice- deficits (Valzelli, 1973) Rats- deficits (Del Arco et al., 2004) Fear conditioning reduced in male and female isolates (Weiss et al., 2004)
Visual learning & memory	Impaired novel object discrimination (Bianchi et al., 2006)
Attention/vigilence	No impairments seen in five choice serial reaction time (Dalley et al., 2002)
Spatial learning	Water maze- improvements (Wongwitdecha and Marsden, 1996b) No change (Schrijver et al., 2004) Impairments (Lu et al., 2003a, Hellemans et al., 2004)
Reasoning and problem solving (attentional set shifting)	Extradimensional shift impairment in spatial/non-spatial ID-ED task (Schrijver and Wurbel, 2001) Reversal impairment, but no ED impairment in bowl-digging (Schrijver et al., 2004) Impairments in reversal learning (Krech et al., 1962) Improved water maze reversal performance (Wongwitdecha and Marsden, 1996b)
Conditioned place preference	No preference for amphetamine treated area (Wongwitdecha and Marsden, 1995)
Anxiety	Anxiogenic on elevated plus-maze (Parker and Morinan, 1986, Wright et al., 1991b, Weiss et al., 2004) Hypo-neophagia (Parker and Morinan, 1986) Reduced cage escape latency (Parker and Morinan, 1986)
Depression	No change in immobility or struggling time in the Porsolt forced swim test in mice (Hilakivi et al., 1989) or rats (Hall et al., 1998a, Hall et al., 2001) Desipramine more effective in isolates (Wongwitdecha et al., 2006)

Table 1.4: Summary of the long-term neurochemical changes associated with rearing rat pups in social isolation

Neurotransmitter	Consequence of isolation rearing
Dopamine	<p>Basal DA: in NAcc unchanged (Jones et al., 1992, Fulford and Marsden, 1998b, Hall et al., 1998d, Howes et al., 2000) or increased (Heidbreder et al., 2000) in isolates which was reversed by handling (Hall et al., 1998d)</p> <p>Foot shock induced DA release in NAcc increased and prolonged (Fulford and Marsden, 1998b)</p> <p>Increased amphetamine-induced DA release in NAcc (Jones et al., 1992).</p> <p>K<sup>+</sup>-induced DA release decreased in NAcc (Hall et al., 1998d).</p> <p>Cocaine-induced DA efflux in NAcc potentiated by isolation (Howes et al., 2000).</p> <p>Number, affinity and efficacy of D<sub>2</sub>Rs in dorsal/ventral striatum unaltered by isolation (Del Arco et al., 2004).</p> <p>Basal DA unchanged in PFC (Dalley et al., 2002), but turnover decreased in FC (Heidbreder et al., 2000)</p>
5-HT	<p>No effect on basal extracellular 5-HT in PFC (Dalley et al., 2002) or NAcc (Howes et al., 2000).</p> <p>Amphetamine-, KCl- and novel environment-induced 5-HT release in PFC attenuated (Dalley et al., 2002).</p> <p>Decreased basal 5-HT turnover in NAcc (Heidbreder et al., 2000).</p> <p>Footshock increased 5-HT release in NAcc (Fulford and Marsden, 1998a).</p> <p>Stress reduced 5-HT release in hippocampus (Muchimapura et al., 2002).</p> <p>Mice: increased 5-HT<sub>1A</sub> binding. (Schiller et al., 2006).</p> <p>Altered presynaptic 5-HT<sub>1B</sub> not postsynaptic hippocampal 5-HT<sub>1A</sub> receptors (Muchimapura et al., 2003).</p> <p>Increased 5-HT<sub>2A</sub> binding in prelimbic, motor and cingulate cortices. Reduced 5-HT<sub>1A</sub> binding in prelimbic cortex and increased in motor &amp; somatosensory cortices and dentate gyrus and CA of hippocampus (Preece et al., 2004) (Wright et al., 1991a).</p> <p>Increased 5-HT fibre density in dorsal caudate putamen, NAcc and amygdala in gerbils (Lehmann et al., 2003).</p> <p>Circadian rhythms altered - 5-HT peak later, tryptophan and 5-HIAA earlier (Greco et al., 1989).</p>

Noradrenaline	No effect on basal or K <sup>+</sup> -induced NA release in hippocampus or hypothalamus (in vitro slices). Altered sensitivity of presynaptic $\alpha$ 2 autoreceptors in hippocampus (Fulford and Marsden, 1997a) (Fulford et al., 1994). Unaltered basal but enhanced K <sup>+</sup> -stimulated dorsal hippocampal NA release. (Fulford and Marsden, 1997b). Increased hypothalamic synaptosomal NA uptake, decreased pons-medulla NA sensitive cAMP production. Decreased $\beta$ -adrenoceptors and increased $\alpha$ -adrenoceptors is in pons-medulla (Kraeuchi et al., 1981).
HPA & Corticosterone	No change in basal plasma corticosterone (Scaccianoce et al., 2006) and ACTH (Schrijver et al., 2002). Corticosterone increased in isolates housed on grid floors only (Heidbreder et al., 2000). Decreased plasma ACTH and impaired negative feedback of HPA axis (Serra et al., 2005). Increased basal ACTH and enhanced stress-induced release of ACTH and corticosterone in males only (Weiss et al., 2004).
GABA	Mice: reduced allopregnanolone (+modulator of GABA) and increased susceptibility to picrotoxin (GABA A antagonist) seizures (Matsumoto et al., 2003). Rats: reduced number of parvalbumin and calbindin positive hippocampal GABAergic interneurons (Harte et al., 2007b).
Glutamate	NMDAR1A mRNA unaltered in striatum, hippocampus and FC (in fawn-hooded and Wistars) (Hall et al., 2002), Decreased mGluR1 and mGluR5 protein in the dorsal PFC (Melendez et al., 2004).
Opiate receptors	No morphine induced place-preference (Wongwitdecha and Marsden, 1996a).
Histamine	Isolated H1 KO mice fail to exhibit PPI impairments (Dai et al., 2005).
Electrophysiology	Reduced LTP in the CA1 - subiculum pathway (Roberts and Greene, 2003). Pyramidal neurones in PFC show abnormal firing and short hyperpolarisation (Peters and O'Donnell, 2005).
Morphology	mPFC volume decreased, no change in neurone number (Day-Wilson et al., 2006). Decreased dendritic length of pyramidal cells from CA1. Density of dendritic spines decreased in pyramidal cells from mPFC and hippocampus (Silva-Gomez et al., 2003) (Comery et al., 1995, Comery et al., 1996). Hippocampal cytoskeletal alterations suggestive of microtubule stabilization. Decreased MAP2 expression (Bianchi et al., 2006).



	Decreased neuronal dendritic arborisation and increased VIP neurons (Pascual et al., 2006).
N-acetyl aspartate	Decreased NAA in temporal cortex, but no changes in hippocampus, FC or striatum (Harte et al., 2004).
CDCrel-1	Decreased in striatum and increased in hippocampus. (Barr et al., 2004).
Synaptophysin	No changes seen (Barr et al., 2004). Decreased in dentate gyrus (Varty et al., 1999b).
BDNF	Decreased hippocampal BDNF (rats adult when isolated) (Scaccianoce et al., 2006).

## **2 Validation of Isolation Rearing Protocol & Effect of Gender**

## **2.1 Aims**

The work in this chapter has two aims:

Firstly to verify that the isolation rearing protocol used in this programme of study produced behavioural changes similar to both those reported in other laboratories and to previous work carried out at Nottingham University.

The second aim was to determine whether gender affected the development of behavioural changes in isolation reared rats.

## **2.2 Introduction**

### **2.2.1 Isolation Protocol**

Several groups have established isolation rearing protocols, as discussed in Chapter 1. These methods use different strains and gender of rat, as well as varying length of the isolation period and the age which isolation commences. As Lister Hooded rats have been used in many isolation rearing studies (see Chapter 1) and have been tested in several cognitive tasks (Andrews, 1996, Ennaceur et al., 2005) this strain will be used in all the studies described. It was also desirable to use a pigmented strain of rat (such as the Lister hooded) as albino strains have been found to have impaired visual acuity (Prusky et al., 2002). Most groups initiate the isolation period on the day of weaning. In the Nottingham University Biomedical Services Unit (BMSU) weaning routinely occurs on post-natal day 24 and therefore this is when the rats were isolated in these studies. However rats that were purchased from Charles River UK (CRUK) could not be guaranteed to be this age and were typically delivered on post-natal day 23-25. In this project both rats from Nottingham University and CRUK were used. A comparison of the effect of isolation rearing animals from the two sources was carried out and no major differences were found in the behavioural studies (Bianchi et al., 2006).

Preliminary studies in our laboratory suggested that hyperactivity in a novel environment and NOD impairments can be detected after 4 weeks of isolation from weaning, but PPI deficits may take longer to develop. This agrees with previously published studies which have also found PPI deficits require a greater duration of isolation housing to develop than increased activity in a novel environment (Bakshi and Geyer, 1999). Therefore our studies generally assessed activity in a novel environment first, typically after 4 or 5 weeks of isolation and did not measure PPI until after 6 weeks of isolation.

### **2.2.2 Behavioural Assessment of Isolation Reared Rats**

Each batch of isolation reared rats used during this project underwent several behavioural tests, with LMA, NOD and PPI being tested in every single set of animals. The previously published work on the effect of isolation rearing in these behavioural tasks has already been discussed in detail in Chapter 1. These tests were chosen to be the start point of behavioural characterisation of the isolation reared rats because they cover the main aspects of the “isolation syndrome” while being tasks which are relatively simple to monitor and which require little handling of the animals.

Hyperactivity in a novel environment is widely reported to be the most robust of the isolation rearing induced behavioural changes as well as one of the earliest to appear after the start of isolation housing (Bakshi and Geyer, 1999). LMA is easily measured in a single one hour test. In addition LMA can double as habituation to the NOD paradigm, if it is conducted the next day, as they are carried out in the same arena.

As one of the main aims of the project was to investigate cognitive changes in the isolation reared rat it was important to ascertain if any cognitive impairment had developed in the animals. Again NOD is a simple, one day task which can rapidly reveal an impairment in visual recognition memory (King et al., 2004, Bianchi et al., 2006).

Finally reduced PPI is indicative of impaired sensorimotor gating which is seen in schizophrenia (Braff et al., 1992). Furthermore the isolation induced PPI impairment can be reversed by treatment with antipsychotic drugs (Varty and Higgins, 1995, Bakshi et al., 1998, Cilia et al., 2001). Together these three tests will provide a screen to ensure that isolation rearing produced the expected behavioural effects in the animals which can then be utilised to address other experimental issues by further appropriate tests.

### **2.2.3 Gender Differences in Schizophrenia**

Gender differences in schizophrenia have been investigated for many years (Dohrenwend and Dohrenwend, 1974), see (Halbreich and Kahn, 2003) for a review. The age of onset of the disease is earlier in men (Castle et al., 1993), usually early 20s, compared with mid to late 20s in women (Hafner et al., 1993, Szymanski et al., 1995), although no differences in ages of onset are found in familial schizophrenia (DeLisi et al., 1994, Konnecke et al., 2000). There is also a second peak in new cases in women around the mid 40s which is not seen in men (Hafner et al., 1993). The incidence of schizophrenia has been found to be greater in men (Aleman et al., 2003, McGrath et al., 2004), although this had been disputed, with some evidence towards a similar overall incidence, with men being affected more earlier in life and women later (Castle et al., 1993). Certainly it has been found that in general women are less severely affected by the disease, requiring fewer and shorter periods of hospitalization, more rapid remissions and better response to antipsychotic drugs (Angermeyer et al., 1989, Szymanski et al., 1995, Salokangas, 2004). However, there is evidence that women undergo deterioration in symptoms later in life, which is not seen in men (Hafner et al., 1993).

Each laboratory which carries out research into isolation rearing uses different protocols, including whether they use male or female rats. The gender differences in schizophrenia have led to the suggestion of a neuroprotective role for oestrogen (Garcia-Segura et al., 2001). If this is the case, it is possible

that female rats may be differentially susceptible to developing isolation-induced behavioural alterations.

#### **2.2.4 Corticosterone Levels in Isolation Reared Rats**

Isolation rearing is an environmental manipulation which could be considered to be a form of chronic mild stress on the animals. The stress response is characterised by increased levels of circulating glucocorticoids, including corticosterone in rodents or cortisol in humans, which may be used as an index of hypothalamo-pituitary-adrenal (HPA) axis activity. As discussed in Chapter 1, the isolation rearing of rats can lead to elevated levels of corticosterone in isolates, if they are raised on wire grids (Heidbreder et al., 2000). This could be due to either the additional stressful effects of standing on the wire grid, compared to isolation housing alone, and/or due to the reduction in handling that grid housing allows. Isolation reared rats which have been housed on sawdust in plastic bottomed cages and received weekly changes of bedding material do not usually demonstrate elevated corticosterone (Schrijver et al., 2002). To confirm this the plasma corticosterone level will be measured in a batch of isolates to further verify our isolation protocol yields similar results to those seen in other laboratories.

## 2.3 Methods

All procedures involving animals were carried out in The University of Nottingham BMSU. Any further details specific to a particular experiment will be covered in the methods section of the relevant chapter. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, under Home Office project licence 40/2715 and personal licence 40/6875 (M V Porkess) and following approval from the local ethics committee. As these experiments involve behavioural testing there is no alternative to using live animals. All animals were housed in University of Nottingham BMSU animal facility, on a 12 hour light-dark cycle (lights on 07:00h) and in a temperature ( $21 \pm 2^{\circ}\text{C}$ ) and humidity controlled environment ( $55 \pm 10\%$ ). All animals had free access to food (Global Diet, Harlan Teklad) and water at all times unless otherwise stated.

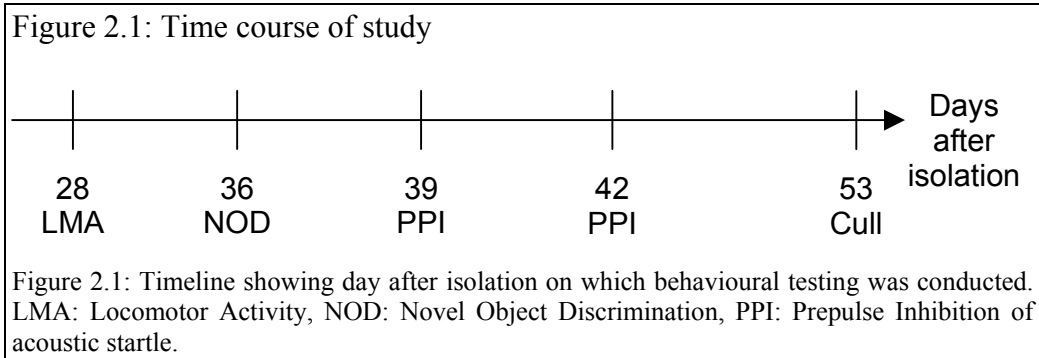
All statistical analyses were carried out using the statistical package SPSS (SPSS Inc.). The statistical methods used to analyse each behavioural test are described in the relevant experimental methods section.

### 2.3.1 Animals

40 male and female Lister Hooded rats (BMSU) were housed in single sex groups of 5 (social) or alone (isolate) from the ages of weaning (PND24-26). Pups were not cross-fostered and each litter was divided equally into socially and isolation housing to avoid any confounding factors of litter on behaviour. In studies using only male rats female pups were culled before PND 7. Social animals were housed in opaque plastic cages 50x32x23cm. Isolated animals were housed alone in cages 40x25x22cm. All animals were housed in the same room and had visual, auditory and olfactory contact with animals caged nearby. After weaning rats were weighed weekly but received no other handling, except that necessitated by husbandry requirements (cage and bedding changed weekly).

### 2.3.2 Behavioural Tests

Animals underwent a series of behavioural tests, according to the timeline shown in Figure 2.1.



#### *Locomotor Activity (LMA)*

On day 28 after isolation rats underwent assessment for LMA in a novel environment for 1 hour. Rats were placed in individual computer-controlled infra-red activity monitor arenas. Each arena consisted of a clear acrylic box, 40×20×25 cm, with a wire mesh lid. Five parallel infrared beams, 7.5 cm apart, crossed the arena at three different heights. The middle layer of beams (3.5cm above floor level) recorded horizontal locomotion, a locomotion count being registered when two adjacent beams were broken simultaneously and in consecutive sequence. The upper layer of beams (7.5cm above the floor) recorded rearing when any portion of the rat interrupted any individual beam. The lower beams, which were beneath the chamber floor (and measure feeding and nose-poking behaviours), were not used in these studies.

#### **Locomotor Activity Statistics**

The measure used was beam breaks per 5 minute epoch. Data were analysed by Repeated Measures Analysis of Variance (RM ANOVA), with a within group effect of time and between group effects of housing and gender. The equality of the variances of the data was checked using Mauchley's Test of Sphericity. If data failed to meet assumptions of sphericity the Greenhouse-Geisser correction was applied. Rearing was analysed using the same tests.



*Novel Object Discrimination*

NOD was tested on day 36 after isolation according to the protocol described by King (King et al., 2004), which is based on the test originally developed by Ennaceur (Ennaceur and Delacour, 1988). The test was carried out in the same arena where LMA was recorded which ensured that rats were habituated on the day prior to NOD testing for 1 hour. On the NOD test day rats were placed into the empty test arena for a further 3 min acclimatisation. Rats were returned to their home cages for 1 min during which time two identical objects (plastic bottles, 8cm high by 5cm diameter, covered with white masking tape and filled with water to weigh them down) were placed in the test arena. The bottles were inverted and attached through holes in the floor of the arena with blu-tack, to prevent rats from knocking them over. The holes in the arena floor were 5cm from the side and 10cm from the end wall, in the front left and back right corners, as depicted in Figure 2.2. Objects were cleaned with 20% v/v ethanol before use to ensure that olfactory cues were removed.

Figure 2.2: Layout of arena during novel object discrimination

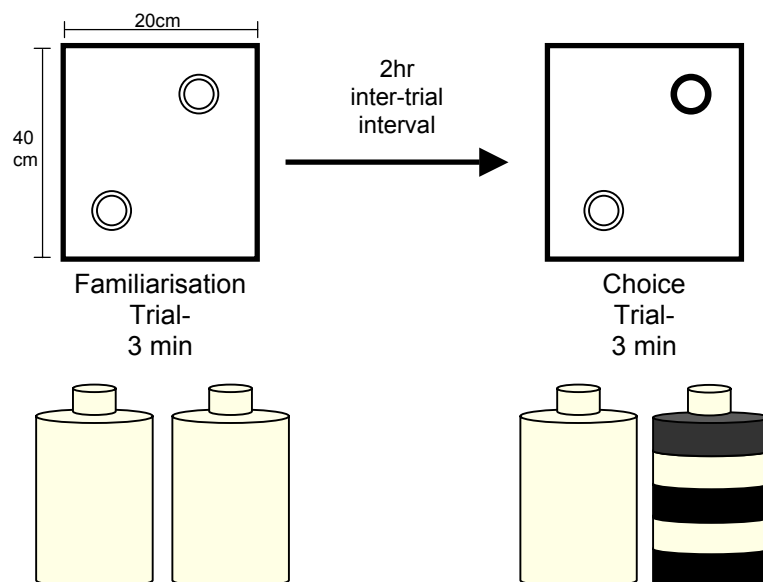


Figure 2.2: Representation of NOD arena during familiarisation and choice trials and schematic representation of the objects used.

Rats were placed in the test arena with the objects for 3 minutes (familiarisation trial, T1) and the time spent exploring each object was recorded separately using stopwatches. The observer stood at least 0.5m from the front of the arena. Exploration was defined as sniffing, licking, chewing, or having moving vibrissae whilst directing the nose towards the object at a distance of  $\leq 1$  cm. Sitting on the object was not counted as exploration. Rats were then returned to their home cages for a 2hour ITI, during which one object was replaced with an identical shaped bottle covered with white masking tape and horizontal black stripes of 1.2cm wide electrical insulating tape (novel object). Following the ITI the rat was returned to the test arena for a final 3 minute session (choice trial, T2) and the time spent exploring each object was again recorded. The position of the novel object (front or back) was balanced across groups. The test arenas were screened from each other so there was no visual contact between rats. There were also no extra-arena cues to ensure the task was non-spatial.

#### **Novel Object Recognition Statistics**

Total exploration times (in seconds) from the familiarisation and choice trials were compared by 2-way ANOVA, using housing and gender as independent variables. Preference for object placement in the familiarisation trial (front or back) was checked by paired two-tailed Student's *t*-tests for each treatment group. Similarly exploration of the novel and familiar objects during the choice trial were also compared by paired two-tailed Student's *t*-tests. Greater exploration of the novel object than the familiar object suggests the familiar object has been remembered and is not as interesting to the rat. To allow comparisons between groups of animals the discrimination ratio (DR) was calculated using the following formula:

$$\frac{\text{Novel object exploration} - \text{Familiar object exploration}}{\text{Total T2 exploration}}$$

Discrimination ratios were analysed by 2-way ANOVA. Total object exploration in the familiarisation and choice trials was also compared using 2-way ANOVA. Total exploration times can be used as an index of activity and may reveal sedative effects of drugs.

*Prepulse Inhibition of Startle*

PPI testing was carried out in four identical purpose built startle chambers (SR-LAB; San Diego Instruments, USA), which consisted of a clear Plexiglas tube, mounted on a platform in a ventilated and illuminated sound proof chamber. The chamber is designed to reduce ultra-sonic vocalisations between animals. A speaker in the top of the chamber produced the background white noise and acoustic pulses. The rat was placed inside the tube and escape was prevented using a Plexiglas slider at each end. The tube was sufficiently large to allow the rat free movement and turning during the test (20cm long x 9cm internal diameter). The startle response of the rat was detected by a piezoelectric accelerometer unit, fitted underneath the tube, digitised and stored by the connected PC using Startle Reflex Testing System software (San Diego Instruments).

The volume of the loud speakers had been previously measured using a sound level meter (Radioshack) and the speakers in all 4 boxes found to be within 3dB of each other. Each day the sensitivity of the accelerometer in each chamber was adjusted to the same baseline ( $\pm 5\%$ ) using a standardization unit (SR-LAB standardization unit, San Diego Instruments, USA) which vibrates with a fixed amplitude and frequency.

The 15min test protocol was adapted from that published by Geyer (Geyer and Swerdlow, 1998) and consisted of:

5min acclimation period with a background 65dB white noise

5x 120dB habituation pulse

10x 120dB pulse

5x 72dB pre-pulse + 120dB pulse

5x 76dB pre-pulse + 120dB pulse

5x 80dB pre-pulse + 120dB pulse

5x 84dB pre-pulse + 120dB pulse presented in a pseudo-random order

5x 120dB pulse

The pre-pulse duration was 20ms, pulse duration 40ms. Pre-pulse and pulse were separated by a fixed inter-stimulus interval (ISI) of 100ms, from start of prepulse to start of pulse. Trials were presented at an inter-trial interval (ITI) of between 10-20 seconds (average ITI 15s) in an unpredictable pseudo-random order. Startle responses were recorded for 100ms from the start of the pulse. Full validation of this protocol is described in Appendix A.

In this study PPI was carried out twice due to anecdotal evidence that animals habituate to the test and therefore might produce more consistent results during the second test.

### **PPI Statistics**

The average startle response (arbitrary units) to 120dB pulses during the pre-pulse presentation phase of the test session was compared by 2-way ANOVA using housing condition and gender as independent variables.

% PPI was calculated for each pre-pulse volume according to the following formula:

$$\%PPI = \frac{(\text{Average 120dB pulse startle} - \text{Average prepulse + pulse startle}) \times 100}{\text{Average 120dB pulse startle}}$$

The % PPI was compared by RM ANOVA, with pre-pulse volume as a within group factor and housing and gender as the between group variables. The equality of the variances of the data was checked using Mauchley's Test of Sphericity. If data failed to meet assumptions of sphericity the Greenhouse-Geisser correction was applied.

If pre-pulse volume was found to have a significant effect, the effect of housing and gender were investigated at individual pre-pulse intensities using 2-way ANOVA. At 72dB the % PPI was often very low or absent (around 0-5%) and therefore the data from this pre-pulse volume was not included in the analysis.

### 2.3.3 Corticosterone RadioImmunoAssay (RIA)

On day 53 after isolation rats were culled by concussion followed immediately by decapitation. Trunk blood was collected in ethylenediamine tetraacetic acid (EDTA) coated tubes (Becton Dickenson, USA), centrifuged at 1074 x g for 5 min (Centaur 2, MSE, UK) at room temperature to separate the plasma. The plasma (supernatant) was decanted into a new tube using a disposable pipette and stored at  $-80^{\circ}\text{C}$  until corticosterone assay.

Plasma corticosterone was measured in duplicate using a  $^{125}\text{I}$ -corticosterone RIA kit (cat #: AA-13F1, ImmunoDiagnostic Systems Ltd., UK). The assay was carried out according to the manufacturer's instructions and used rabbit anti-corticosterone anti-serum with goat anti-rabbit gamma globulin antibody to precipitate the corticosterone-anticorticosterone complex. A  $\gamma$ -counter (Cobra II Auto-Gamma, Packard, Meriden CT) was used to count the bound  $^{125}\text{I}$ -corticosterone. Samples were diluted 1:10 in 0.154M saline to ensure corticosterone concentrations lay within the linear portion (20-80% maximal binding) of the standard curve. Standards were prepared at 0.5-62.5ng/ml corticosterone and known-unknowns were analysed to ensure inter-assay reliability. The lowest detectable corticosterone concentration (the limit of sensitivity) was given as 0.39ng/ml. The cross-reactivity of the anti-corticosterone anti-serum was tested against other hormones by the test manufacturer. The largest cross-reactivity was with deoxycorticosterone (3.3%). All other hormones tested demonstrated cross-reactivity of  $\leq 1\%$ .

#### *Corticosterone Statistics*

Corticosterone levels were determined from the standard curve and the dilution factor accounted for. The corticosterone levels (ng/ml plasma) were analysed by 2-WAY ANOVA, using housing and gender as independent variables.

## 2.4 Results

### 2.4.1 Body Weight

All rats gained weight over the course of the experiment (RM ANOVA  $F_{(2.8,100.5)}=2040.1$   $p<0.001$ ) (Figure 2.3). Female rats weighed significantly less than males at all time points (RM ANOVA  $F_{(1,36)}=431.81$   $p<0.001$ ). Housing had no overall significant effect on body weight (RM ANOVA  $F_{(1,36)}=0.676$   $p=0.416$ ) and there was no housing x gender interaction. However a significant time x gender interaction indicated females did not gain weight as quickly as males (RM ANOVA  $F_{(2.8,100.5)}=310.3$   $p<0.001$ ) and a significant time x gender x housing interaction revealed that female isolates gained weight more slowly than the other rats (RM ANOVA  $F_{(2.8,100.5)}=5.65$   $p=0.002$ ).

### 2.4.2 Locomotor Activity

Locomotor activity reduced over the duration of the test as the rats habituated to the arena (RM ANOVA  $F_{(6.7, 242.8)}=48.5$   $p<0.001$ ). Isolation reared rats were significantly more active in the novel environment than socially housed rats (RM ANOVA  $F_{(1,36)}=34.42$   $p<0.001$ ), (Figure 2.4). Female rats were also more active overall than males (RM ANOVA  $F_{(1,36)}=4.47$   $p=0.041$ ).

Due to a malfunction in one test box rearing data was not recorded for one male social and one male isolated rat. Isolated rats showed increased rearing activity compared to social controls (RM ANOVA  $F_{(1,34)}=12.83$ ,  $p=0.001$ ), Figure 2.5. Females rats also showed significantly greater rearing than males (RM ANOVA  $F_{(1,34)}=21.32$ ,  $p<0.001$ ).

Figure 2.3: Females were significantly lighter than males and female isolates gained weight more slowly.

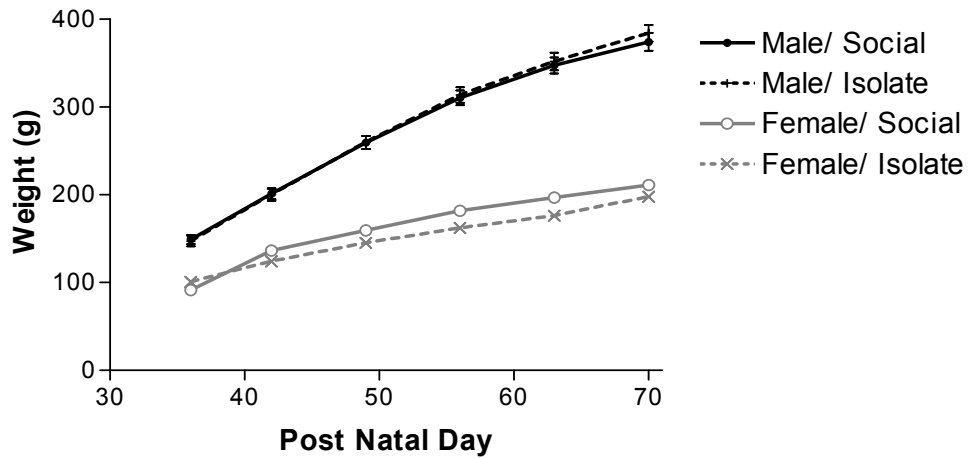


Figure 2.3: Body weight of social and isolated rats during the post-weaning isolation period. Results are plotted as mean body weight  $\pm$  s.e.m.  $n=10$ . Females were significantly lighter than males (RM ANOVA  $F_{(1,36)}=431.81$   $p<0.001$ ) and female isolates gained weight the most slowly (RM ANOVA  $F_{(2,8,100.5)}=5.65$   $p=0.002$ ). Overall there was no effect of housing on body weight (RM ANOVA  $F_{(1,36)}=0.676$   $p=0.416$ ).

Figure 2.4: Isolation reared rats were more active than controls in a novel environment and female rats were more active than males.

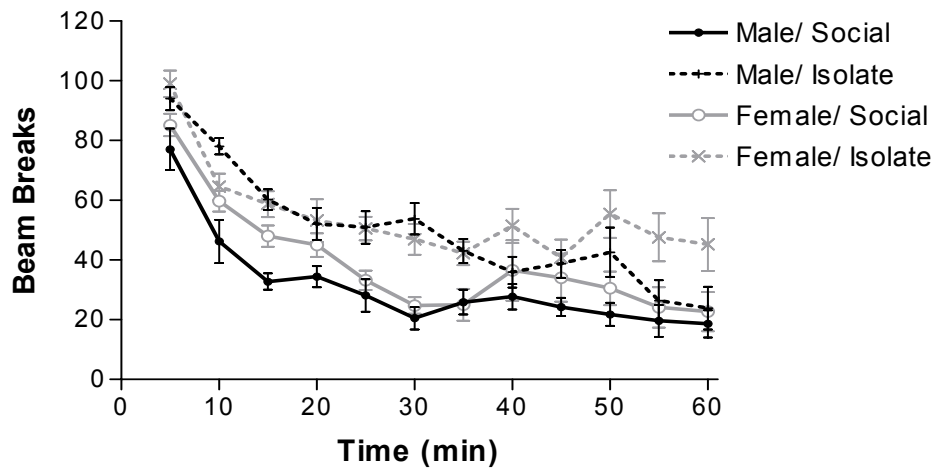


Figure 2.4: Locomotor activity of social and isolated male and female rats placed in a novel environment, measured by total beam breaks in consecutive 5 minute time bins. For clarity beam breaks are plotted at the end of each time bin but counts represent beams breaks during each 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m.  $n=10$ . Isolated rats were more active than socials (RM ANOVA  $F_{(1,36)}=34.42$   $p<0.001$ ) and females were more active than males (RM ANOVA  $F_{(1,36)}=4.47$   $p=0.041$ ).

Figure 2.5: Isolation reared rats reared more than social in a novel environment and female rats reared more than males.

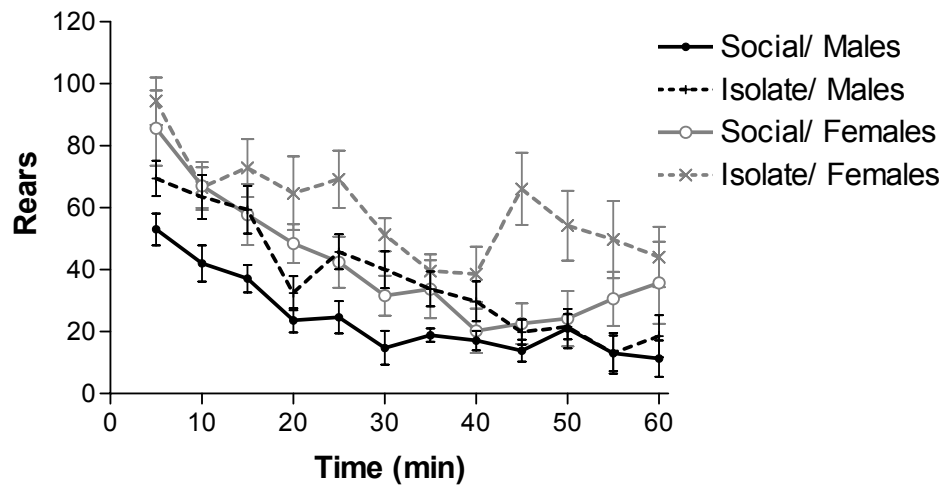


Figure 2.5: Rearing activity of social and isolated male and female rats placed in a novel environment, measured by total beam breaks in consecutive 5 minute time bins. For clarity beam breaks are plotted at the end of each time bin but counts represent beams breaks during each 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m.  $n=10$ . Isolated rats reared more than socials (RM ANOVA  $F_{(1,34)}=12.83$   $p=0.001$ ) and females reared more than males (RM ANOVA  $F_{(1,34)}=21.32$   $p<0.001$ ).

### 2.4.3 Novel Object Discrimination

In the familiarisation trial (T1) no differences were found in total exploration of the identical objects (2-way ANOVA housing effect  $F_{(1,36)}=1.821$   $p=0.186$ , gender effect  $F_{(1,36)}=2.176$   $p=0.149$ , no interactions) (see Table 2.1). There was also no preference for either object according to its position, with the front and back objects being explored equally by all treatment groups.

During the choice trial (T2) male socially housed animals spent significantly more time exploring the novel object (paired Student's  $t$ -test  $p<0.001$ ), but this was not seen in isolated males (paired Student's  $t$ -test  $p=0.286$ ) (Figure 2.6). Female social rats displayed a trend towards greater exploration of the novel object (paired Student's  $t$ -test  $p=0.066$ ) and in isolation reared females this was significant. (paired Student's  $t$ -test  $p=0.021$ ). However, when the discrimination ratios were compared by 2-way ANOVA there was no significant difference between the groups (see Table 2.1 for statistical values). The total object exploration in each trial and the discovery ratios are



summarised in Table 2.1. In T2 the female rats had greater total exploration than the males.

Table 2.1: Exploration during the familiarisation and choice trials of NOD test and discrimination ratios.

Trial	Average total exploration (s) $\pm$ s.e.m.				2-way ANOVA result	
	Social/ Male	Isolate/ Male	Social/ Female	Isolate/ Female	Effect of Isolation	Effect of Gender
T1	41.2 $\pm$ 5.1	53.8 $\pm$ 2.7	40.3 $\pm$ 4.3	40.6 $\pm$ 6.3	$F_{(1,36)}=1.82$ $p=0.186$	$F_{(1,36)}=2.18$ $p=0.149$
T2	36.4 $\pm$ 3.2	35.0 $\pm$ 5.1	42.3 $\pm$ 2.6	50.3 $\pm$ 5.2	$F_{(1,36)}=0.62$ $p=0.44$	$F_{(1,36)}=6.43$ $p=0.016 *$
DR	0.398 $\pm$ 0.070	0.158 $\pm$ 0.104	0.214 $\pm$ 0.108	0.199 $\pm$ 0.077	$F_{(1,36)}=1.96$ $p=0.170$	$F_{(1,36)}=0.63$ $p=0.434$

#### 2.4.4 Prepulse Inhibition of Acoustic Startle

PPI of acoustic startle was tested twice. In both PPI test sessions social and isolated rats showed a pre-pulse intensity dependent increase in PPI (RM ANOVA, PPI 1:  $F_{(2,72)}=34.63$ ,  $p<0.001$ , PPI 2:  $F_{(2,72)}=69.16$ ,  $p<0.001$ ). Isolation reared rats exhibited impaired PPI across all prepulse intensities (RM ANOVA, PPI 1:  $F_{(1,36)}=11.92$ ,  $p=0.001$ , PPI 2:  $F_{(1,36)}=5.85$ ,  $p=0.021$ ). However, no significant effect of gender was observed in either PPI test session (RM ANOVA, PPI 1  $F_{(1,36)}=0.120$   $p=0.281$ , PPI 2:  $F_{(1,36)}=1.75$   $p=0.194$ ). Figure 2.7 shows the data from the first PPI session, session 2 data is not shown. As the data from the two PPI sessions was extremely comparable only one session of PPI was utilized in all future experiments conducted as part of this thesis.

A 2-way ANOVA on startle amplitude at 120dB pulse revealed no effect of housing in either PPI test session (PPI 1:  $F_{(1,36)}=0.931$   $p=0.341$ , PPI 2:  $F_{(1,36)}=0.024$   $p=0.878$ ) but a significant effect of gender (PPI 1:  $F_{(1,36)}=17.014$   $p<0.001$ , PPI 2:  $F_{(1,36)}=14.447$   $p=0.001$ ) (data not shown). This gender effect is

probably due to the weight difference in the animals, with the males being significantly heavier than the female rats at an equivalent age, resulting in a greater amplitude of startle response being detected by the accelerometer

Figure 2.6: In the choice trial social male and isolated female rats spent more time exploring the novel object than the familiar.

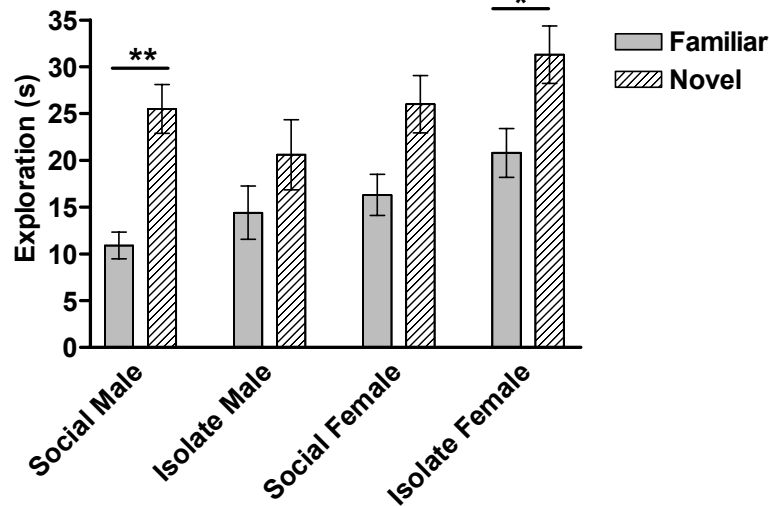


Figure 2.6: Choice trial (T2) exploration times (s) of the familiar and novel objects. Results shown as mean exploration times (s)  $\pm$  s.e.m. n=10. Student's paired *t*-test between familiar and novel exploration times \* $p < 0.05$ , \*\* $p < 0.01$ .

Figure 2.7: Isolation reared rats have attenuated PPI in the first PPI test session

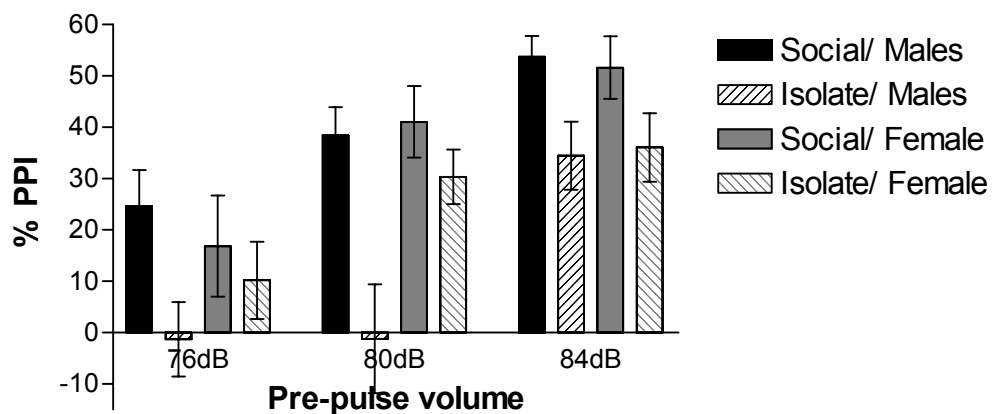


Figure 2.7: Data shown are mean % PPI  $\pm$  s.e.m exhibited by socially and isolation housed male and female rats (n=10) at pre-pulse intensities of 76-84dB. % PPI increased with increasing pre-pulse intensity (RM ANOVA  $F_{(2,72)}=34.63$ ,  $p < 0.001$ ). Housing caused a significant reduction in % PPI (RM ANOVA  $F_{(1,36)}=11.92$ ,  $p = 0.001$ ), but there was no significant overall effect of gender (RM ANOVA  $F_{(1,36)}=1.20$   $p = 0.281$ ).

### 2.4.5 Plasma Corticosterone

Females were found to have higher corticosterone than males (2-way ANOVA  $F_{(1,36)}=73.3$   $p<0.001$ ). However, housing condition during rearing had no effect on the plasma corticosterone level (2-way ANOVA  $F_{(1,36)}=0.43$   $p=0.515$ , Figure 2.8).

Figure 2.8: Female rats had a higher plasma concentration of corticosterone than males. Isolation rearing had no effect on corticosterone levels in either male or female rats.

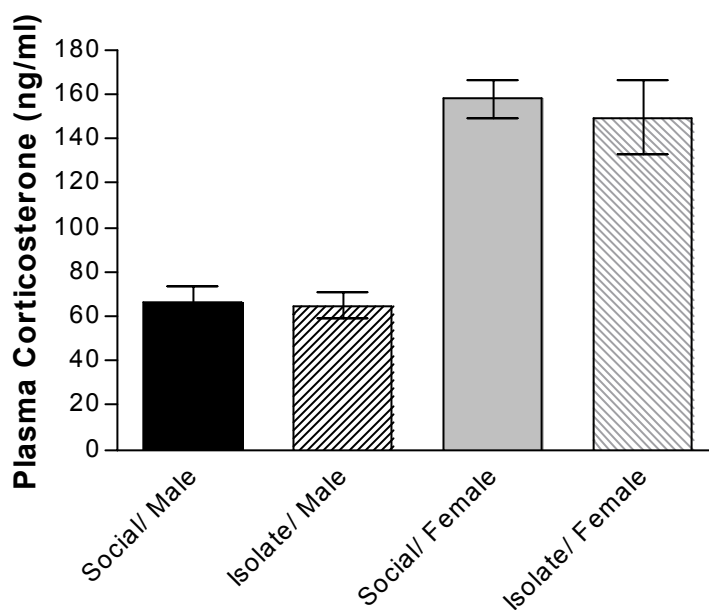


Figure 2.8: Data shown are mean  $\pm$  s.e.m. plasma corticosterone levels from socially housed and isolated rats ( $n=10$ ). Females had higher corticosterone levels than males (2-way ANOVA  $F_{(1,36)}=73.28$   $p<0.0001$ ), but isolation rearing had no effect on corticosterone levels (2-way ANOVA  $F_{(1,36)}=0.432$   $p=0.515$ ), with no significant interactions.

## 2.5 Discussion

Overall the data in this chapter shows that isolation rearing caused hyperactivity in a novel environment and decreased PPI with no change in basal plasma corticosterone, independent of gender. However, NOD was impaired by isolation rearing only in male rats.

LMA in a novel environment was increased in isolation reared rats, independent of gender, indicating a behavioural effect of isolation rearing in both male and female rats. This was an initial confirmation that in our hands isolation rearing had a similar effect to that reported by other groups using similar protocols (Weiss et al., 2000, Cilia et al., 2005b) and agrees with previous studies carried out at Nottingham (Wright et al., 1991a, Lapiz et al., 2000, Bianchi et al., 2006). Female rats were more active than males, which is also well reported in the literature (Beatty, 1979).

In this study isolation reared male rats were unable to discriminate the novel object using a 2 hour ITI while socially housed males could, indicating an impairment in recognition memory in the isolation reared rats. However, when the discrimination ratios were analysed there was no significant difference between the groups showing neither isolation rearing nor gender had an effect on novel object discrimination. This is due to the large variability in the responses of the animals. The responses in behavioural assays are notoriously variable which requires very large n numbers to achieve adequate statistical power. Increasing the number of animals in this study may have revealed a significant effect of isolation rearing and/or gender by 2-way ANOVA but would not have been ethically or practically feasible. Novel object discrimination is largely dependent on the perirhinal cortex and to some extent the hippocampus. This area of the parahippocampal gyrus is required for recognition of familiarity (reviewed in (Eichenbaum et al., 2007)). The volume of the medial temporal lobe is reduced in schizophrenia, but this is not limited to one subregion, with small reductions in perirhinal cortex, entorhinal cortex, parahippocampal cortex and the hippocampus all contributing to the reductions

recorded (Sim et al., 2006). As these regions are connected and involved in many learning and memory processes reductions in size may correlate with cognitive impairments in schizophrenic patients. Isolation rearing may be modelling at least one aspect of the cognitive deficits seen in schizophrenia as visual recognition memory is frequently impaired in schizophrenic patients (Nuechterlein et al., 2004, Caligiuri et al., 2005). Therefore the deficit in NOD in isolation reared rats may have translational relevance to common cognitive deficits seen in schizophrenia.

The socially housed female rats tended to show increased exploration of the novel object, but this just failed to reach significance ( $p=0.066$ ). The female isolates were able to discriminate the novel from the familiar object, unlike the males, indicating that isolation rearing does not have the same effect on recognition memory in female and male rats. However, female rats have been reported to be able to recognise the novel object for a longer time than male rats (Sutcliffe et al., 2007), so it maybe that if the task were made more difficult, such as by increasing the ITI, it would be found that isolation reared female rats were also impaired compared to socially housed controls. Alternatively it may be that female rats require a longer period of isolation to develop disturbances in recognition memory than male rats. In this study the rats had been isolated for 5 weeks when NOD was carried out. An interesting further study would be to see whether females developed NOD impairments if isolated for a longer period. If, as some evidence suggests, oestrogen in the female is having a protective effect (Garcia-Segura et al., 2001) and preventing the development of recognition memory impairments, it is possible that a greater duration of isolation rearing would overcome the protective effects of oestrogen. This could also be further investigated by isolation rearing ovariectomised female rats. It has been suggested that oestradiol mediates its protective effect in schizophrenia by dampening DA transmission in the striatum (Hafner et al., 1991). However, this may also have affected the extent of the hyperactivity in a novel environment, which is related to mesolimbic DA activity (Lapiz et al., 2003). Interestingly, chronic restraint stress also causes NOD impairments in male but not in female rats nor in ovariectomised females (Luine, 2002, Bisagno et al., 2003, Bowman et al., 2003). This suggests that at least part of the protective effect of oestrogen on cognition must be

developmental rather than acute. In a radial arm maze test of working memory female rats did not perform as well as males. However, isolation rearing affected the male rats more severely so that they performed less well than isolation reared females (Einon, 1980). Although in Einon's study the socially housed animals were maintained in environmentally-enriched cages it supports the proposal that female rats suffer less severe cognitive consequences of chronic stress and isolation rearing than males.

The oestrogen levels and vaginal cytology of the female rats were not measured in this study so it is impossible to know what stage of the oestrous cycle they were in. However, it has recently been shown that oestrous cycle has no effect on performance in NOD (Sutcliffe et al., 2007).

Both male and female rats exhibited PPI deficits when tested after 6 weeks of rearing in social isolation. No differences were seen between male and female rats, which is consistent with some reports in humans (Ludewig et al., 2003). However, other studies suggest that sensorimotor gating is lower in women and varies according to phase of the menstrual cycle (Swerdlow et al., 1997). Previous PPI studies in female rats showed no differences between the genders, except when the females were in the pro-oestrous (high oestrogen) phase of their cycle when PPI was reduced compared to that recorded in other stages of the oestrous cycle and also to PPI levels in male rats (Koch, 1998).

No differences in plasma corticosterone were seen between isolated and socially housed rats. This agrees with previously reported studies (Holson et al., 1991, Heidbreder et al., 2000) using similar housing conditions. Female rats had higher plasma concentrations of corticosterone than males. The levels reported in both the males and females are similar to those previously reported (Jones et al., 1989) (Holson et al., 1991). The increased corticosterone in response to restraint stress is also unaltered in isolation reared rats compared to socially housed controls (Schrijver et al., 2002) leading to the conclusion that isolation reared rats have alterations in neither basal nor stress-induced corticosterone release, although this may depend on housing conditions (Holson et al., 1991). If more severe housing conditions are used, such as no

auditory contact or wire cages, both basal and stress induced corticosterone levels are increased (Greco et al., 1989, Heidbreder et al., 2000). It is important to note that in this study plasma corticosterone was only measured at one time point. Plasma corticosterone levels show diurnal variations, with increases from early afternoon, peaking after onset of darkness (Kwak et al., 1992). The animals were culled between 9-11am, with order of treatment group randomised, to minimise the natural fluctuations.

It has been found that schizophrenic patients have higher basal cortisol levels (Gallagher et al., 2007) and decreased cortisol release in response to stress (Corcoran et al., 2003), so in this respect the current isolation rearing protocol does not model the clinical situation. However, cortisol levels have been found to correlate with symptom severity (Kaneko et al., 1992) and with thought disorder in male patients (Halari et al., 2004). Treatment with the atypical antipsychotics olanzapine and quetiapine, but not with the typical antipsychotic haloperidol, reduces both cortisol and ACTH in schizophrenic patients (Cohrs et al., 2006).

## 2.6 Conclusion

The isolation protocol used induced detectable behavioural changes after 5-6 weeks:

- Hyperactivity in a novel environment
- A trend towards impaired NOD
- Attenuated PPI of acoustic startle
- No elevation of basal plasma corticosterone levels.

While both male and female isolation reared rats developed behavioural alterations, only the male rats showed evidence of a cognitive deficit in NOD. Therefore all future studies will use only male rats.

In the Chapter 3 the cognitive effects of isolation rearing will be examined further using a test of behavioural flexibility.



**3 Effect of Isolation Rearing on Attentional Set Shifting**


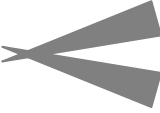
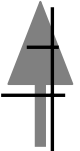
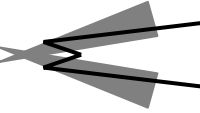
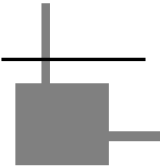
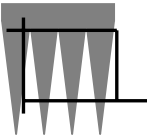
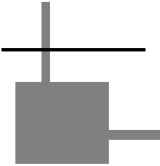
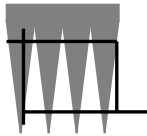

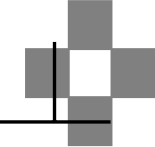
### 3.1 Aim

The aim of the work in this chapter was to determine the effect of isolation rearing on behavioural flexibility, using the bowl digging task of attentional set shifting. This task was first validated using rats treated with a sub-chronic PCP dosing regimen which has previously been shown to cause impairments in attentional set shifting ability.

### 3.2 Introduction

The MATRICS group identified 7 cognitive domains that are impaired in schizophrenia (Nuechterlein et al., 2004). Amongst these is visual memory, which can be measured in rodents using the NOD task. However, it is the deficits experienced by schizophrenics on tasks of executive function and behavioural flexibility that are most linked to functional impairment and poor outcome (Bilder et al., 2000). The Wisconsin card sort test (WCST) described in Chapter 1 is often used to measure the type of behavioural flexibility known as attentional set shifting. The Cambridge Automated Neuropsychological Testing Battery (CANTAB) (Cambridge Cognition, Ltd.) includes a computerised version of an attentional set shifting task called the intra-dimensional/extra-dimensional (ID/ED) set shifting task. In this computerized task the subject must carry out a series of rule learning discriminations between white line and coloured shape images (the dimensions). The subject must learn which stimuli is correct using feedback from the computerised system. Once 6 consecutive correct trials have been completed the subject moves on to the next stage. The first stage is a simple discrimination between two stimuli from the same dimension, e.g. two different shapes, a choice is made by touching the image. Table 3.1 shows some stages of a test session with symbols similar to those used in the CANTAB test (adapted from (Jazbec et al., 2007). The stage in which attentional set must be switched (i.e. where the previously irrelevant dimension becomes relevant) is called the extra-dimensional (ED) shift.

Table 3.1: Representation of a CANTAB ID/ED attentional set shifting test.

CANTAB images		Discrimination
		Simple Discrimination (SD) Images from only one dimension (shape) presented.
✓	x	
		Compound discrimination (CD) Line images added but should be ignored.
✓	x	
		Intra-dimensional Shift (IDS) New set of images, but the solid shape dimension remains salient.
✓	x	
		Intra-dimensional Reversal (IDR) Same images as previous discrimination, but now opposite shape is correct.
x	✓	
		Extra-dimensional Shift (EDS) New set of images. The line dimension is now correct.
x	✓	

Although schizophrenics perform poorly at most stages of this task their performance is most impaired at the extra-dimensional shift stage of the task (Pantelis et al., 1999, Jazbec et al., 2007) where they fail to shift attention away from the previously relevant dimension (i.e. show perseveration) (Haut et al., 1996). Although this deficit is not specific to schizophrenia, as Parkinson's disease patients also demonstrate ED-shifting impairments (Downes et al., 1989, Owen et al., 1993), inability to carry out ED-shifting is correlated with negative symptom severity (Pantelis et al., 1999). In first episode schizophrenics ED-shifting impairment was seen in only 25% of patients, but correlated with the duration of untreated psychosis (Joyce et al., 2002). However, in a study of patients with chronic schizophrenia only 25% of patients were able to complete the ED-shift (Pantelis et al., 1999). This suggests that as the disease progresses attentional set-shifting performance deteriorates (Joyce et al., 2002). It has also been found that schizophrenics are more easily distracted by the addition of the irrelevant dimension in the first compound discrimination (CD) stage of the test, taking longer than controls to reach criterion (Jazbec et al., 2007).

Performance in ID/ED tasks is highly dependent on the frontal cortex. Patients with frontal lobe damage find the ED-shift particularly difficult (Owen et al., 1991, Haut et al., 1996, Pantelis et al., 1999), although they do not have any problems with ID-shifts. Studies in monkeys have shown that lesions to selective areas of the prefrontal cortex can have very specific effects on ID/ED performance, with orbitofrontal cortex damage leading to impairments in reversal shifts while dorsolateral PFC damage impairs ED-shifting (Dias et al., 1996). As it is hypothesised that schizophrenics demonstrate hypofrontality, a reduction in activity in the frontal cortex, this could explain their poor performance on this prefrontal cortex mediated task (Hill et al., 2004). Consistent with this proposal imaging studies have found schizophrenics have reduced activation of the prefrontal cortex whilst carrying out the WCST (Weinberger et al., 1992, Volz et al., 1997).

A rodent version of the ID/ED test has been developed (Birrell and Brown, 2000), in which rats must identify which of two pots contains a hidden food reward based on the odour and the medium with which the pot is filled. In order to ascertain whether isolation reared rats have impairments in executive function, they were tested in the bowl-digging attentional set-shifting task. This task will reveal any differences in learning to discriminate and follow one dimension, reversal learning and the ability to switch attention to a previously irrelevant cue (extra-dimensional attentional set-shifting).

As discussed in Chapter 1, treatment with the NMDA receptor antagonist PCP has been proposed as a pharmacological model of schizophrenia due to the similarity between PCP-induced psychosis and schizophrenia (Steinpreis, 1996). Both acute and chronic treatment with PCP have been found to lead to deficits in many cognitive tasks such as delayed alternation (Moghaddam and Adams, 1998), delayed non-matching to position (Campbell et al., 2004), and watermaze acquisition (Didriksen et al., 2007). Acute PCP has been shown to disrupt ED-shift performance in the bowl digging task of attentional set shifting (Egerton et al., 2005) 24 hours after treatment. However, a sub-chronic PCP treatment regimen (5mg/kg twice a day for 7 days) has also been found to induce a robust and long lasting impairment in the ability of rats to perform the ED-shift (Rodefer et al., 2005) and also impairs reversal learning performance (Abdul-Monim et al., 2003), NOD (Grayson et al., 2007) and conditional discrimination (Dunn and Killcross, 2006). A 7-day washout period between the final dose of PCP and the start of behavioural testing ensures the behavioural effects seen are not due to direct pharmacological effects of PCP, but rather due to adaptive changes brought about by the sub-chronic treatment. This is advantageous when testing putative nootropic compounds as it avoids direct pharmacological interactions and therefore this dosing protocol will be used to validate the bowl-digging task before isolation reared rats are tested.

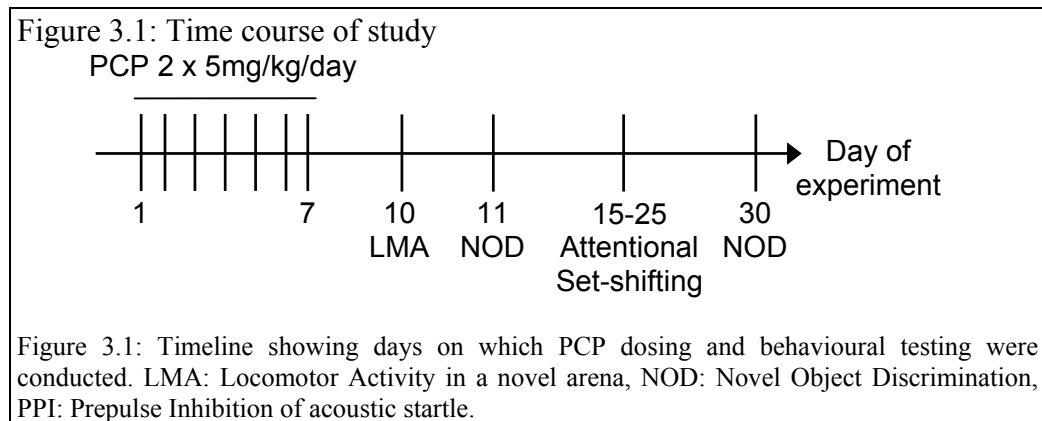
### 3.3 Methods

#### 3.3.1 Validation of ID/ED Protocol using Sub-chronic PCP Treatment

##### *Animals*

20 male LH rats (BMSU, University of Nottingham) were housed in groups of 4 in standard cages. Rats were approximately 180g at the start of dosing. Rats were dosed with 5 mg/kg phencyclidine HCl (Sigma UK) or saline (0.9% NaCl) at 1ml/kg, intraperitoneal (i.p.) injection twice a day (morning and late afternoon) for 7 days. From one day prior to the start of habituation to testing in the attentional set shifting paradigm rats were placed on a restricted diet of approximately 15g/rat/day of standard rodent chow (Global diet, Harlan Teklad) such that body weight remained constant ( $\pm 5\%$ ) for the 6 days of testing. The food was given at 4pm each day.

Behavioural studies were carried out according to the timeline shown in Figure 3.1.



##### *Locomotor Activity in a Novel Arena*

3 days after the final PCP dose rats underwent 1 hour of LMA testing exactly as previously described in Chapter 2. This test also acted as habituation to NOD which was carried out the following day in the same arena.

### *Novel Object Discrimination*

Four days after the end of the PCP dosing regimen rats underwent testing in NOD. NOD was carried out as described in Chapter 2, with the exception that a reduced ITI of 1 hour was used. The reduced ITI was used because previous work has found PCP-induced recognition memory deficits can be detected at using this ITI (Watson et al., 2005). This NOD testing was repeated 23 days after the end of the PCP dosing period, after the completion of ID/ED testing.

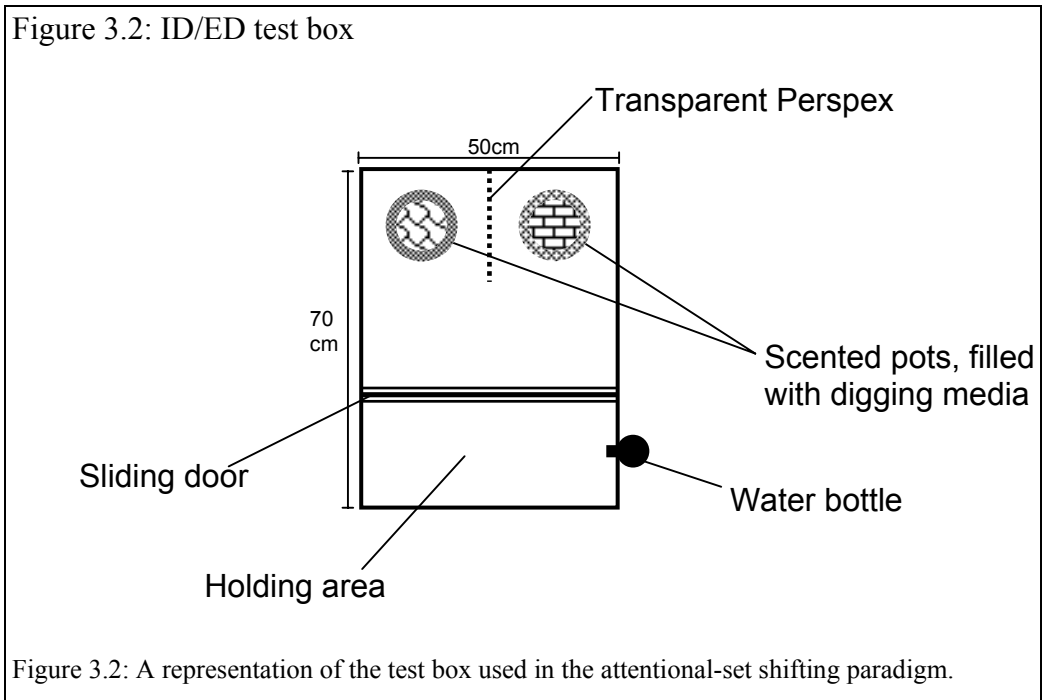
### *Attentional Set Shifting*

Due to time constraints only 16 of the 20 animals (8 saline, 8 PCP, selected at random) underwent attentional set shifting testing. The behavioural testing was carried out on days 15-25, 7 days after the end of PCP dosing to allow the PCP to have been fully cleared from the animals. As the half-life of PCP in male rats is approximately 80 minutes this seven day wash out period is more than sufficient to ensure full clearance of the drug (Nabeshima et al., 1984).

### **Apparatus**

The test arena was a black, opaque Perspex box 50 x 70 x 50cm depicted in Figure 3.2. The rear third of the test box was separated off using a sliding opaque door to create a holding area. A bottle in the holding area provided access to water between each trial. In the test area two holes in the base allowed an 11cm diameter terracotta pot to be sunk securely into the test box floor, with a 4cm rim at the top of each pot remaining above the test floor. The exposed rim of each flowerpot was scented with home fragrance oil (Body Shop, UK) which was allowed to absorb into the terracotta for at least 24 hours before testing to prevent the oil from transferring on to the rats during testing. The two pots were separated by a transparent Perspex divider which allowed rats to see from one pot to the other, but not move rapidly between them. Each pot was filled with a digging medium in which rats could dig to search for food rewards consisting of halved Honey Loops (Kelloggs, UK). The media chosen all consisted of small pieces, with no strong odour, in which rats could dig safely without risk of injury. All media were inedible, although most rats tested most of the media. All digging media and scented oil combinations are detailed

in Table 3.2. The media and odours were picked after preliminary trials showed no preference between pairs of media or scents.



### Behavioural Testing

To ensure the rats were motivated to work for the food rewards animals were placed on food restriction from 1 day prior to the start of habituation. On the first day of food restriction animals were given a few honey loops in their home cages to familiarise them with the new foodstuff. Rats underwent 6 habituation sessions (2 per day for 3 days) in which they were placed into the holding area of the test box. The sliding door was lifted and the rat allowed to run through into the test area. The sliding door was then replaced, shutting the rat in the test area which contained two unscented flower pots filled with cage bedding (sawdust) and generously loaded with honey loops. The rats were left for 15 minutes exploring the box and freely eating the honey loops. As the habituation sessions progressed the loops were placed deeper into the sawdust to encourage the rats to associate the pots with digging and food rewards.

On the 4<sup>th</sup> day the rats underwent discrimination training, in which the rat must learn that only one pot contains a food reward and a cue must be followed to find the reward. As in the habituation sessions the rat was placed in the holding



area. When the door was raised the animal ran into the test area to find the pots either contained different digging media (media discrimination training) or sawdust but were scented differently (odour discrimination training). If the rat dug in the correct pot and retrieved a honey loop he was allowed to consume the reward then returned to the holding area. The first 4 trials of each discrimination were discovery trials in which animals were allowed to dig in the other pot if their first dig was incorrect. However, in subsequent trials an incorrect initial dig resulted in the rat being returned to the holding area without receiving a food reward. In all discriminations the criterion for progression to the next stage was 6 consecutive correct digs. All animals were trained on both media and odour discrimination, in a random order.

On the 5<sup>th</sup> day the full intra/extra dimensional set-shifting test occurred. The animals progressed through 7 discriminations in which different odour/media combinations were used. The study was balanced such that half the animals from each group started following digging medium as the relevant cue dimension and half began with odour. The odour/media combinations were kept constant, but the order in which these combinations were presented and the initial correct cue were varied and balanced across test groups (see Tables 3.2 and 3.3). This ensured that any slight differences in cue salience or simple preference by the animals could not skew the results. The order of the discriminations which the rats complete is shown in Table 3.3, along with examples of the cues used. The main measure recorded was the number of trials to achieve criterion of 6 consecutive correct trials. The time taken for animals to complete each discrimination was also recorded. If a rat refused to dig in a pot for 20 minutes, or went to sleep, it was returned to the home cage for at least a 30 minute rest period, in accordance with the Home Office Project licence. Preliminary studies suggested that rats became tired after completing four or five discriminations. In order to minimise rats becoming tired in the middle of a discrimination all rats were rested for 30 minutes after completing the compound reversal stage. This usually enabled the animals to tackle the second half of the test, from the intra-dimensional shift to the extra-dimensional reversal, without requiring a further rest period.

Table 3.2: Cue combinations and order of presentation used in attentional set shifting studies.

Pair	Media	Odour
Training	Shredded Paper	Rose
	Wood chips	Ginger grapefruit
1	Wax chips	Passion fruit
	Plastic beads	Sandalwood
2	Pebbles	Lavender
	Gravel	Strawberry
3	Paper cat litter	Green tea
	Catsan	White Musk
Order in which pairs are presented		
I	1→2→3	
II	2→3→1	
III	3→1→2	
IV	2→1→3	

Table 3.3: An example of the discriminations performed by one rat on test day, beginning with odour as the relevant dimension. **Rewarded cue in bold.**

Discrimination	Odour	Medium
Simple	<b>Passion fruit</b> , sandalwood	Sawdust in both pots
Compound	<b>Passion fruit</b> , sandalwood	Wax chips, plastic beads
Compound Reversal	Passion fruit, <b>sandalwood</b>	Wax chips, plastic beads
Animals rested before intra-dimensional shift		
Intra-dimensional Shift	<b>Lavender</b> , strawberry	Pebbles, gravel
Intra-dimensional Reversal	Lavender, <b>strawberry</b>	Pebbles, gravel
Extra-dimensional Shift	Green tea, white musk	<b>Catsan</b> , paper cat litter
Extra-dimensional Reversal	Green tea, white musk	Catsan, <b>paper cat litter</b>

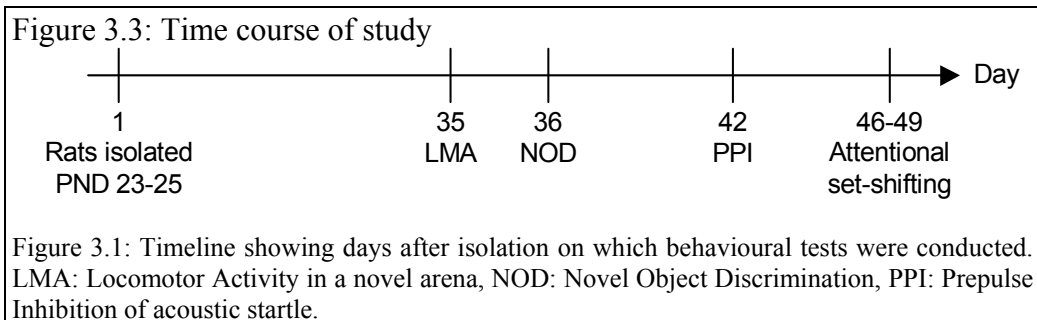
### Statistical Analysis

All data were analysed using SPSS. The number of trials to criterion of 6 consecutive correct digs was the main measure.  $\text{Log}_{10}$  transformation of data was carried out before analysis to correct for differences in variance. Training data were analysed by 2-way ANOVA. The data from the main test day discriminations were analysed using repeated measures ANOVA. Sphericity of the data was checked using Mauchley's Test of Sphericity. If data failed to meet assumptions of sphericity the Greenhouse-Geisser correction was applied. If a significant effect of discrimination was observed more detailed analyses were carried out which specifically examined the effects at the ID-shift and ED-shift stage. The actual time (in minutes) taken to complete each discrimination was also analysed by repeated measures ANOVA, in the same way as the number of trials to criterion.

### 3.3.2 Effect of Isolation Rearing on Attentional Set Shifting

#### *Animals*

Male Lister Hooded rats (CRUK), PND 23-25 were housed in groups of 4 (social,  $n=8$ ) or alone (isolates,  $n=8$ ) and housed as previously described (Chapter 2) for 5 weeks. Behavioural tests were carried out according to the timeline shown in Figure 3.3.



LMA, NOD and PPI were carried out as previously described (in Chapter 2). Attentional set shifting was carried out exactly according to the sub-chronic PCP validation of the protocol above.

### 3.4 Results

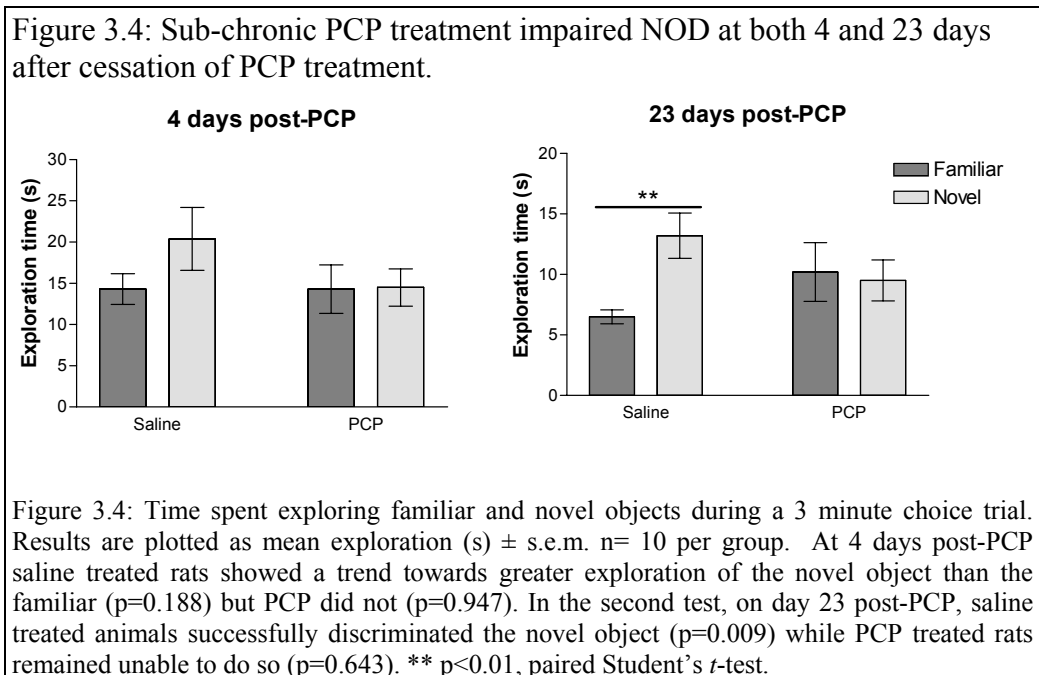
#### 3.4.1 Validation of ID/ED Protocol using Sub-chronic PCP Treatment

##### *Locomotor Activity*

PCP had no effect on either horizontal movement (RM ANOVA  $F_{(1,18)}=0.484$   $p=0.495$ ) or vertical rearing ( $F_{(1,18)}=0.002$   $p=0.966$ ) in a 1 hour test in a novel environment (data not shown).

##### *Novel Object Discrimination*

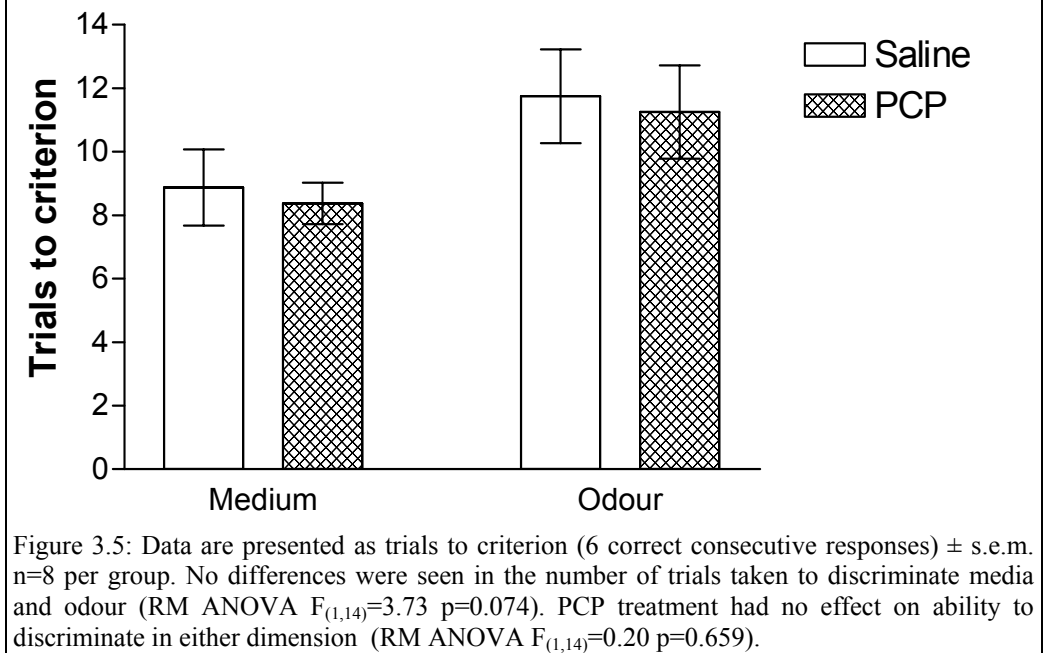
When tested 4 days after cessation of PCP treatment neither saline nor PCP treated rats were able to discriminate the novel object after a 1hr ITI, (Figure 3.4) (paired Student's *t*-test saline  $p=0.188$ , PCP  $p=0.947$ ). At 23 days after the final dose (and after attentional set shifting) saline treated animals could successfully discriminate the novel object (paired Student's *t*-test  $p=0.009$ ) but PCP treated animals were unable to discriminate (paired Student's *t*-test  $p=0.643$ ), indicating an impairment in recognition memory. The discrimination ratios also revealed a significant effect of PCP treatment after 23 days (mean  $\pm$  s.e.m saline =  $0.145 \pm 0.09$ , PCP =  $0.027 \pm 0.1$ , Student's *t*-test  $p=0.005$ ).



*Attentional Set Shifting***Discrimination Training**

On the fourth day of the ID/ED protocol animals underwent discrimination training. No difference was found in the number of trials to criterion between the media and odour dimensions during training, although there was a trend towards odour discrimination requiring more trials to learn than medium (RM ANOVA  $F_{(1,14)}=3.73$   $p=0.074$ ) (Figure 3.5). Drug treatment was found to have no effect on ability to discriminate either dimension (RM ANOVA  $F_{(1,14)}=0.20$   $p=0.659$ ).

Figure 3.5: PCP had no effect on the number of trials to reach criterion in either medium or odour training.



### Discrimination Testing

On the attentional set-shifting test day animals performed seven discriminations. A significant effect of discrimination was observed, indicating that all animals found some discriminations more difficult than others (RM ANOVA  $F_{(6,9)}=7.29$   $p=0.005$ ) (Figure 3.6). Furthermore, across all the discriminations there was no total effect of PCP treatment (RM ANOVA  $F_{(1,14)}=2.33$   $p=0.150$ ).

However, when the intra-dimensional and extra-dimensional shift results are compared by a multivariate analysis a significant overall effect of drug was found ( $F_{(2,13)}=8.89$   $p=0.004$ ) with a specific effect of PCP at the ED shift ( $F_{(1,14)}=19.09$   $p=0.001$ ) which was not seen at the ID shift. This shows that the PCP treated animals took more trials to reach criterion in the ED-shift, but not the ID-shift. In contrast PCP caused no differences in reversal learning performance, indicating that a specific ED shift deficit was produced in these animals.

Figure 3.6: PCP treated rats took significantly more trials to reach criterion at the ED-shift than controls.

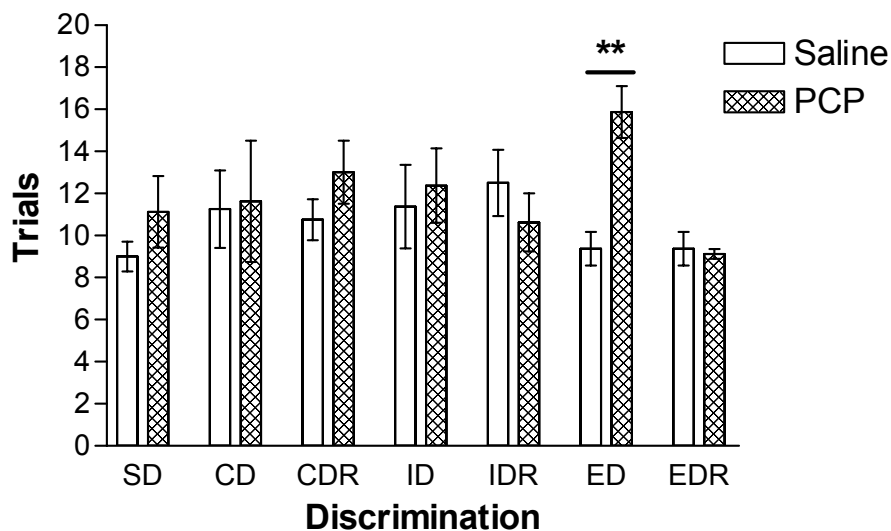
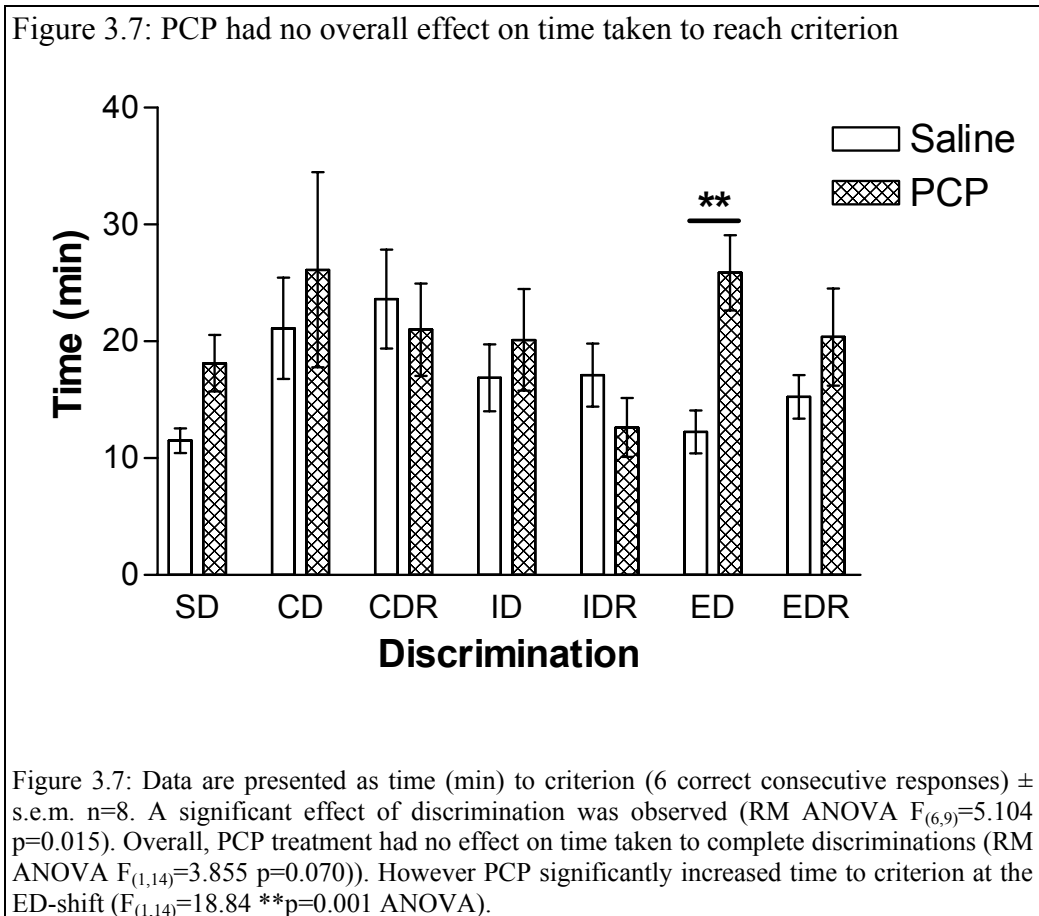


Figure 3.6: Data are presented as trials to criterion (6 correct consecutive responses)  $\pm$  s.e.m.  $n=8$ . A significant effect of discrimination was observed (RM ANOVA  $F_{(6,9)}=7.29$   $p=0.005$ ). Overall, PCP treatment had no effect on ability to discriminate (RM ANOVA  $F_{(1,14)}=2.32$   $p=0.150$ ). However PCP significantly increased trials to criterion at the ED-shift ( $F_{(1,14)}=19.09$   $p=0.001$ ). \*\*  $p<0.01$ , ANOVA

As the data from the training session showed a trend towards odour being more difficult to discriminate than medium some further analyses were carried out. Firstly, the total trials from SD to completion of IDR were compared to check for differences in the ability to follow either medium or odour (2-way ANOVA: dimension effect  $F_{(1,12)}=2.14$   $p=0.169$ , treatment effect  $F_{(1,12)}=0.37$   $p=0.557$ , no interaction). No differences were found indicating that both saline and PCP treated rats took the same number of trials to complete the IDR stage, regardless of whether they were following the odour or medium dimension. A further 2-way ANOVA was carried out on the trials to complete the ED-shift (dimension effect  $F_{(1,12)}=2.77$   $p=0.122$ , treatment effect  $F_{(1,12)}=20.25$   $p\leq 0.001$ , interaction  $F_{(1,12)}=0.08$   $p=0.779$ ), which showed that PCP treatment significantly increased the number of trials to criterion irrespective of the dimension followed.



### Time to Criterion

Overall there was a significant effect of discrimination on the time taken to reach criterion (RM ANOVA  $F_{(6,9)}=5.104$   $p=0.015$ ), indicating that some discriminations look longer to complete than others (Figure 3.7). However, there was no significant effect of PCP treatment on the overall time to complete the task (RM ANOVA  $F_{(1,14)}=3.855$   $p=0.070$ ). However, as seen with the number of trials, PCP pre-treated rats took longer to complete the ED shift, but not the ID shift (Multivariate ANOVA PCP effect at ID-shift  $F_{(1,14)}=0.15$   $p=0.704$ , ED-shift  $F_{(1,14)}=18.84$   $p=0.001$ ).

### 3.4.2 Effect of Isolation Rearing on Attentional Set Shifting

#### *Locomotor Activity in a Novel Environment*

After five weeks of isolation rearing rats demonstrated a different horizontal activity profile compared to socially housed animals, when placed in a novel environment (Figure 3.8). Activity was increased during the first half of the test, but reached the same level as the controls after approximately 30 minutes (such that there was RM ANOVA Time x Housing effect  $F_{(4,5,63,6)}=3.258$   $p=0.013$ ). The total number of beam breaks over the entire test hour was also significantly increased in isolates compared to group housed controls (Student's *t*-test  $p=0.019$ ), as expected. A trend towards increased vertical rearing in isolation reared rats was also seen, but this failed to reach significance from group housed controls (RM ANOVA  $F_{(1,14)}=3.865$   $p=0.069$ ) (data not shown).

#### *Novel Object Discrimination*

Socially housed animals successfully discriminated the novel from the familiar object (paired Student's *t*-test  $p=0.004$ ) during the choice trial (T2) in the NOD paradigm, as seen in Figure 3.9. In contrast, isolation reared rats were unable to discriminate between the two objects (paired Student's *t*-test  $p=0.786$ ). When the discrimination ratios were compared isolation reared rats demonstrated impaired novel object discrimination compared to socially housed controls (mean  $\pm$  s.e.m social =  $0.391 \pm 0.051$ , isolates =  $-0.021 \pm 0.095$ , Student's *t*-test  $p=0.002$ ). The isolation reared rats showed a trend towards increased total



exploration of both objects in the familiarisation trial (T1), but this just failed to reach significance (Student's *t*-test  $p=0.055$ ). Similarly, no difference was seen in the total exploration during the T2 choice trial (Student's *t*-test  $p=0.739$ ).

Figure 3.8: Isolation reared rats were more active in a novel environment than socially housed controls.

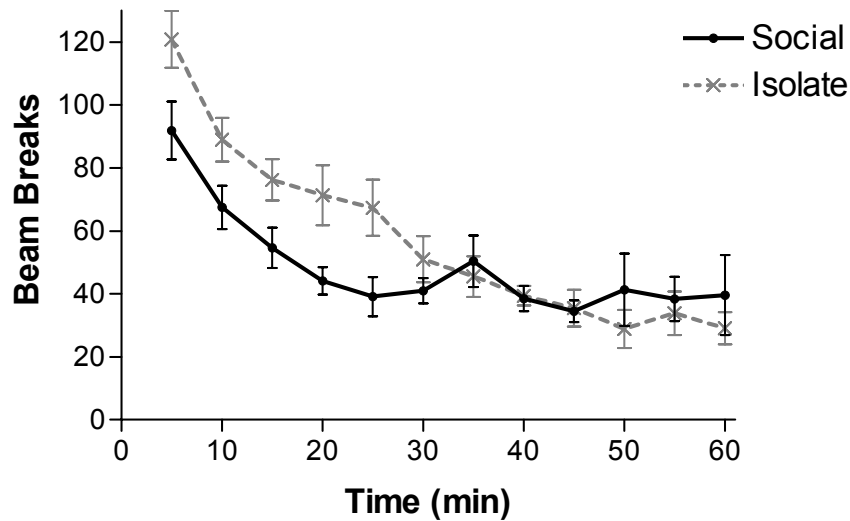


Figure 3.8: Locomotor activity of social and isolated rats in a novel environment, measured by beam breaks in a 5 minute time bin. Beam breaks are plotted at the end of each time bin but represent breaks during the 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m.  $n=8$ . Isolation reared rats were more active than controls over the first 30 minutes of the test (RM ANOVA  $F_{(11,154)}=3.258$   $p=0.013$ ).

Figure 3.9: Isolation reared rats were impaired at novel object discrimination

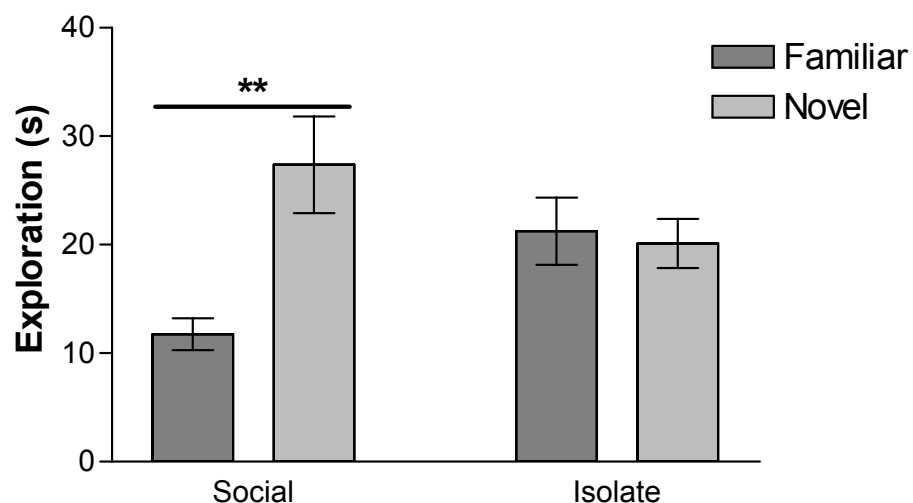
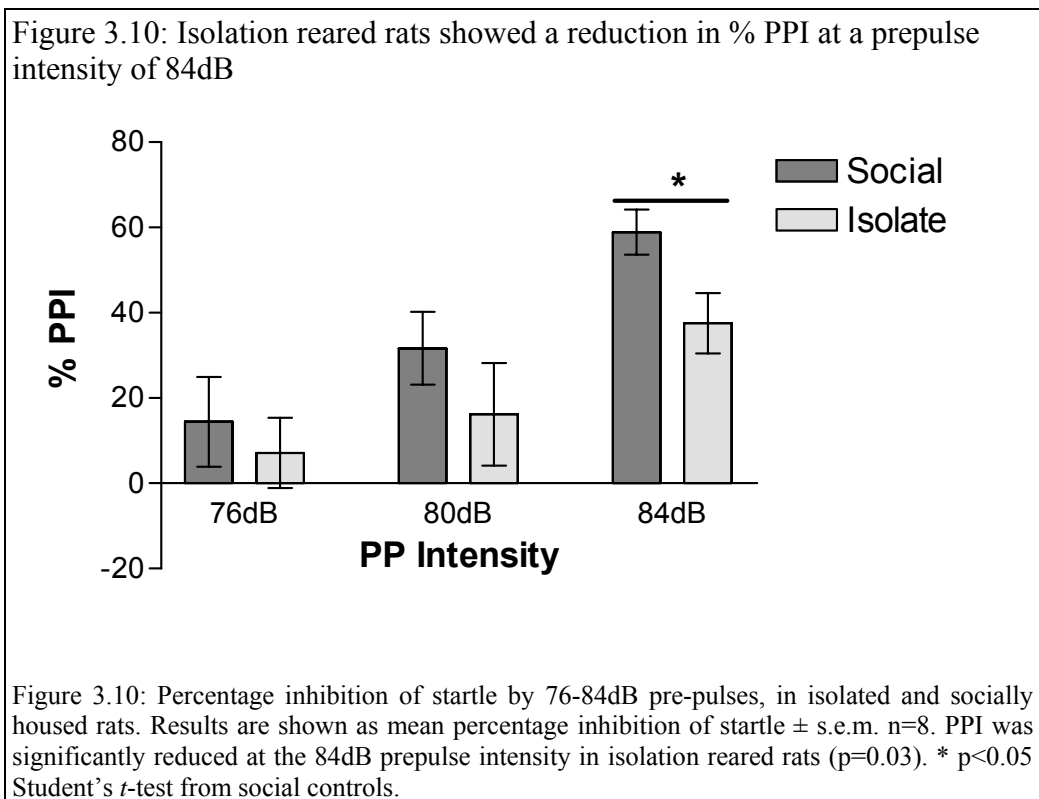


Figure 3.9: Time spent exploring familiar and novel objects during a 3 minute choice trial. Results are plotted as mean exploration (s)  $\pm$  s.e.m.  $n=8$ . Socially housed animals successfully discriminated the novel object ( $p=0.004$ ) while isolated rats were unable to do so ( $p=0.786$ ). \*\*  $p<0.01$  Paired Student's *t*-test.

*Prepulse Inhibition of Acoustic Startle*

Overall, isolation reared rats did not show an impairment in PPI (RM ANOVA  $F_{(1,14)}=1.094$   $p=0.201$ ), as seen in Figure 3.10. However, pre-pulse intensity was found to have a significant effect on PPI (RM ANOVA  $F_{(2,28)}=25.019$   $p\geq 0.001$ ) and therefore the results at individual pre-pulse intensities were examined in more detail. It was found that at 84dB isolation reared rats demonstrated a reduction in PPI (Student's  $t$ -test  $p=0.03$ ) compared to group housed controls.



*Attentional Set Shifting***Discrimination Training**

Before the attentional set shifting test animals were trained to discriminate between odours and media. No differences were found in either the number of trials taken to reach criterion between odour and medium training (RM ANOVA  $F_{(1,14)}=0.101$   $p=0.333$ ), or the trials taken by social and isolation reared rats (RM ANOVA  $F_{(1,14)}=0.94$   $p=0.349$ ) (data not shown).

**Discrimination Testing**

Figure 3.11 shows the trials taken to reach criterion during each stage of the set shifting paradigm on the test day. No significant effect of discrimination was found, indicating the animals took the same number of trials to reach criterion on all trials (RM ANOVA  $F_{(3,9)}=0.80$   $p=0.595$ ). No overall effect of housing was found (RM ANOVA  $F_{(1,14)}=0.02$   $p=0.904$ ). Further analysis of the ID and ED data by multivariate analysis found no effect of housing either overall ( $F_{(2,13)}=0.153$   $p=0.860$ ) or at the ID or ED shift stage.

Figure 3.11: Housing had no effect on the number of trials to reach criterion at any discrimination.

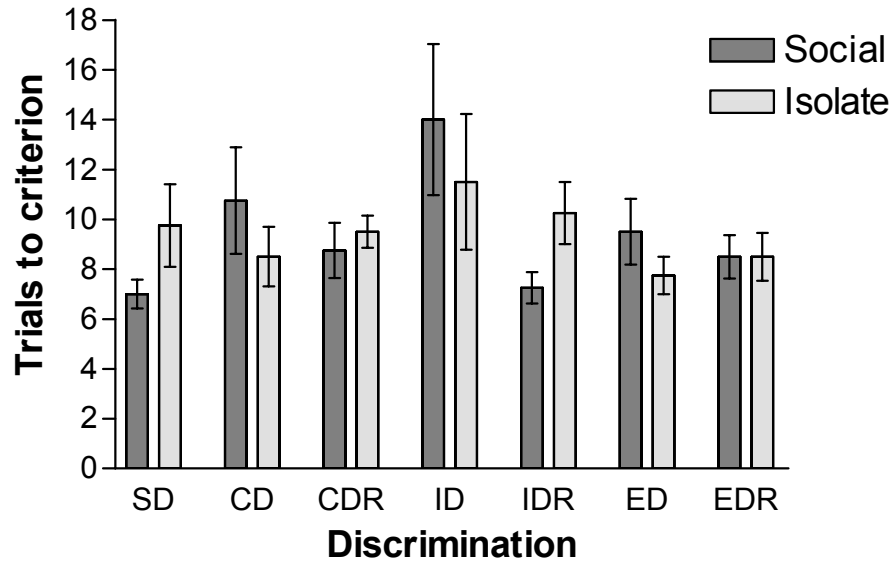


Figure 3.11: Data are presented as trials to criterion (6 correct consecutive responses)  $\pm$  s.e.m.  $n=8$ . No significant effect of discrimination was observed (RM ANOVA  $F_{(3,9)}=0.80$   $p=0.596$ ). Overall, isolation rearing had no effect on ability to discriminate (RM ANOVA  $F_{(1,14)}=0.02$   $p=0.904$ ).

### Time to Criterion

Overall there was no significant effect of discrimination on the time taken to reach criterion (RM ANOVA  $F_{(6,9)}=1.44$   $p=0.299$ ). There was also no significant effect of housing on the overall time to complete the task (RM ANOVA  $F_{(1,14)}=0.06$   $p=0.812$ ) (data not shown).

### 3.5 Discussion

The results in this chapter show that sub-chronic PCP treatment causes a specific impairment in ED-shifting. However, isolation rearing, which produced the expected hyperactivity in a novel arena, deficit in novel object discrimination and attenuation of PPI compared with social controls, caused no deficits in any aspect of the attentional set shifting paradigm, including the ED-shift.

In the sub-chronic PCP study no differences were seen in the basal locomotor activity in response to a novel arena in PCP treated rats, 3 days after cessation of treatment. Sub-chronic PCP treatment caused a long-lasting impairment in recognition memory, as measured by NOD, agreeing with previous data (Grayson et al., 2007). This deficit in NOD is long-lasting, still being present 23 days after the cessation of PCP treatment and after the completion of ID/ED testing. As PCP has a half-life of approximately 80 minute (Nabeshima et al., 1984) it will have been completely cleared from the body at this point, therefore the NOD deficit must be due to a long lasting effect of PCP treatment, rather than a direct pharmacological antagonism of NMDA receptors. Similar sub-chronic PCP treatment regimens have also been found to impair reversal learning performance (Abdul-Monim et al., 2006) and conditional discrimination (Dunn and Killcross, 2006), seven days after cessation of PCP treatment.

In the bowl-digging training no differences were found in the ability of the PCP and saline pre-treated rats to learn to discriminate, when either medium or odour was the relevant dimension. There was a trend ( $p=0.068$ ) towards odour requiring more trials to reach criterion than media, indicating it is possibly more difficult to learn to follow odour than medium. However further analysis of the data from the test sessions showed no effect of dimension on indices of the difficulty of the task. In addition, animals from each treatment group were equally divided between starting dimension and order of cue presentation to control for any potential differences in cue saliency and difficulty.

In the main bowl-digging test session the PCP treated rats were selectively impaired on the ED-shift discrimination, no differences being found between the saline and PCP pre-treated animals at any other discrimination stage. This replicates other studies in rats (Rodefer et al., 2005) and also models the situation found in first-episode schizophrenic patients (Joyce et al., 2002).

Isolation rearing induced behavioural changes in this batch of animals as confirmed by increased activity during the first 30 minutes of the LMA test, impairment in NOD and a reduction in PPI of acoustic startle response. However, in the attentional set shifting task no differences were found between the social and isolation reared animals during any of the discriminations, or during the training period.

As PCP and isolation rearing were not investigated in the same study it is not possible to compare directly between the two methods of modelling the cognitive deficits of schizophrenia. However, the controls in both studies performed to a similar level in the ED-shift in terms of number of trials to reach criterion. However, when the time taken to complete the ED-shift is compared, the social controls in the isolation rearing study took much longer to complete the discrimination ( $22 \pm 5.5$ min) than the saline treated controls in the PCP study ( $12.3 \pm 1.8$ min). This could be due to the age of the rats, the PCP treated rats started ID/ED when they were approximately 200-300g, whereas the isolation reared rats underwent ID/ED at 10-12 weeks old and weighed approximately 350-450g. Age has been found to cause impairments in ID/ED testing (Barense et al., 2002, Rodefer and Nguyen, 2006), so it is possible that the socially housed controls were beginning to demonstrate a slight age-related impairment, which was overcome in the isolation reared animals by their decreased habituation to novelty, i.e. the task was still new and interesting to them. Isolation reared rats have been found to be impaired at ED-shifting in a task requiring switching between spatial and non-spatial cues (Schrijver and Wurbel, 2001). The same laboratory used a bowl-digging paradigm and found isolated rats were impaired at reversal learning (but they did not measure ED-shifting) (Schrijver et al., 2004). In both of Schrijver's studies the rats were isolated for 10 weeks from weaning to the start of the behavioural testing. The

rats used in the current experiment underwent attentional set shifting testing after approximately 7-8 weeks, so it possible that this cognitive deficit develops after between 8-10 weeks isolation.

The bowl-digging task is a long procedure in which animals tire as the task progresses. It is possible that the lack of effect of isolation rearing in attentional-set shifting could be due to the amount of attention the animal is paying to the task. Isolation reared rats have been found to have only small impairments in the five choice serial reaction time task (5-CSRTT), with no reduction in accuracy (Dalley et al., 2002). While the effects of sub-chronic PCP have not been studied in 5-CSRTT, it has been found that other PCP regimens disturb performance to a much greater extent than is seen with isolation reared rats (Le Pen et al., 2003, Amitai et al., 2007). In a 3-CSRTT acute PCP reduced response accuracy by 30% and significantly increased reaction time (Jin et al., 1997). Thus it is possible that the ED-shifting impairment seen in PCP treated animals is in part due to a lack of attention during this complicated part of the task. As well as becoming tired, the animals may also become satiated after consuming large numbers of honey loops. Satiated animals are less likely to participate in the task, eventually refusing to dig and requiring a rest period. Isolation reared rats have been found to continue to perform food reward related behaviours even when satiated (Morgan, 1973, Morgan et al., 1975, Jones et al., 1991) which could be a potential confound.

In the PCP study the saline treated controls did not find the ED-shift any more difficult than the previous ID-shift or ID-reversal. This could suggest that the controls had not adequately learned an attentional set and therefore were not actually switching set in the ED-shift stage. To be certain that an attentional set has been formed the animals should find the ED-shift more difficult than an ID-shift. It is possible that exposing the animals to more ID-shifts before the ED-shift could help the animal focus in on the correct dimension more closely. If it is the case that the control animals have not formed an attentional-set, this could lead to the conclusion that, as the PCP treated animals found the ED-shift more difficult than the ID-shift, PCP is actually improving the ability to learn

an attentional-set, rather than impairing the ability to switch set. However, this would seem unlikely given the level of impairment demonstrated by sub-chronic PCP treated rats in many other cognitive tasks.

### **3.6 Conclusion**

The work in this chapter demonstrated that isolation reared rats are not impaired in the bowl-digging task of attentional set shifting when tested after 7 weeks of isolation. As attentional set-shifting shows direct translational validity to the cognitive deficits seen in schizophrenia this shows that isolation rearing is not modelling all of the impairments seen in this disease.

The work in this chapter also confirms that rearing pups in isolation from weaning produces robust impairment of recognition memory and PPI which are relevant to the impairments seen in schizophrenia. Therefore the next chapter will investigate the effects of the atypical antipsychotic drug clozapine on PPI.



4 **The Effect of Acute Clozapine on PPI in Isolation Reared Rats**

## 4.1 Aim

The work in this chapter aimed to ascertain the effects of acute treatment with the antipsychotic drug clozapine on isolation rearing induced deficits in prepulse inhibition of acoustic startle.

## 4.2 Introduction

Various animal models of the core symptoms of schizophrenia, including isolation rearing, have been used to assess the potential therapeutic benefit of putative anti-psychotic compounds. As discussed in Chapter 1, the predictive validity of an animal model is crucial if it is to be used in the development of novel anti-psychotic therapies. Currently the most commonly used drugs are the atypical anti-psychotics, exemplified by clozapine which was the first discovered. Whereas previous anti-psychotics, such as haloperidol mainly act as antagonists at the D<sub>2</sub> dopamine receptor, the atypical anti-psychotics have affinity for several other receptors. For example, clozapine has high affinity for D<sub>4</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>6</sub>, M<sub>1</sub>, Histamine H<sub>1</sub> receptors and  $\alpha_1$  adrenoceptors, amongst others, with lower affinity for GABA, D<sub>2</sub>, NMDA,  $\alpha_2$  and  $\beta$ -adrenoceptors (Ashby and Wang, 1996). It is believed that this mixed pharmacology may be responsible for the different therapeutic profile, with atypical anti-psychotic drugs being more active against the positive and negative symptoms of schizophrenia. In this chapter the predictive validity of the isolation rearing model was evaluated using an acute dose of clozapine on PPI of acoustic startle as several previous studies have examined the impact on this particular behavioural change (Geyer et al., 2001a).

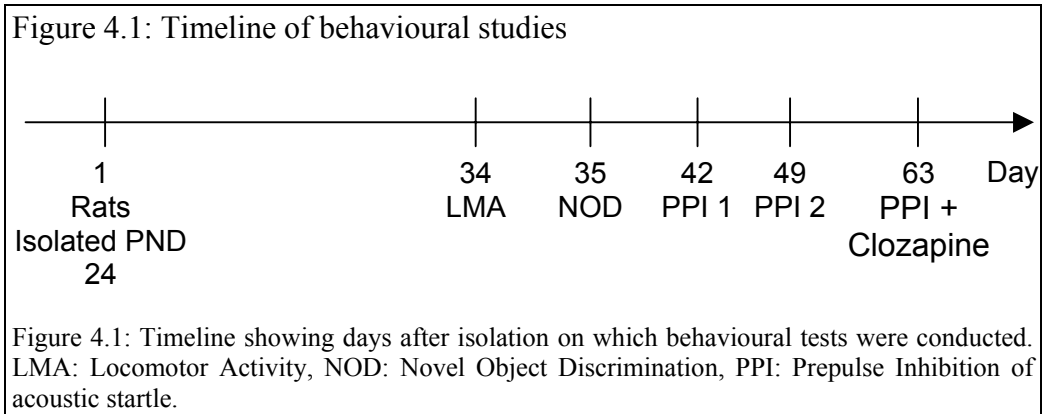
### 4.3 Methods

#### *Animals*

Male Lister Hooded rats (BMSU, Nottingham), PND 24, were housed in groups of 3 or 4 (social) (n=18) or alone (isolates) (n=19) with the husbandry conditions previously described (Chapter 2) for 5 weeks.

#### *Behavioural Testing*

LMA, NOD and PPI were carried out as described in Chapter 2, according to the timeline depicted in Figure 4.1.

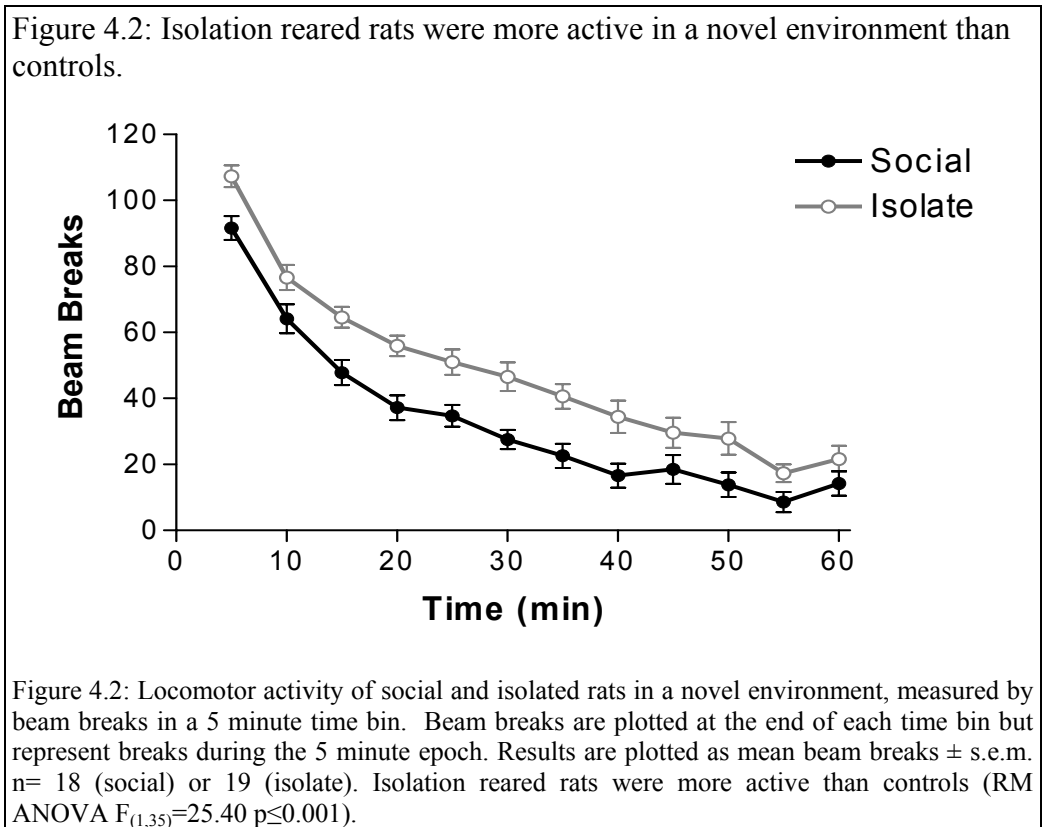


On PND 87 (9 weeks after isolation rearing commenced) the effect of acute clozapine on PPI was tested. 5mg/kg Clozapine (Sigma UK) was dissolved in vehicle (1M HCl, diluted with 0.9% NaCl and buffered to pH 5.6 using NaOH). Rats were dosed with clozapine or vehicle at 1ml/kg, 45 minutes prior to PPI testing.

## 4.4 Results

### *Locomotor Activity*

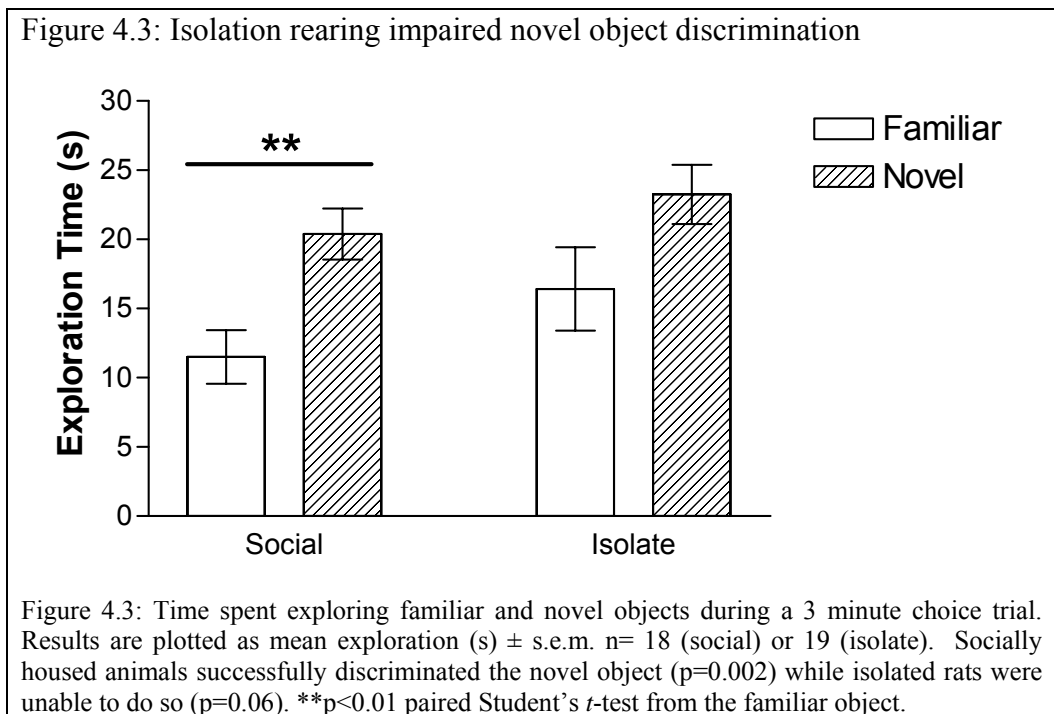
Over the course of the one hour test all animals habituated to the LMA box, as shown by a significant reduction in activity (RM ANOVA, time effect  $F_{(11,385)}=111.45$   $p \leq 0.001$ ) (Figure 4.2). Isolation reared rats were more active in the novel environment than socially housed controls (RM ANOVA  $F_{(1,35)}=25.40$   $p \leq 0.001$ ). Isolation reared rats also exhibited more rearing than social controls (RM ANOVA  $F_{(1,35)}=44.83$   $p \leq 0.001$ ), data not shown.



### *Novel Object Discrimination*

In the NOD familiarisation trial (T1) there was no difference in total object exploration time, irrespective of rearing condition (Student's  $t$ -test  $p=0.166$ ). Neither isolation reared nor social control rats showed a preference for object position in the familiarisation trial (paired Student's  $t$ -test: social  $p=0.824$ , isolate  $p=0.616$ ), data not shown.

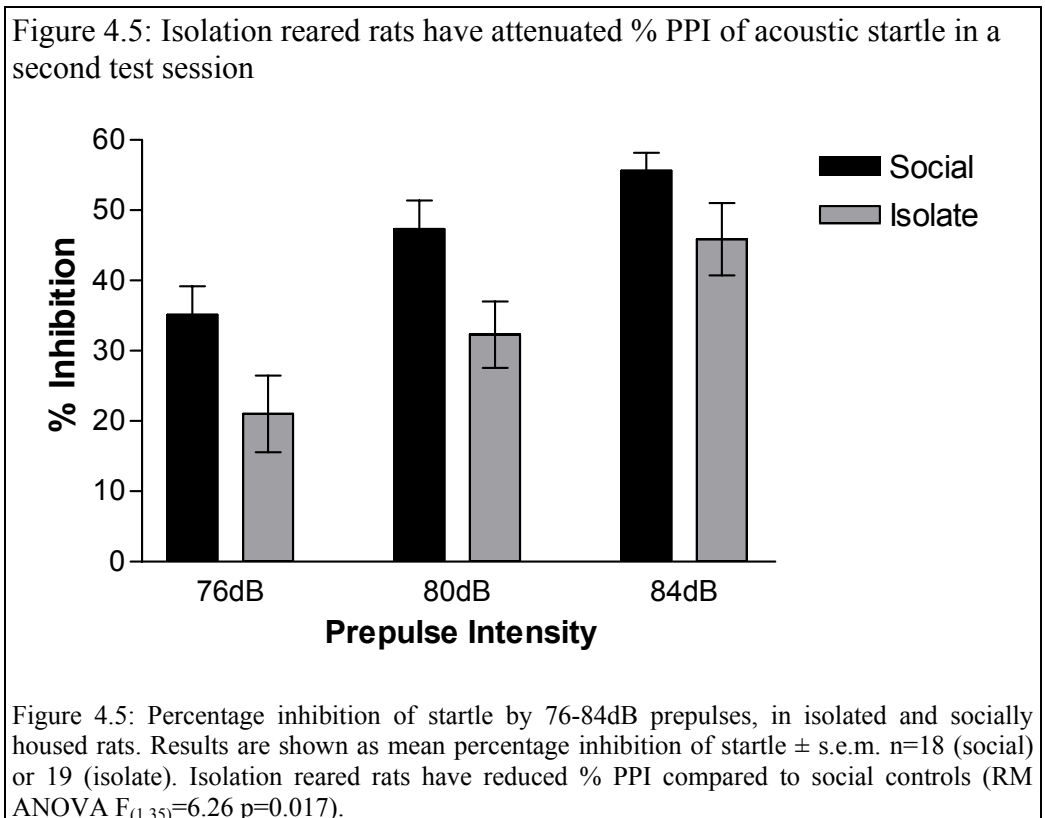
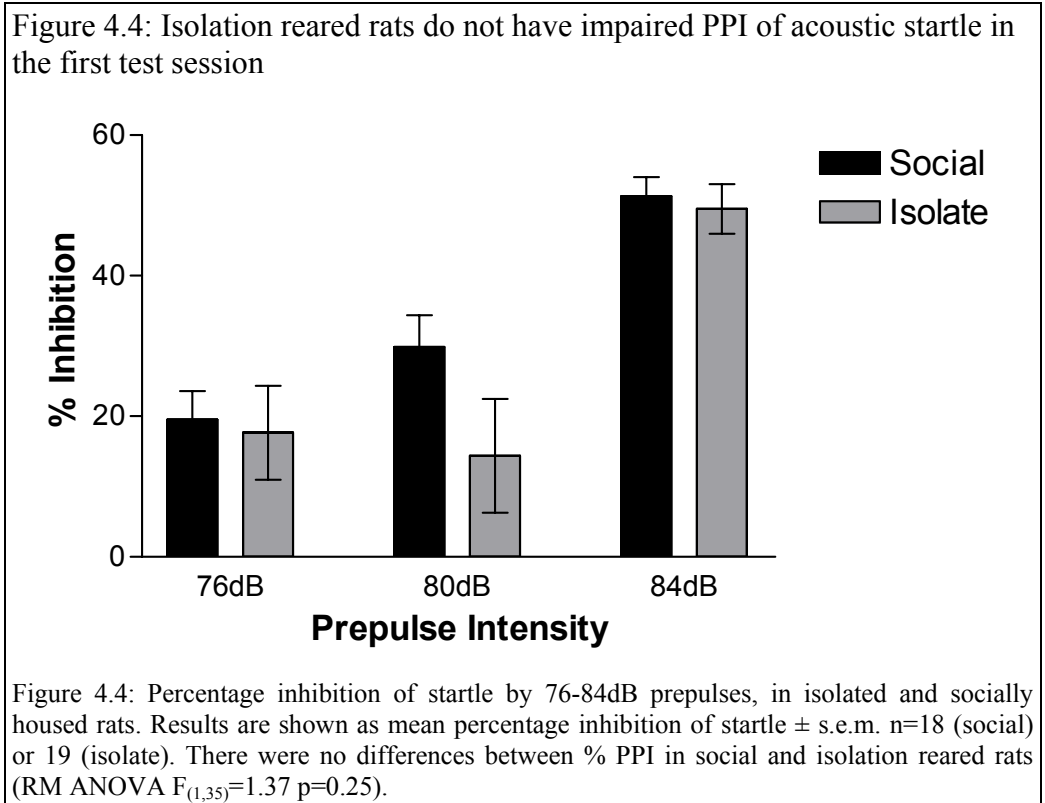
In the choice trial (T2) only social rats successfully discriminated the novel object (paired Student's *t*-test: Social  $p=0.002$ , Isolate  $p=0.06$ ) rats, as shown in Figure 4.3. However when the discrimination ratios were analysed no difference was found between isolation reared and socially housed animals (DR: mean  $\pm$  s.e.m social= $0.320 \pm 0.071$ , isolate= $0.208 \pm 0.063$  Student's *t*-test  $p=0.246$ ). No differences were found in total exploration during the choice trial (Student's *t*-test  $p=0.125$ ).



#### *PPI of Acoustic Startle*

In the first acoustic startle PPI test session, following 6 weeks isolation, the magnitude of % PPI increased progressively with pre-pulse intensity (RM ANOVA  $F_{(2,70)}=29.80$   $p\leq 0.001$ ) (Figure 4.4). However, no differences were seen between social and isolation reared rats (RM ANOVA  $F_{(1,35)}=1.37$   $p=0.25$ ). The rats were then re-tested a week later to see if PPI deficits had developed in the animals after this additional isolation period. In the second test % PPI also increased as prepulse volume increased (RM ANOVA  $F_{(2,70)}=24.06$   $p\leq 0.001$ ) (Figure 4.5) but now PPI was attenuated in isolation reared rats compared to socially housed controls (RM ANOVA  $F_{(1,35)}=6.26$   $p=0.017$ ). In contrast, housing condition had no effect on startle amplitude in

either PPI test (Student's *t*-test, PPI 1:  $p=0.306$ . PPI 2:  $p=0.961$ ), data not shown.



*Effect of Acute Clozapine Treatment on PPI of Acoustic Startle*

After a further 2 weeks of isolation the effect of acute clozapine on PPI was tested. Rats received 5mg/kg clozapine or vehicle, 45minutes before PPI testing. As expected % PPI increased as pre-pulse intensity increased (RM ANOVA  $F_{(2,66)}=33.01$   $p \leq 0.001$ ). However, isolation reared rats were no longer impaired compared to socially housed animals (RM ANOVA  $F_{(1,33)}=1.07$   $p=0.309$ ) (Figure 4.6). Clozapine caused a significant increase in PPI, both overall (RM ANOVA  $F_{(1,33)}=7.13$   $p=0.012$ ) and specifically increasing PPI in socially housed animals only (RM ANOVA housing x treatment interaction  $F_{(1,33)}=4.32$   $p=0.046$ ).

Housing condition had no effect on amplitude of startle in this PPI test (2-way ANOVA  $F_{(1,33)}=0.66$   $p=0.442$ ). Clozapine treatment showed a trend towards decreasing startle amplitude which failed to reach significance (2-way ANOVA  $F_{(1,33)}=3.50$   $p=0.070$ ) (Figure 4.7).

Figure 4.6: Clozapine treatment increases % PPI in socially housed animals only.

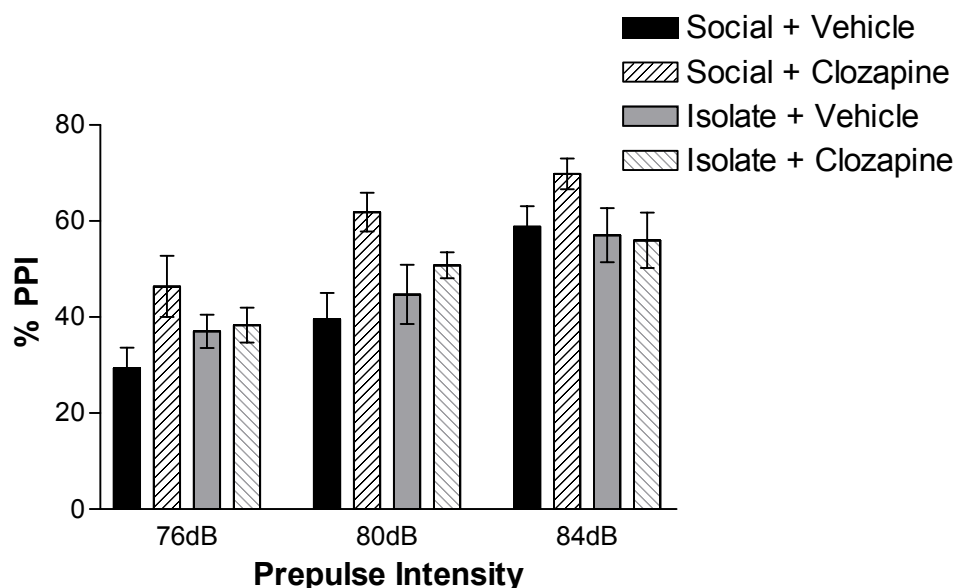


Figure 4.6: Percentage inhibition of startle by 76-84dB prepulses, in isolated and socially housed rats, treated with vehicle or 5mg/kg clozapine. Results are shown as mean percentage inhibition of startle  $\pm$  s.e.m.  $n=10$  (isolate/vehicle) or 9 (all other groups). Isolation reared rats do not have reduced % PPI compared to social controls. Clozapine significantly increases PPI overall and specifically in socially housed animals (RM ANOVA, treatment effect  $F_{(1,33)}=7.13$   $p=0.012$ , housing x treatment interaction  $F_{(1,33)}=4.32$   $p=0.046$ ).

Figure 4.7: Neither housing condition nor drug treatment had a significant effect on startle amplitude

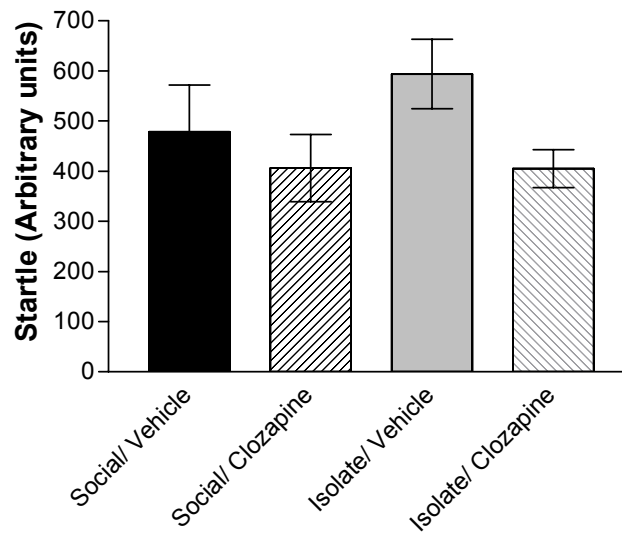


Figure 4.7: Startle amplitude (arbitrary units) in response to 120dB pulse alone, in social or isolation housed rats, treated with vehicle or clozapine. Results are shown as mean  $\pm$  s.e.m.  $n=10$  (isolate/ vehicle) or 9 (all other groups). Neither housing nor drug treatment had a significant effect on startle (2-way ANOVA, Housing:  $F_{(1,33)}=0.66$   $p=0.442$ , Treatment:  $F_{(1,33)}=3.50$   $p=0.070$ ).



## 4.5 Discussion

Overall the data in this chapter show that acute clozapine treatment increases PPI and has a greater effect in social than isolation reared rats.

The reported effects of acute clozapine on PPI in naive rats are inconsistent. Previous groups have reported no change in PPI using 8mg/kg (Johansson et al., 1995) or increases in PPI in rats with 20mg/kg clozapine in rats (Depoortere et al., 1997) and mice (2mg/kg) (Fejgin et al., 2007), as was found in this study where a 5mg/kg dose was used. It should be noted that in the current study clozapine caused a near-significant reduction in basal startle response to the 120dB pulse ( $p=0.07$ ), indicative of a sedative effect of the drug, which can confound the interpretation of PPI results.

In isolation reared rats the typical anti-psychotics haloperidol and raclopride (Geyer et al., 1993, Varty and Higgins, 1995) and the atypical antipsychotics clozapine (5mg/kg), risperidone, olanzapine and seroquel (Varty and Higgins, 1995, Bakshi et al., 1998), but not iloperidone (Barr et al., 2006), have reversed isolation rearing induced PPI deficits. Clozapine has been found to reverse the PPI deficits found in other models of disrupted sensorimotor gating, including those produced by neonatal ventral hippocampal lesions (Le Pen et al., 2003) and PCP treatment of capuchin monkeys (Linn et al., 2003) and Wistar rats (Yamada et al., 1999). However, there may be differences in sensitivity to clozapine between strains of rat, as the PCP induced PPI deficit in SD rats is reversed in some laboratories (Ballmaier et al., 2001), but not all (Wiley, 1994, Wiley and Kennedy, 2002). As long-term treatment with clozapine alleviates the PPI impairment in schizophrenic patients (Kumari et al., 1999, Oranje et al., 2002, Kumari et al., 2007) the response of isolation reared rats to chronic dosing with clozapine may be more relevant to the clinical situation.

Although clozapine had no effect on isolation reared rats in these studies, PPI was increased in drug-naïve socially reared animals. As clozapine has affinity at many receptors some attempts have been made to identify the specific roles

of these receptors in clozapine's effects on PPI. PPI disruption by the H<sub>1</sub> antagonist pyrilamine is attenuated by clozapine (Roegge et al., 2007). Clozapine has high affinity for the H<sub>1</sub> receptor (Ashby and Wang, 1996) and therefore this may play a role in PPI restoration. Clozapine also has a high affinity for the 5-HT<sub>2A</sub> receptor (Ashby and Wang, 1996) and the ED<sub>50</sub> of a range of antipsychotics to reverse PCP-induced PPI deficits correlates with their affinity for the 5-HT<sub>2A</sub> receptor, but not 5-HT<sub>2C</sub>, or dopamine D<sub>2</sub> receptors (Yamada et al., 1999). Interestingly the 5-HT<sub>2A</sub> antagonist MDL100907 partially reduced an isolation rearing induced PPI deficit, indicating 5-HT<sub>2A</sub> receptors are involved in PPI restoration in isolation reared rats (Geyer et al., 1999). However, antagonism at the  $\alpha$ -adrenoceptor is probably not involved in PPI restoration by clozapine as the  $\alpha$ -adrenoceptor antagonist idazoxan, in combination with raclopride, did not reverse PCP-induced PPI deficits (Ballmaier et al., 2001).

As other laboratories have managed to reverse isolation-induced PPI deficits, with similar doses of clozapine (Varty and Higgins, 1995), it maybe that isolation rearing itself is simply not sufficiently reproducible to be used as a reliable anti-psychotic screen on its own. It should be noted that in this study the isolation reared/vehicle treated rats did not show PPI deficits during the third (clozapine) PPI test. The first PPI test (before clozapine dosing) also failed to find PPI deficits, so it is possible that the isolation syndrome may have required longer to develop fully in this group of isolation reared animals. Close examination of the NOD results shows there were no difference between the discrimination ratios of isolation reared and socially housed animals further suggesting a partial failure of the isolation procedure in this cohort of rats.

As the isolation rearing induced prepulse inhibition deficits can be variable future studies in this thesis will examine the impact on a battery of behavioural changes that have translational relevance to the core symptoms seen in schizophrenia including those directly related to cognitive dysfunction, which is currently resistant to antipsychotic medication in schizophrenics.

#### **4.6 Conclusion**

- Acute clozapine increases PPI only in social rats.
- The isolation reared rats did not demonstrate reproducible deficits in PPI at the time point measured and a battery of behavioural observations is required to assess any impact of isolation rearing.

The work in the next chapter will investigate whether components of the isolation syndrome including cognitive impairment is reversed by treatment with novel putative nootropic drugs.

**5 The Effects of Nootropic Drugs on Isolation Reared Rats**

## 5.1 Aim

This chapter will investigate the effects of two cognitive enhancing drugs that act via different pharmacological mechanisms on isolation rearing induced NOD impairments and ascertain whether isolation reared rats show impairments in other cognitive tasks.

- The 5-HT<sub>6</sub> antagonist, Ro 04-6790, in the water maze.
- Aniracetam, an AMPA potentiator, in passive avoidance.

## 5.2 Introduction

As previously shown in Chapters 2, 3 and 4, isolation rearing often causes impairments in recognition memory. However, no impairments were found in attentional set shifting. In this chapter the effect of isolation rearing was examined in two other types of memory, spatial learning and emotional memory. Further to this the efficacy of two putative cognitive enhancing compounds on reversing isolation rearing induced cognitive deficits was investigated. The two nootropic compounds were the 5-HT<sub>6</sub> antagonist, Ro 04-6790, and the AMPA potentiator aniracetam.

### 5.2.1 Effect of Ro 04-6790 on the Performance of Isolation Reared Rats in the Water Maze

Several previous studies have looked at the effect of isolation rearing on spatial learning in the water maze. However, as discussed in Chapter 1, the results have been inconsistent with isolation rearing causing no change (Lapiz et al., 2003, Schrijver et al., 2004), improvements (Wongwitdecha and Marsden, 1996b) and deficits (Hellemans et al., 2004) in acquisition of platform location. However, in this study the aim was to investigate more than just the acquisition of a spatial location, but also to move the platform location and see how the animals adapted. In this reversal learning stage of the protocol the platform was

moved daily, for 3 days to see if the animals were able to adopt a new searching strategy.

In this study the animals were also treated with a 5-HT<sub>6</sub> antagonist 4-amino-N-(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790). This compound has previously been shown to have cognition enhancing properties in the NOD paradigm (King et al., 2004), increasing the length of ITI at which rats can discriminate the novel object. The atypical antipsychotic clozapine has a high affinity for the 5-HT<sub>6</sub> receptor (Ashby and Wang, 1996), which has led to speculation that this receptor might have a role in clozapine's antipsychotic actions. Therefore this study will also investigate the effect of Ro 04-6790 on isolation rearing induced hyperactivity in a novel environment and reversal learning in the water maze.

### **5.2.2 Effect of Aniracetam on Passive Avoidance in Isolation Reared Rats**

As discussed in Chapter 1, there is considerable evidence for glutamatergic dysfunction in schizophrenia. Positive modulators of the AMPA receptor, including the so called AMPAkinases, have shown promise as cognitive enhancers in pre-clinical studies (Bartolini et al., 1996, Nakamura et al., 1998, Lebrun et al., 2000). These AMPA modulators have been found to slow AMPA receptor deactivation by stabilizing the glutamate bound conformation of the receptor (Jin et al., 2005). The aim of this study was to see if an AMPA modulator, aniracetam (1-*p*-anisoyl-2-pyrrolidinone), has any effect on isolation rearing induced cognitive deficits. As with the previous studies described in this thesis the isolation reared rats were tested in NOD and the effects of aniracetam on this cognitive task were assessed. However, as well as testing recognition memory, this study also investigated the effect of isolation rearing on the type of fear-related memory known as passive avoidance. In passive avoidance the animal receives an electric shock from the grid floor in a black chamber of the test box. The following day the animal is placed in the connected white chamber and allowed to freely move into the dark chamber in

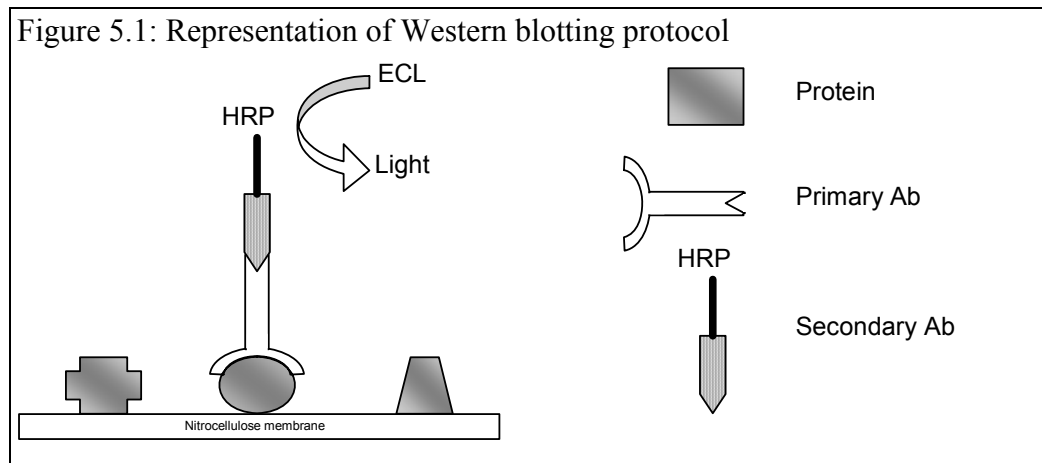
his own time. Animals that associate the black chamber with the electric shock will remain in the white chamber for longer and may refuse to enter the black chamber at all. Passive avoidance is a form of fear related memory which is mediated by the amygdala (Davis, 1992). In relation to this it is relevant to know that schizophrenics have problems recognising facial emotions, which has also been attributed to amygdala dysfunction (Namiki et al., 2007).

The role of the ionotropic glutamate receptors in schizophrenia has been under investigation due to the similarity between the psychotic symptoms of schizophrenia and the effects of the NMDA antagonist PCP. NMDA receptors are made up of 4 or 5 subunits, known as NR1 and NR2A-D. The NR1 subunit is required for a functional receptor, but the expression of the NR2 subunits has been found to be region specific and change through development (Sheng et al., 1994, Kim et al., 2005). Studies have found reductions in NR1 expression in superior temporal cortex and the superior frontal cortex, but not the prefrontal cortex in schizophrenic patients (reviewed by (Meador-Woodruff and Healy, 2000). NR1 mRNA expression is also reduced in the hippocampus of schizophrenic patients (Gao et al., 2000), as well as a reduction in vesicular glutamate transporter (VGLUT) mRNA (Eastwood and Harrison, 2005). VGLUT is selectively expressed in pre-synaptic glutamatergic synapses and therefore gives an indication of a decline in number or activity of glutamatergic synapses in the hippocampus in schizophrenia (Harrison et al., 2003) (Harrison and Eastwood, 2003). The expression of a synaptic protein associated with excitatory neurons, complexin II, has been found to be reduced in the hippocampus of schizophrenic patients (Sawada et al., 2005), also suggesting a decrease in glutamatergic function in the hippocampus.

In rats NR2B expression is high at birth and declines during adulthood, whereas NR2A expression appears around PND7 and increases over 2-3 weeks to adult levels (Sheng et al., 1994, Kim et al., 2005), which suggests they may be vulnerable to a development manipulation such as isolation rearing. Isolation reared rats have been shown to exhibit impairments in learning and memory which are processes dependent on synaptic plasticity which is mediated by NMDA receptors (Kim et al., 2005). At resting membrane

potential NMDA receptors are blocked by  $Mg^{2+}$ . A small depolarisation removes the  $Mg^{2+}$  and allows the NMDA channel to open and  $Ca^{2+}$  to pass through (Moghaddam, 2003). This depolarisation is provided by AMPA receptor activation (Rao and Finkbeiner, 2007). As NMDA and AMPA receptors are so closely linked the effects of isolation rearing and aniracetam treatment on levels of NR1, NR2A and NR2B protein were investigated in the hippocampus.

The levels of NR subunit protein were investigated by Western blotting (or immunoblotting), which is a widely used method for detection and semi-quantitative analysis of proteins. This method separated proteins by electrophoresis on an acrylamide gel. The proteins are then transferred (blotted) onto a membrane and detected using an antibody specific to the protein, as depicted in Figure 5.1. A secondary antibody conjugated to a detection system then binds to the first (primary) antibody. In these studies the secondary antibody was conjugated to horse-radish peroxidase (HRP) and detected using enhanced chemi-luminescence (ECL) reagents in which the oxidation of luminol is catalysed by the HRP in a light emitting reaction. This light is then detected using photographic film.



It was essential to establish a suitable dose of aniracetam which would have pro-cognitive effects. Therefore the first stage of the aniracetam studies was a small dose response study on the effect of acute aniracetam in novel object recognition, using rats of a similar age to those which would be used in the isolation rearing studies.



### 5.3 Methods

#### 5.3.1 Effect of Ro 04-6790 on the Performance of Isolation Reared Rats in the Water Maze

##### *Animals*

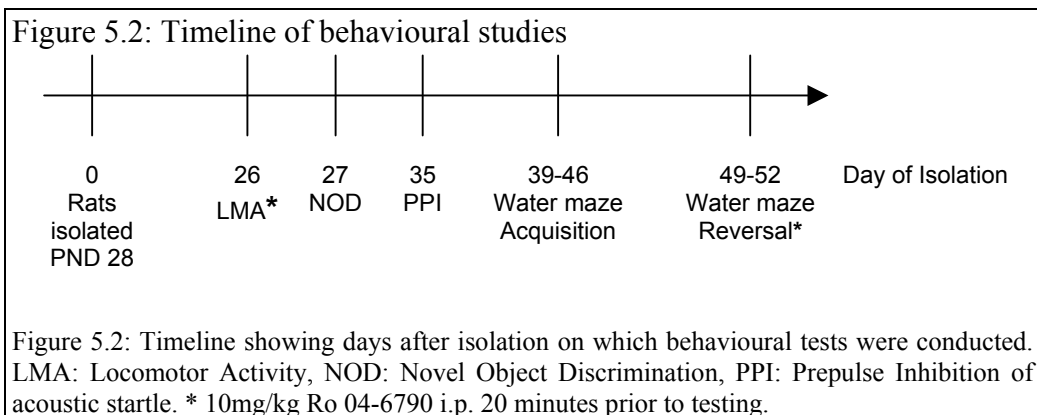
Thirty-two male Lister Hooded rats (BMSU, Nottingham), PND 24, were housed in groups of 3 or 4 (social) or alone (isolates) (n=16) and housed as previously described (see Chapter 2) for 5 weeks.

##### *Drugs*

The 5-HT<sub>6</sub> antagonist Ro 04-6790 (donated by F. Hoffman-La Roche) was administered at 10mg/kg in a 0.9%NaCl vehicle at 1ml/kg, i.p., 20 minutes prior to behavioural testing in locomotor activity and reversal learning in the water maze. This dose of Ro 04-6790 was chosen as several previous studies in our laboratories have shown 10mg/kg to exert cognition enhancing effects in both novel object discrimination and the water maze (Woolley et al., 2001, Woolley et al., 2003, King et al., 2004).

##### *Behavioural Testing*

Locomotor activity in a novel arena, novel object discrimination and prepulse inhibition of acoustic startle were carried out as described in Chapter 2, according to the timeline depicted in Figure 5.2.



### *Water Maze*

The water maze protocol was adapted from that originally described by Morris et al (Morris, 1984). The maze consisted of a fibreglass circular pool (2m diameter, 0.7m high) situated in the centre of a test room, underneath a video camera linked with software to track animals' movements around the maze (Ethovision Animal Tracking system, Noldus). There was sufficient space around the maze to walk right around it, but the room was small enough for the rat to see the walls from the centre of the maze. Distinctive visual cues (black patterns on white cards) were placed on the walls, as extra-maze cues to assist spatial orientation.

The maze was filled with water at room temperature and made opaque using 50ml of white opacifier (Taski Calcacio 7244048, F Hoffman-La Roche). A 10cm x 10cm Perspex platform was 1.5cm below the water surface and positioned 43cm from the poolside, in one of four positions, designated North, East, South and West. The platform could be reliably positioned using fixed runners on the base of the maze.

Rats commenced water maze training on PND 67, in the 6<sup>th</sup> week of isolation. The water maze protocol was nine days long, consisting of habituation, acquisition, a probe test and reversal learning phases.

#### **Habituation**

On day one (Friday) of water maze training rats underwent a two minute habituation trial, in which the platform was not present, to eliminate subsequent thigmotaxic behaviour.

#### **Acquisition**

On days 2-6 (Monday- Friday) rats underwent acquisition learning. The platform was placed in a fixed position for each animal, but balanced across groups. Each rat underwent three 90s trials a day (with an ITI of 30s), being placed into the water facing the sidewall of the maze, starting from each of the three positions not occupied by the platform, in a pseudorandom order each day. After each trial rats were either placed or allowed to stay on the platform for 20s before being removed from the maze and dried.

On day six the rats also underwent a 60s probe test (no platform present) to ascertain how well they had learnt the position of the platform and the time spent swimming in each quadrant of the maze was recorded. Time spent swimming in the quadrant where the platform had previously been positioned was taken as an indicator of memory of the platform position during acquisition.

### **Reversal Learning**

On days 7-9 the protocol was changed to a reversal learning paradigm in which the platform was placed in a new position on each of the 3 consecutive reversal test days. The rats needed to adopt a new strategy to find the platform, such as by swimming in circles round the maze, rather than across it. As in the acquisition phase, rats received three 90s trials each day, with 20s on the platform after each trial. The platform was moved in the same order for each rat: opposite the original position, then clockwise from the original, followed by anti-clockwise from the original. On each day of reversal learning the platform remained in the same place for the three trials.

### **Statistical Analysis**

The acquisition trial duration (time taken to find the platform) was analysed by repeated measures ANOVA with housing condition as the between group variable and trial number as the within group variable. The equality of the variances of the data was checked using Mauchley's Test of Sphericity. If data failed to meet assumptions of sphericity the Greenhouse-Geisser correction was applied. This test was applied across all 5 days and also within the trial for each day. The same RM ANOVA method was also used to analyse the reversal learning section of the paradigm.

The time spent in each quadrant during the probe test was also analysed by RM ANOVA, using quadrant as a within group factor and housing as a between group factor. The effect of housing and drug treatment on average swim speed in the reversal learning trials were analysed by 2-way ANOVA.

### **5.3.2 Effect of Acute Aniracetam on NOD and PPI in Social Rats**

#### *Animals*

To determine a suitable dose of aniracetam for use in the chronic dosing study, 24 male Lister Hooded rats (BMSU, Nottingham), 350-400g, were housed in groups of 4.

#### *Drugs*

Animals received vehicle (1ml/kg 10% hydroxypropyl  $\beta$  cyclodextrin (Sigma, UK)), 15mg/kg or 30mg/kg aniracetam (Sigma, UK), i.p. one hour prior to behavioural testing in NOD and PPI. Animals received the same drug treatment in both NOD and PPI.

#### *Novel Object Discrimination*

NOD was carried out as described in Chapter 2, using a 2 hour ITI. Drug treatment was given one hour before the start of the familiarisation trial (T1).

#### *PPI of Acoustic Startle*

PPI was also carried out as described in Chapter 2, one week after NOD testing. Drug treatment was given one hour before the start of PPI acclimation.

### **5.3.3 Effect of Sub-chronic Aniracetam in Isolation Reared Rats**

#### *Animals*

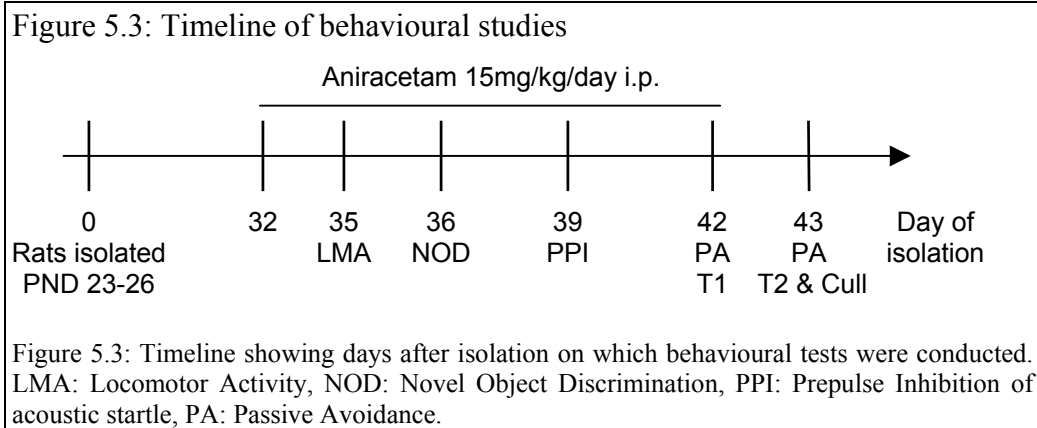
40 male Lister Hooded rats (Charles River, UK), (PND 23-26) were housed in groups of 4 (social) or alone (isolates) (n=20) for 5 weeks with minimal handling. Housing conditions were as described in Chapter 2.

#### *Drugs*

Animals received either vehicle (1ml/kg 10% hydroxypropyl  $\beta$  cyclodextrin (Sigma, UK)) or 15mg/kg aniracetam (Sigma, UK), i.p. daily at 09:00, or 1 hour prior to behavioural testing, on days 32-42 after isolation.

*Behavioural Testing*

LMA, NOD and PPI were carried out as described in Chapter 2, according to the timeline depicted in Figure 5.3. Passive avoidance (PA) was carried out as described below. All behavioural testing was carried out between 09:00 and 14:00.

*Passive Avoidance*

Passive avoidance was assessed in a rat shuttle box (LE916, PanLab, Spain) and controlled by computer using the manufacturer's software (Shutavoid, PanLab). The shuttle box consisted of a white and a black chamber (each 25 x 25 x 24cm) with a door between. Each chamber had a transparent Perspex door to allow the experimenter to view the experiment and easily access the animals. Both chambers had grid floors through which an electric shock could be administered. The grid floors also incorporated weight transducers to allow the system to monitor the location of the animals.

In the first, habituation, trial (T1) the rat was placed into the white chamber and allowed 30 seconds to habituate. After 30 seconds the door between the chambers was opened and the latency for the animal to move into the black chamber was recorded. Once the rat had moved all 4 feet into the black chamber the door was closed. Immediately after the door was closed a 1s 0.4mA shock was delivered via the grid floor. After receiving the shock the rat was left for 10s then removed from the shuttle box and placed back in the home cage.

After 24 hours memory retention was tested in a second trial (T2). The animal was placed back into the white chamber and allowed 30s habituation, as in the first trial. The door between the chambers was then opened and the time taken for the rat to move into the dark chamber was recorded, up to a maximum of 300s.

#### **Statistical Analysis**

In both T1 and T2 the latency to enter the dark compartment was compared by 2-way ANOVA, with housing and drug treatment as factors.

#### *Western Blotting*

On day 42 after isolation rats in the aniracetam study were culled by stunning followed by decapitation. The brains were removed and the left hippocampus dissected out, at 4°C, weighed, then frozen in liquid nitrogen and held at -80°C until use.

#### **Sample Preparation and Protein Determination**

Samples were homogenised, on ice, using a polytron (Ultra-turrax T8, IKA Labortechnik, Germany) in 1ml lysis buffer (20mM Tris(hydroxymethyl) amidomethane (Tris,) 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA), 320mM sucrose, 0.1% Triton X100, 1mM NaF, 10mM  $\beta$  glycerophosphate, pH 7.6 plus protease inhibitor cocktail (Sigma UK, P8340)) per 60mg of tissue. Samples were rotated for 10 minutes at 4°C then centrifuged (Harrier 18/80, Sanyo/MSE) at 15115g for 10 minutes at 4°C. 150 $\mu$ l of the supernatant was transferred to a new tube and 150 $\mu$ l of 2x sample buffer (0.125M Tris, 20% v/v glycerol, 2% w/v sodium dodecyl sulphate (SDS), 10% v/v  $\beta$  mercaptoethanol and 0.001% bromophenol blue) added.

A separate aliquot of homogenate was taken to determine protein concentration using the Lowry method (Lowry et al., 1951). Briefly, nine protein standards of 0-0.4mg/ml bovine serum albumin (Sigma, UK) were made and run in the assay alongside the samples, which were diluted 1:10 in distilled water to a final volume of 200 $\mu$ l. The Lowry AB test solution was made up of 200ml of solution A (0.4% w/v NaOH, 0.2% SDS and 2% Na<sub>2</sub>CO<sub>3</sub>) and 100 $\mu$ l solution

B (1% w/v CuSO<sub>4</sub> and 2% NaK Tartrate). One ml of the Lowry AB solution was added to each sample and standard and incubated at room temperature for 10 minutes. A 1:1 dilution of Folin reagent (Sigma, UK) with distilled water was made and 100µl added to tube. This was left at room temperature for 45 minutes then 200µl of each sample and standard was plated into a 96 well plate in duplicate. The plates were read on a microplate reader (Dynatech MRX using Revelation software) at 750nm absorbance.

Following calculation of the protein levels, all sample protein levels were normalised to 1µg/µl using a 1:1 mixture of 2x sample buffer and lysis buffer and stored at -20°C.

#### **Electrophoresis and Blotting**

Samples were defrosted, heated to 95°C for 5 minutes, mixed briefly and spun in a microcentrifuge for 1 minute. Samples were then loaded on to SDS-7% polyacrylamide gels for electrophoresis. The amount of sample loaded was different for each protein being investigated and is summarised in Table 5.1, along with the dilutions of antibodies used. A set of standard marked proteins of known molecular weight (Precision Plus Protein Standards, BioRad, UK) were run alongside the samples to show the distance travelled by protein of each weight. The gels were run in electrophoresis buffer (25mM Tris, 192mM glycine, 3mM SDS) at room temperature. Samples were separated for 40 minutes at 200V using BioRad MiniProtean III equipment.

Once the sample proteins had been separated they were transferred onto a nitrocellulose membrane (Amersham, UK) in transfer buffer (25mM Tris, 192mM glycine 20% v/v methanol) at 4°C, for 1 hour at 100V, also using BioRad Mini Protean III equipment.

The nitrocellulose membrane was then stained with Ponceau S solution (Sigma UK) which stains all protein pink. This step is a quick check to ensure the running and transfer steps have worked correctly as band of protein should be clearly visible on the membrane. The Ponceau stain was washed off the

membrane using water and Tris buffered saline with Tween (TBS-T: 25mM Tris, 125mM NaCl, 0.1% v/v Tween 20 pH 7.6). Once all pink colouration had been removed the non-specific binding on the membranes was blocked using 5% (w/v) skimmed milk powder in TBS-T gently agitated for 1 hour at room temperature. The lower half of the membrane, which contained the low molecular weight proteins, was then incubated with 1/200,000 dilution of anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab9484, AbCam, UK) which is a ubiquitous glycolytic enzyme (MW 36kDa) and is used as a house-keeping protein to ensure slight differences in protein loading can be corrected for. The upper half of each membrane was incubated with an antibody to an NR subunit, at the dilutions shown in Table 5.1. All antibodies were diluted in 5% skimmed milk. The membranes and antibody solutions were sealed in polythene bags and incubated overnight at 4°C while shaking. A separate set of samples was run and blotted for each NR subunit. Each antibody detected protein at a weight that corresponded to the known weight of the protein in question when compared to marker proteins of known weight run on the same gel. The NR2A and NR2B antibodies both detected a single band of protein, whereas the NR1 antibody detected a double band, the whole of which was used for quantification of optical densities. The antibodies were chosen based on previously published characterisation (Hanaoka et al., 2003, Lindahl and Keifer, 2004).

The following day the excess antibody was removed with 9 washes in TBS-T over a one hour period, at room temperature. The secondary antibody was then added, again in 5% milk, and incubated at room temperature for one hour, while shaking. The secondary antibodies were conjugated with horse radish peroxidase (HRP) which catalyses the enhanced chemical luminescence (ECL) reagents in a light emitting reaction. Following secondary antibody incubation the membranes were again washed 9 times over one hour, with a final wash in water. The membranes were then incubated with the ECL reagents (Amersham, UK), as per manufacturer's directions, and exposed to autoradiography film (Kodak, UK). The levels of NR2B protein were low so to enhance the signal, for this protein only, ECL-plus reagents (Amersham, UK) were used which



increase the light emitted by the reaction, allowing a shorter exposure to film than if standard ECL reagents were used.

The density of the protein bands was determined using a densitometer (BioRad, UK) and the manufacturer's software, Quantity One. To account for small variations in protein loading band density was first normalised to the GADPH level. All densities were then expressed as a percentage of the mean control (social/vehicle) density for that gel. Samples were distributed such that each gel contained similar numbers of samples from each treatment group. This required three gels for each protein analysed.

#### Statistical Analysis

Data were analysed by 2-way ANOVA with housing condition and drug treatment as the factors.

Table 5.1: Amount of sample loaded and antibody used in western blotting studies

	NR1	NR2A	NR2B
Sample loaded ( $\mu\text{g}$ )	15	15	10
Approx. weight (kDa)	110	180	180
Primary antibody	Mouse Anti-NMDAR1 (MAB363, Chemicon, USA) 1/2000	Rabbit Anti-NMDAR2A (AB1555P, Chemicon, USA) 1/2000	Rabbit Anti-NMDAR2B (AB1557P, Chemicon, USA) 1/1000
Secondary antibody	Goat Anti-mouse (Dako, UK) 1/2000	Goat Anti-rabbit (Dako, UK) 1/2000	Goat Anti-rabbit (Dako, UK) 1/2000
ECL & film exposure time	ECL, 5 min exposure	ECL, 10 min exposure	ECL Plus, 30 min exposure

## 5.4 Results

### 5.4.1 Effect of Ro 04-6790 on the Performance of Isolation Reared Rats in the Water Maze

#### *Locomotor Activity*

As expected, when placed in a novel arena LMA decreased over the test period as animals habituated, such that there was a significant effect of time (RM ANOVA  $F_{(11,308)}=68.39$   $p<0.001$ ). Isolation reared rats were more active than socially housed animals (RM ANOVA  $F_{(1,28)}=9.30$   $p=0.005$ ) but treatment with Ro 04-6790 had no effect on activity (RM ANOVA  $F_{(1,28)}=2.59$   $p=0.119$ ). Furthermore no drug, housing or time interactions were observed (Figure 5.4).

Isolation had no effect on rearing behaviour (RM ANOVA  $F_{(1,27)}=2.33$   $p=0.139$ ). Treatment with Ro 04-6790 showed a trend towards decreasing rearing but this failed to reach significance (RM ANOVA  $F_{(1,27)}=3.54$   $p=0.071$ ), data not shown.

Figure 5.4: Isolation reared rats are more active than socials, but treatment with Ro 04-6790 has no effect on activity

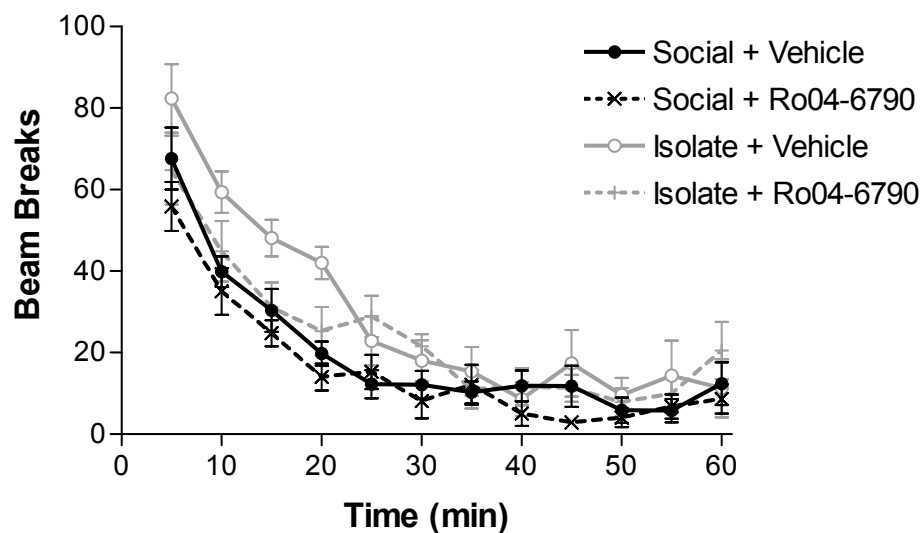


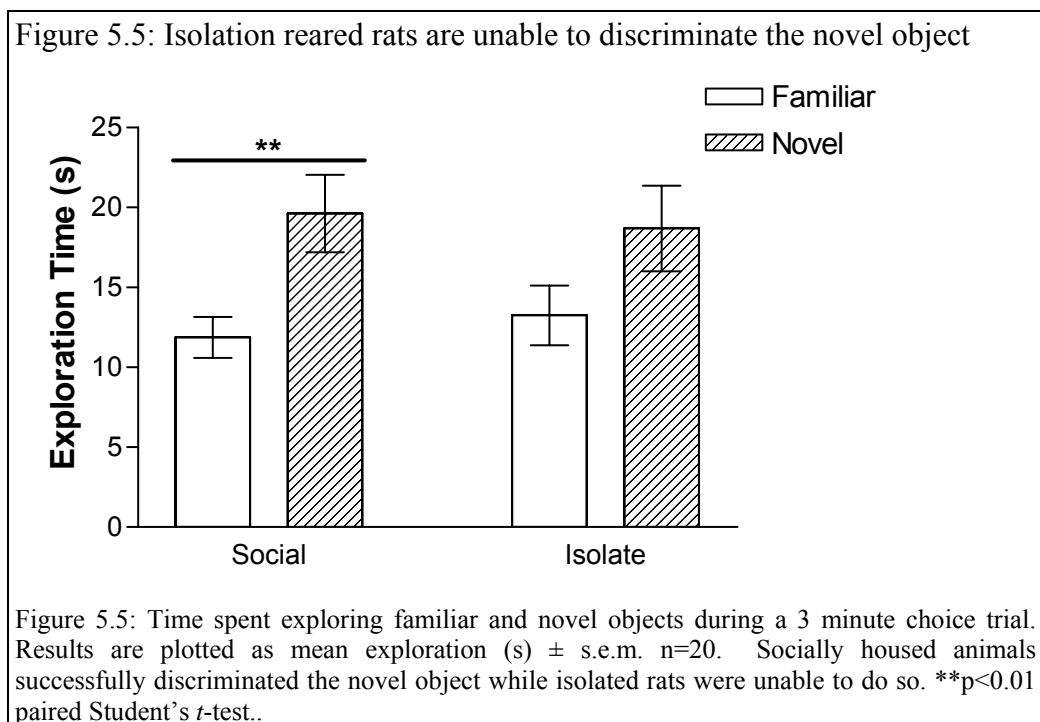
Figure 5.4: Locomotor activity of social and isolated rats, treated with either vehicle or Ro 04-6790, in a novel environment, measured by beam breaks in a 5 minute time bin. Beam breaks are plotted at the end of each time bin but represent breaks during the 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m.  $n=8$ . Isolation reared rats were more active than controls (RM ANOVA  $F_{(1,28)}=9.30$   $p=0.005$ ) but Ro 04-6790 treatment had no effect on activity (RM ANOVA  $F_{(1,28)}=2.59$   $p=0.119$ ).

*Novel Object Discrimination*

In the familiarisation trial (T1) the isolation reared rats showed an unexpected preference for the object at the back of the test box (see Table 5.2 for statistical significance values). However in the choice trial (T2) no object location preference was seen and the position of the novel object was varied across groups. No differences were found in total exploration in either of the trials (Student's *t*-test: T1  $p=0.861$ , T2  $p=0.930$ ). In the choice trial the socially housed animals successfully discriminated the novel from the familiar object (paired Student's *t*-test  $p=0.001$ ), but the isolation reared rats did not (paired Student's *t*-test  $p=0.074$ ) (Figure 5.5). Isolation reared rats showed a decreased discrimination ratio which just failed to reach significance (DR mean  $\pm$  s.e.m. social =  $0.279 \pm 0.040$ , isolate =  $0.049 \pm 0.123$ , Student's *t*-test  $p=0.084$ )

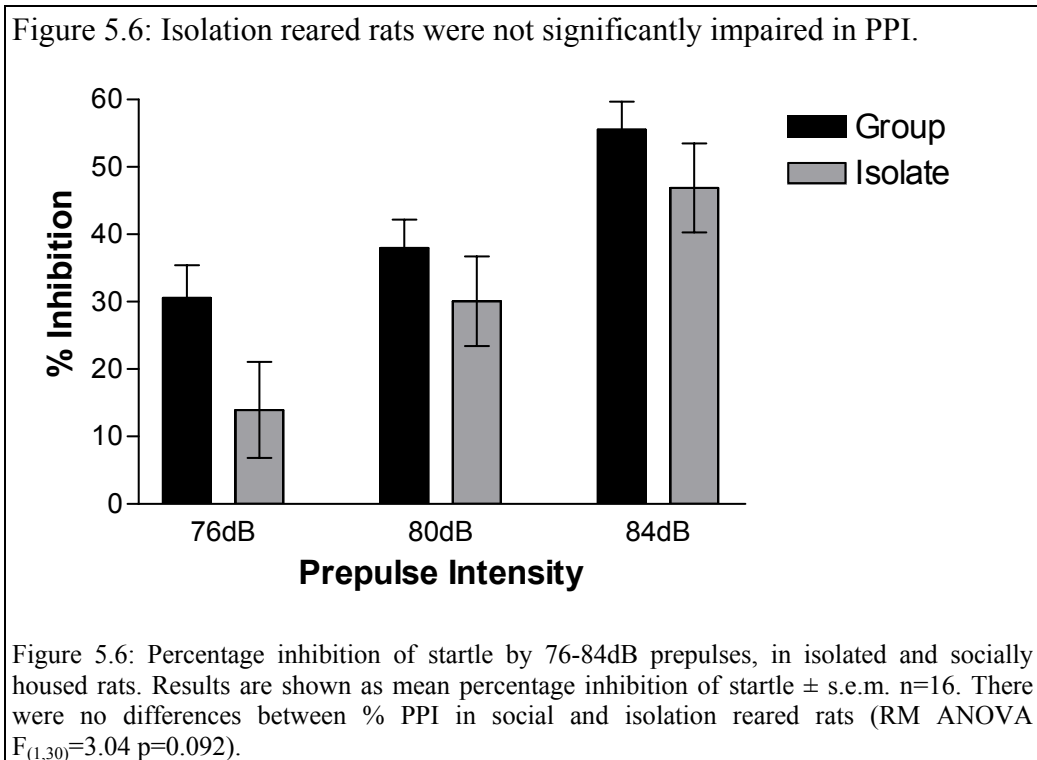
Table 5.2: Statistical values for paired Student's *t*-tests on NOD

	Social	Isolates
T1: Front/back	$p=0.371$	$p=0.025$ *
T2: Front/back	$p=0.628$	$p=0.625$
T2: Familiar/ novel	$p=0.001$ **	$p=0.074$



### *PPI of Acoustic Startle*

As seen in Figure 5.6, when the prepulse volume increased the % PPI of acoustic startle also increased (RM ANOVA  $F_{(2,60)}=22.26$   $p\leq 0.001$ ). However, 35 days of isolation rearing did not have a significant overall effect on PPI (RM ANOVA  $F_{(1,30)}=3.04$   $p=0.092$ ), although there was a trend towards reduced PPI in the isolates at 76dB (Student's  $t$ -test  $p=0.063$ ). There was no significant effect of housing on startle response to the 120dB pulse (Student's  $t$ -test  $p=0.799$ ).



### *Water Maze Acquisition*

When all 15 acquisition trials were analysed there was no difference between the trial duration of isolated and socially reared rats (RM ANOVA  $F_{(1,30)}=0.87$   $p=0.358$ , Figure 5.7). There was a significant effect of trial, indicating that the trial duration was shorter as the study progressed (RM ANOVA  $F_{(14,420)}=20.49$   $p<0.001$ ). When each test day was analysed individually a difference in trial duration between social and isolated rats was found only on day 2 (RM ANOVA  $F_{(1,30)}=4.17$   $p=0.05$ ). However, on all subsequent days isolation reared rats found the platform as quickly as socially housed animals.

Figure 5.7: Isolation rearing has no effect on trial duration in water maze acquisition.

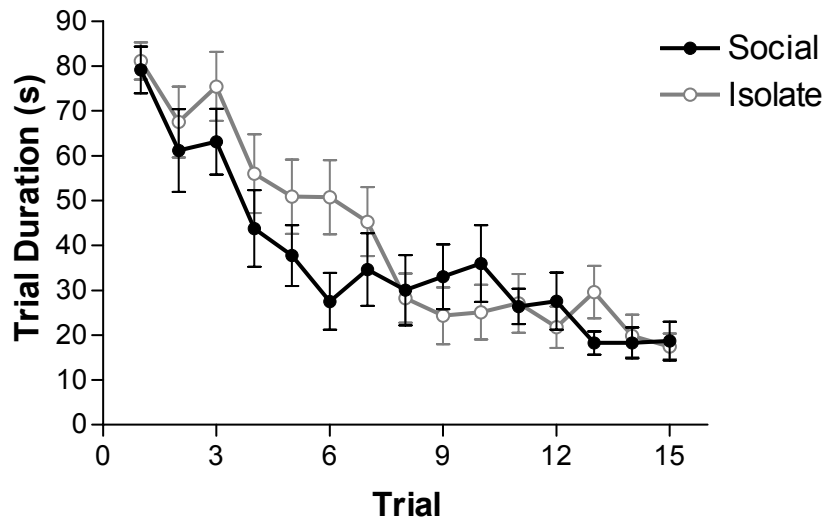


Figure 5.7: Time taken to find the submerged platform during the fixed platform position acquisition period, in social and isolated rats. Three trials were conducted each day for five days. Results are shown as mean time to reach platform (s)  $\pm$  s.e.m. (n=20). No overall difference between social and isolated rats was found in time to reach the platform (RM ANOVA  $F_{(1,30)}=0.87$   $p=0.358$ ).

Figure 5.8: Housing had no effect on the time spent in the training quadrant during the probe test after 5 days of training.

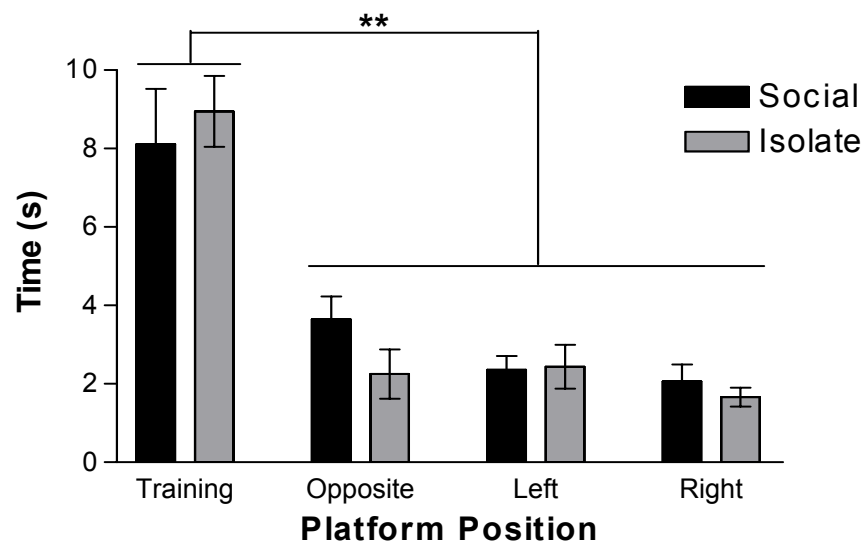


Figure 5.8: Time spent swimming in each quadrant of the maze during the probe test, in social and isolated rats. Results are shown as mean time (s)  $\pm$  s.e.m. (n=20). All animals spent longer in the training quadrant ( $p<0.001$ \*\* RM ANOVA), but isolation rearing had no effect on time spent in any quadrant.

*Water Maze Probe Test*

Rats underwent a probe test after 5 days of acquisition training in the water maze. In the probe test a significant effect of quadrant was observed, indicating animals spent more time in the quadrant which contained the platform during training (RM ANOVA  $F_{(3,90)}=32.00$   $p<0.001$ ) (Figure 5.8). However, no effect of housing, or quadrant x housing interaction was observed (RM ANOVA Housing:  $F_{(1,30)}=0.32$   $p=0.581$ , Interaction  $F_{(3,90)}=0.73$   $p=0.536$ ). Importantly, this shows that all animals had learnt the position of the platform to the same extent following the 15 acquisition trials and any subsequent dissimilarity in performance is not due to a difference in previous learning.

*Water Maze Reversal Learning*

Rats underwent three days of reversal learning, with three trials per day. Ro 04-6790 was administered 20 minutes prior to the first trial on each day. On the first day of reversal learning there was a significant effect of trial on time taken to find the platform (RM ANOVA  $F_{(2,56)}=6.13$   $p=0.004$ , Figure 5.9). There was a trend towards isolation reared rats taking longer to find the platform, but this just failed to reach significance (RM ANOVA  $F_{(1,28)}=3.58$   $p=0.069$ ). Treatment with Ro 04-6790 had no overall effect on time taken to find the platform (RM ANOVA  $F_{(1,28)}=0.57$   $p=0.458$ ), but there was a significant interaction between housing and drug treatment (RM ANOVA  $F_{(1,28)}=7.82$   $p=0.009$ ) indicating that Ro 04-6790 impaired performance in socially housed animals, but improved performance in isolation reared rats.

On days 2 and 3 of reversal learning no differences were seen between social and isolation reared rats and there was no observed effect of treatment with Ro 04-6790. However, across all 9 trials the interaction between drug treatment and housing condition remained present, again indicating that Ro 04-6790 had a different effect in isolation reared rats than socially housed control rats (RM ANOVA  $F_{(1,26)}=5.24$   $p=0.031$ ).

The effect of Ro 04-6790 on speed of swimming was also analysed, to check for sedative effects of the drug which might have resulted in longer trial times. However, no effect of Ro 04-6790 or housing was seen on swim speed averaged across the reversal learning trials (2-way ANOVA Housing:  $F_{(1,28)}=0.96$   $p=0.335$ , Drug treatment:  $F_{(1,28)}=0.01$   $p=0.909$ , Interaction:  $F_{(1,28)}=0.13$   $p=0.725$ ).

Figure 5.9: Ro 04-6790 had opposite effects on day 1 reversal learning performance in social and isolation reared rats.

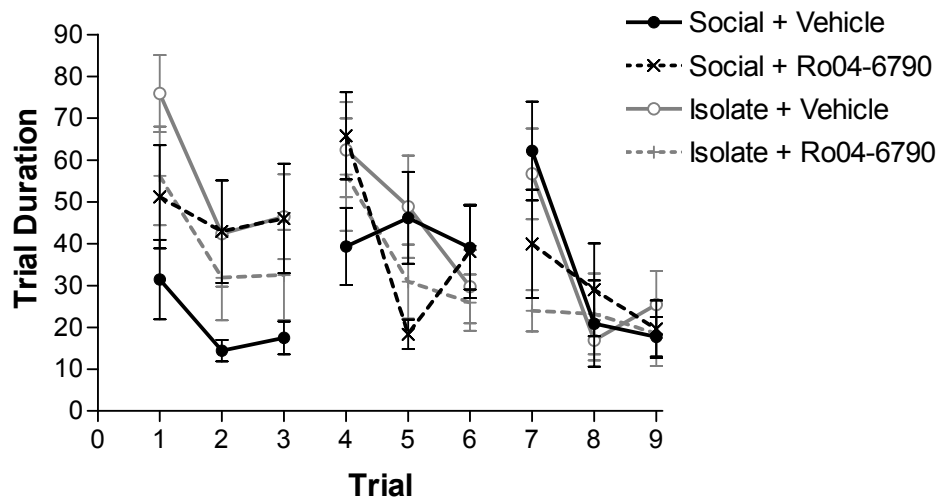


Figure 5.9: Time spent swimming to the platform during the reversal learning phase, in social and isolated rats receiving either vehicle or Ro 04-6790. Results are presented as mean trial duration (s)  $\pm$  s.e.m. (n=10). On Day 1 (trials 1-3) isolation reared rats showed a trend towards impaired performance (RM ANOVA  $F_{(1,28)}=3.58$   $p=0.069$ ). Ro 04-6790 improved performance in isolation reared rats, but impaired performance in socially housed rats (RM ANOVA  $F_{(1,28)}=7.82$   $p=0.009$ ).

### 5.4.2 Effect of Acute Aniracetam on NOD and PPI in Social Rats

In order to establish a suitable dose of aniracetam for sub-chronic administration a pilot study was conducted to find a dose with efficacy in novel object discrimination.

#### *Novel Object Discrimination*

In the familiarisation trial (T1) there were no differences in total exploration (1-way ANOVA  $F_{(2,19)}=1.04$   $p=0.373$ ) and no group showed preference for the object in either position (see Table 5.3 for significance values).

In the choice trial (T2) there was a trend towards aniracetam treatment increasing total exploration, but this did not reach significance (1-way ANOVA  $F_{(2,19)}=2.81$   $p=0.084$ ), data not shown. Neither the vehicle nor the high dose (30mg/kg) aniracetam group were able to discriminate the novel from the familiar object, but the group that received 15mg/kg aniracetam were successfully able to discriminate between the objects in T2 (see Table 5.3 and Figure 5.10). When discrimination ratios (see Table 5.3) were compared by 1-way ANOVA 15mg/kg aniracetam was found to significantly improve NOD compared to vehicle treated rats ( $F_{(2,20)}=3.58$   $p=0.047$ ).

Table 5.3: Statistical significance values for 2 hour ITI NOD after treatment with aniracetam, paired Student's *t*-test and discrimination ratios.

	Vehicle	15mg/kg aniracetam	30mg/kg aniracetam
T1: front/ back	$p=1.000$	$p=0.162$	$p=0.472$
T2: familiar/ novel	$p=0.841$	$p=0.004^{**}$	$p=0.146$
DR (mean $\pm$ s.e.m.)	$0.096 \pm 0.105$	$0.458 \pm 0.085$	$0.223 \pm 0.107$



Figure 5.10: Treatment with 15mg/kg aniracetam improved NOD.

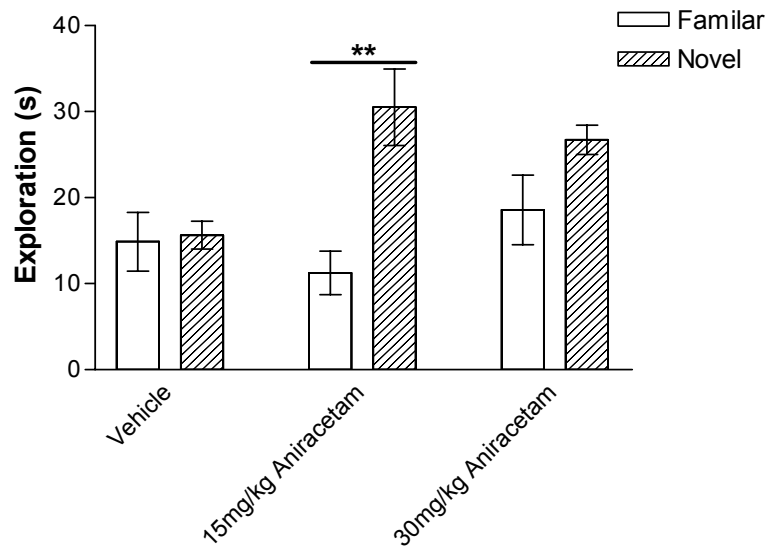


Figure 5.10: Time spent exploring familiar and novel objects during a 3 minute choice trial. Results are plotted as mean exploration (s)  $\pm$  s.e.m.  $n=8$ . Only 15mg/kg aniracetam treated animals are able to discriminate the novel object (\*\*  $p \leq 0.01$ , paired Student's  $t$ -test).

#### *PPI of Acoustic Startle*

The level of PPI increased as prepulse volume increased (RM ANOVA  $F_{(2,40)}=32.13$   $p \leq 0.001$ ). However, acute aniracetam treatment had no effect on %PPI at either 15 or 30mg/kg (RM ANOVA  $F_{(2,20)}=0.33$   $p=0.724$ ) (Figure 5.11).

Figure 5.11: Acute aniracetam had no effect on % PPI.

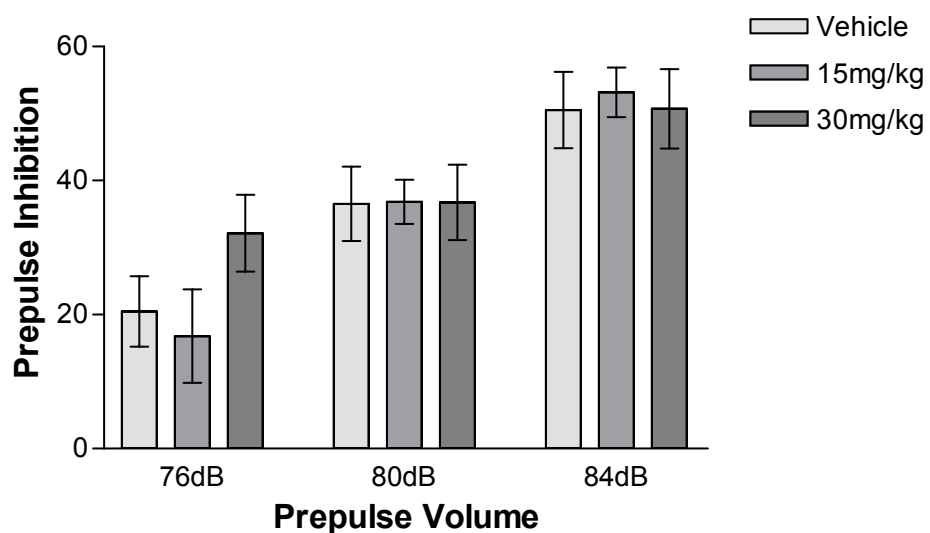


Figure 5.11: Percentage inhibition of startle by 76-84dB prepulses in rats treated with vehicle, 15mg/kg or 30mg/kg aniracetam. Results are shown as mean percentage inhibition of startle  $\pm$  s.e.m.  $n=8$ . Overall aniracetam had no effect on % PPI (RM ANOVA  $F_{(2,20)}=0.33$   $p=0.724$ ).

### 5.4.3 Effect of Sub-chronic Aniracetam in Isolation Reared Rats

Having found a suitable dose of aniracetam which appeared to enhance NOD without any marked change in activity this dose was used in a sub-chronic administration study to try to reverse the isolation-induced deficits in cognition.

#### *Locomotor Activity*

LMA declined over the 1 hour test period in all animals, as they habituated to the arena (RM ANOVA  $F_{(11,26)}=44.21$   $p \leq 0.001$ ) (Figure 5.12). Isolation reared rats were significantly more active than socials (RM ANOVA  $F_{(1,36)}=6.86$   $p=0.013$ ) but drug treatment had no effect (RM ANOVA  $F_{(1,36)}=0.18$   $p=0.672$ ) and no interactions were observed.

Neither housing nor aniracetam treatment had an effect on rearing activity in a novel environment (RM ANOVA Housing:  $F_{(1,36)}=0.34$   $p=0.561$ , Treatment:  $F_{(1,36)}=0.02$   $p=0.881$ ), data not shown.

Figure 5.12: Isolation reared rats are more active than social controls in a novel environment but aniracetam has no effect.

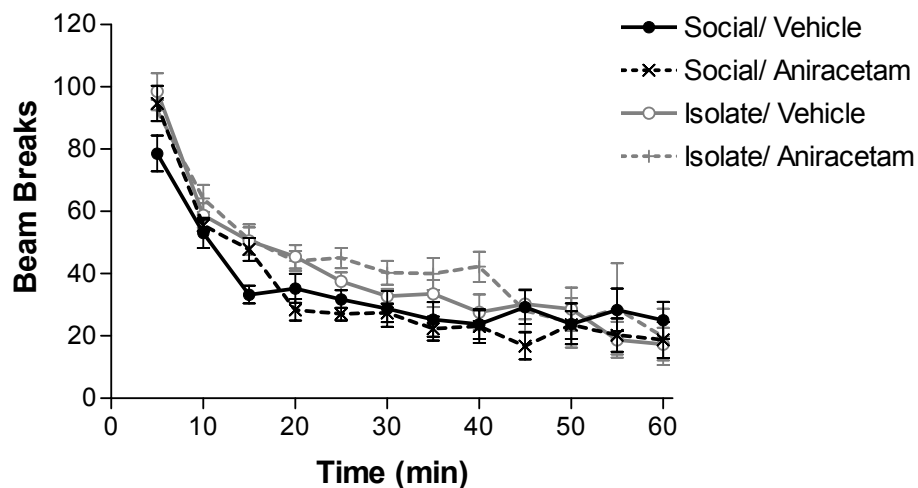


Figure 5.12: Locomotor activity of social and isolated rats treated with vehicle or sub-chronic aniracetam (15mg/kg for 4 days), measured by beam breaks in a 5 minute time bin. Beam breaks are plotted at the end of each time bin but represent breaks during the 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m.  $n=10$ . Isolation reared rats were more active than controls (RM ANOVA  $F_{(1,36)}=6.86$   $p=0.013$ ) but aniracetam had no effect (RM ANOVA  $F_{(1,36)}=0.18$   $p=0.672$ ).

*Novel Object Discrimination*

In the familiarisation trial no differences were seen in total exploration (2-way ANOVA Housing:  $F_{(1,36)}=2.37$   $p=0.133$ , Treatment:  $F_{(1,36)}=0.03$   $p=0.860$ ). The social/ vehicle group displayed a preference for the object at the back of the test box (see Table 5.4 for statistical values), which was not seen in any other group. In T2 the position of the novel object is always randomised to minimise any effect of position preference. The exploration times of the objects in T2 were compared by position and no preference was found (paired Student's  $t$ -test  $p=0.156$ ).

In the choice trial (T2) neither the social nor the isolated vehicle treated groups were able to successfully discriminate the novel object. Both group housed and isolation reared rats receiving aniracetam were able to discriminate the novel object (see Figure 5.13 and Table 5.4 for significance values). However when discrimination ratios (see Table 5.4) were analysed no significant differences were found (2-way ANOVA housing  $F_{(1,36)}=0.69$   $p=0.413$ , drug treatment  $F_{(1,36)}=0.96$   $p=0.333$ ).

Table 5.4: Statistical significance values for 2 hour ITI NOD after isolation rearing and treatment with sub-chronic aniracetam, paired Student's  $t$ -test.

	Social/ vehicle	Social/ aniracetam	Isolate/ vehicle	Isolate/ aniracetam
T1: front/ back	$p=0.003$ **	$p=0.890$	$p=0.689$	$p=0.088$
T2: familiar/ novel	$p=0.213$	$p=0.033$ *	$p=0.415$	$p=0.005$ **
DR (mean $\pm$ s.e.m.)	$0.23 \pm 0.11$	$0.22 \pm 0.91$	$0.14 \pm 0.13$	$0.28 \pm 0.07$

Figure 5.13: Aniracetam treated rats were able to discriminate the novel object

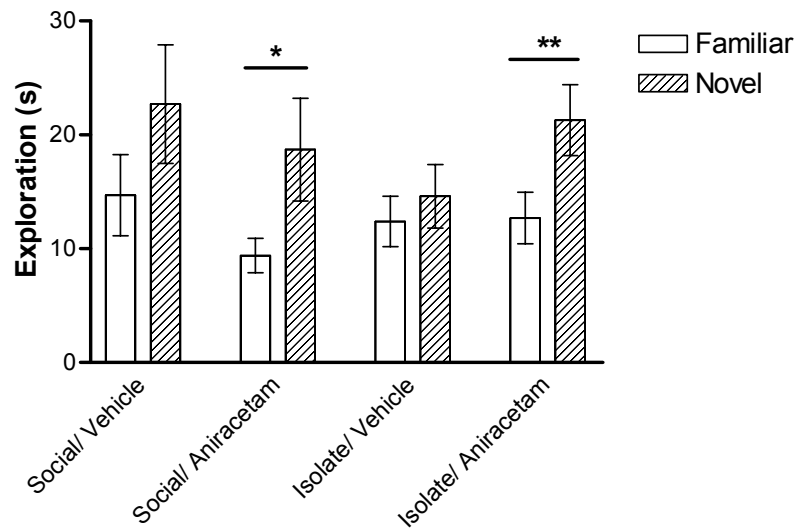


Figure 5.13: Time spent exploring familiar and novel objects during a 3 minute choice trial in social and isolation reared rats treated with sub-chronic vehicle or aniracetam. Results are plotted as mean exploration (s)  $\pm$  s.e.m. (n=10). Aniracetam treated rats successfully discriminated the novel object (paired Student's *t*-test), whereas vehicle treated rats did not, regardless of housing conditions. \* $p < 0.05$ , \*\*  $p \leq 0.01$ .

#### *PPI of Acoustic Startle*

As expected % PPI increased as the prepulse volume increased (RM ANOVA  $F_{(2,72)}=19.32$   $p < 0.001$ ) (Figure 5.14). Isolation rearing caused a significant reduction in PPI (RM ANOVA  $F_{(1,36)}=8.00$   $p=0.008$ ). Aniracetam treatment also showed a trend towards reducing PPI but this just failed to reach significance (RM ANOVA  $F_{(1,36)}=3.78$   $p=0.060$ ). Furthermore there was no significant housing x drug interaction.

There was a trend towards aniracetam reducing startle response to the 120dB pulse, however this failed to reach significance (2-way ANOVA  $F_{(1,36)}=3.71$   $p=0.062$ ). No other effects on startle response were observed.

Figure 5.14: Isolation rearing impaired prepulse inhibition but sub-chronic aniracetam had no effect.

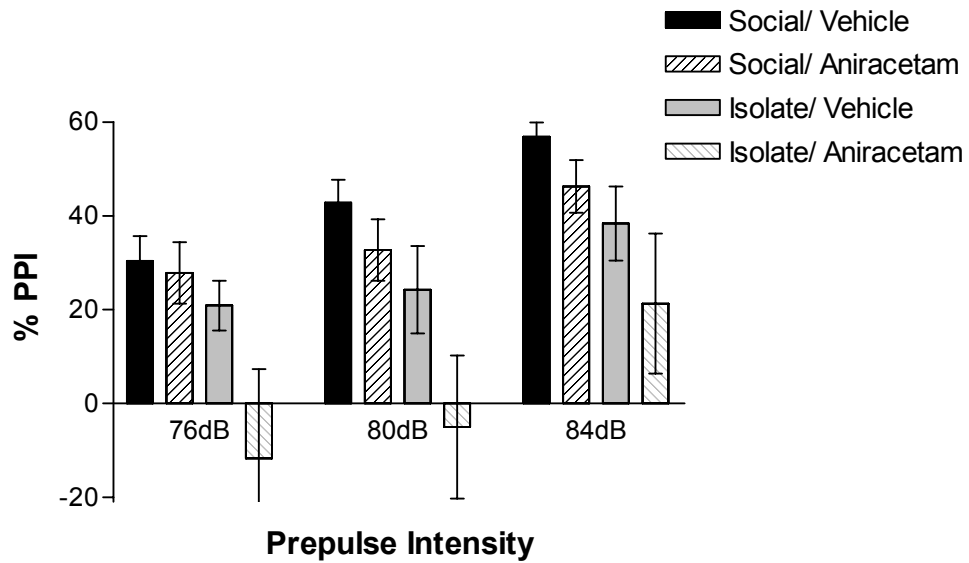


Figure 5.14: Percentage inhibition of startle by 76-84dB prepulses, in isolated and socially housed rats treated with sub-chronic vehicle or aniracetam. Results are shown as mean percentage inhibition of startle  $\pm$  s.e.m.  $n=10$  per group. Isolation rearing reduced PPI (RM ANOVA  $F_{(1,36)}=8.00$   $p=0.008$ ) but aniracetam treatment had no significant effect.

### *Passive Avoidance*

In the first passive avoidance trial no difference was seen in time taken to enter the black chamber (2-way ANOVA Housing:  $F_{(1,36)}=0.10$   $p=0.760$ , Drug:  $F_{(1,36)}<0.001$   $p=0.988$ ) (Figure 5.15).

In the second passive avoidance trial, 24 hours after receiving a shock in the black chamber, isolation reared rats entered the black chamber after a shorter time than the socially housed animals (2-way ANOVA Housing effect  $F_{(1,36)}=4.48$   $p=0.041$ ) (Figure 5.16). Aniracetam treatment had no effect on time taken to enter the black chamber (2-way ANOVA  $F_{(1,36)}=0.002$   $p=0.961$ ) and no drug x housing interaction was observed.

Figure 5.15: No differences were seen in time taken to enter the dark chamber in the first passive avoidance trial

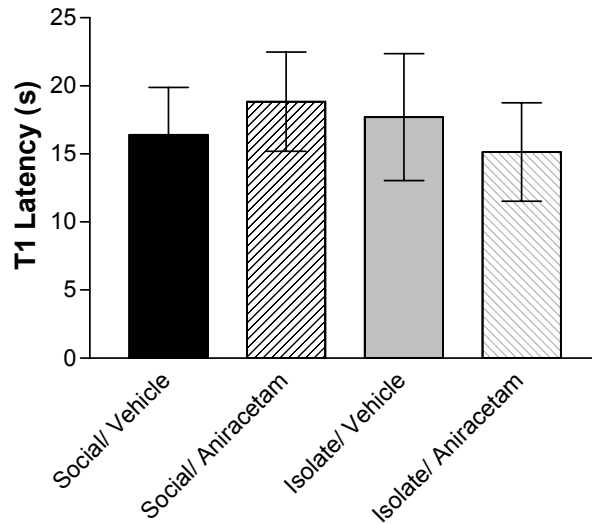


Figure 5.15: Time taken for rats to move from the white to the black chamber in the first passive avoidance trial. Data are shown as mean  $\pm$  s.e.m., n=10. No effect of housing or aniracetam treatment was found.

Figure 5.16: Isolation reared rats entered the black chamber more quickly than the social control but aniracetam treatment had no effect.

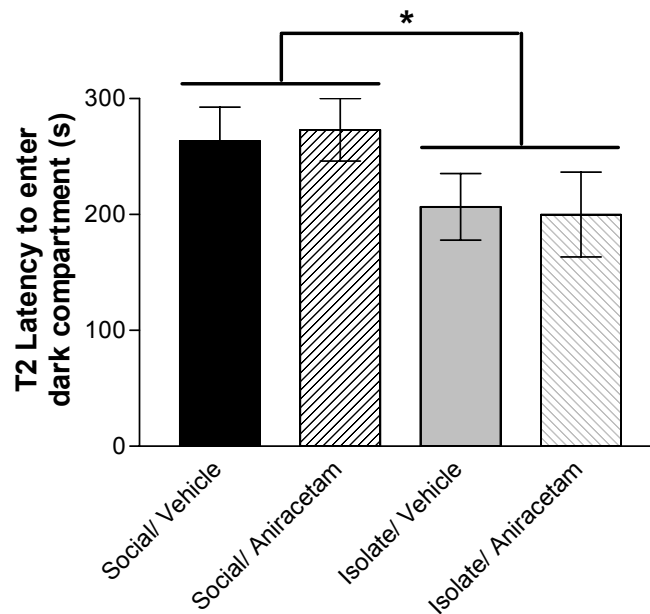


Figure 5.16: Time taken for rats to move from the white to the black chamber in the passive avoidance retention trial (T2). Data are shown as mean  $\pm$  s.e.m., n=10. Isolation reared rats entered the dark chamber more quickly than social controls (2-way ANOVA  $F_{(1,36)}=4.48$   $p=0.041$ ) but no effect of aniracetam treatment was found. \*  $p<0.05$  2-way ANOVA.

*Western Blotting for NR1, NR2A and NR2B Protein Levels*

The western blotting protocol used a light-emitting detection system, which was exposed to photographic film for quantification. The protein detected by the antibodies appeared as a dark band on the photographic film. The optical densities (OD) of these dark bands was then quantified. In order to control for slight variations in the amount of protein loaded in each well, the OD of each NR subunit band were normalised using a house-keeping protein, GAPDH, which is ubiquitously expressed in high levels in the brain. This normalisation is based on the assumption that neither isolation rearing nor aniracetam treatment effect GAPDH levels. To check this the GAPDH band OD's were analysed by 2-way ANOVA. Neither isolation rearing nor aniracetam treatment had an effect on levels of GAPDH, (see Table 5.5 for significance values).

Following normalisation to GAPDH levels to control for protein loading, neither isolation rearing nor aniracetam treatment had an effect on any of the NMDA subunit protein levels measured in the left hippocampus. The statistical significance values are shown in Table 5.5 and the data are shown in Figure 5.17 A, B and C. Representative sections of photographic films are shown in Table 5.6

Table 5.5: 2-way ANOVA results for GAPDH optical densities and normalised NMDA subunit levels in the hippocampus

	Housing effect	Treatment effect	Interaction
GAPDH (NR1 gels)	$F_{(1,38)}=0.004$ $p=0.945$	$F_{(1,38)}=0.34$ $p=0.561$	$F_{(1,38)}=0.38$ $p=0.541$
GAPDH (NR2A gels)	$F_{(1,38)}=0.68$ $p=0.416$	$F_{(1,38)}=1.07$ $p=0.307$	$F_{(1,38)}=0.13$ $p=0.723$
GAPDH (NR2B gels)	$F_{(1,38)}=2.21$ $p=0.146$	$F_{(1,38)}=0.66$ $p=0.423$	$F_{(1,38)}=0.12$ $p=0.730$
NR1	$F_{(1,38)}=0.28$ $p=0.597$	$F_{(1,38)}=0.001$ $p=0.969$	$F_{(1,38)}=0.82$ $p=0.372$
NR2A	$F_{(1,38)}=0.38$ $p=0.539$	$F_{(1,38)}=0.28$ $p=0.600$	$F_{(1,38)}=1.71$ $p=0.198$
NR2B	$F_{(1,38)}=0.18$ $p=0.671$	$F_{(1,38)}=1.58$ $p=0.216$	$F_{(1,38)}=1.15$ $p=0.290$

Table 5.6: Example Photographic films from NR subunit western blots


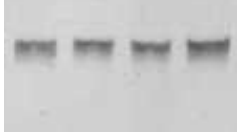

Subunit	Example films
NR1	 I/A I/V S/A S/V
NR2A	 I/A I/V S/A S/V
NR2B	 I/A I/V S/A S/V
S= Social, I= isolate, V=vehicle, A=aniracetam	



Figure 5.17: Neither housing nor aniracetam treatment had an effect on NR subunit protein levels in the hippocampus

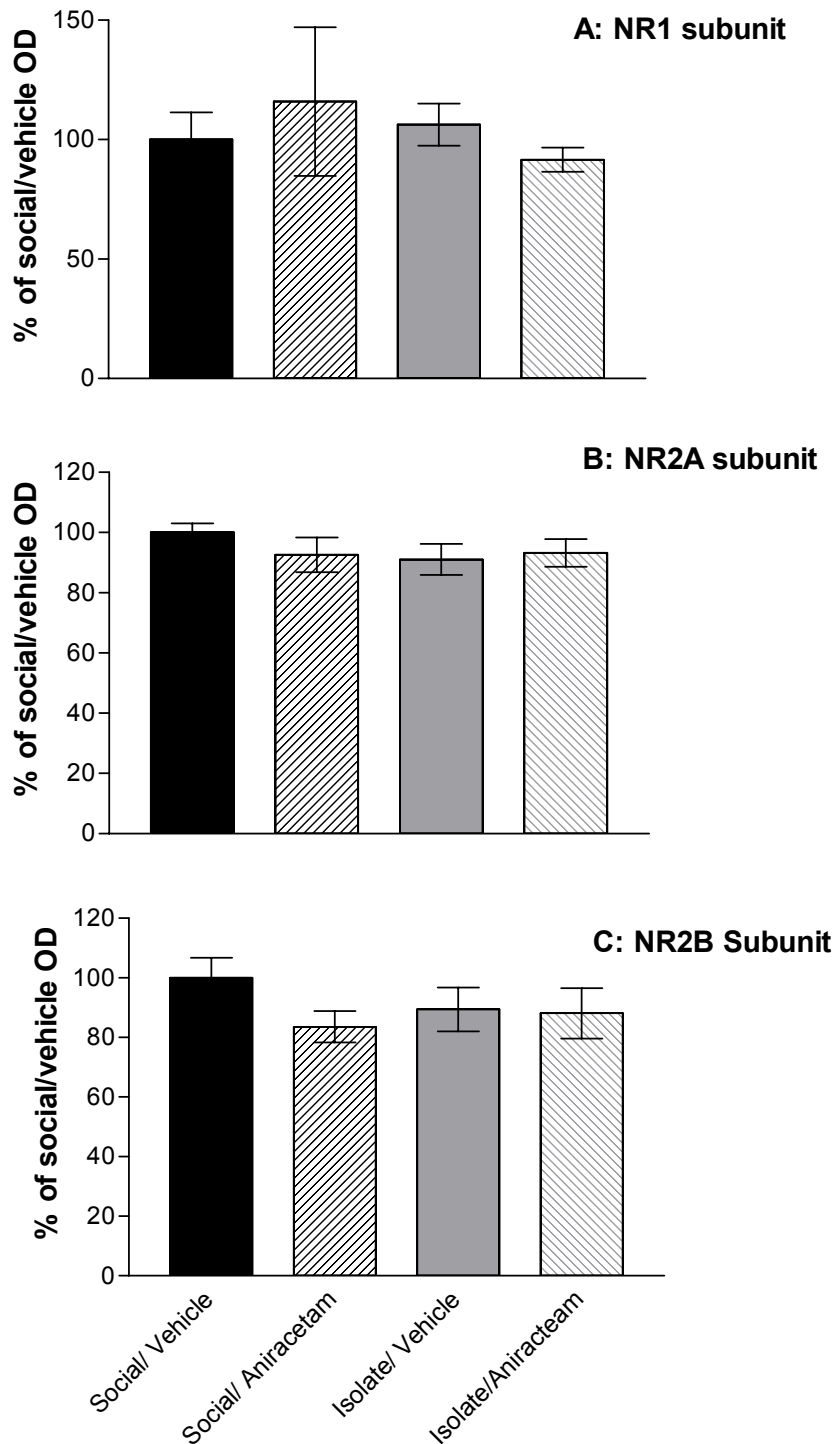


Figure 5.17: Level of NMDA subunit protein expression (A:NR1, B:NR2A and C:NR2B) in hippocampal tissue of social or isolation reared rats treated with sub-chronic vehicle or aniracetam. Data are normalised to GAPDH and expressed as percentage of the social/ vehicle group  $\pm$  s.e.m, n=10 per group. No effect of housing or drug treatment was observed in any subunit.

## 5.5 Discussion

### 5.5.1 Effect of Ro 04-6790 on the Performance of Isolation Reared Rats in the Water Maze

In this study isolation reared rats were hyperactive in a novel environment and demonstrated impairments in novel object recognition and reversal learning in the water maze. Treatment with the 5-HT<sub>6</sub> antagonist Ro 04-6790 had no effect on activity but did attenuate the reversal learning impairment in the isolation reared rats.

Few published studies have investigated the effects of 5-HT<sub>6</sub> antagonists on locomotor activity, but one paper showed Ro 04-6790 caused a dose dependent reduction in activity which reached significance at 30mg/kg (Bentley et al., 1999). In the current study a dose of 10mg/kg was used, which had previously been shown to improve cognition and did not significantly affect locomotor activity in either the Bentley study or the experiment described herein. The aim of this study was to see if Ro 04-6790 had a specific effect on isolation rearing induced hyperactivity. The results showed that at 10mg/kg Ro 04-6790 did not have any effect on exploratory activity in social or isolated rats either in the open field or on swim speed in the water maze.

Isolation rearing failed to impair PPI in this cohort of rats. As discussed in Chapter 1, even with ideal housing conditions for isolation rearing, such as complete silence and restricted personnel, only 85% of isolation reared cohorts developed PPI deficits (Cilia et al., 2005b). The overall success rate of isolation rearing in our laboratory over the last 3 years has been of a similar level (82%). One possible interpretation is that the lack of PPI deficits in this rat cohort means they have not fully developed an isolation syndrome and therefore cannot be presented as isolation reared rats, but they do demonstrate several other behavioural alterations (increased activity, a trend towards NOD impairments, reversal learning impairments) and so should not be discounted so readily. The effect of the 5-HT<sub>6</sub> antagonist on PPI was not investigated. However other laboratories have found Ro 04-6790 to have no effect on PPI

deficits induced by PCP, apomorphine or lysergic acid diethylamide (LSD) (Leng et al., 2003). Another 5-HT<sub>6</sub> antagonist Ro 4368554 does reverse apomorphine induced PPI deficits, but not those induced by scopolamine (Mitchell and Neumaier, 2008).

As seen previously the isolation reared rats demonstrated a trend towards impaired recognition memory in the NOD test. Ro 04-6790 was not tested against these deficits as its efficacy improving recognition memory has been widely shown in both time-induced (King et al., 2004) and pharmacologically-induced NOD impairments (Woolley et al., 2003), although not in isolation reared rats. Recently another 5-HT<sub>6</sub> antagonist PRX-07037 has been shown to reverse isolation rearing induced deficits in recognition memory (King et al., 2007). Other 5-HT<sub>6</sub> antagonists have also shown efficacy in NOD with SB-399885 and Ro 4368554 reversing scopolamine induced deficits (Lieben et al., 2005, Hirst et al., 2006) (Schreiber et al., 2007).

During water maze acquisition isolation reared rats showed a small impairment on day 2 only. However, on days 3-5 of acquisition and during the post-acquisition probe test no difference was seen between isolation reared rats and the social controls. This agrees with some previous studies which have found no significant difference in water maze acquisition between social and isolated rats (Lapiz et al., 2003, Schrijver et al., 2004), although other groups have seen improvements (Wongwitdecha and Marsden, 1996b) or impairments (Hellemans et al., 2004).

During the reversal learning phase the isolation reared rats took longer to locate the platform on the first day of reversal learning, which was improved by Ro 04-6790 treatment. Conversely the performance of the socially housed animals was impaired by Ro 04-6790 treatment. As there was no difference in the locomotor effect of Ro 04-6790 in social and isolated rats and swim speed in the water maze was the same it is unlikely that the difference in latency to find the platform was due to any sedative effects of the drug. The 5-HT<sub>6</sub> antagonists Ro 04-6790, SB-399885-T, SB-271046-A and an antisense oligonucleotide to the 5-HT<sub>6</sub> receptor have all been shown to improve retention

of platform position in the water maze, while having no effect on the acquisition phase (Rogers and Hagan, 2001, Woolley et al., 2001) and SB-399885 also improved acquisition in aged rats (Hirst et al., 2006). No studies have looked at the effects of 5-HT<sub>6</sub> antagonists in reversal learning in the water maze, but in a bowl-digging test of attentional set-shifting sub-chronic treatment with SB-399885-T and SB-271046-A specifically improved performance at the first reversal stage of the paradigm and also reduced overall trials to completion (Hatcher et al., 2005). This shows that it is possible for 5-HT<sub>6</sub> antagonists to improve behavioural flexibility, but does not explain why it would only do so in the isolated animals in the current study. The effect of isolation rearing on 5-HT<sub>6</sub> receptor density are currently unknown and may shed some light on this issue. Interestingly no differences were found in 5-HT<sub>6</sub> receptor density in the dlPFC of schizophrenic patients compared with normal controls (East et al., 2002b).

There is currently no published data on the effects of 5-HT<sub>6</sub> antagonists on human cognitive performance, in either healthy controls or schizophrenic patients, although some compounds are in Phase I or II clinical trials. Two studies have looked for associations between schizophrenia and 5-HT<sub>6</sub> genes and found no evidence of any links (Shinkai et al., 1999, Vogt et al., 2000). However, reduced levels of 5-HT<sub>6</sub> receptor mRNA have been found in the hippocampus of schizophrenic patients (East et al., 2002a) and response to clozapine and risperidone has been linked to polymorphisms in the 5-HT<sub>6</sub> receptor (Yu et al., 1999, Lane et al., 2004) suggesting some role for 5-HT<sub>6</sub> receptors in this disease. At this stage it is not possible to predict whether 5-HT<sub>6</sub> antagonists would show efficacy in schizophrenic patients, but the results of this study suggest that they may be efficacious against the cognitive symptoms of schizophrenia.

### **5.5.2 Effect of Acute Aniracetam on NOD and PPI in Social Rats**

In a pilot study in group housed rats acute aniracetam treatment improved novel object recognition at 15mg/kg but not at the higher dose of 30mg/kg. Neither dose had any effect on prepulse inhibition. From this data it was decided that the isolation reared rats would receive 15mg/kg aniracetam daily.

The high dose (30mg/kg) of aniracetam used in this study was less effective in novel object discrimination than the lower dose of 15mg/kg. This effect has been noticed previously with acute aniracetam improving novel object discrimination in age-impaired and scopolamine-impaired rats at 50mg/kg p.o., but not at 25 or 100mg/kg (Bartolini et al., 1996). Bartolini used much higher doses of aniracetam, but the animals were dosed orally and aniracetam has poor bioavailability when dosed by the oral route (Ogiso et al., 1998) which may explain why higher doses were required to achieve pro-cognitive effects. These results suggest an inverted U-shaped curve for efficacy of aniracetam in cognitive tasks. However, another study of the effect of aniracetam on novel object discrimination found that aniracetam treatment improved discrimination from 10-100mg/kg i.p., with no suggestion of reduced drug effect at high doses (Lebrun et al., 2000).

It should also be noted that both the vehicle and 30mg/kg aniracetam treated rats were unable to discriminate the novel object in this study. This may indicate a general impairment in the ability of these rats to carry out the novel object task, thus invalidating the effect at 15mg/kg. Despite this the 15mg/kg dose was still thought to be the most appropriate dose to use in the sub-chronic dosing study.

### **5.5.3 Effect of Sub-chronic Aniracetam in Isolation Reared Rats**

In this study a cohort of isolation reared rats were more active in a novel environment and showed attenuated prepulse inhibition of acoustic startle, consistent with induction of the isolation syndrome. Aniracetam had no

significant effect on either of these behaviours in both social and isolated rats. However, there was a trend towards aniracetam impairing PPI which was not seen when dosed acutely. The isolated rats were also impaired at novel object recognition while aniracetam treated rats were able to discriminate the novel object. However the effect of aniracetam was not significant when the discrimination ratios were analysed. In the passive avoidance test, isolation reared rats had a reduced latency to re-enter the compartment where they had received the shock, suggesting they may have impaired memory of the shock or the context. Aniracetam treatment had no effect on this in either social or isolation reared rats. Finally neither isolation rearing nor aniracetam treatment had an effect on the level of the NMDA receptor subunits NR1, NR2A or NR2B protein expressed in the hippocampus.

Aniracetam treatment had no effect on locomotor activity. There is no previously published data on the effects of AMPA potentiators on activity levels. There is also almost no published data on AMPA potentiators' effects on prepulse inhibition, although one study found CX546 treatment partially reversed PPI deficits in mGluR5 deficient mice (Lipina et al., 2007). In the study reported here the trend was for sub-chronic aniracetam to impair PPI. This was not seen with acute aniracetam treatment. Interestingly as there is some evidence for a down regulation of AMPA receptors during chronic treatment with AMPA potentiators (Lauterborn et al., 2003) it is possible that this could disrupt sensorimotor gating if a sufficient dose was administered.

Aniracetam treated isolation reared rats were able to discriminate the novel object, although further studies are required to be certain that aniracetam is improving novel object discrimination in isolation reared rats. Most cognitive studies carried out using aniracetam have used an acute dose. However, sub-chronic aniracetam has been found to reverse impairments in radial arm maze performance induced by lesion of the entorhinal cortex (Zajackowski and Danysz, 1997). These results suggest that sub-chronic treatment with aniracetam is also able to enhance cognition.

Isolation reared rats exhibited a reduced latency to re-enter the shock compartment during the second passive avoidance trial, consistent with previous reports (Valzelli, 1973, Del Arco et al., 2004), suggesting they have not remembered that they received a shock there previously. This could be due to memory impairment or reduced attention to context. Alternatively, as isolates demonstrate increased activity in novel environments, the reduced latency could simply be a confound of hyperactivity in a novel environment. However, no differences were seen in the time taken to enter the dark compartment in the first trial. Isolates also show reductions in contextual fear conditioning (indicated by reduced time spent in freezing body posture) (Weiss et al., 2004). Aniracetam had no effect on passive avoidance in either social or isolation reared rats. It is possible that this lack of effect was due to the dosing schedule used in the study as animal received aniracetam one hour prior to the first trial, when they received the shock, but did not received another dose before the second trial, revealing the drug's effects on acquisition and consolidation, but not retrieval. Another AMPA modulator, S18986, has been found to improve novel object recognition only when given before the choice trial, but not when it is dosed before the first, familiarisation, trial (Lebrun et al., 2000). One possibility is that the AMPA modulators act by enhancing memory retrieval and this would explain why no effect was seen in the passive avoidance test presented herein. An acute dose of aniracetam has been found to improve contextual fear conditioning in mice when dosed before the training session, but a much higher dose was used (100mg/kg i.p.) than in this study (Smith and Wehner, 2002). S18986 has been shown to reverse scopolamine-induced impairments in passive avoidance, but when dosed before both trials (Rosi et al., 2004). To be certain of the lack of effect of aniracetam on isolation rearing-induced impairment of passive avoidance it would be necessary to repeat the study including a dose of aniracetam on the day of the second trial. While it is clear that the dosing schedule used produced no effect of aniracetam on isolation reared rats, it is possible that an effect in social animals was masked by a ceiling effect. The animals were allowed 300s to move into the dark compartment because in a pilot study most animals completed the task within this time. However, in the isolation rearing/ aniracetam study some rats did not pass through in that time. If the observation time allowed had been

increased it may have revealed more subtle differences in the time taken to enter the dark side.

Aniracetam treated isolation reared rats were able to discriminate the novel object and therefore AMPA potentiators may have a role as a treatment for cognitive deficits in schizophrenia. However, recent studies with the AMPAkinase CX516 had no effect on positive, negative or cognitive symptoms in schizophrenia when given alone (Marenco et al., 2002), or when given in combination with antipsychotics (Tuominen et al., 2005, Goff et al., 2007). This was despite initial promising results in combination with clozapine (Goff et al., 2001). AMPA receptor subunit expression was not significantly altered in the dlPFC of medicated schizophrenics compared to control patients (O'Connor et al., 2007).

AMPAkinases do improve some areas of cognition in humans, with CX516 showing efficacy in visual association, odour recognition, acquisition of a visuospatial maze and location and identity of playing cards, but cued recall of verbal information was not improved (Ingvar et al., 1997). Therefore it is not that human cognition cannot be improved by treatment with AMPA potentiators, just that the deficits seen in schizophrenia are resistant to treatment in this way.

No differences were seen in the levels of either NR1, NR2A or NR2B receptors in the hippocampus in isolation reared or aniracetam treated animals. Western blotting is only a semi-quantitative method of looking at protein levels and is not suited to detecting small changes. In this study the whole hippocampus was used and it is possible that this masked any small changes occurring in specific sub-regions. Also the method used to prepare the hippocampal samples resulted in the analysis of whole cell lysate. As the active receptors would have been on the cell membranes it would have been more appropriate to separate out the membranes for analysis. This would also have removed any receptor subunits being held intracellularly which may have masked any small changes in active receptor levels. As it has previously been found that cells from mouse forebrain contain pools of unassembled NR1 subunits it is possible that these



could have confounded the results (Chazot and Stephenson, 1997). The effect of isolation rearing in rats appears to have a strain dependant effect on NR1 mRNA expression in the hippocampus, with Wistar rats showing no change, but NR1 mRNA levels increasing in isolated Fawn Hooded (FH) rats (Hall et al., 2002). However, FH rats show lower levels of NR1 mRNA than Wistars in almost all brain regions, such that isolation rearing actually normalised NR1 expression levels in the hippocampus.

Taken together the experiments reported in this chapter demonstrate the ability of isolation rearing to detect pro-cognitive effects of drugs with diverse pharmacological mechanisms.

## **5.6 Conclusion**

The 5-HT<sub>6</sub> antagonist Ro 04-6790 has no effect on isolation rearing induced hyperactivity in a novel environment, but does reverse isolation-induced impairments in reversal learning in the water maze and therefore may show efficacy against cognitive deficits in schizophrenia.

The AMPA modulator aniracetam showed trends towards improving recognition memory in social and isolation reared rats but further work is required. Aniracetam had no effect on isolation induced deficits in passive avoidance or PPI.

The next chapter will attempt to model schizophrenia by investigating the effects of combining isolation rearing with exposure to the active compound in cannabis,  $\Delta^9$ -tetrahydrocannabinol.

**6 The Effects of  $\Delta^9$ -Tetrahydrocannabinol on Isolation Reared Rats**

## 6.1 Aim

Some studies have suggested an increased risk of schizophrenia in individuals who have used high levels of cannabis during adolescence. To investigate this potential interaction further the long-term effects of the major active compound in cannabis,  $\Delta^9$ -tetrahydrocannabinol (THC) were investigated in isolation reared rats, using two dosing schedules.

## 6.2 Introduction

While the exact causes of schizophrenia remain unknown, it is believed that risk of developing the disease is controlled by a combination of genetic and environmental factors. One environmental factor that has been the focus of much research is the impact of cannabis misuse. Cannabis, or marijuana, is an illegal substance derived from the *Cannabis sativa* plant. Cannabis is widely abused, especially amongst young people (McArdle, 2006). Cannabis intoxication can cause mild psychosis which is usually transient (McGuire et al., 1994, Emrich et al., 1997, Kalant, 2004) as well as impairment in a number of cognitive tasks (Ranganathan and D'Souza, 2006). However, there is increasing evidence that as well as the transient effects, cannabis can have longer-term repercussions, such as cognitive impairment and increased risk of developing schizophrenia (Moore et al., 2007).

In 1987 a 15-year longitudinal study of conscripts to the Swedish army found that those who used cannabis frequently had a 6-times greater risk of developing schizophrenia than those who had never used cannabis (Andreasson et al., 1987). However, Andreasson's work received criticism for not adequately addressing three possible interpretations of the results: the possible effects of abuse of other substances; pre-morbid personality traits which predisposed subjects to both schizophrenia and cannabis abuse and use of cannabis as 'self-medication' of the symptoms of pre-diagnosed schizophrenia (Zammit et al., 2002). In 2002 a follow up paper was published, standing by

the original findings, in which abuse of other substances and the presence of mental illness were excluded as possible confounds to the interpretation (Zammit et al., 2002). This work remains controversial, with several rapid responses to the paper appearing on the British Medical Journal website (Birkett et al., 2002-2004). While most of the respondents acknowledge the association between cannabis use and schizophrenia they point out that causality is very difficult to prove, with one of the main arguments being that while cannabis use varies across the world, the rates of schizophrenia are very similar worldwide (Bromet and Fennig, 1999, Hall and Degenhardt, 2007).

In 2002 a 3-year study in the Netherlands also found that cannabis use at baseline predicted the level of psychotic symptoms at follow up after 1 and 3 years (van Os et al., 2002) and a similar result was found in New Zealand (Fergusson et al., 2003). Recent reviews of the available longitudinal studies also found an increased, dose-dependent, risk of psychosis in cannabis users, with frequent users being most at risk (Henquet et al., 2005, Moore et al., 2007). However, subtle behavioural abnormalities exist in people who go on to develop schizophrenia well before they show symptoms of the disease so it is highly likely that many of the subjects in these studies could already have been on the path to schizophrenia before they ever abused cannabis (Walker et al., 1994, Mittal and Walker, 2007). It has also been found that schizophrenics who used cannabis had an earlier age of onset than those who did not (Veen et al., 2004). Within the central nervous system (CNS)  $\Delta^9$ -tetrahydrocannabinol (THC), the major active constituent of cannabis, acts primarily on CB<sub>1</sub> receptors. These G-protein coupled receptors are wide spread in the brain, including areas implicated in schizophrenia and cognitive processes, such as nucleus accumbens and prefrontal cortex. CB<sub>1</sub> receptors are largely located on pre-synaptic nerve terminals where they are thought to inhibit the release of a variety of neurotransmitters including GABA, glutamate, noradrenaline and dopamine (Schlicker and Kathmann, 2001). Normally CB<sub>1</sub> receptors are activated by endogenous cannabinoids (endocannabinoids), such as anandamide and 2-arachidonyl glycerol (2-AG) (van der Stelt and Di Marzo, 2003, Childers, 2006). While a biological mechanism by which cannabis exposure could cause schizophrenia has not been elucidated it is hypothesised

that the close links between the endocannabinoid and dopaminergic systems may have a role (Laviolette and Grace, 2006b). Of particular note, CB<sub>1</sub> receptor density is increased in the PFC and the endocannabinoid anandamide is increased in the cerebrospinal fluid (CSF) of schizophrenic patients (Ujike and Morita, 2004).

While it seems that there is some association between cannabis and schizophrenia the nature of the link is still unclear. Clearly not all people who use cannabis develop schizophrenia. This raises the question, are some people more vulnerable to the effects of cannabis due to genetic or environmental factors? For example, a polymorphism of the COMT gene can affect the response to cannabis; individuals with a valine<sup>158</sup> allele are more likely to exhibit schizophrenic symptoms after adolescent cannabis use than those with two methionine alleles (Caspi et al., 2005). The studies described in this thesis so far have demonstrated that isolation rearing causes behavioural changes which resemble some of the core symptoms of schizophrenia (prepulse inhibition deficits, recognition memory impairments) while other aspects of schizophrenia may not be seen in isolation reared rats (such as attentional set shifting deficits). Isolation rearing is also not as robust a model as would be required for program screening novel compounds for efficacy, with only 80-85% of cohorts demonstrating prepulse inhibition deficits (see general discussion and (Cilia et al., 2005b)). Therefore, this final set of experiments investigated the effect of exposure to THC during the period of isolation rearing to identify any interaction and impact on the resultant neurodevelopmental alteration produced.

Two dosing regimens were used, both of which administered THC at around the time of weaning. In the first study four doses of 2mg/kg THC were given, starting on the first full day of isolation. A low dose was used to avoid the sedative effects of THC that occur when doses exceed 10mg/kg (Cota et al., 2003). The second study increased both the dose (5mg/kg) and the number of times the drug was given (eight) and began drug administration before weaning. The drug was administered around the time of weaning, while neurodevelopmental changes were still in progress.

## 6.3 Methods

### 6.3.1 Effect of Isolation Rearing and THC (4 x 2mg/kg)

#### *Animals*

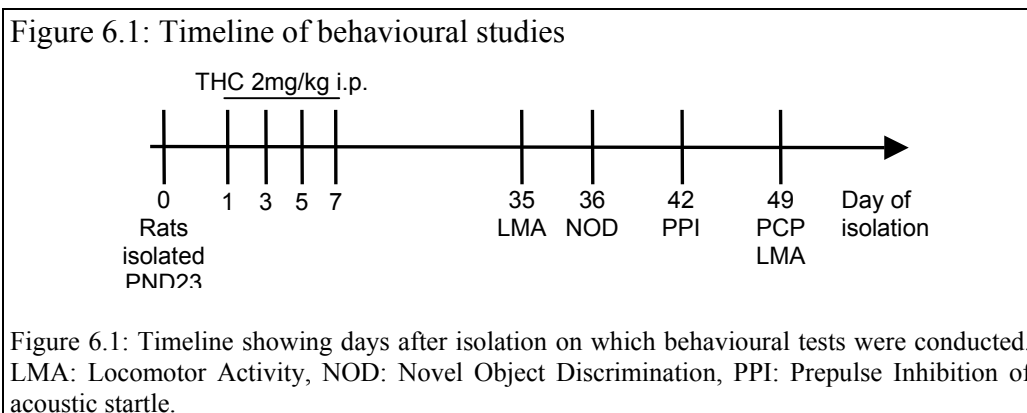
40 Lister Hooded rats (BMSU, Nottingham University and CRUK) were weaned on PND 23. Rats were either housed in alone (isolate) or in groups of 3 or 4 (social). Housing conditions were exactly as described in Chapter 2. THC dosing and behavioural testing was carried out according to the timeline shown in Figure 6.1.

#### *Drugs*

On PND 24, 26, 28 and 30 rats received either 2mg/kg THC (Sigma, UK) or 1ml/kg vehicle (0.25% v/v Tween 80 in 0.9% NaCl), i.p. This dosing regimen was chosen to administer the THC during the very early isolation period, but not handle the rats everyday as excessive handling can prevent the development of isolation-induced behavioural alterations (Rosa et al., 2005).

On day 49 after isolation (PND 72) all rats received 3mg/kg phencyclidine HCl (Sigma, UK), dissolved in 0.9%NaCl, i.p. to measure any differences in response to the NMDA receptor antagonist.

#### *Behavioural Testing*



The locomotor activity, novel object discrimination and prepulse inhibition tests were carried out as previously described in Chapter 2. On day 49 after isolation the effects of PCP treatment on locomotor activity were examined. Rats were placed in the activity boxes for 20 minutes to habituate to the environment. All animals then received 3mg/kg PCP and were immediately returned to the activity boxes and monitored for a further 2 hours. This dose of PCP was chosen following a preliminary study in which 2mg/kg and 5mg/kg of PCP were administered to drug naive rats and the locomotor response observed. The locomotor response to 5mg/kg PCP was very high and may not have allowed any sensitisation to be detected, so 3mg/kg was chosen.

## 6.4 Results

### 6.4.1 Effect of Isolation Rearing and THC (4 x 2mg/kg)

#### *Body Weight*

Over the duration of the study all animals gained weight, with isolation reared rats gaining significantly more weight than socially housed animals (RM ANOVA  $F_{(7,252)}=4.81$   $p=0.012$ ) (Figure 6.2) and THC treated animals gaining less weight than the vehicle controls (RM ANOVA  $F_{(7,252)}=8.09$   $p=0.001$ ). No time x housing x drug interaction was observed. When data was collapsed over the whole time period a significant effect of THC treatment remained (RM ANOVA  $F_{(1,36)}=6.19$   $p=0.018$ ) but no effect of housing or interactions were found.

#### *Locomotor Activity in a Novel Environment*

Overall isolation reared rats were more active than the socially housed animals (RM ANOVA  $F_{(1,36)}=11.02$   $p=0.002$ ) (Figure 6.3), but THC treatment had no effect on activity (RM ANOVA  $F_{(1,36)}=0.50$   $p=4.83$ ) and no interactions were observed. A similar pattern was observed in rearing behaviour, with isolated rats rearing more (RM ANOVA  $F_{(1,36)}=17.21$   $p<0.001$ ) but no effect of THC treatment (data not shown).

Figure 6.2: THC treated rats gain weight more slowly than controls, regardless of housing condition

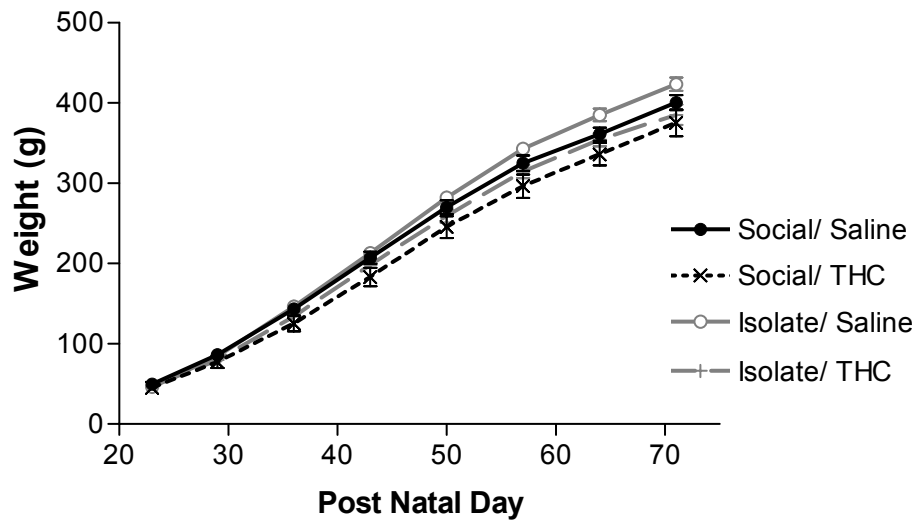


Figure 6.2: Weight of 4x 2mg/kg THC and vehicle treated rats housed in isolation or social conditions. Data are presented as mean  $\pm$  s.e.m, n=10 per group. THC treatment reduced the amount of weight gained by both social and isolated animals (RM ANOVA  $F_{(7,252)}=4.81$   $p=0.012$ ).

Figure 6.3: Isolation reared rats are more active in a novel environment, but THC treatment has no effect.

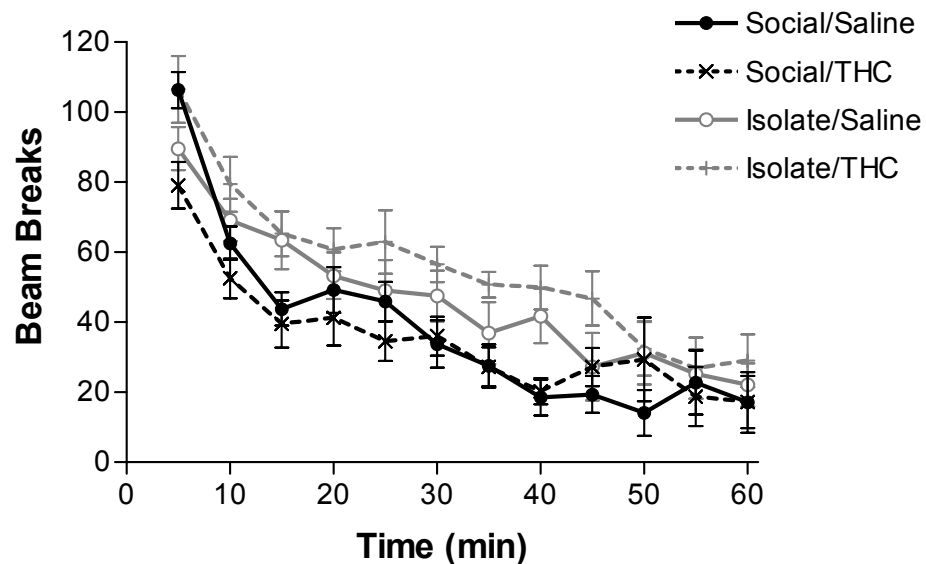


Figure 6.3: Activity of isolated or socially reared animals treated with 4x 2mg/kg THC or vehicle measured by beam breaks in a 5 minute time bin. Beam breaks are plotted at the end of each time bin but represent breaks during the 5 minute epoch. Results are plotted as mean beam breaks  $\pm$  s.e.m, n=10. Isolation reared rats were more active than socials (RM ANOVA  $F_{(1,36)}=11.02$   $p=0.002$ ).



*Novel Object Discrimination*

During the familiarisation trial (T1) no animals showed a preference for the front or back object (see Table 6.1 for paired Student's *t*-test statistical values) and no differences were seen in total object exploration (2-way ANOVA Housing:  $F_{(1,36)}=1.22$   $p=0.277$ , Treatment:  $F_{(1,36)}=0.53$   $p=0.471$ ).

When presented with a novel and familiar object during the choice trial (T2) all groups of animals successfully discriminated the novel object from the familiar object (Figure 6.4 and Table 6.1). Therefore, in this particular study, using a 2 hour inter-trial interval, neither isolation rearing nor previous THC treatment impaired recognition memory. No differences were seen in discrimination ratios either (2-way ANOVA housing  $F_{(1,36)}=0.20$   $p=0.661$ , treatment  $F_{(1,36)}=0.36$   $p=0.553$ , Table 6.1). No differences were seen in total exploration of the objects in this trial (2-way ANOVA housing:  $F_{(1,36)}=2.27$   $p=0.141$  treatment:  $F_{(1,36)}=0.42$   $p=0.255$ ) (data not shown).

Table 6.1: NOD statistical values for social and isolated animals after THC or vehicle treatment and discrimination ratios.

	Social/ Vehicle	Social/ THC	Isolate/ Vehicle	Isolate/ THC
T1: Front vs. back	$p=0.874$	$p=0.069$	$p=0.578$	$p=0.437$
T2: Novel vs Familiar	$p=0.012$ *	$p=0.017$ *	$p=0.008$ **	$p=0.006$ **
DR (mean $\pm$ s.e.m.)	$0.31\pm 0.098$	$0.28\pm 0.071$	$0.29\pm 0.074$	$0.23\pm 0.059$

*Prepulse Inhibition*

Overall isolation rearing attenuated prepulse inhibition of acoustic startle (RM ANOVA  $F_{(1,36)}=5.05$   $p=0.031$ ) (Figure 6.5). THC treatment had no effect on PPI (RM ANOVA  $F_{(1,36)}=0.34$   $p=0.566$ ) and no interactions were observed. Neither housing nor THC treatment effected the basal startle amplitude to the pulse alone (2-way ANOVA housing:  $F_{(1,36)}=0.14$   $p=0.714$ , treatment:  $F_{(1,36)}=0.96$   $p=0.33$ ) (data not shown).

Figure 6.4: Neither isolation rearing nor THC treatment affected recognition memory

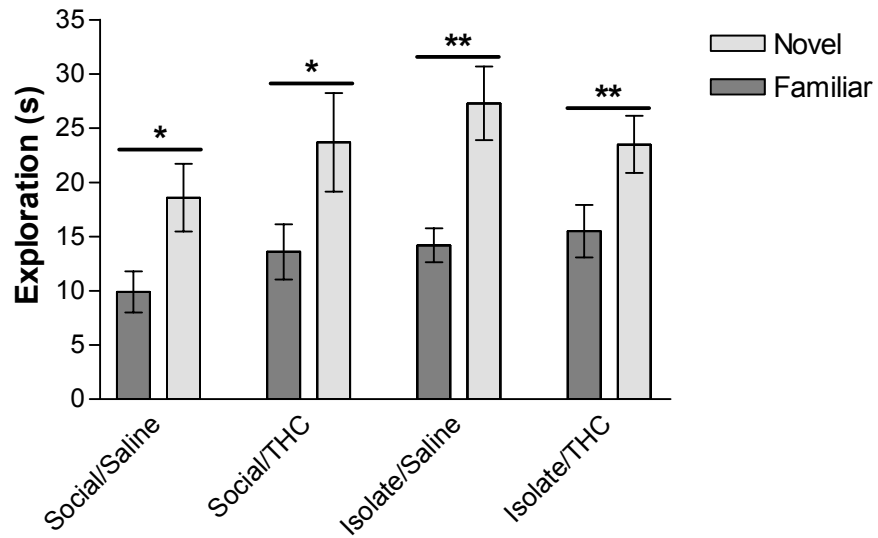


Figure 6.4: Time (s) (mean  $\pm$  s.e.m.) spent exploring the familiar and novel object in the NOD choice trial. Neither isolation rearing nor 4x 2mg/kg THC treatment impaired discrimination of the novel object. \* $p < 0.05$ , \*\* $p < 0.001$  paired Student's *t*-test.

Figure 6.5: Isolation reared rats have impaired prepulse inhibition, which is unaffected by THC treatment.

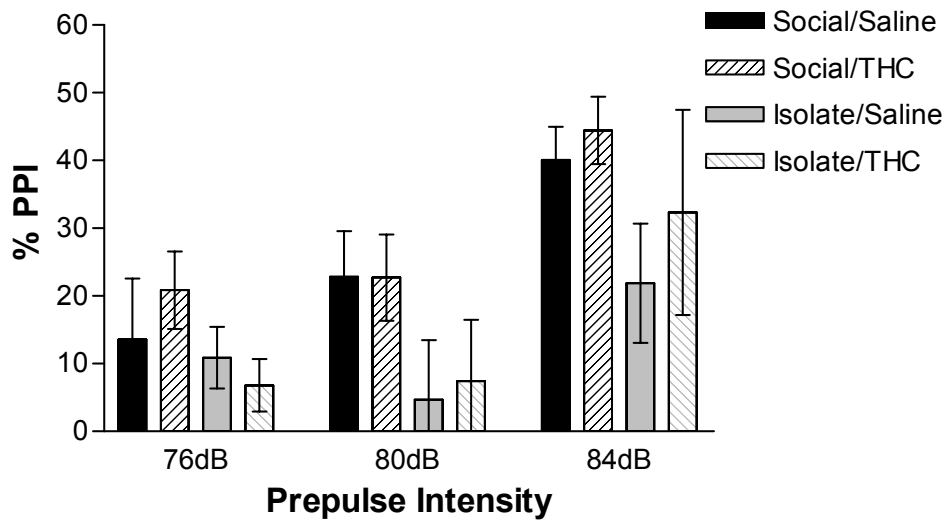


Figure 6.5: % PPI of isolation reared and socially housed rats after 4x 2mg/kg THC or vehicle treatment, data shown as mean  $\pm$  s.e.m.,  $n=10$  per group. Isolation rearing decreased % PPI (RM ANOVA  $F_{(1,36)}=5.05$   $p=0.031$ ) but THC had no effect.

*Effect of PCP on Locomotor Activity*

The activity of the animals was monitored for 2 hours after systemic PCP administration to assess whether there was any interaction between isolation rearing and cannabis on NMDA receptor function. PCP treatment only elevated activity levels above basal during the first 20 minutes following drug treatment so this period of the activity response was selected for analysis. Neither isolation rearing nor previous THC treatment had any effect on PCP-induced locomotor activity during the first 20 minutes post-PCP (RM ANOVA housing:  $F_{(1,36)}=0.14$   $p=0.711$ , treatment:  $F_{(1,36)}=0.21$   $p=0.647$ ) (Figure 6.6).

Figure 6.6: Locomotor response to PCP is unaffected by either isolation rearing or previous THC treatment.

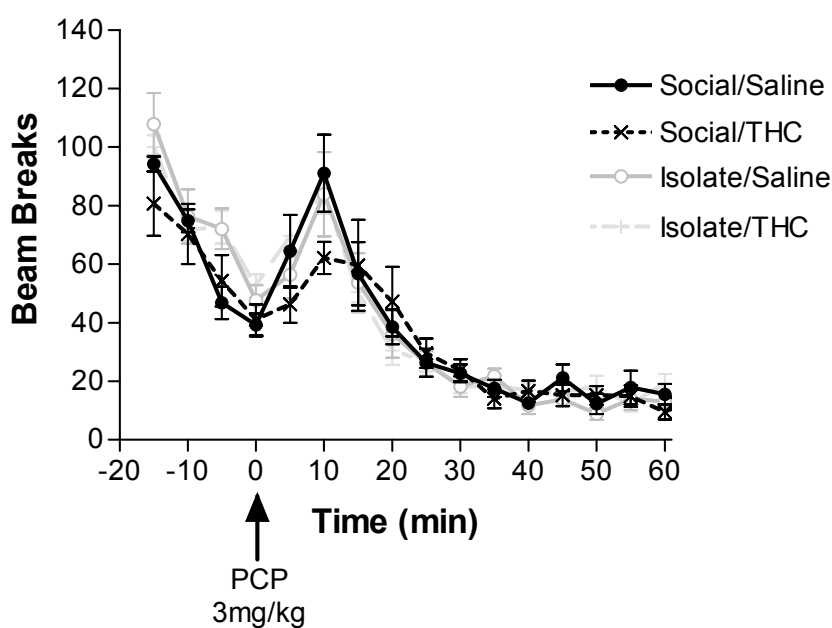


Figure 6.6: Activity of social or isolated rats previously treated with 4 x 2mg/kg THC or vehicle following PCP administration. Activity was measured by beam breaks in 5 minute time bins, plotted at the end of each bin, as mean  $\pm$  s.e.m.  $n=10$  per group. Neither isolation nor THC had any affect on activity levels.

## 6.5 Discussion- Part I

### 6.5.1 Effect of Isolation Rearing and THC (4 x 2mg/kg)

The results showed that THC treatment caused a reduction in body weight in both social and isolation reared rats. Isolation rearing caused the expected increase in activity on placement in a novel environment and attenuated prepulse inhibition of acoustic startle but neither behaviour was affected by THC treatment. Neither isolation rearing nor THC treatment had an effect on recognition memory or the locomotor response to PCP treatment. The lack of effect on PCP-induced locomotor activity suggests that THC has no long-term effects mediated by NMDA receptor function.

Cannabis has long been known to have effects on appetite and energy metabolism. THC and other CB<sub>1</sub> agonists increase in appetite and body weight gain via a range of central and peripheral mechanisms (reviewed in (Matias and Di Marzo, 2007), whereas in this study the THC treated animals did not gain weight as quickly as the controls. Previous studies have found that treatment with THC or the CB agonist CP 55,940 can cause a reduction in weight and food intake during the dosing period (Manning et al., 1971, Biscaia et al., 2003). In Manning's study although food intake returned to normal after dosing, the THC treated rats remained lighter than the vehicle controls up to the end of the study period 30 days later. At this point the rats that had received i.p. THC showed signs of peritonitis on post-mortem examination. The present study was not specifically designed to look at effects of THC on food intake, activity or metabolic rate, so the exact cause of the lack of weight gain cannot be explained, but peritonitis is a possible cause. It is also possible that the THC treatment caused a down-regulation of CB<sub>1</sub> receptors which reduced appetite for longer than the THC treatment period. THC has a low efficacy at the CB<sub>1</sub> receptor and acts as a partial, rather than full, agonist (Childers, 2006). If the endocannabinoid activity was high, during this phase of rapid growth in the pubertal period examined herein it is possible that THC was acting as a competitive antagonist, thereby reducing appetite.

While isolation rearing caused an increase in locomotor activity and impaired prepulse inhibition, THC had no effect on any of the behavioural end points in this study. The doses used were relatively low, so a second study was carried out using a higher dose of THC (5mg/kg instead of 2mg/kg) as some studies have found the effects of cannabis on schizophrenia are dose dependent (Zammit et al., 2002, Moore et al., 2007). It is also possible that the THC was not given early enough to affect the long-term development of the CB<sub>1</sub> receptor or the subsequent down stream changes. To address this possibility THC administration was also commenced earlier in the second study.

The effect of THC dosing was also addressed in two extra behavioural assays, passive avoidance and attentional set shifting. As shown in a previous chapter isolation rearing alone does not appear to affect attentional set-shifting, which is thought to have translational relevance to a core symptom of schizophrenia. Heavy cannabis use impairs executive function more than infrequent use (Pope and Yurgelun-Todd, 1996) so it is possible that the higher doses used in this study could impair attentional set-shifting. Repeated exposure to THC (14 days, 10mg/kg 2xday) has been shown to reduce DA metabolism in the PFC, a brain region involved in executive function and attentional set-shifting (Jentsch et al., 1998). The CB<sub>1</sub> receptor and cannabinoid system have also been implicated in learning and extinction of aversive memories (Marsicano et al., 2002) and therefore the effect of the isolation rearing / THC combination on passive avoidance was also investigated.

## 6.6 Methods

### 6.6.1 Effect of Isolation Rearing and THC (8 x 5mg/kg)

#### *Animals*

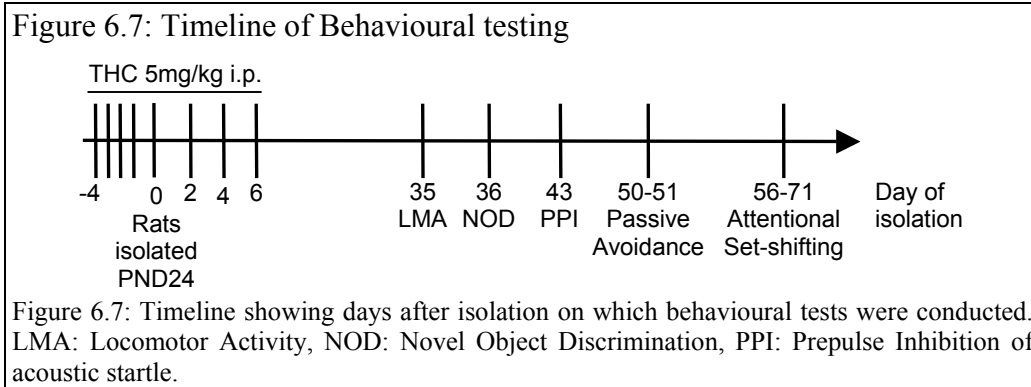
38 male Lister Hooded rats (BMSU, Nottingham University) were bred on site. Female pups were culled on PND 10, leaving only males. All litters consisted of 5-8 pups. Pups were assigned to groups and on PND 20 began treatment with THC or vehicle as described below. Rats were weaned on PND 24 and either housed in groups of 3-4 (social) or alone (isolate) as previously described in Chapter 2.

#### *Drug Treatment*

Rats received either 5mg/kg THC (Sigma, UK) or 2ml/kg vehicle (0.025% Tween 80 in 0.9% NaCl), i.p. on PND 20, 21, 22, 23, 24, 26, 28 and 30. This dosing regimen was chosen as the PND 24-30 injections were given at the same age as the THC doses in the first study described in this chapter, but a higher dose was used. In order to have more time to effect brain development THC was not only given at a higher dose, but also at an earlier age, starting dosing before weaning on PND 20-23.

#### *Behavioural Testing*

Behavioural tests were carried out according to the timeline depicted in Figure 6.7. All tests were carried out as previously described in Chapters 2, 3 and 5, with one change to the passive avoidance protocol. Previously when the rats were returned to the test box on the second day they were allowed 5 minutes to move from the light compartment into the dark (shock) compartment. If they did not re-enter the dark compartment within the allocated time the test was stopped. However, some rats did not run through within this time and therefore in this study the time allowed was increased to 10 minutes.



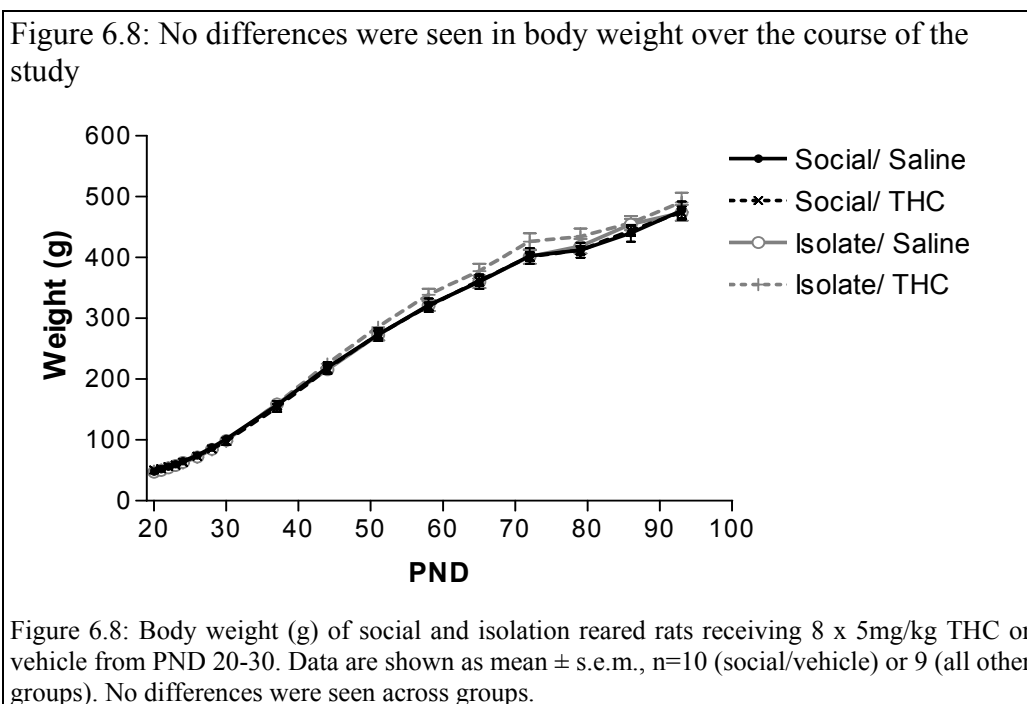
## 6.7 Results

### 6.7.1 Effect of Isolation Rearing and THC (8 x 5mg/kg)

The results from one rat from the social/THC group were omitted from all tests as substantial cortical atrophy and ventricular enlargement were found during brain dissection.

#### *Body Weight*

Both during the drug treatment period and over the whole time course of the study no differences were found in the weights of the animals (whole study RM ANOVA Housing:  $F_{(1,29)}=1.26$   $p=0.271$ , Treatment  $F_{(1,29)}=0.09$   $p=0.771$ ) (Figure 6.8).



#### *Locomotor Activity in a Novel Environment*

Over the whole test session neither isolation rearing nor THC treatment had an effect on activity in a novel environment (RM ANOVA housing:  $F_{(1,33)}=1.61$   $p=0.213$ , treatment:  $F_{(1,33)}=0.30$   $p=0.587$ , no interactions) (Figure 6.9A). During the first 30 minutes isolation reared rats demonstrated a tendency towards increased activity, but this failed to reach significance (RM ANOVA Housing:  $F_{(1,33)}=3.38$   $p=0.075$ ).



Isolation reared rats performed significantly more rearing behaviour than socially housed animals, over the whole test session (RM ANOVA Housing:  $F_{(1,33)}=4.84$   $p=0.035$ , Treatment:  $F_{(1,33)}=0.20$   $p=0.657$ , no interactions) (Figure 6.9B).

Figure 6.9: Neither isolation nor THC treatment increased locomotor activity (A), but isolation reared rats performed more rearing than socials (B).

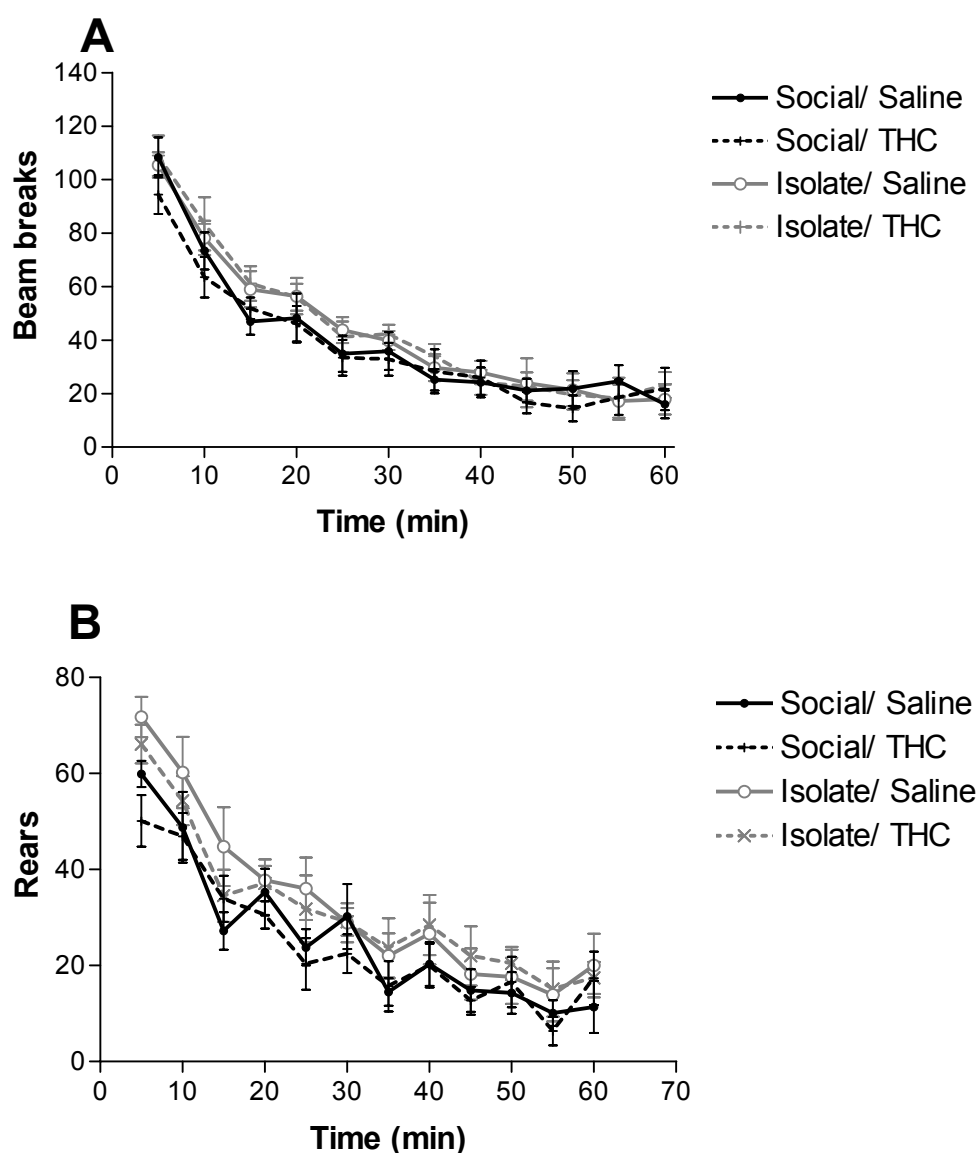


Figure 6.9: Horizontal activity (A) or rearing (B) measured by beam breaks during a 5 minute time bin. Results are presented as mean  $\pm$  s.e.m. at the end of the time bin,  $n=10$  (social/vehicle) or 9 (all other groups). No differences were seen in horizontal activity, but isolation increased rearing behaviour ( $p<0.05$ ).

*Novel Object Discrimination*

During the familiarisation trial (T1) all groups performed the same total exploration time (2-way ANOVA Housing:  $F_{(1,33)}=3.12$   $p=0.087$ , Treatment:  $F_{(1,33)}=1.27$   $p=0.268$ ). The isolate/vehicle group showed a slight preference for the object at the back of the arena (see Table 6.2 for paired  $t$ -test significance values), but all other groups showed no preference for object position. In the choice trial (T2) the position of the novel object is varied and no groups showed a preference for object position (see Table 6.2). Only the social/vehicle group were able to discriminate the novel object, neither social or isolated rats that received THC were able to discriminate the novel object (Figure 6.10). The discrimination ratios (Table 6.2) showed a trend towards a significant effect of housing, although this just failed to reach significance (2-way ANOVA housing  $F_{(1,36)}=3.99$   $p=0.054$ , treatment  $F_{(1,36)}=2.835.10$   $p=0.102$ ). In the choice trial there was a trend towards increased total exploration by the isolation reared rats (2-way ANOVA Housing:  $F_{(1,33)}=3.67$   $p=0.064$ ) but this did not reach significance (data not shown).

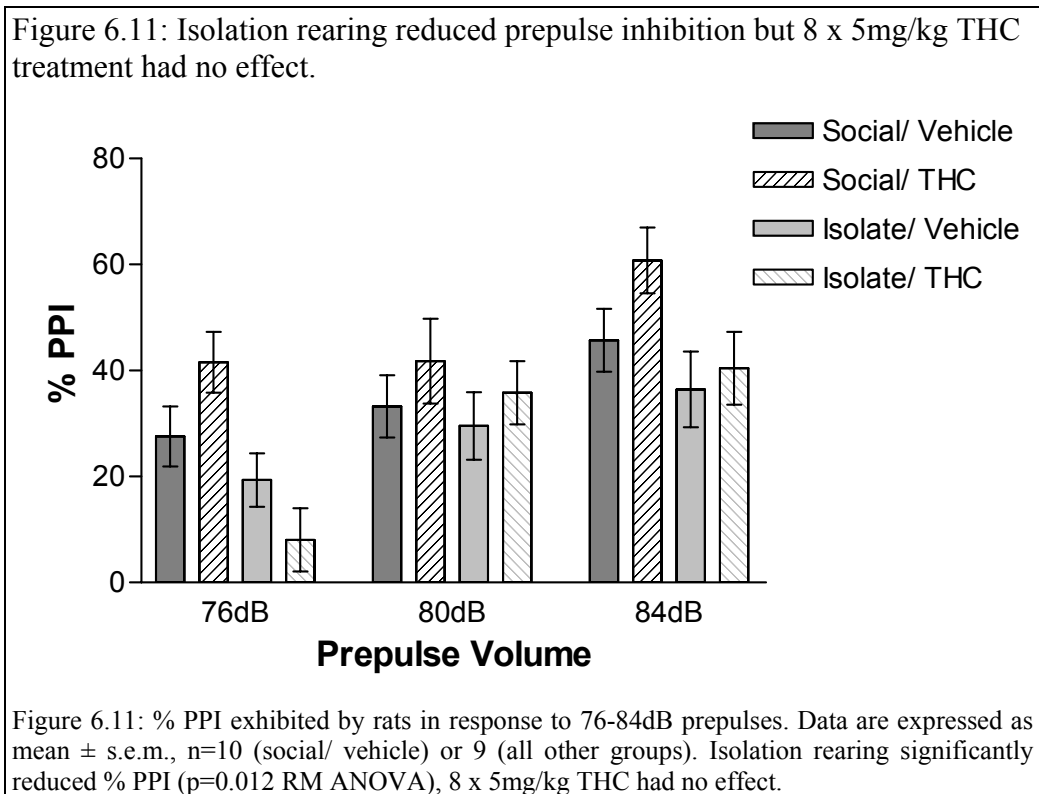
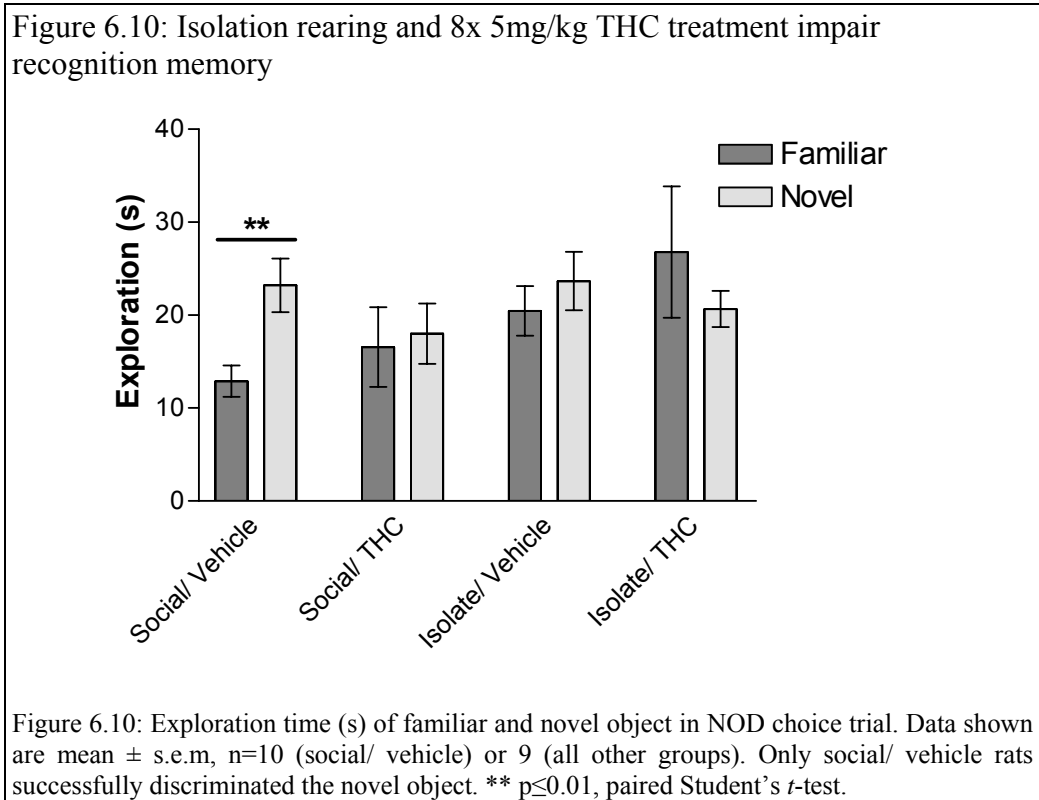
Table 6.2: Statistical significance values for NOD in THC treated, isolation reared rats. \*  $p<0.05$ , \*\* $p\leq 0.01$ , paired Student's  $t$ -test.

	Social/ Vehicle	Social/ THC	Isolate/ Vehicle	Isolate/ THC
T1: front vs. back	$p=0.290$	$p=0.605$	$p=0.011$ *	$p=0.834$
T2: front vs. back	$p=0.787$	$p=0.492$	$p=0.305$	$p=0.143$
T2: novel vs. familiar	$p=0.010$ **	$p=0.801$	$p=0.401$	$p=0.387$
DR (mean $\pm$ s.e.m.)	$0.29\pm 0.068$	$0.10\pm 0.118$	$0.07\pm 0.085$	$-0.04\pm 0.080$

*Prepulse Inhibition of Acoustic Startle*

Increasing prepulse volume caused a significant increase in prepulse inhibition (RM ANOVA  $F_{(2,32)}=33.79$   $p<0.001$ ) (Figure 6.11). Isolation rearing significantly attenuated prepulse inhibition, but THC treatment had no effect (RM ANOVA Housing:  $F_{(1,33)}=6.99$   $p=0.012$  Treatment:  $F_{(1,33)}=1.43$   $p=0.241$ , no interactions). Neither housing nor drug treatment had an effect on basal

startle response to the 120dB pulse (2-way ANOVA Housing:  $F_{(1,33)}=0.29$   $p=0.593$  Treatment:  $F_{(1,33)}=2.13$   $p=0.154$ ), data not shown.



*Passive Avoidance*

On day 1 of passive avoidance neither housing nor drug treatment had an effect on the time taken to enter the dark compartment, where the foot-shock was administered (2-way ANOVA Housing:  $F_{(1,33)}=0.13$   $p=0.726$  Treatment:  $F_{(1,33)}=0.63$   $p=0.432$ , no interactions) (data not shown).

On day 2 of passive avoidance, during the retention trial, isolation reared rats re-entered the dark compartment significantly more quickly than socially housed animals (2-way ANOVA Housing:  $F_{(1,33)}=10.91$   $p=0.002$ ). Overall, THC treatment had no effect ( $F_{(1,33)}=0.62$   $p=0.437$ ). However, a significant interaction between housing and treatment was seen, indicating that THC treatment had opposite effects in social and isolation reared rats (2-way ANOVA Housing x Treatment interaction  $F_{(1,33)}=4.82$   $p=0.035$ ), with socials being impaired by THC treatment and moving into the dark compartment more quickly, while isolates who had received THC took longer to re-enter the dark compartment than those who had received vehicle (Figure 6.12).

Figure 6.12: THC treatment impaired passive avoidance in social rats, but improved performance in isolation reared animals

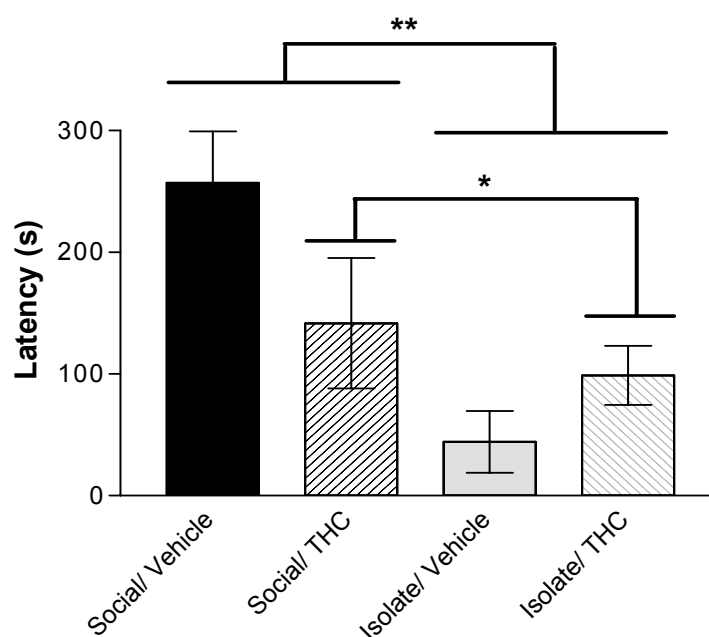
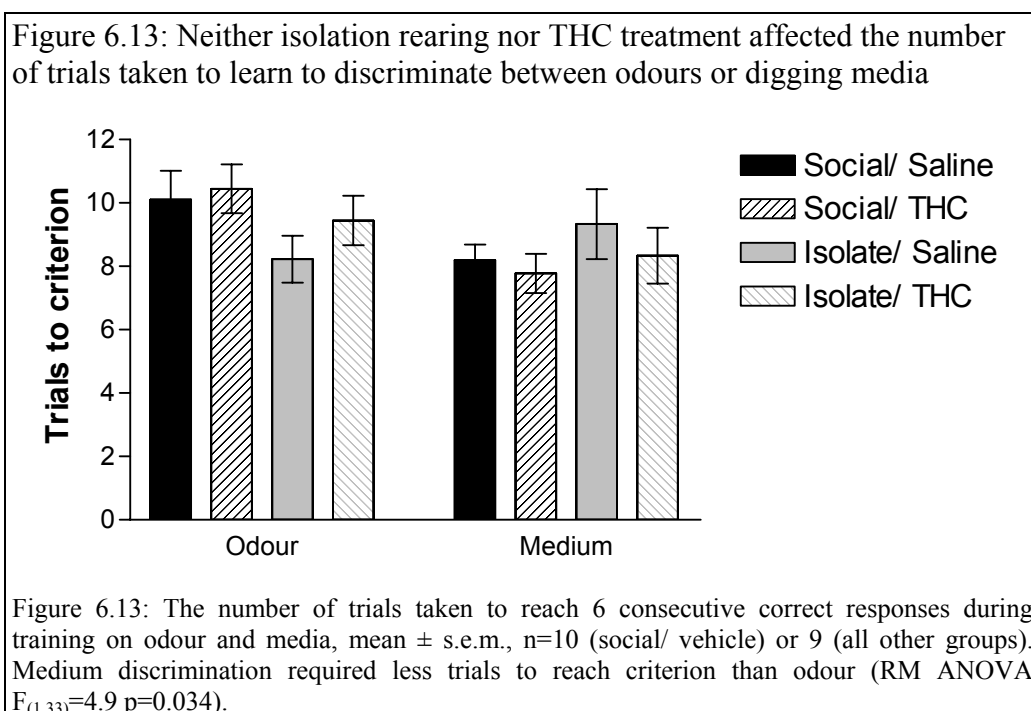


Figure 6.12: Time (s) taken to re-enter the dark (shock) compartment in passive avoidance (mean  $\pm$  s.e.m.).  $n=10$  (social/ vehicle) or 9 (all other groups). Isolation reared rats took less time to pass into the dark compartment (\*\* $p<0.01$ ) and 8 x 5mg/kg THC treatment had opposite effects in social and isolation reared rats (\* $p<0.05$ , 2-way ANOVA).

*Attentional Set Shifting***Odour and Medium Discrimination Training**

On the fourth day of the ID/ED protocol animals underwent discrimination training. Statistical analysis revealed a significant effect of dimension on number of trials to reach criterion (6 consecutive correct trials), indicating that rats learnt to discriminate between two media more easily than between two odours (RM ANOVA, dimension effect  $F_{(1,33)}=4.9$   $p=0.034$ , see Figure 6.13) Previous treatment with 8x5mg/kg THC and isolation rearing were found to have no effect on ability to discriminate either dimension (RM ANOVA Housing effect:  $F_{(1,33)}=0.38$   $p=0.542$  THC effect:  $F_{(1,33)}=0.01$   $p=0.940$ ). The training results indicate that learning media discrimination was easier than odour discrimination. However the study was carefully balanced such that equal numbers of animals from each treatment group followed the same starting dimension, which would counteract this effect.



**Attentional Set Shifting Test Day**

On the test day there was no significant difference in the number of trials to criterion between all the discriminations (RM ANOVA  $F_{(6,28)}=1.90$   $p=0.117$ ) (Figure 6.14), indicating that overall the animals did not find any particular discriminations more difficult than any others. Across all the discriminations there was no effect of either housing or drug on performance in the attentional set shifting task (RM ANOVA Housing:  $F_{(1,33)}=1.03$   $p=0.317$ , Treatment:  $F_{(1,33)}=0.59$   $p=0.446$ , no interactions). 2-way ANOVA analysis of individual discriminations revealed no effect of housing or THC treatment at any discrimination.

Analysis of the time taken to complete the set-shifting task revealed an overall significant effect of discrimination, indicating that some discriminations took longer to complete than others (RM ANOVA  $F_{(4,6,152.8)}=6.22$   $p<0.001$ ) (Figure 6.15). However, across all discriminations there was no effect of housing or THC treatment (RM ANOVA Housing:  $F_{(1,33)}=1.48$   $p=0.232$  Treatment:  $F_{(1,33)}=0.53$   $p=0.473$ , no interactions). Individual analysis of each discrimination revealed only one significant effect: in the compound discrimination isolation reared rats took significantly longer to reach criterion than socials (2-way ANOVA Housing  $F_{(1,33)}=5.81$   $p=0.022$ ) but THC treatment had no effect.

Figure 6.14: Neither isolation rearing nor THC treatment had a significant effect on number of trials to reach criterion during attentional set-shifting

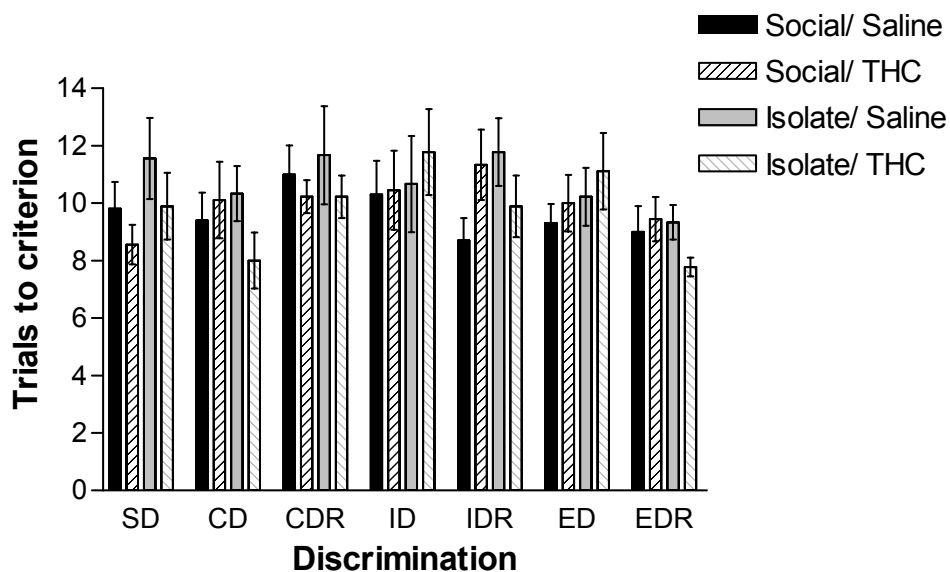


Figure 6.14: Trials to reach criterion (mean  $\pm$  s.e.m) for each discrimination during attentional set shifting. n=10 (social/ vehicle) or 9 (all other groups). Neither housing nor drug treatment had any significant effect.

Figure 6.15: Time taken to reach criterion was not significantly affected by isolation rearing or THC treatment

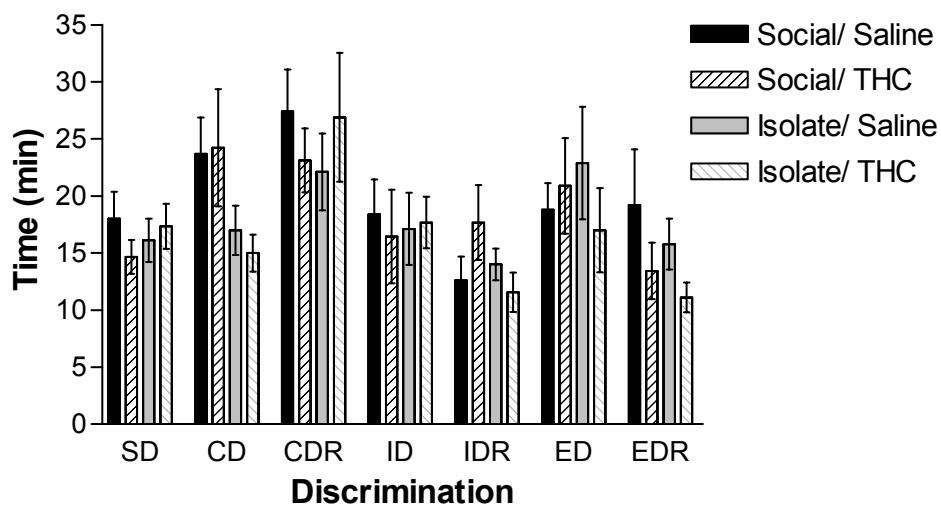


Figure 6.15: Time (s) to reach criterion for each discrimination during attentional set shifting. Data are presented at mean  $\pm$  s.e.m., n=10 (social/ vehicle) or 9 (all other groups). There was no effect of isolation rearing or THC treatment (RM ANOVA).

## 6.8 Discussion- Part II

In the second isolation rearing/THC study there was no difference in the body weight gain of the animals, or performance in the attentional set-shifting paradigm, irrespective of rearing condition or drug treatment. Isolation rearing also failed to produce a significant increase in the horizontal locomotor activity in a novel environment, although isolation reared rats did perform significantly more rears up the arena walls than the social controls. Both isolation rearing and THC treatment impaired novel object recognition, but only isolation rearing had an effect in prepulse inhibition of acoustic startle. In passive avoidance isolation reared rats re-entered the dark (shock paired) compartment more quickly than socials on the retention trial. THC had opposite effects in social and isolated rats, impairing the performance of social animals, but increasing re-entry latency in isolates. Table 6.3 shows a comparison of the behavioural effects of isolation rearing with the two THC treatment regimens used in this chapter.

Table 6.3: Comparison of the effects of isolation rearing and THC treatment

	4 x 2mg/kg			8 x 5mg/kg		
	Housing	THC	Interaction	Housing	THC	Interaction
Body weight	x	✓	x	x	x	x
Horizontal LMA	✓	x	x	x	x	x
Rearing	✓	x	x	✓	x	x
NOD	x	x	-	✓	✓	-
PPI	✓	x	x	✓	x	x
Passive avoidance	-	-	-	✓	x	✓
Attentional set-shifting	-	-	-	x	x	x

Table 6.3: ✓ A significant effect was observed ( $p < 0.05$ ); x no significant effect was observed ( $p > 0.05$ ); - effect was not measured.



In contrast to the first, lower dose THC study, the second study found no effect of THC treatment on body weight. This is surprising as the proposed explanations for weight loss in the first study (peritonitis caused by i.p. dosing or down regulation of CB<sub>1</sub> receptors in the hypothalamus) would also be expected to result in weight loss in the second study. It has been found that THC can have opposing effects on appetite in humans and animals, depending on the dose. Low doses seem to cause appetite stimulation and higher doses caused an initial decrease in appetite, followed by an increase (Cota et al., 2003). The difference between the effects of THC on body weight in these two studies cannot currently be explained.

#### *Locomotor Activity*

In both of these studies THC treatment alone had no effect on the locomotor activity in a novel environment. While acute THC has been shown to reduce activity (Jarbe et al., 2002), most studies which have looked at the long-term effects of THC, or other CB<sub>1</sub> agonists, on activity have also found no effect (Schneider and Koch, 2003, Schneider et al., 2005, Kolb et al., 2006, O'Shea et al., 2006). In the second study isolation rearing also had no effect on locomotor activity. Increased activity is usually one of the most robust behavioural alterations demonstrated by isolation reared rats, so a lack of effect could call into question whether these animals have been adequately isolated. However, as in Chapter 5 where the Ro 04-6790 treated cohort of isolation reared rats did not develop PPI impairments, this group of isolation reared rats did show deficits in recognition memory and sensorimotor gating, supporting the validity and success of the isolation paradigm. Thus both of these cohorts of isolation reared rats developed behavioural deficits in two out of the three core behaviours tested (LMA, NOD and PPI). It is possible that handling during the THC dosing was sufficient to prevent some behavioural changes from developing (Krebs-Thomson et al., 2001). However, in the high dose THC cohort isolation rearing increased rearing activity, so isolation was not completely without effect on activity in a novel environment.

### *Novel Object Recognition*

The low dose THC cohort of isolation reared rats did not develop any alterations in recognition memory, such that all groups of animals could successfully discriminate the novel object. As discussed above, it is possible that the handling of the animals during the dosing period provided sufficient stimulation to prevent the development of the recognition memory deficit usually seen in isolation reared rats. However, it is surprising that in the two studies different behavioural traits did not develop as expected; the recognition memory deficit (in the low THC) and neophobia in the high THC cohort. This further demonstrates that isolation rearing is not sufficiently robust a model to look at the effects on only one behavioural phenotype.

In the high dose THC study isolation rearing caused an impairment in recognition memory which just failed to reach significance when discrimination ratios were analysed ( $p=0.054$ ). THC treated rats were unable to discriminate the novel object, but this was not significant when the discrimination ratios were analysed. This further shows that the variability of the novel object discrimination assay requires a large number of animals to be used to achieve significant results. The cannabinoid agonists WIN 55,212-2 and CP 55,940 have been shown to disrupt recognition memory when given acutely before the familiarisation trial (T1), as has the endocannabinoid anandamide (Schneider and Koch, 2002, Kosiorek et al., 2003). Schneider has also carried out a considerable amount of research into the long-term effects of cannabinoids on development and found that WIN 55,212-2 given in the pre-pubertal period (PND 15-40) or in adulthood had no long-term effects on recognition memory. However, when WIN 55,212-2 was administered in the peri-pubertal period (PND 40-65) the rats showed recognition memory deficits at adulthood (Schneider and Koch, 2003, Schneider et al., 2005). The rats in the study reported here were dosed between PND 20-30 and received fewer doses of cannabinoid but still developed recognition memory deficits. While this appears to contradict Schneider's results, it does demonstrate that the timing and exact dosage of cannabinoid can be important in determining the effect on recognition memory. However, rats dosed with 21 increasing doses of

another synthetic cannabinoid, CP 55,940, starting at either PND4 (perinatal), 30 (adolescent) or 56 (adult) all showed deficits in object recognition and decreased social interaction, regardless of the age at dosing (O'Shea et al., 2006). It should also be noted that THC has a relatively low efficacy at the CB<sub>1</sub> receptor and actually acts as a partial agonist, whereas the efficacy of some of the synthetic cannabinoid agonists for the CB<sub>1</sub> receptor is much greater (Childers, 2006) which could account for discrepancies in findings.

#### *Prepulse Inhibition of Acoustic Startle*

Both sets of isolation reared rats developed deficits in prepulse inhibition, indicating impairments in sensorimotor gating. Neither THC dosing regimen used in these studies had any effect on PPI. Schneider has also measured PPI in the pre-pubertal, peri-pubertal and adult rats dosed 20-25 times with WIN 55,212-2. The pre-pubertal and peri-pubertal dosing schedules both caused an attenuation of PPI when the rats reached adulthood, but rats dosed as adults did not develop PPI impairments (Schneider and Koch, 2003, Schneider et al., 2005), indicating that there is a critical period of vulnerability to the effects of THC.

Acute cannabinoid administration impairs PPI in both rats (Schneider and Koch, 2002) and mice (Nagai et al., 2006). In isolation reared rats acute THC (1 & 3mg/kg) treatment further attenuated isolation rearing-induced PPI impairments, but this deficit was prevented by pre-treatment with SR141716 (a CB<sub>1</sub> antagonist) (Malone and Taylor, 2006). However, in Malone's study acute THC had no effect on PPI in socially housed animals. This could be due to strain differences, as Malone used Sprague Dawley rats while Schneider used Wistars, or due to drug differences as Schneider used the synthetic cannabinoid WIN 55,212-2. Malone's finding of THC impairing PPI only in isolates suggests THC maybe more likely to reduce PPI in rats with abnormal sensorimotor gating processes. As the rats in these studies were at the start of their isolation period and had not had time to develop sensorimotor gating deficits this may explain why THC had no effect in this behavioural test.

THC (0.25-10mg/kg) reduced time spent in the open arms of the elevated-plus maze in mice. Interestingly the synthetic CB<sub>1</sub> agonists CP 55,940 and WIN 55,212-2 both increased time spent on the open arms and the antagonists SR141716 and AM251 both decreased open-arm time. This could be explained by the relatively low efficacy of THC compared to WIN 55,212-2 and CP 55,940, which may effectively render THC an antagonist, or poorer CB receptor selectivity (Patel and Hillard, 2006).

#### *Passive Avoidance*

As seen previously isolation rearing reduced the latency to re-enter the dark compartment in the retention trial of a passive avoidance test (see Chapter 5). Interestingly in this test THC treatment had opposite effects in social and isolated rats, reducing latency in socials, but increasing latency in isolates. Acute THC has previously been found to impair retrieval and acquisition of passive avoidance in rats (Mishima et al., 2001). It has been shown that functional CB<sub>1</sub> receptors are required for the extinction of aversive memories, since CB<sub>1</sub> receptor knockout mice show reduced extinction in a fear-conditioning paradigm. In agreement with this, treatment of wild-types with the CB<sub>1</sub> antagonist SR171614A reproduced the knockout phenotype (Marsicano et al., 2002). In the same study it was also shown that endocannabinoid levels were increased in the basolateral amygdala during presentation of the tone in the extinction trials. However, it has also been found that WIN 55,212-2 treatment potentiated the response of mPFC neurons to olfactory cues associated with a footshock (Laviolette and Grace, 2006a). These two studies indicate the cannabinoids can have opposing effects on fear motivated memory in different situations. Therefore it is possible that in social rats THC treatment reduced fear-associated memory, but in isolation reared rats this association was increased.

#### *Attentional Set-shifting*

As seen in Chapter 3, isolation rearing had no effect on any aspect of attentional set shifting and neither did THC treatment. There is considerable

evidence of acute cannabinoids disrupting various cognitive paradigms including set-shifting on a maze (Hill et al., 2006) and both spatial (Varvel et al., 2001, Da Silva and Takahashi, 2002, Fadda et al., 2004, Cha et al., 2006, Niyuhire et al., 2007, Robinson et al., 2007a) and non-spatial (Varvel et al., 2001, Da Silva and Takahashi, 2002, Cha et al., 2006) variations of the water maze, and working memory in the 8-arm radial maze (Mishima et al., 2001). Furthermore, sub-chronic THC disrupted spatial learning in the water maze more when given to adolescent (PND30) than adult rats (Cha et al., 2007). Infusion of the CB<sub>1</sub> antagonist SR141716A into the hippocampus enhanced acquisition of the water maze, further confirming a role for CB<sub>1</sub> receptors in cognitive processes (Robinson et al., 2007b).

The long-term cognitive effects of cannabinoid treatment have been investigated, with no long-term effects found in the water maze 4 weeks after completion of a 21 day THC regimen in both adult and adolescent rats (Cha et al., 2006). However, WIN 55,212-2 disrupted motivation in an operant lever-pressing task only in rats treated between PND40-65, not in rats treated as adults (Schneider and Koch, 2003). Neonatal THC (PND 4-14) has also been found to disrupt working, but not spatial, memory in adulthood (O'Shea and Mallet, 2005). Interestingly a single, very low dose of THC (0.001mg/kg) significantly impaired acquisition and reversal learning in the water maze and working memory in a water T-maze, 3 weeks after dosing in mice (Tselnicker et al., 2007, Senn et al., 2008). There is no literature on the long-term effects of THC on attentional set-shifting, but the data presented here show that this dosing regimen has no long term effects on behavioural flexibility measured with this paradigm.

Cannabis use has been found to be associated with schizophrenia although the nature of this association is unclear and controversial (Arseneault et al., 2002, van Os et al., 2002, Zammit et al., 2002, Fergusson et al., 2003, Arseneault et al., 2004, Hall and Degenhardt, 2007). In some cognitive tasks, including the WCST, cannabis use has either no effect or improves performance in schizophrenics, while impairing the performance of healthy controls (Jockers-Scherubl et al., 2007, Wobrock et al., 2007). In addition to this treatment with

the CB<sub>1</sub> antagonist SR141716A had no effect in a placebo controlled trial in schizophrenic patients (Meltzer et al., 2004). Opposing this is the evidence that THC treatment increased positive and negative symptoms as well as inducing learning deficits in schizophrenic patients (D'Souza et al., 2005). What is clear is that considerable further work needs to be carried out, both in humans and animals, in order to further elucidate the exact nature of the interaction between cannabis and schizophrenia.

The results presented in this chapter suggest that combining isolation rearing and THC treatment does not enhance the robustness of the isolation rearing paradigm as a model of aspects of schizophrenia. A recent review suggests that pre-natal exposure to cannabis results in impaired executive function in humans and cognitive deficits and hyperactivity in animals (Huizink and Mulder, 2006). It would be interesting to see if this early-life intervention would have more effect when combined with isolation rearing than the regimens used in these studies.

## **6.9 Conclusion**

Neither of the two regimens of THC dosing interacted with isolation rearing to produce a more robust model of schizophrenia, although subtle interactions, dependent on dosing regime, were observed.

## 7 **General Discussion**

## 7.1 Summary of Main Findings

The main findings of the experiments reported in this thesis are as follows:

Isolation rearing from weaning causes increased locomotor activity in a novel environment and impaired sensorimotor gating, as measured by prepulse inhibition of acoustic startle. Isolation reared rats also demonstrate cognitive deficits in novel object discrimination, reversal learning in the water maze and passive avoidance. However no deficits were seen in spatial learning or attentional set-shifting.

The reversal learning deficit was reversed by treatment with a 5-HT<sub>6</sub> antagonist, Ro 04-6790 but further work is required to ascertain whether the AMPA modulator aniracetam can reverse novel object recognition deficits. The non-pharmacological nature of isolation rearing makes it particularly useful for assessing the effects of a range of drugs acting via different mechanisms.

Treatment with THC around the time of weaning did not affect development of isolation rearing induced behaviours. High doses of THC can cause long-term impairments in recognition memory and passive avoidance but have no effect on attentional set shifting.

These findings show that isolation rearing from the age of weaning causes alterations in a variety of behavioural paradigms, including several different cognitive tasks. These behavioural changes mean that isolation rearing meets the criteria for face validity as a model of schizophrenia. While isolation reared rats did not demonstrate deficits in attentional set shifting, the impairment demonstrated in reversal learning in the water maze suggests that processes of behavioural flexibility have been affected by the isolation procedure. As other laboratories have demonstrated that isolation rearing can impair attentional set shifting (Schrijver and Wurbel, 2001) it is possible that subtle differences in both the isolation rearing procedure and the attentional set shifting paradigms may have prevented the deficits from being detected in these studies.



## **7.2 Predictive Validity and Reliability of Isolation Rearing**

Acute dosing with the atypical antipsychotic clozapine had no effect on PPI of acoustic startle in isolation reared rats, despite increasing PPI in socially housed control animals. The same dose of clozapine has been found to reverse isolation induced PPI deficits in other laboratories (Varty and Higgins, 1995). However, in the study reported here, the vehicle treated isolation reared rats did not exhibit PPI impairments. This demonstrates that the predictive validity of isolation rearing as a screen for novel antipsychotic drugs is highly dependent on the reliability of the procedure to produce robust behavioural alterations.

During the last three years the behavioural profile of thirteen batches of male isolation reared Lister hooded rats, have been characterised in our laboratory and the outcome is summarised in Table 7.1, demonstrating that reproducible alterations are produced when a stringent protocol is applied to the same strain of rat. By using comparable isolation procedures (8 weeks of isolation from PND 28) Cilia et al (Cilia et al., 2001, Cilia et al., 2005b) showed that 23 of 27 cohorts (85%) of isolated male Lister hooded rats exhibited significant reductions in the PPI produced by a single prepulse either 5 or 10 dB above baseline, demonstrating the robust, reproducible nature of this behavioural alteration which is independent of any change in basal startle (Domeney and Feldon, 1998). A comparable reproducibility of this PPI deficit (82% compared with 85% of isolation cohorts, Table 7.1) has been recorded in our laboratory.

Table 7.1: Behavioural characterisation of 13 separate isolation rearing studies

Isolation duration (weeks to first behavioural test)	Hyperactivity in a novel arena	Novel object discrimination impairment	Prepulse inhibition of startle deficit
4 (5 studies, PPI examined in 3)	100	100	66
5 (8 studies)	88	50	88
Overall	92	69	82

Numbers given indicate the percentage of experiments where a significant change in behaviour was recorded from that in group housed controls. Hyperactivity = 60 min test in a novel arena; Novel object discrimination used a 3 min test with the choice trial 2 hours after familiarisation to 2 identical objects. Prepulse inhibition = 120dB pulse, 72, 76, 80 & 84dB prepulses over 65dB background noise, 100ms inter-stimulus interval, 40 pseudo-random trials with a 10-20s variable inter-trial interval.

The ideal animal model of schizophrenia would include alterations in behaviour, cognition and neurochemistry, which respond to currently available antipsychotics as is seen in the clinical situation. There are many animal models that are proposed for use as a screen for antipsychotic drugs and comparing them using the published literature is difficult as negative findings are not usually published. For instance, the chronic intermittent PCP regimen seems to closely mimic the neurochemical changes seen in schizophrenia (Cochran et al., 2002, Cochran et al., 2003, Reynolds et al., 2005). However the small amount of behavioural or cognitive data that has been published suggests that there is no long lasting effect on cognition (Watson et al., 2005). The ideal animal model described above is unlikely to be a reality, as no animal model will be able to accurately reproduce all the symptoms of a human psychiatric disorder, especially a disease with symptomology as heterogeneous as is seen in schizophrenia. However, both previously published work and that reported in this thesis show that isolation rearing causes robust alterations in behavioural paradigms, coupled with neurochemical and morphological alterations (see General Introduction). It is the cognitive deficits in schizophrenia that are currently inadequately treated and the isolation reared rat provides a research tool to investigate the effects of putative nootropic drugs on environmentally induced cognitive impairments. The lack of pharmacological manipulation in isolation reared rats allows them to be utilised for assessing the activity of drugs working by various different mechanisms.

## **7.3 Future Work**

### **7.3.1 Isolation Rearing Combined with Pharmacological Models of Schizophrenia**

In Chapter 6 of this thesis we attempted to improve the reliability of isolation rearing by treating young rats with THC during the early stages of isolation. It was thought that this combination of environmental and pharmacological manipulations might increase the severity of the isolation induced behavioural deficits. However, at no stage did THC interact with isolation rearing to increase the size of a behavioural deficit. The effects of THC in adult rats varied between the two dosing regimens, indicating specific vulnerabilities depending on age and strength of THC dosing. It is possible that further investigation may shed more light on this.

It would also be relevant to investigate the effects of combining isolation rearing with treatment with an NMDA receptor antagonist, such as phencyclidine. Recent preliminary work by Watson at Nottingham University suggests that PCP on PND7, followed by five weeks isolation rearing from weaning causes a potentiation of isolation rearing induced hyperactivity (personal communication). While this effect has not been replicated it suggests that PCP and isolation rearing can also interact and this may produce a more robust behavioural phenotype.

### **7.3.2 Isolation rearing in Combination with Genetic Models of Schizophrenia**

As discussed in Chapter 1, schizophrenia is a polygenetic disorder with many genes each conferring a small level of risk. These genetic effects combine with early life environmental occurrences to increase the risk of developing schizophrenia later in life. It would therefore be interesting to investigate the effects of an environmental manipulation such as isolation rearing in an animal with a high genetic loading for schizophrenia. Most gene manipulations, such

as knockouts, are performed in mice for ease of breeding. Most of the behavioural tests described in this thesis can also be carried out in mice (prepulse inhibition, novel object recognition, water maze, passive avoidance). Even the attentional set-shifting paradigm has been translated to mice (Garner et al., 2006). Isolation rearing has also been investigated in mice with many similar results to those seen in rats (Valzelli, 1973, Valzelli et al., 1974, Varty et al., 2006).

Many of the schizophrenia risk genes have been manipulated in mice and these transgenic animals would seem to be a next step in animal modelling of schizophrenia. For instance, neuregulin 1 was mentioned in Chapter 1 as a possible risk gene for schizophrenia. The homozygous knockout of this gene in mice is not viable, but mice with a heterozygous genotype exhibit impaired prepulse inhibition and hyperactivity which can be reversed by clozapine (Stefansson et al., 2002), as well as social recognition deficits (O'Tuathaigh et al., 2007b) and an increased sensitivity to THC (Boucher et al., 2007). However, there are no differences in spatial learning or working memory (O'Tuathaigh et al., 2007b). Isolation rearing of these mice may cause further behavioural alterations that more closely match the behavioural phenotype seen in schizophrenia. Mice with genetic manipulations of COMT, dysbindin or RGS4 have also been created. Most of the mice exhibit some schizophrenia relevant behaviours, but not all and as such isolation rearing may enhance the schizophrenia-like phenotype further (O'Tuathaigh et al., 2007a).

### **7.3.3 Isolation Rearing and Social Cognition**

MATRICES identified the domain “social cognition” as a key area where schizophrenics exhibit deficits. The effect of isolation rearing on this aspect of cognition was not investigated at any point during this the work undertaken in this thesis, but this is clearly an area for further investigation. Although increased aggression has been noted in isolation reared rats (Valzelli and Garattini, 1972, Wongwitdecha and Marsden, 1996c) and mice (Valzelli, 1973), the effects on social recognition memory have not been investigated.

The effects of cognition enhancing drugs and antipsychotics on these behaviours are also of interest.

Consistent with findings in schizophrenic patients, isolation rearing has previously been shown to reduce mPFC volume by 7% without changing the number of neurons present, using a stereological technique (Day-Wilson et al., 2006). At Nottingham University we were able to use a 7 Tesla magnetic resonance imaging (MRI) scanner to measure brain region volume in isolation reared rats. Preliminary results confirmed those of Day-Wilson and found a significant 5% reduction in right mPFC volume (Porkess et al., 2007). The effect of isolation rearing on other brain regions and correlations between region size and behavioural effects is still under investigation, but further work on this could shed light on structural changes in the brains of isolation reared rats. Functional MRI (fMRI) can be used to investigate regional changes in brain activation following drug administration which could elucidate differences in responses of isolation reared rats.

#### **7.4 Final Conclusion**

In conclusion, isolation rearing of rats from the age of weaning causes behavioural and cognitive changes, some of which have relevance to deficits seen in schizophrenia. Some of the cognitive deficits are sensitive to treatment with nootropic agents and could therefore be used as a tool to aid development of novel treatments for the cognitive symptoms of schizophrenia.

## 8 Appendices

## **8.1 Appendix A: Pharmacological Validation of PPI Paradigm**

### **8.1.1 Introduction**

The ability of the PPI protocol to detect reductions in PPI was tested with an NMDA receptor antagonist, phencyclidine (PCP), and a 5-HT<sub>1A</sub> receptor agonist 8-hydroxy-2-(di-n-propylamino)-tetraline (8-OH-DPAT) which have both been shown to attenuate prepulse inhibition of acoustic startle in other laboratories (Geyer et al., 2001b).

### **8.1.2 Methods**

#### *Animals*

32 Male Lister Hooded (LH) rats (BMSU, University of Nottingham, derived from Charles River UK stock) weighing between 150-300g were used. Animals were housed in groups of 3 or 4 in cages 50x32x23cm, according to the husbandry conditions described in Chapter 2.

#### *Prepulse Inhibition of Acoustic Startle*

PPI was carried out exactly as described in Chapter 2.

#### *Drugs*

Animals were randomly assigned to either PCP or 8-OH-DPAT groups (n=16 per group). On each of 4 weeks animals received either vehicle or one of three drug doses, according to a latin square arrangement, such that each rat received all doses of drug in different sequences.

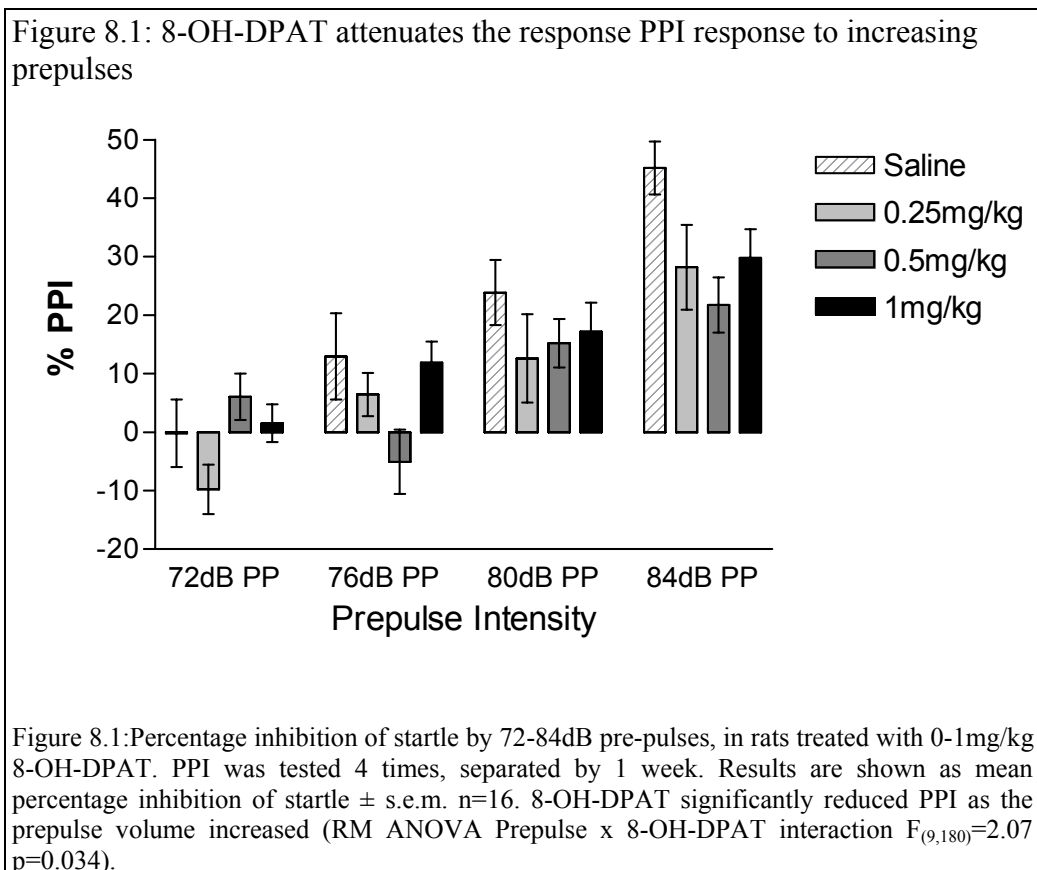
Phencyclidine HCl (Sigma, UK) was administered at 0.3, 1 and 3mg/kg, accounting for salt:base ratio of 1.15, in a vehicle of saline (1ml/kg, i.p.) 5 minutes prior to PPI testing.

Doses of 0.25, 0.5 and 1mg/kg 8-OH-DPAT hydrobromide (Sigma, UK), accounting for salt:base ratio of 1.327, or saline vehicle were given (1ml/kg, s.c.) 10 minutes prior to PPI testing.

### 8.1.3 Results

#### 8-OH-DPAT

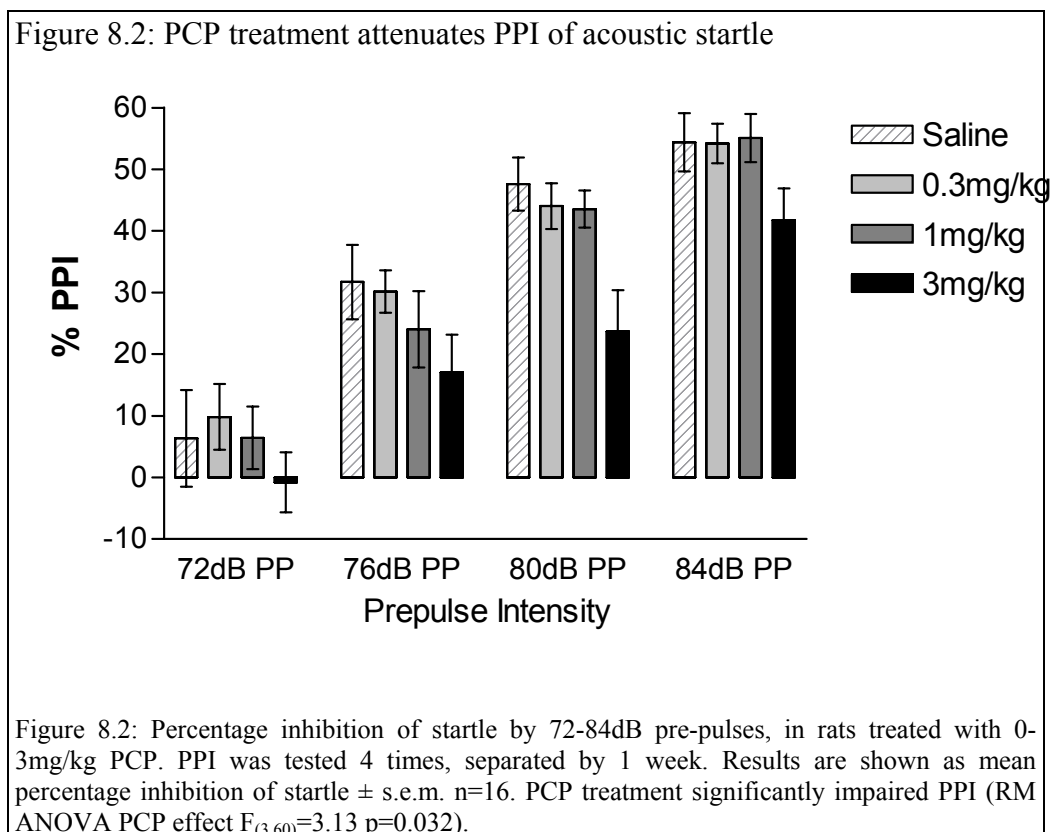
The effects of 8-OH-DPAT on PPI of acoustic startle can be seen in Figure 8.1. Animals showed an increase in % PPI as the prepulse intensity increased (RM ANOVA Prepulse intensity  $F_{(3,58)}=52.04$   $p<0.001$ ). Overall there was no main effect of 8-OH-DPAT (RM ANOVA  $F_{(3,60)}=1.11$   $p=0.353$ ) but a significant interaction between prepulse intensity and 8-OH-DPAT showed that as increasing prepulse caused larger %PPI in saline treated animals, this was not seen in rats receiving 8-OH-DPAT (RM ANOVA Prepulse x 8-OH-DPAT interaction  $F_{(9,180)}=2.07$   $p=0.034$ ). There was no significant effect of drug on basal pulse-alone startle amplitude (1-way ANOVA,  $F_{(3,60)}=1.413$   $p=0.2478$ , data not shown).





### PCP

Saline treated animals showed a prepulse intensity dependent increase in PPI (RM ANOVA Prepulse effect  $F_{(3,58)}=94.03$   $p<0.001$ ) reaching  $54.4 \pm 4.7\%$  at 84dB prepulse (see Figure 8.2). PPI was significantly impaired by PCP treatment (RM ANOVA PCP effect  $F_{(3,60)}=3.13$   $p=0.032$ ). There was no significant effect of drug on basal pulse-alone startle amplitude (1-way ANOVA,  $F_{(3,60)}=0.172$   $p=0.9151$ , data not shown).



#### 8.1.4 Conclusion

Both 8-OH-DPAT and PCP caused attenuation of PPI of acoustic startle. Therefore the PPI protocol used is capable of detecting impairments in PPI and can be used to assess sensorimotor gating in isolation reared rats.

## **8.2 Appendix B: Effect of Scopolamine on Passive Avoidance**

### **8.2.1 Introduction**

The ability of the passive avoidance protocol to detect changes in retention of fear-related memory was tested with a muscarinic receptor antagonist, scopolamine, which has previously been shown to impair memory (Vannucchi et al., 1997).

### **8.2.2 Methods**

#### *Animals*

7 Hooded Lister rats (Nottingham University BMSU), weighing 250-500g were used. Animals were housed in groups of 2-3 according to the husbandry conditions described in Chapter 2.

#### *Passive Avoidance Protocol*

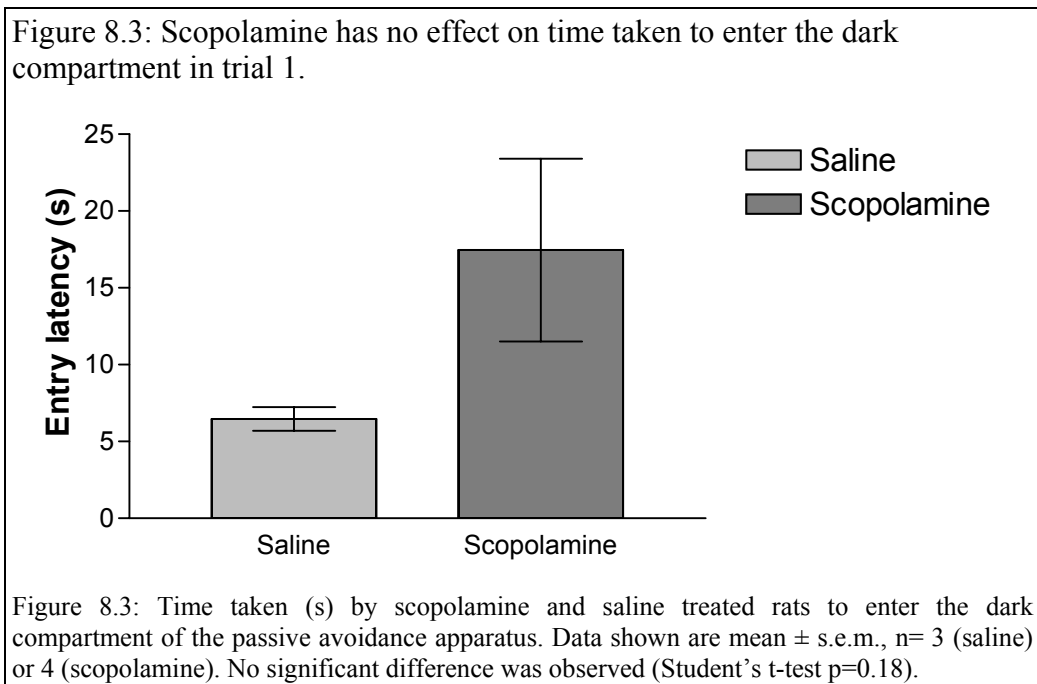
Passive avoidance was carried out exactly as described in Chapter 5.

#### *Drugs*

Rats received either scopolamine hydrobromide (Sigma, UK), dosed at 0.5mg/kg (n=4), or vehicle (saline, 1ml/kg) (n=3) 20 minutes prior to the first passive avoidance trial.

### 8.2.3 Results

In the first trial there was no significant difference in time taken to enter the dark compartment by scopolamine and saline treated rats (Student's t-test  $p=0.18$ ), Figure 8.3).



In the retention trial (T2) scopolamine treated rats showed a tendency towards re-entering the dark (shock-paired) compartment more quickly than the saline treated animals (Figure 8.4). However, this failed to reach statistical significance (Student's t-test  $p=0.098$ ), probably due to the low number of animals used in this pilot study.

Figure 8.4: Scopolamine treated rats show a tendency towards faster re-entry into the shock-paired compartment

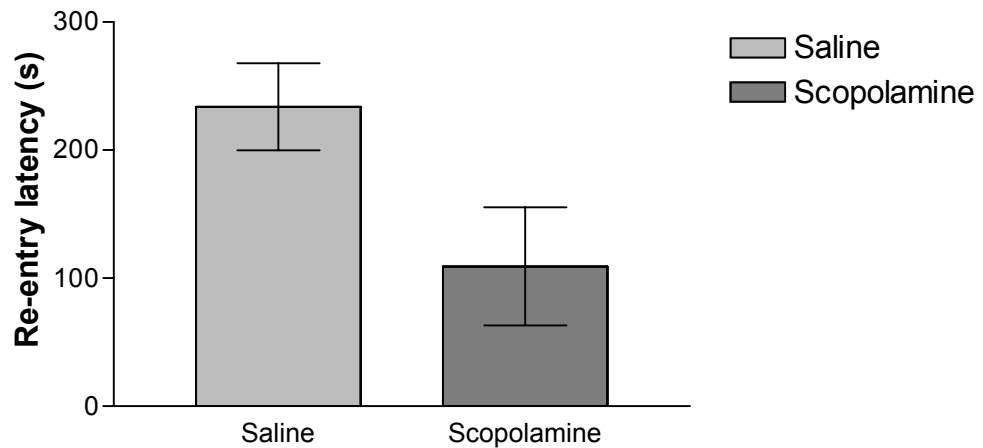


Figure 8.4: Time taken (s) by scopolamine and saline treated rats to re-enter the dark compartment of the passive avoidance apparatus in T2. Data shown are mean  $\pm$  s.e.m.,  $n=3$  (saline) or  $4$  (scopolamine). No significant difference was observed (Student's t-test  $p=0.098$ ).

#### 8.2.4 Conclusion

Although a significant difference in latency to re-enter the dark compartment was not seen, this was almost certainly due to the small number of animals used in this pilot study. This suggests that an increased number of animals would have shown a scopolamine-induced impairment in passive avoidance. Based on the data shown it was concluded that the equipment was functioning properly and protocol used was capable of detecting deficits in passive avoidance.

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