Isolation-rearing from Weaning to Investigate Depressive-like Behaviour in the Rat



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"Dá fhada an lá tagann an tráthnóna"

Abstract

Depression is a heterogeneous condition characterised by low mood and a lack of motivation and enjoyment of regular activities. The response rate to current treatments coupled with adverse side effect profiles requires new avenues of investigation into the development of novel therapeutics to treat the condition. Rearing rats in isolation from weaning causes behavioural, cognitive and neurochemical changes which persist into adulthood; some of the symptoms produced have relevance to depression.

In the current thesis, rats raised in social isolation from weaning consistently developed a hyperactive phenotype compared to group-housed littermates when placed into a novel environment. They also developed deficits in associative learning assessed by the conditioned fear response task. They displayed some anxiety-like behaviours in the open field and novelty-suppressed feeding task and deficits in visual memory in the novel object discrimination task, although these were not reliable across cohorts. There was a reduction in levels of hippocampal neurogenesis in a number of cohorts and for the first time it was demonstrated that rats reared in isolation exhibited changes in gut bacteria, opening up a potential new avenue of investigation into potential treatments.

The efficacy of novel versus established antidepressant treatments was evaluated in isolated rats. Chronic fluoxetine had some anxiolytic effects in the open field, attenuated isolation induced changes in associative memory and increased neurogenesis but also had inconsistent effects on activity. Treatment with acute ketamine increased freezing time in the conditioned freezing response task, indicating an improvement in associative memory. The final study examined, for the first time, the effect of treatment with the JNK-1 inhibitor DJNKI in isolation reared rats. DJNKI had some positive cognitive effects in both the novel object discrimination task and the conditioned freezing response task.

In conclusion, the isolation rearing model induced varying levels of depression-like deficits, which were responsive to some treatments. The model is a useful tool for investigating the symptoms of depression and evaluating novel treatment options.

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List of Abbreviations

5-HT 5-hydroxytryptamine

ACh Acetylcholine

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Arc Activity related cytoskeletal protein

BDNF Brain-derived neurotrophic factor

BrdU Bromodeoxyuridine

BMSU Biomedical services unit

CaMKII Calcium/calmodulin dependent protein kinase II

CB Cannabinoid

CFR Conditioned freezing response

CMS Chronic mild stress

CNS Central Nervous System

COMT Catechol-O-methyl-transferase

CRUK Charles River United Kingdom

CSF Cerebrospinal fluid

CORT Corticosterone

DA Dopamine

DAAO D-amino acid oxidase

DCX Doublecortin

dlPFC Dorsolateral prefrontal cortex

DR Discrimination ratio

DST Dexamethasone suppression test

EDTA Ethylenediamine tetraacetic acid

EGTA Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid

EPM Elevated plus maze

FDA Food & Drug Administration

FST Forced swim test

FLX Fluoxetine

fMRI Functional magnetic resonance imaging

GABA γ-aminobutyric acid

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

GlyT1 Glycine transporter 1

HPA Hypothalamic-pituitary-adrenal

I.C.V. Intracerebroventicular

IL Interleukin

i.p. Intraperitoneal

ITI Inter-trial interval

JNK c-Jun N-terminal kinase

KET Ketamine

LH Lister hooded

LI Latent inhibition

LMA Locomotor activity

mCPP m-chlorophenylpiperazine

mGluR Metabotropic glutamate receptor

MAOI Monoamine-oxidase inhibitor

MRI Magnetic resonance imaging

mRNA Messenger ribonucleic acid

MW Molecular weight

mTOR Mechanistic target of rapamycin

NAA N-acetyl aspartate

NAcc Nucleus accumbens

NIMH National Institute of Mental Health

NMDA N-methyl-D-aspartate

NOD Novel object discrimination

NSF Novelty-suppressed feeding

OBX Olfactory bulbectomy

OD Optical density

OF Open field

PCP Phencyclidine

PFC Prefrontal cortex

PI3K Phosphatidylinositol-4,5-bisphosphate 3-kinase

PLC Phospholipase C

PND Post natal day

p.o. per os (oral)

PPI Prepulse inhibition

PSD Post-synaptic density

RM ANOVA Repeated measures analysis of variance

s.c. Subcutaneous

SD Sprague Dawley

SDS sodium dodecyl sulphate

SEM. Standard error of the mean

SERT Serotonin transporter

SI Social Isolation

SIH Stress-induced Hyperthermia

SNP Single nucleotide polymorphism

SNRI Selective noradrenaline reuptake inhibitor.

SSRI Selective serotonin reuptake inhibitor

TCA Tri-cyclic antidepressant

THC Δ9-tetrahydrocannabinol

TNF-α Tumor-necrosis factor α

Tris Tris(hydroxymethyl) amidomethane

UCMS Unpredictable chronic mild stress

VGLUT Vesicular glutamate transporter

VTA Ventral tegmental area

WKY Wistay-Kyoto

WM Working memory

Publications

Papers:

Doherty, F.D., O'Mahony, S.M., Peterson, V.L., O'Sullivan, O., Crispie, F., Cotter, P.D., Wigmore, P., King, M.V., Cryan, J.F., Fone, K.C.F., 2017. Post-weaning social isolation of rats leads to long-term disruption of the gut microbiota-immune-brain axis. *Brain, behavior, and immunity*. (Article in press)

Abstracts:

Dunphy-Doherty F, King MV, Wigmore P & Fone KCF (2016) Characterising the predictive validity of rearing rats in isolation from weaning as a model for psychiatric disorders- Journal of Psychopharmacology, 30, A69

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Chapter 1:

General Introduction

1.1 Introduction

Depression is now one of the most common illnesses in the world, the total number of people living with depression in the world is estimated at 350 million. It currently has a lifetime prevalence of 16% (Kessler et al., 2003). 121 million people will suffer from a depressive episode over a 12 month period and currently in the UK, 1 in 5 people are reported to suffer with depression or anxiety. The WHO states (Figure 1.1) that depression will be the leading global burden of disease by the year 2030 (Lépine and Briley, 2011).

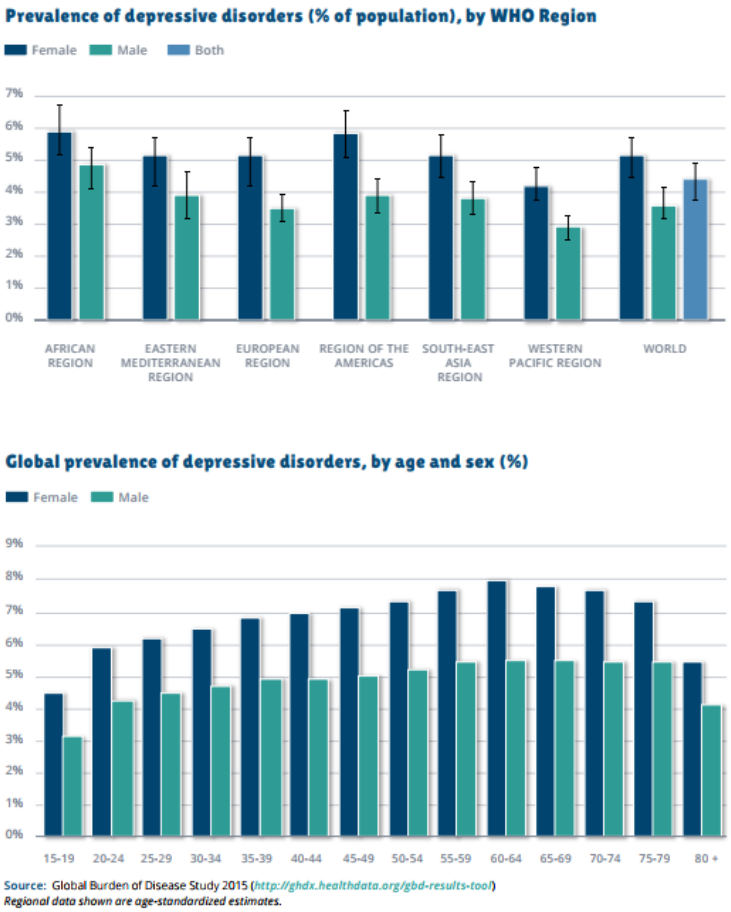


Figure 1.1: Global prevalence of depressive disorders by (A) region and by (B) age and sex (Global burden of disease study, 2015)

Depression is a lifelong illness with a high prevalence for relapse, the most common form being Major Depressive Disorder (MDD). This form of depression is characterised by low mood or a loss of interest and pleasure in life, lasting for a prolonged period of time (DSM-5, 2013). A clinical diagnosis of depression requires 5 out of 9 symptoms (Figure 2) appearing and persisting longer than two week periods (Ostergaard et al., 2011).

- Depressed mood or a loss of interest or pleasure in daily activities for more than two weeks.
- Mood represents a change from the person's baseline.
- Impaired function: social, occupational, educational.
- Specific symptoms, at least 5 of these 9, present nearly every day:
    1. **Depressed mood or irritable** most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad or empty) or observation made by others (e.g., appears tearful).
    2. **Decreased interest or pleasure** in most activities, most of each day
    3. **Significant weight change (5%) or change in appetite**
    4. **Change in sleep**: Insomnia or hypersomnia
    5. **Change in activity**: Psychomotor agitation or retardation
    6. **Fatigue or loss of energy**
    7. **Guilt/worthlessness:** Feelings of worthlessness or excessive or inappropriate guilt
    8. **Concentration**: diminished ability to think or concentrate, or more indecisiveness
    9. **Suicidality:** Thoughts of death or suicide, or has suicide plan

Figure 1.2: DSM-V criteria for MDD (DSM-5, 2013)

Between 30-50% of patients diagnosed with MDD will not fully respond to standard medication, these people are said to have treatment-resistant depression (TRD) (Fava and Davidson, 1996). Depression persists in approximately 70% of patients after first-line treatment, and at least 30% of patients remain depressed even after proceeding through four rounds of distinct treatments (Gaynes et al., 2008). There is also a delay of onset of action to effectively treat the depressive symptoms, this can persevere for up to 6 weeks (Malagie et al., 2001). During this time, patients often report with side-effects of the medication which can lead to non-compliance. There is evidence to suggest that a proportion of patients report improvement in symptoms earlier than this, but this improvement can often be ascribed to a placebo effect (Quitkin et al., 1987).

1.2 Theories of D**epression**Several hypotheses have been proposed to explain the aetiology of MDD, the monoamine hypothesis is still the most prevailing, as most currently available antidepressants work on monoamine transporters or receptors. This hypothesis claims that MDD is the result of a central deficiency of monoamines in the synaptic cleft, and antidepressant efficacy is achieved through increasing monoamine concentration in the synapse by inhibiting the removal or promoting synthesis and release of these monoamines. This has been posited since the 1960s, (Schildkraut, 1965) suggested that depression is caused by a functional deficit of monoamine transmitters within certain brain areas, whereas mania is the consequence of a functional excess. (Coppen, 1967) further supported this idea and although the idea of depression being driven by biochemical processes was a controversial idea at the time they believed the evidence of the effectiveness of electric shock therapy now referred to as electro-convulsive therapy (ECT), the effect of the drug reserpine on catecholamines and serotonin and particularly the efficacy of monoamine oxidase inhibitors (MAOIs) on depressed patients was enough evidence to support the role of

monoamines in the aetiology of depression. The MAOI's are often credited as being the first pharmacological treatment for depression, their discovery much like the discovery of the first antipsychotic is one of luck. Patients being treated for with antitubercular compounds were found to have a number of side-effects which included improved sleep, psychomotor stimulation and euphoria (Pletscher, 1991). Since then the monoamine hypothesis has been the driving force for the development of new antidepressants although trying to account for the complicated and heterogeneous symptoms of MDD to a deficiency of a neurotransmitter molecule may be an oversimplification and fail to appreciate the complexity of the disorder. There have been inconsistencies with this hypothesis, the most prominent being the delayed onset of efficacy and the inadequate response rate of typical antidepressants. There is a rapid increase in 5-HT concentration in the synaptic cleft following administration of selective serotonin reuptake inhibitors (SSRIs) which is inconsistent with the delayed onset of action of antidepressants observed in clinical populations. Studies have also demonstrated that lowering the concentration of 5-HT in the synaptic cleft using tryptophan depletion or increasing the serotonin transporter (SERT) failed to induce depressive symptoms in healthy subjects (Kasper and McEwen, 2008; Ruhe et al., 2007). However other studies have demonstrated relapse of depressive symptoms following tryptophan depletion in responders(Leyton et al., 2000). Some studies have observed that long-term antidepressant treatment results in decreases in 5-HT levels in the brains of healthy subjects (Bosker et al., 2010; Marsteller et al., 2007; Siesser et al., 2013). Stahl (2013) goes as far to state that ''there is no clear and convincing evidence that monoamine deficiency accounts for depression''. These inconsistencies challenged the monoamine hypothesis and lead to the development of alternative theories for the cause of depression; the monoaminergic receptor hypothesis, signalling hypothesis, neuroplasticity hypothesis, immune and genetic hypotheses (Racagni and Popoli, 2008). These hypotheses progressed towards a more comprehensive and reasonable understanding of MDD and antidepressant efficacy. The monoamine receptor hypothesis states that the desensitisation of monoamine autoreceptors is the actual mechanism of antidepressant efficacy (Stahl, 2013). As it takes up to two weeks for the downregulation of the 5-HT_{1A} autoreceptor to occur following SSRI treatment, which being an autoreceptor would account for the delay in change of 5-HT and clinical response, this hypothesis would go some way towards explaining the delayed onset of action of antidepressants. However, post-mortem studies using molecular imaging failed to find evidence supporting 5-HT_{1A} alterations in patients with MDD (Ruhé H. G., 2014). 5-HT_{1A} antagonists also failed to achieve antidepressant efficacy in clinical trials, although work has shown they enhanced the rate of increase in 5-HT following SSRI treatment.

There is strong evidence, both pre-clinical and clinical, to suggest the role of stress in the development of depression (Biegler, 2008; Binder and Nemeroff,

2010; Pizzagalli, 2014; Risch et al., 2009; Young and Korszun, 2010). Furthermore, stress is suggested to be linked with treatment resistance (Amital et al., 2008) and higher rates of relapse (Harkness et al., 2014; Monroe and Harkness, 2005) of MDD. The main component of the link between stress and MDD relates to the dysregulation of the hypothalamic pituitary adrenal (HPA) axis. When the HPA axis is activated there is a release of corticotrophin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus which in turn leads to release of adrenocorticotrophic hormone (ACTH) from anterior pituitary cells. The release of ACTH stimulates the adrenal cortex which leads to the release of cortisol in man or corticosterone in rat (Juruena et al., 2003). Circulating levels of cortisol in the blood then act on glucocorticoid receptors in the hippocampus, hypothalamus and pituitary inducing inhibition feedback (Figure 1.1) (Juruena et al., 2003).

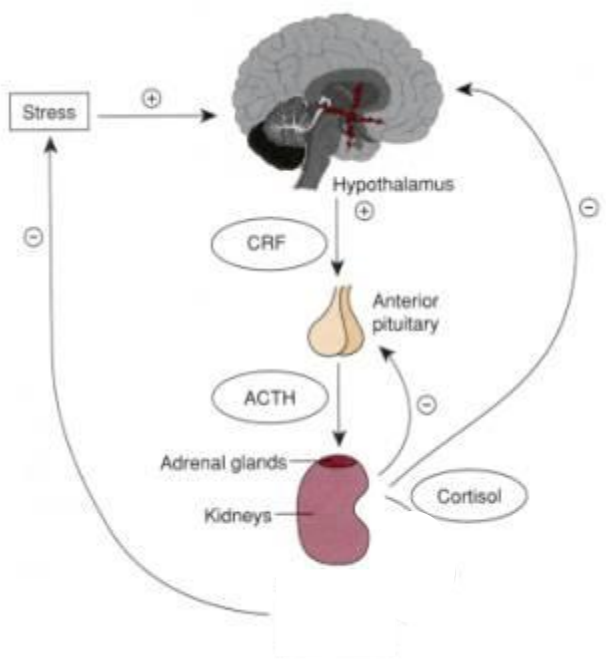


Figure 1.3: Diagram of the hypothalamic pituitary adrenal (HPA) axis. CRF: Corticotrophin releasing factor, ACTH: Adrenocorticotrophic hormone.

In cases of overactive or prolonged activation of the HPA axis there is a disruption of this feedback inhibition loop which can often result in hypercortisolemia, a key symptom often found in patients with severe forms of depression (Gillespie and Nemeroff, 2005). Atrophy and neuroplasticity hypofunction of the hippocampus and PFC, and hypertrophy and neuroplasticity hyperfunction of amygdala are some of the functional and morphological changes of the brain induced by hypercortisolemia and are consistent with neuroimaging findings of abnormalities (Andrade and Rao, 2010). Brain region specific deficits may be the underlying cause of separate MDD symptoms. For instance changes in the PFC-amygdala/hippocampus circuit, may lead to low

mood and depressive emotions. Abnormalities in the PFC-nucleus accumbens (NAc) circuit may serve as the neural substrate of anhedonia (Phillips et al., 2015); while alterations of medial and dorsolateral PFC may mediate the cognitive dysfunction of MDD (Thomas and Elliott, 2009). Patients with severe depression have increased cortisol in urine, plasma and CSF; as well as an exaggerated cortisol response to ACTH and hypertrophy of both the adrenal and pituitary glands (Gold et al., 1988). Higher levels of circulating cortisol induced by chronic stress could have a neurotoxic effect on hippocampal neurons through activation of the glucocorticoid receptor and its downstream effects. This would result in decreased neurogenesis, synaptogenesis and increased apoptosis of neurons (de Kloet et al., 2005; Holsboer, 2000; Holsboer and Barden, 1996). This loss of neurons further leads to functional deficits of long-term potentiation (LTP) or long-term depression (LTD) in the hippocampus (Egeland et al., 2015; Holsboer, 2000). This loss of neurons in the hippocampus can potentially worsen through the disinhibition of the negative feedback loop of the HPA axis leading to further morphological and functional deficits, commonly seen in depression (Liu et al., 2017). These neurochemical changes would together result in negative influence on neuroplasticity through blunted neurogenesis, disrupted synaptogenesis, diminished dendritic spines and reduced synaptic connections. The roots of the glutamate hypothesis can be traced back to the early 1990s, when findings showed that N-Methyl-D-Aspartate receptor (NMDA-R) antagonists, such as phencyclidine, possess antidepressant-like action in animal models of depression (Trullas and Skolnick, 1990). The glutamate theory further developed after the discovery of the role of glutamate in the brain as the major excitatory neurotransmitter. In total there are approximately two to three hundred thousand serotonergic neurons it has been estimated, out of a hundred billion total neurons (Baker et al., 1991). In humans, the neocortex represents about 85% of the total brain mass. Stereological methods have estimated that around 80% of neurons in the neocortex are excitatory and form 85% of all synapses, while about 20% of the neurons are inhibitory and form 15% of the synapses (Douglas and Martin, 2007).

Research has demonstrated associations between cognitive processes in the brain and functional and structural changes in glutamate neurotransmission. A wealth of studies have shown that activity-dependent changes in the synaptic strength of central glutamate synapses correlate with the processes of learning and memory, and that interfering with these changes impairs memory (Citri and Malenka, 2008; Diamond et al., 2004; Neves et al., 2008; Wong and Ghosh, 2002). The most extensively studied forms of synaptic plasticity at glutamate synapses are long-term potentiation (LTP) and long-term depression (LTD). It is widely accepted that plasticity occurring at glutamate synapses allows the encoding of new information, which has been further validated by the findings that both sensory stimulation and learning processes *in vivo* induce LTP in the hippocampus (Holtmaat and Svoboda, 2009; Whitlock et al., 2006).

The initial concept of glutamate involvement has evolved and now encompasses a neuroplasticity hypothesis as it integrates intracellular signalling, mechanisms of gene expression, neurotrophic mechanisms, remodelling of neuronal cells, synaptic function/plasticity and neurogenesis (Pittenger and Duman, 2008; Racagni and Popoli, 2008; Sanacora et al., 2008). The neuroplasticity theory is supported by evidence from three areas. Decreased neuroplasticity in hippocampus and PFC in depressed patients, decreased concentration of neurotrophic factors (BDNF, NGF) in bothe the hippocampus and plasma of subjects with depression and that antidepressants increase the concentration of neurotrophic factors and improve neuroplasticity in the hippocampus and PFC (Serafini, 2012). These improvements include several forms of neuroplasticity including neurogenesis, synaptogenesis and neuronal maturation (Lee & Kim, 2010). Supporting this hypothesis is evidence that shows how neuroplasticity can be influenced by environmental factors, for example the positive effects that can be brought about by physical exercise, antidepressants, environmental enrichment or the negative effects that are observed after chronic stress in imaging studies of depressed patients or in animal models of stress and mood disorders (Abraham, 2008; Abraham and Bear, 1996; Sanacora et al., 2012).

As the potential role of neurogenesis in depression is one of the features which is examined throughout the following studies the role of neurogenesis and its potential involvement in the pathophysiology of depression will be discussed.

1.3 Neurogenesis

Tanzi (1893) proposed his hypothesis of neuronal learning and memory. This was based on the theory that existing connections were reinforced while the number of neuronal contacts remained the same. He hypothesized that practice and experiencing events shortened spatial gaps between functionally similar neurons, which facilitated their actions and increased their functional capacity (Tanzi, 1893). Around the same time another theory of learning and plasticity was proposed by Ramon y Cajal (1894). This theory stated that 'cerebral gymnastics' was capable of increasing the connections between neurons by favouring the development of cell processes, which in turn augmented the capacity of the brain. These theories established a dogma that new cells could not be produced by the adult brain as nerve cells could not multiply.

The first example of experimental evidence to support the theory that the adult brain could generate new neurons came with Altman (1962). Using the ^{3}H-thymidine method, he was able to identify adult mammalian neurogenesis in a lesioned rat brain. Proliferating cells in the dentate gyrus and new neurons in the neocortex of young adult rat and cat brains were also identified (Altman, 1963). Altman and Das (1965, 1966, 1967) reported new neurons in the olfactory bulbs, hippocampal formation and cerebral cortex of adult rats and guinea pigs. Following these studies Nottebohm (1989) examined songbirds ability to learn new songs and demonstrated that new brain cells could grow in the area of the

brain responsible for this learning. These neurons would continue to fluctuate, peaking in the springtime (mating season, when new songs were learned) and reducing in the summertime. Nottebohm concluded that the new neurons were temporary and were replacing the old neurons that died. This was the first example of functional integration of new neurons in the adult central nervous system (Paton and Nottebohm, 1984). Kaplan and Hinds (1977) examined neurogenesis in rodents using the same method as Altman and were able to confirm new neurons were produced in the hippocampus, olfactory bulbs and neocortex of adult rats. They also identified new glia in the rat neocortex (Kaplan and Hinds, 1980) as well as evidence for neurogenesis in the adult rat visual cortex (Kaplan, 1981). Kaplan and Bell (1984) found evidence for dividing neurons in the hippocampi of young and old rats. Since then a series of papers have been published by researchers including Elizabeth Gould, Fred Gage, and Peter Eriksson on the existence, function, and implications of adult mammalian neurogenesis (Gould and Gross, 2002).

In the late 1990s, the occurrence of adult neurogenesis in the mammalian brain had been established (Altman and Das, 1965; Eriksson et al., 1998; Gould et al., 1999). This process involves the production of neurons (throughout life) and has been shown to occur in specific regions. Eriksson et al. (1998) showed neurons within the adult dentate gyrus of the hippocampus in brains of cancer patients who were examined post mortem. Similar studies were able to demonstrate the occurrence of adult neurogenesis in the sub-ventricular zone of the lateral ventricles, from which they then migrate to the olfactory bulbs (Curtis et al., 2003). A recent study(Sorrells et al., 2018) has claimed there is little evidence for adult neurogenesis, although it has an effect in early human life there is a sharp drop off as we reach adulthood. There is still no clear evidence as to how much of an influence adult hippocampal neurogenesis has.

1.3.1 Role of the Dentate Gyrus

The dentate gyrus receives multiple sensory inputs that are conveyed by cortical and subcortical afferents. The major input to the dentate gyrus is the perforant pathway, which originates from layer 2 of the entorhinal cortex. It is divided into the medial perforant pathway, which processes spatial information and the lateral component, which processes nonspatial information. The dentate gyrus also receives subcortical inputs from the septum, the supramammillary region and the posterior hypothalamus and from several monoaminergic nuclei of the brain stem (locus coeruleus, raphe, ventral tegmental area). In contrast to the richness of its inputs, the dentate gyrus projects only to the CA3 subfield of the hippocampus proper through the mossy fibres and the projections from CA3 to CA1 through the Schaffer collaterals. The entorhinal cortex is the major input and output system with which the hippocampus communicates with the rest of the brain. This is done via projections to the dentate gyrus through the perforant pathway and through projections from CA1 to the entorhinal cortex (Amaral,

1995). Its connectivity places it in an ideal position to control the flow of information within the hippocampus (Koehl and Abrous, 2011).

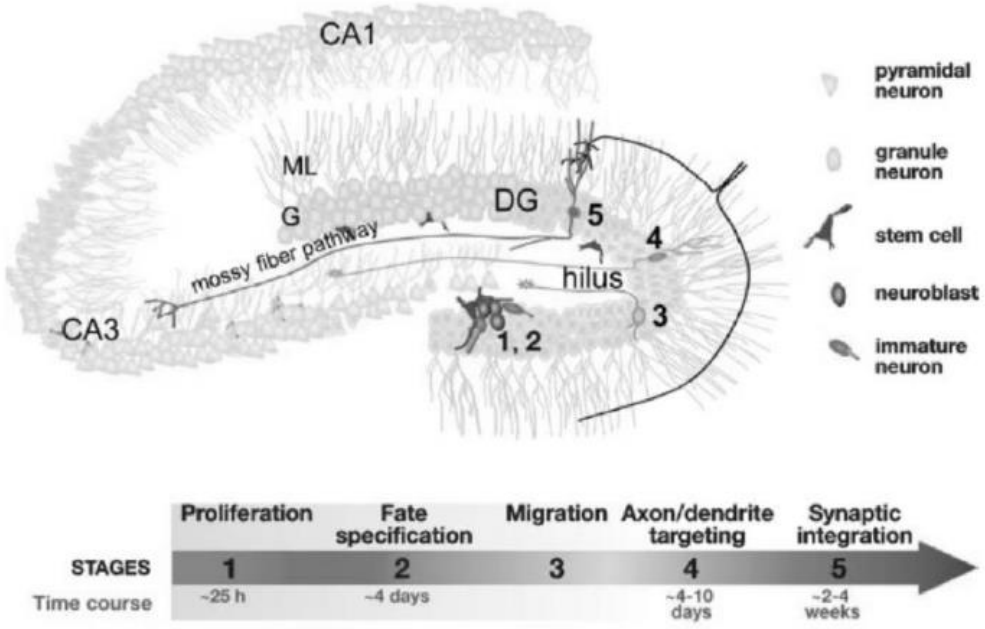


Figure 1.3.1: Five stage timeline for neurogenesis in the subventricular zone of the dentate gyrus (DG) and the new cells integration into the hippocampus. Taken from "Adult neurogenesis in the mammalian central nervous system," (Ming & Song, 2005)

1.3.2 Neurogenesis Role in Depression

In its simplest form, the neurogenic hypothesis of depression states that impaired adult hippocampal neurogenesis triggers depression and restoration of hippocampal neurogenesis restores it(Petrik et al., 2012). The neurogenic hypothesis of depression quickly became popular due to a wealth of correlative studies (Eisch and Petrik, 2012). Humans with depression had decreased hippocampal volume (Fotuhi et al., 2012; Sheline et al., 1999), adult born neurons are added to the ventral dentate gyrus, a key regulator of mood and anxiety (Sahay and Hen, 2007). .Hippocampal neurogenesiss in animals is measured using immunohistochemmical techniques most commonly markers of immature neurons are stained in post-mortem tissue and the number of positive cells are quantified. In laboratory animals, antidepressants enhanced hippocampal neurogenesis with a timeframe similar to that of the delay in onset between antidepressant administration and clinical efficacy in humans (Malberg et al., 2000), reflecting the time of neuronal maturation in the adult hippocampus (Ming and Song, 2011). In non-human primates, stress decreased neurogenesis and levels were normalized by antidepressants (Czeh et al., 2001). These studies gave the neurogenic hypothesis of depression its initial support (Petrik et al., 2012): Many chronic stressors cause depression and anxiety-like behaviours in

rodents and result in impaired neurogenesis. These include repeat restraint stress (Pham et al., 2003), chronic unpredictable mild stress (Song et al., 2006), social defeat stress (Van Bokhoven et al., 2011), corticosterone administration (Cameron and Gould, 1994) and social isolation (Dranovsky et al., 2011). It is still debatable whether or not impairment of neurogenesis alone is sufficient to induce depressive-like behaviours (Petrik et al., 2012). It has been observed that three of the most effective treatments of affective disorders in humans, namely electroconvulsive shock, fluoxetine and lithium, can all enhance neurogenesis (Chen et al., 2000; Madsen et al., 2000; Malberg et al., 2000). Adult-generated hippocampal neurons are needed for proper mood control and for antidepressant efficacy, Santarelli et al. (2003) demonstrated that ablation of hippocampal neurogenesis in mice indeed impairs antidepressant efficacy, however follow up studies showed that AHN is not required for fluoxetine efficacy in different mouse strains (Holick et al., 2008; Huang and Herbert, 2005). Currently there is no evidence to support the idea that boosting neurogenesis alone is sufficient for treating depression (Miller and Hen, 2015). The role for neurogenesis in depression is indefinable, whether decreases in neurogenesis are a symptom of depression or if they are causative is still up for debate. To evaluate the aetiology of depression using invasive techniques it is essential to use animal models.

1.4 Animal models of depression

Animal models need to have face, construct and predictive validity as set out by Willner (1984) for the disorder they investigate. Face validity demonstrates a similarity between the clinical symptoms present in the condition and the behavioural phenotype that is observed in the animal. Construct validity is that the biological underpinnings are similar in both clinical populations and in the animal model. Predictive validity is the reduction/attenuation of symptoms using clinically effective treatments/doses and/or the lack of response to ineffective treatments in animal models. As mentioned above, depression has high individual variability in symptom profiles of patients and it can therefore be difficult to ever establish true face validity in an animal model. In some cases, changes in behaviours can go in opposite directions (hypersomnia/insomnia) and single models cannot often take this into account but rather choose a subset of specific changes. Depression is also a relapsing condition, this cannot always be taken into account in animal models, and often changes produced from the model are permanent and can only be changed with treatment/interventions. Currently no animal models of MDD sufficiently fulfil the criteria for face/construct/predictive validity, but there are some behavioural task that can be used as indices to assess predictive validity of specific phenotypes e.g. behavioural despair (forced swim test), anhedonia (sucrose preference test) but nothing which sufficiently models the full repertoire of depression. The table below is taken from (Czéh et al., 2016) and summarises the clinical symptoms, translationally related phenotype in animal models and the tests used to measure these phenomenon. The original animal models of depression were based on the depletion of monoamines by the administration of drugs like reserpine (a VMAT2 inhibitor) which prevents storage of NA and 5-HT. Reserpine was originally used as a treatment for hypertensive vascular disease however, it was found to precipitate depression in some patients. The depression produced by reserpine was reversed after the treatment was terminated (Muller, Pryor, Gibbons, & Orgain, 1955). Reserpine non-selectively inhibits vesicular release of monoamines, diminishing brain levels (Nagakura et al., 2009). Key behavioural features include locomotor hypo-motility, a reduction in body temperature which can be reversed using an irreversible, non-selective, monoamine oxidase inhibitor (MAO), such as iproniazid and also by tricyclic antidepressants (Danysz et al., 1991) suggesting good predictive validity for existing antidepressants. Although this model is effective in inducing 'depressive-like' behaviours in the rat there are several limitations. For instance the mechanism by which reserpine lowers mood-related behaviours is unclear (O'Neil and Moore, 2003). It is now realised that these early models are only likely to be able to predict the therapeutic potential of drugs which restore monoamine function and do not encompass other neurochemical dysfunction.

Few rodent tasks directly replicate the human condition although changes in plasma cortisol and corticosterone may have close approximation. However, for

most phenotypes a task is used which is thought to have some translational equivalence such as immobility in the FST reflecting behavioural despair. Yet for other symptoms, such as suicidal ideation, no equivalence exists (Table 2). No single animal model will adequately reproduce all the various aspects of depression, especially if it is primarily altering in monoamine neurotransmission. To improve modelling of this disease, novel therapies need to be tested that further investigate the effect depression has on molecular and cellular processes in the brain (Deussing, 2006). Current research has turned to the negative effect depression has on neurogenesis in the hippocampus and molecular signalling pathways downstream of monoamine neurotransmitter receptors as possible therapeutic targets in the future in particular for the cognitive defects seen in depressed patients.

Depressive symptoms and potential biomarkers in patients	Related physiological or behavioral phenotype in experimental animals	Tests to measure behavior or method to assess physiological changes
DSM-5 criteria		
Depressed mood		
Markedly diminished interest or pleasure in activities	Anhedonic-like behavior	Sucrose preference Intracranial self-stimulation Conditioned place-preference Female urine sniffing
Significant weight loss or weight gain	Weight gain or loss	Body weight measurements
Insomnia or hypersomnia	Altered diurnal rhythm	Measuring diurnal activity, sleep EEG
Psychomotor agitation or retardation	Altered locomotor activity	Open field test Home cage activity Treadmill running
Fatigue or loss of energy	Behavioral despair, altered locomotor activity	Forced swim test Tail suspension test Learned helplessness Reduced home cage activity Treadmill running Nest building
Feelings of worthlessness or excessive or inappropriate guilt	–	? Grooming? * ? Assessing fur condition? *
Diminished ability to think or concentrate, or indecisiveness	Cognitive deficits	Morris water maze Y-maze Attentional set-shifting Novel object exploration Modified hole board
Recurrent thoughts of death, recurrent suicidal ideation	–	–
Further typical symptoms		
Anxiety, agitation or restlessness – for example, excessive worrying, pacing, hand-wringing or an inability to sit still	Anxiety-related behavior	Open field Elevated plus maze Dark-light box Novelty-induced hypophagia Marble burying
Dysfunctional social behavior, frequent negative social interactions	Altered social behavior	Time spent on social interaction/avoidance
Angry outbursts, irritability or frustration, even over small matters	Impulsivity	Continuous performance task, stop-signal task, go/no-go and delay-discounting paradigms
Unexplained physical problems, such as back pain or headaches	Increased pain sensitivity	Pain threshold measurements
Potential "biomarkers"		
Disturbed HPAaxis regulation	Disturbed HPA-axis regulation	Serum corticosterone levels Dexamethasone suppression test
Hippocampal volume decrease	Hippocampal volume decrease	*in vivo* MRI *post mortem* histology
Inflammatory biomarkers	Serum markers for inflammation	Pro-inflammatory cytokines (IL-1, IL-6, TNF-alpha, IFN-gamma)

Table 2: Clinical symptoms, related phenotypes and tests to measure changes in depression. Taken from Czéh et al. (2016)

1.4.1 Genetic models

Research into genetic models involves silencing or overexpressing particular genes thought to play a role in a disease. Earlier genetic models of depression were based around the monoamine theory, this resulted in the development of several knockout mouse lines to investigate changes associated with particular

'risk' genes. Heisler et al. (1998) and Parks et al. (1998) both used a 5-HT_{1A} receptor knockout model which displayed an increase in anxiety-like behaviour in paradigms such as the EPM. Based on the stress/HPA axis theory of depression Timpl et al. (1998) used a CRH-R1 knockout model that displayed a reduced anxiety profile and an impaired stress response. In relation to the neurotrophic theory of depression some researchers have examined BDNF knockout mice although these displayed no changes in anxiety-like behaviour in the EPM, or in the forced swim test or sucrose consumption task of despair and anhedonia respectively (Barkus, 2012). The use of genetic manipulations to examine depression in animal models has a number of limitations. Many genetic models rely upon the use of mice, this contrasts with most behavioural tests of depression which utilize rats. Furthermore, most genetic manipulations have an effect on the entire system rather than being able to target a specific brain region or pharmacological site which can lead to complications and a lack of specificity.

1.4.2 Strain/Breeding Models

Genetic inbred lines of rodents have also been utilised to attempt to gain insight into the neurobiology of depression. Rodent breeding models are based on strains of animal that contain specific behavioural or physiological features generated through several generations of breeding. Some lines of rodent were originally developed for another purpose but are now more commonly used to model depressive-like behaviours. The Wistar-Kyoto (WKY) rat strain is one example. It was originally developed as a control strain for the spontaneous hypertensive rat (Okamoto and Aoki, 1963). The WKY rat was found to have several physiological and behavioural phenotypes akin to depression including stress hyperactivity, HPA axis dysregulation and depressive-like behaviour in the forced swim test (Overstreet, 2012). More recently the WKY rat has also been adopted as a model to examine treatment resistant depression (Tizabi et al., 2012). The Wistar rat strain has also been selectively bred for a high and low anxiety phenotype in the elevated plus maze (Liebsch et al., 1998).

1.4.3 The Olfactory Bulbectomy Model

Bilateral olfactory bulbectomy (OBX) is a surgical model of depression which results in behavioural changes that resemble several core symptoms observed in patients with major depression, as well as similar neurochemical and immunological alterations (Kelly et al., 1997; Song and Leonard, 2005; van Riezen et al., 1976). Following olfactory bulbectomy, there is degeneration of neurones in the hippocampus, amygdala, cortex and other subcortical regions (Carlsen et al., 1982), similar to pathology seen in MDD patients (Duman et al., 2001). As anosmia alone does not cause the same behavioural changes it is not the loss of olfaction from the bulbectomy that is the major factor that contributes to the behavioural changes observed, (Song and Leonard, 2005). The changes are thought to result from dysfunctions or compensatory mechanisms of the cortical–hippocampal–amygdala circuits that involve changes in synaptic

strength and/or loss of spine density in these limbic areas. These same regions are dysfunctional in patients with major depression (Price and Drevets, 2012). The following behavioural changes have been observed after bilateral OBX: hyperactivity, changes in social behaviour, deficits in learning and memory, although no indication of anhedonic-like behaviour has been reported (Kelly et al., 1997; Song and Leonard, 2005).

Alterations in neurotransmitter systems are also associated with OBX. Increased secretion of corticosterone during the night cycle is also observed, this can be suppressed by the administration of dexamethasone. Importantly chronic, but not acute, administration of antidepressants attenuates most of the behavioural and neurotransmitter changes that can occur after bulbectomy, these include TCAs (amitriptyline and desipramine), SSRIs (paroxetine, sertraline, and fluvoxamine), and atypical agents (mianserin) (Kelly et al., 1997; Song and Leonard, 2005).

1.4.5 Stress Models

Another one of the more common hypotheses of depression as discussed above is related to stress and the dysregulation of the HPA axis (Gold, 2015) and from this one of the more common forms of modelling depression involves the use of stress exposure. These models are quite well known for their face and construct validity what they lack is in their predictive validity. One of these models is the learned helplessness model (LH), it is based around the idea that core symptoms of depression are feelings of helplessness. This model was one of the earliest adopted for its use for depression research (Seligman et al., 1968). Learned helplessness is a deficit in behaviour induced by prior exposure to an aversive stimuli. Learned helplessness is commonly established using the following method, there are three groups, with two control groups. The first control group is exposed to electric shocks, exposure to which can be controlled by escaping or lever pressing. The animals of the second group receive shocks of the same amount, duration and pattern in an unpredictable and unavoidable manner. A third group of animals are not exposed to stress. Thus, animals exposed to uncontrollable shocks can be compared with animals exposed to controllable shocks and unstressed controls. Studies that employ this design indicate that the deficits that follow exposure to stress are not caused by stress per se; rather, the uncontrollability of the stress was the critical determinant (Drugan et al., 1996).

1.4.6 Chronic Mild Stress (CMS)

The chronic mild stress model is one of the most extensively validated and realistic models of depression focusing on one of the core symptoms of MDD; anhedonia (Willner, 2005). The CMS paradigm involves exposing rodents to a series of mild stressors (damp bedding, tilted cages, food/water deprivation, isolation, disruption of light/dark cycle) over a period of at least 2 weeks. One advantage of this model is that it induces long-lasting behavioural and neurochemical changes that resemble the dysfunctions observed in depressed

patients (Wiborg, 2013; Willner, 1997, 2005). Most importantly, the CMS paradigm induces anhedonic-like behaviour which could be reversed by chronic, but not acute, antidepressant treatment. The inducing conditions and the repetition of stress are said to closely resemble conditions in human life that may result in MDD. These features of the model thus provide etiological validity. If the stress paradigm is sustained, the depressive-like state can be maintained for months due to the unpredictable nature of the stress regimen the animals are incapable of habituating. If chronic antidepressant treatment is given in conjunction with stress exposure the animals may recover from the depressive-like state (Kudryashov et al., 2016; Wiborg, 2013).

1.4.7 Early-life stress models

Early life stress models are based on the idea that exposure to potential traumas or stressors at critical times throughout early development can lead to long lasting changes into adulthood potentially contributing or resulting in the development of psychiatric illness. The models are based on initial studies performed in rodents (Levine, 1957, 1967; Weininger, 1953) and in non-human primates (Harlow and Zimmermann, 1959). Evidence suggests that negative factors within an environment both pre- and post-natally can lead to the development of psychiatric disorders (Heim et al., 1997). One of the more common models used in for early-life stress is maternal separation. Many studies performed in rats have shown that single or repeated separation of the pups from the dam leads to both acute and/or long-term changes in behaviour and physiology, although there is a high level of inconsistency. (Daly, 1973; Lehmann and Feldon, 2000). These inconsistencies may be due to variations in the application of the model; repeated maternal separation promotes maternal care (Enthoven et al., 2008) while maternal deprivation stress leads to long lasting changes both behaviourally and physiologically (Oomen et al., 2010). Pups exposed to maternal separation develop HPA axis abnormalities and a variety of anxiety related changes such as enhanced immobility in the FST (Desbonnet et al., 2010). A major disadvantage of this model is the large variability in changes reported in different cohorts of pups. An alternative form of early-life stress involves separation of rat pups at the point of weaning preventing social play.

This work described in this thesis focuses on the effects of social isolation of rats from weaning, so this model will now be discussed in greater detail. Rearing rodents in social isolation immediately after weaning between post-natal day (PND) 21-28 produces behavioural changes akin to anxiety and depressive-like behaviours compared with group housed controls (Einon and Morgan, 1977; Heidbreder et al., 2000; Lapiz et al., 2003; Valzelli, 1973). Collectively the behavioural changes observed are consistent with the proposal that rats reared in social isolation are unable to process environmental stimuli appropriately.

Even from early experiments performed in the 1960s and 1970s, 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) leading to the description of the 'isolation-induced stress syndrome' (Holson et al., 1991; Valzelli, 1973). Some of the behavioural and neurochemical alterations seen in isolation reared rats appear to have translational relevance to prevalent developmental neuropsychological disorders, in particular to several core symptoms of schizophrenia but also to changes seen in depression. This has led to isolation rearing being proposed as an animal model to investigate the neurobiology of these disorders. The majority of such studies have been performed with male rats as the behavioural phenotype produced is more pronounced in male rats versus females (Fone & Porkess, 2008) for example isolation-reared male Sprague–Dawley rats show a greater anxiogenic profile in the elevated plus maze, together with a higher ACTH and corticosterone elevation in response to a stressor, than females (Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004a) but where another species or gender has been examined this will be stated.

1.4.8 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 must be carefully controlled and minimised. To avoid any confounding effect 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 PND28) in a critical period around the time of puberty (Einon and Morgan, 1977) although there may be strain differences. For instance, the reductions in social interaction (Ferdman et al., 2007) and prepulse inhibition of acoustic startle (Wilkinson et al., 1994), or the increases 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 example, basal corticosterone levels may only be elevated by more austere housing conditions, such as wire floored cages where no handling is performed at all during the isolation period (Heidbreder et al., 2000; Holson et al., 1991) 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 when required for daily drug injection), may 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 symptom domain seen in the disease being modelled should be examined (Powell and Miyakawa, 2006).

1.5 Behavioural Effects & Cognitive Deficits in Isolation Rearing

1.5.1 Hyperactivity in a Novel Environment

One of the earliest and most reproducible behavioural changes reported in isolation reared rats is neophobia, a deficit in the ability to habituate to a novel environment, characterised by hyperactivity compared with group housed controls, studies were conducted in open fields or Perspex boxes and activity was measured using photo beam analysis to track movement (Bianchi et al., 2006; Gentsch et al., 1981b; Gentsch et al., 1982; Sahakian et al., 1977; Watson et al., 2012). Although this may be strain dependent, isolation-induced hyperactivity being greater in Lister-hooded strain compared to Sprague Dawleys (Geyer et al., 1993; Weiss et al., 1999). However this hyperactivity is sensitive to alterations, being reduced in bright light (Hall et al., 1998) and periods of handling have been shown to be sufficient to prevent its development (Holson et al., 1991). The consistency and that it is also one of the earliest behavioural features to appear, manifesting after two weeks of isolation (Einon and Morgan, 1977) makes this a suitable marker with which to confirm the development of the ‘isolation syndrome’ prior to performing more complex behavioural or neurochemical analyses.

1.5.2 Anxiety/Depressive-like Behaviour

Some groups have reported modest increases in anxiety-related behaviours on the elevated plus maze in isolation reared rats (Hellemans et al., 2004; Parker and Morinan, 1986; Wright et al., 1991) although this is not a reliable finding (Fone et al., 1996). Isolation reared rats also show behaviours consistent with increased anxiety in tasks involving mild aversion, such as a reduced latency to escape from a novel open top cage and reduced consumption of novel food (Parker and Morinan, 1986). Other groups have examined whether rearing rodents in isolation may result in the development of behavioural despair, such as the Porsolt forced swim test (Porsolt et al., 1978; Porsolt et al., 1977). In mice

although short periods of social isolation (for between 24 h to 5 days) near the age of weaning (Hilakivi et al., 1989) may reduce immobility time in the forced swim test 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 (Hall et al., 2001; Hall et al., 1998). This suggests that prolonged social isolation may not affect the ability of the rodent to cope with a short period of severe stress.

1.5.3 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) and in Lister-hooded rats in our laboratory (McIntosh et al., 2013; Watson et al., 2012) consistent with impairment in associative learning.

1.5.4 Novel Object Discrimination

Isolation reared rats show impaired recognition memory in the novel object discrimination paradigm (Bianchi et al., 2006; King et al., 2004; McIntosh et al., 2013; McLean et al., 2008; Watson et al., 2012) This deficit can reliably be considered to be caused by cognitive impairment rather than increases in exploration due to hyperactivity, as the time spent exploring both objects in the first (familiarisation) trial is unchanged in isolates and, with short inter-trial delay, isolates can discriminate objects in the choice trial but display premature forgetting as the inter trial interval is increased (King et al., 2004). Also in an object contact task, isolation reared rats explored the same number of novel objects as social reared animals (Einon and Morgan, 1977). The apparent reduction in object recognition could also reflect cognitive inflexibility, a study conducted by Amitai et al. (2014) found that isolation reared rats required more sessions, to acquire criterion performance in a reversal learning task, and were slower to adjust their task strategy after reward contingencies were switched. They also completed fewer trials and exhibited lower levels of overall activity in the probabilistic reversal-learning task than did the socially reared rats. This again suggests that there is no confounding impact of hyperactivity on cognitive impairment seen in isolation reared rats.

1.5.5 Water Maze

Several groups have examined the impact of isolation rearing on spatial learning in the water maze but results are inconsistent. Many groups find no changes in isolates abilities to find the hidden platform (Lapiz et al., 2003; Schrijver et al., 2004), although some report slight improvements (Wongwitdecha and Marsden,

1996) or impairments in learning (Hellemans et al., 2004) accompanied by reduction in LTP in the CA1 area of rat hippocampus (Lu et al., 2003). However, the difference in acquisition in the isolation reared compared to group housed rats may be due to the effect of the environmental enrichment present in the group housed cage rather than the social isolation itself (Schrijver et al., 2002). One group has shown that isolation rearing improved reversal performance in the water maze over social housed controls (Wongwitdecha and Marsden, 1996). 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). Quan et al. (2010) demonstrated that in the MWM showed isolation-reared rats performed worse in probe trials and memory retention tests and that LTP tests showed that the prefrontal cortical postsynaptic potential slopes were significantly lower in isolated rats than group housed ones. A variety of other changes are seen in isolated rats. MRI studies have shown reductions in medial PFC volume (Schubert et al., 2009) alterations in ketamine induced glutamate release assessed by MRS (Napolitano et al., 2014) showing changes in glutamatergic function and a variety of abnormalities in synaptic markers including: synaptophysin (Varty et al., 1999) and growth factors, BDNF in the hippocampus and cytoskeletal alterations (Bianchi et al., 2006). Taken together, this collection of behavioural and neurochemical changes which represent many seen in MDD were the reasons for selecting and further validating this model in the current thesis.

Behaviour	Consequence of isolation rearing
Explorotory behaviour	Males may be slightly heavier (Fiala et al., 1977) No change in food consumption (Hellemans, Benge, & Olmstead, 2004) Increased food hoarding behaviour (Heidbreder et al., 2000)
Prepulse inhibition of acoustic startle	Impairment in PPI without change in control startle amplitude (Bakshi, Swerdlow, Braff, & Geyer, 1998; Barr, Powell, Markou, & Geyer, 2006; Cilia, Reavill, Hagan, & Jones, 2001; Day-Wilson, Jones, Southam, Cilia, & Totterdell, 2006; Varty, Paulus, Braff, & Geyer, 2000)
Latent inhibition	No effects (Weiss, Domeney, Moreau, Russig, & Feldon, 2001; Wilkinson et al., 1994) (Wilkinson et al., 1994, Weiss et al., 2001b)
Anhedonia	Increased sucrose consumption (increased motivation) (Hall, Humby, Wilkinson, & Robbins, 1997) Hyperphagia (Fiala et al., 1977) Increased reward sensitivity (Jones, Marsden, & Robbins, 1990) Increased anticipatory behaviour (Morgan, Einon, & Nicholas, 1975) Increased self-administration with low but impaired acquisition with high doses of cocaine (Howes, Dalley, Morrison, Robbins, & Everitt, 2000) Increased ethanol preference in mice (Advani, Hensler, & Koek, 2007) and Long Evans rats (Deehan, Cain, & Kiefer, 2007)
Social interaction	Increased interactions and aggression (N. Wongwitdecha & Marsden, 1996) Progressive increase in muricidal behaviour in isolation reared rats(Valzelli & Garattini, 1972) Increase in defensive burying of a shock probe (Arakawa, 2007)
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, Zhu, Terasmaa, Mohammed, & Fuxe, 2004) Fear conditioning reduced in male and female isolates (Weiss et al., 2004a)
Visual learning & memory	Impaired novel object discrimination (Bianchi et al., 2006; McIntosh, Ballard, Steward, Moran, & Fone, 2013; Watson et al., 2015; Watson, Marsden, Millan, & Fone, 2012a)
Attention/vigilence	No impairments seen in five choice serial reaction time (Dalley, Theobald, Pereira, Li, & Robbins, 2002)
Spatial learning	Water maze- improvements (Wongwitdecha & Marsden, 1996) No change (Schrijver, Pallier, Brown, & Würbel, 2004) Impairments (Hellemans et al., 2004; Lu, Shepard, Hall, & Shaham, 2003)
Reasoning and problem solving (attentional set shifting)	Extradimensional shift impairment in spatial/non-spatial ID-ED task (Schrijver & Würbel, 2001) Reversal impairment, but no ED impairment in bowl-digging (Schrijver et al., 2004) Impairments in reversal learning (Krech, Rosenzweig, & Bennett, 1962) Improved water maze reversal performance (Wongwitdecha and Marsden, 1996b)
Conditioned place preference	No preference for amphetamine treated area (Noppamars Wongwitdecha & Marsden, 1995)
Anxiety	Anxiogenic on elevated plus-maze (Parker & Morinan, 1986; Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004b; Wright, Upton, & Marsden, 1991) 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, Ota, & Lister, 1989)or rats (Hall, Huang, Fong, Pert, & Linnoila, 1998; Hall,

	Sundstrom, Lerner, & Pert, 2001)Desipramine more effective in isolates (N. Wongwitdecha, Kasemsook, & Plasen, 2006)

Table 1.1: Summary of the long-term behavioural phenotype changes associated with isolation rearing of rat pups from weaning (Adapted from Fone & Porkess, 2008)

1.6 Aims of this Study

The aim this study was to validate further the post-weaning social isolation model and to characterise and assess its suitability as a model for depressive-like behaviours. This was done using a battery of behavioural tasks assessing composite symptoms often observed in preclinical models of MDD. These were chosen based on previous evidence observed in isolation reared rats and because of their translational relevance to symptoms observed in patients with depression. They included measures of hyperactivity, a symptom typically observed in isolated rats, anxiety measures in tasks such as the open-field, elevated plus maze and novelty-supressed feeding. Cognition; the novel object discrimination task and the conditioned fear response task were utilised. In addition, stress response was examined using a restraint stress paradigm.

The second aim of the study was to assess treatment options for MDD at alleviating or attenuating the symptoms observed in this model. The treatments chosen were based on previous studies,the SSRI fluoxetine was chosen as it is a first line treatment in clinical populations for MDD (Bech et al., 2000) and one of the most common SSRI's adopted in animal studies of depression (Dulawa, Holick, Gundersen, & Hen, 2004).The NMDA-R antagonist ketamine was also chosen due to recent studies demonstrating its potential use as a rapid acting antidepressant in depression (Zarate et al., 2012). Finally DJNKI a peptide which inhibits JNK-1 was also be examined to assess its theoretical potential and value as an antidepressant. This was chosen as work conducted by colleagues has shown the peptide has promising efficacy in animal models of depression (Mohammad et al., 2018).

The final part of the study examined changes in post-mortem, plasma corticosterone levels to examine stress response, cytokine levels to examine immunological function/response, assessing levels of neurogenesis in the hippocampal dentate gyrus to determine the role it plays in cognitive deficits in depression and facilitation of antidepressant efficacy and finally microbiome analysis was carried out to assess any changes seen in gut bacteria of isolated rats which might be a cause of phenotypic alterations seen.

Chapter 2:

Characterising the physiological and behavioural changes in isolation rearing model of depression

2.1. Introduction

Exposure to early-life stress is an established risk factor for the development of several common psychiatric conditions; including anxiety-related disorders, schizophrenia, post-traumatic stress disorder and depression (Heim et al., 1997). Furthermore, stress is the strongest predictor of developing depression in clinical populations (Kendler et al., 1993), and developmental rodent models of psychiatric illness often contain a stress component to attempt to provide translational relevance (Nestler and Hyman, 2010). In rodents adverse early-life events modify brain development, affecting resultant adult behaviour and often producing neurobiological sequelae that resemble changes seen in depression and schizophrenia which may therefore contribute to development of these common psychiatric disorders (Lapiz et al., 2003; Pryce et al., 2005).

Social isolation of rat pups from weaning (hereafter referred to as SI) is an established neurodevelopmental model producing robust behavioural, neurochemical and anatomical changes reminiscent of several core features of depression (Fone and Porkess, 2008; Jones et al., 2011). These include attenuated exploratory habituation to a novel arena (Gentsch et al., 1982; Meffre et al., 2012; Sahakian et al., 1977), enhanced 'anxiety-related' behaviour in the elevated plus maze (Parker and Morinan, 1986; Weiss et al., 2004) and reduced social interaction (McIntosh et al., 2013; Watson et al., 2016; Wongwitdecha and Marsden, 1996). SI rats also show impaired learning and memory in multiple cognitive domains including; visual learning and memory (novel object discrimination) pre-attention processing (pre-pulse inhibition of acoustic startle) and associative learning (conditioned freezing response, CFR) (Bakshi et al., 1998; Bianchi et al., 2006; Fone and Porkess, 2008; Gaskin et al., 2016; McIntosh et al., 2013; Meffre et al., 2012). SI also modifies the hypothalamic pituitary-adrenal (HPA) axis stress response. However basal levels of adrenocorticotrophic hormone and corticosterone have been reported to be increased (Weiss et al., 2004) or unaltered (Scaccianoce et al., 2006; Schrijver et al., 2002), which might depend on the strain of rat (Malkesman et al., 2006) or the severity of the isolation procedure (e.g. use of sawdust bedding or housing on a grid floor) (Fone and Porkess, 2008). One group (Serra et al., 2005) reported SI caused hypersensitivity of the pituitary gland to intraventricular injection of corticotrophin-releasing hormone and impaired glucocorticoid receptor mediated negative feedback of the HPA axis, analogous to suppression of feedback inhibition seen in severe depression. Furthermore, Muchimapura et al. (2002) demonstrated that SI can affect the stress response by altering neurodevelopment of the serotonergic innervation of the hippocampus.

Despite a wealth of research, the mechanisms underlying the developmental changes produced by SI are not established. One possible contributory factor, which has so far received little investigation, is that isolation rearing modifies the microbiota-gut-brain axis thereby disrupting normal neural, endocrine, metabolic and immunological bicommunication between gut microbiome and

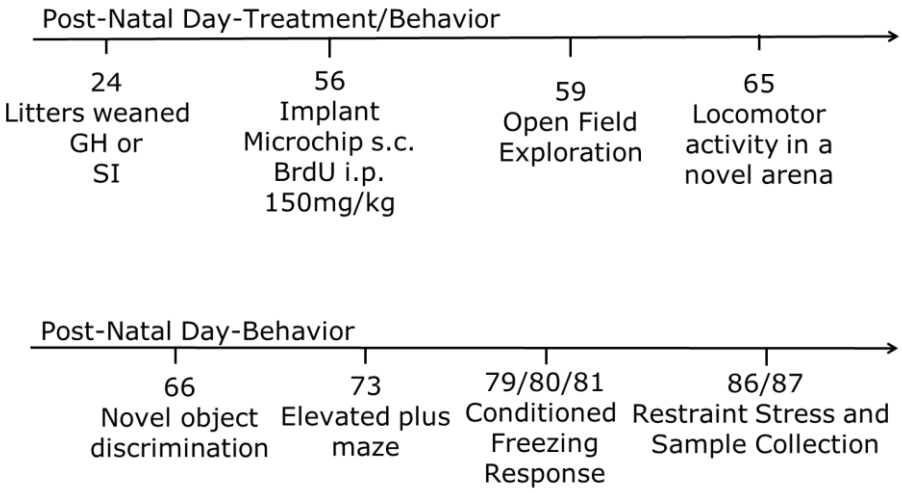
the brain (Jones et al., 2006). This could then adversely affect neuronal development and adult behaviour (see reviews, (Cryan and Dinan, 2012; Parashar and Udayabanu, 2016; Sarkar et al., 2016)). Indeed recent research has implicated alterations in the composition of gut microbiota as a potential factor in the aetiology of a number of central nervous system disorders; including autism (Williams et al., 2011) and anxiety and depression (Foster and McVey Neufeld, 2013; Naseribafrouei et al., 2014). Early studies of microbiota established that germ-free mice show increased stress reactivity (Sudo et al., 2004), repetitive self-grooming and reduced social interaction which are all reversed by colonization of the gut (Desbonnet et al., 2014). Conversely studies also demonstrate that exposure of rodents to a stressor alters microbiota resulting in an associated increase in pro-inflammatory cytokines (Bailey et al., 2011; Galley and Bailey, 2014).

For example, maternal separation of rat pups not only enhances anxiety-related behaviour and cytokine levels but also changes gut microbiota (O'Mahony et al., 2009) and treatment with probiotics has a comparative effect to the SSRI citalopram, attenuating some of these changes (Desbonnet et al., 2010). However, there is limited data on the role of the microbiota and to this end we characterised the impact of housing rats in SI from weaning on a behavioural tasks evaluating performance in cognitive, 'anxiety-like' and stress paradigms and correlated these with changes in gut microbiota, hippocampal and prefrontal cortical (PFC) cytokine, plasma corticosterone levels and hippocampal neurogenesis. Associations are made between changes in specific behavioural phenotypes and gut microbiota in rat littermates either housed in small social groups (GH) or in SI and the potential translational relevance of these findings to common neurodevelopmental psychiatric disorders is discussed.

2.2. Methods**2.2.1 Animals**

Twenty-four male Lister hooded rats (from four equal sized litters, this allowed for an even split for groups) were obtained from Charles River UK on post-natal day 24 and weaned on arrival. Each litter was randomly divided such that half the pups were group-housed (GH) and the other half reared alone (SI) this was done so that any effect of litter could be accounted for. Each group had 3 animals from each of the 4 litters. The group size n=12 was decided upon based on previous research conducted in the lab which indicated there was enough statistical power (Bianchi et al., 2006; McIntosh et al., 2013; Watson et al., 2012a). Rats were housed in plastic cages (32x51cm, n= 3 GH or 25x42cm, n= 1 SI) containing sawdust bedding with metal grid lids, and had visual, auditory and olfactory contact with conspecifics in the same holding room. Rats were weighed once weekly (when bedding was changed). Food and water were available *ad libitum* and rats were kept on a twelve hour light/dark cycle (lights on 07.00h) at constant temperature (21 ± 2 °C) and humidity (45 ± 15%). On post-natal day 56 rats received 5-bromo-2'-deoxyuridine (BrdU, a marker of cell proliferation, Sigma Aldrich, 150mg/kg, i.p.) and housed for a further week before behavioural testing began (Fig. 2.2.1). All behavioural studies were conducted between 08.00 and 17.00h with SI and GH rats being analysed in a random order to minimise any temporal effect, and before each test the arena was cleaned with 20% ethanol and dried to remove odour cues. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986), with approval of the University of Nottingham Local Ethical Review Committee, and conformed to the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010). Behavioural studies were conducted during the light phase of the circadian cycle to enable comparison with all our previous work with this paradigm and the majority of other publications. Although there may be sex differences in behavioural and neuroendocrine responses following SI, this was not the primary focus of the current studies which were therefore performed on male rats to minimise the number of animals required according to the 3R's principle.

A



B



Figure 2.2.1: (A) Protocol diagram showing the time course of experimental housing and behavioural testing across post-natal day age of the Lister hooded rats used in the study. **(B)** Pictures of the behavioural apparatus used in each behavioural study Top row: Open field, Locomotor Acitivity Box, Novel Object Arena. Bottom Row: Elevated Plus-maze, Operant Box for CFR and Restraint tube.

2.2.2 Open Field Behaviour

As an ethological index of anxiety, behaviour was recorded in a novel circular open field (on post-natal day 59, Fig. 2.2.1.). Rats were placed in the centre of the unfamiliar arena (75cm diameter with 45cm high walls, lined with grey card) at 120Lux for 15min and behaviour recorded using computerised tracking (Noldus, Ethovision XT ver7.1). Ambulatory speed and distance travelled in the peripheral and central zone (15cm from the wall defined on Ethovision) together with time spent rearing and body grooming and the number of faecal boli were measured as indices of 'anxiety levels'.

2.2.3 Spontaneous locomotor activity in a novel cage

Isolation rearing-induced hyperactivity in response to a novel environment was measured to confirm development of the isolation syndrome and as a further index of neophobia, this allows for a second measure of exploratory behaviour in a novel environment while also allowing the animals to habituate to the arena as it is adopted for the novel object task 24 hours later as described previously (Gaskin et al., 2016; King et al., 2009; Watson et al., 2016)(Fig. 2.2.1). Each rat was placed in an unfamiliar perspex arena (39cm x 23.5cm x 24.5cm for 1h) to record horizontal locomotor activity and rearing using a computerised infra-red activity system (Photobeam Activity System, PAS).

2.2.4 Novel object discrimination (NOD)

Twenty four hours following locomotor assessment (and consequent habituation to the arena) novel object discrimination (NOD) was assessed to evaluate visual recognition memory (Lyon et al., 2012) as described before (Jones et al., 2011; Millan et al., 2010). In turn, each rat was tested for NOD using the following sequence. Each rat was re-acclimatised to the same Perspex arena previously used for locomotor activity for 3min, immediately placed back in the home cage for 1min and then returned to the arena to receive two consecutive 3min object exploration trials separated by a 2h inter-trial interval where the rat was returned to the home cage. During the first (familiarisation) trial, two identical white bottles (8cm high, 5cm diameter) were placed in opposite corners of the arena (5cm from the side and 10cm from the end wall). During the second (choice) trial, one object (chosen in a pseudo-random balanced design) was replaced with a novel unfamiliar object (white bottle with black horizontal stripes). The choice of the objects was based on previous studies conducted within our laboratory where the novel object was successfully discriminated compared to the familiar (Bianchi et al., 2006; McIntosh et al., 2013) although many previous studies vary the type of objects used, these can vary in size/shape and complexity (Antunes & Biala, 2012) the simple and previously validated objects were decided upon for the current study. During each trial the time spent exploring each object (sniffing or active vibrissae with the nose within 1cm of the object) was recorded by stop watches. Climbing on or chewing at the object was not counted as exploration. The discrimination ratio (novel/(novel + familiar times)) was derived from the raw data to further determine object preference.

2.2.5 Elevated plus maze

Exploration of the elevated plus maze was measured to evaluate any change in ‘anxiety-like behaviour’ associated with isolation rearing, as described previously by us (Bull et al., 2004; Fone et al., 1996). Each rat was placed in the centre of the maze facing a closed arm and left to explore for 5min. The 70cm high black Perspex maze (with foam mats beneath to prevent injury from falls) consisted of four 45cm long arms, two opposing arms being open (no walls) and two enclosed by 10cm high walls. Each arm was divided into equal dimension

outer and inner zones and behaviour monitored using software (Noldus Ethovision XT ver7.1) to determine time and entries into each arm and zone and distance travelled. In addition the number of unprotected head-dips (head-dip below the floor of the open arm) and stretch attend postures (extending two front feet into the centre of an open arm without immediately entering; a risk assessment behaviour) were recorded manually from video recordings. The percentage time and entries in the open arms (open/(open+closed) was calculated from the raw data, as a further index of anxiety.

2.2.6 Conditioned freezing response (CFR)

To determine any change in associative learning and memory, a two compartment Geller box was used to record CFR behaviour, a reliable index of associative learning, memory and extinction, as described previously (Jones et al., 2011; Woods et al., 2012). On the conditioning day (post-natal day 79, Fig. 2.2.1) each rat was placed in the light side of a computerised shuttle box (24.8 x 25.2 x 24.3cm, Panlab) for 30s before the compartment door temporarily opened to allow transition to the dark side and latency to enter was recorded. Thirty seconds later a conditioned stimulus (5s light and 3Hz, 89dB tone) signalled the start of an unavoidable foot shock (unconditioned stimulus, 1s, 0.4mA during the last 1s of the light and tone). Each rat received three shocks separated by 59s. Freezing behaviour (in a hunched position with inactive vibrissae and totally immobile except for respiratory movement) was measured after the first two shocks. Twenty four and 48h after conditioning rats were returned to the dark side of the shuttle box with no access to the light side for 300s, without further shocks, and time spent freezing recorded again. Immediately after the 48h trial the light and tone were presented again without the foot shock and freezing recorded during the next 300s.

2.2.7 Restraint Stress

Exposure to restraint stress has been regularly used as a psychological stress to evaluate HPA axis function (Hesketh et al., 2005) which may be modified by previous exposure to early-life SI (Fone et al., 1996). To elicit this response rats were restrained in a Perspex tube (27 x 7 x 7cm) for 1h on post-natal day 86 or 87 (Fig. 2.2.1).

2.2.8 Post-Mortem

Immediately following restraint rats were humanely killed by concussion followed by immediate exsanguination and mixed arterio-venous blood was collected, centrifuged (13,000g, 4^{o}C, 5min) and plasma decanted and frozen at -80°C for plasma corticosterone determination (in duplicate from the linear portion of the curve, 20-80% maximal binding region) by ELISA (Enzo Life Sciences). The brain was hemisected to remove the whole hippocampus and prefrontal cortex (PFC) from the left side and snap frozen in liquid N_2 for measurement of cytokines. The other half of the brain was placed in 4% paraformaldehyde for 24h then into 30% sucrose overnight and flash-frozen 24h

later for immunohistochemical analysis. Caecal samples were collected under aseptic conditions for microbiota analysis.

2.2.9 Mircobiota analysis

All microbiota analysis was carried out by collaborators at University College Cork. Caecal samples were prepared for 16S sequencing on the Illumina Miseq system essentially as described in the Illumina 16S library preparation workflow. In brief, the V3 and V4 region of the 16S bacterial rRNA gene were amplified using 16S primers (Sigma Aldrich Ireland ltd., Wicklow, Ireland). Kapa Robust was used to amplify the products and the number of cycles used was 30. The resulting PCR products were cleaned up using Ampure XP beads (Labplan ltd., Co. Kildare, Ireland). Dual indices and Illumina sequencing adapters (Nextera XT Index Kit, Illumina, Inc., San Diego, California, USA) were attached to the amplicons. Following another clean-up step amplicons were quantified, normalized and pooled. The resulting pool was once again cleaned before being run at the Teagasc sequencing facility on the Illumina Miseq platform.

2.2.10 Bioinformatic Analysis

Resulting FastQ forward and reverse reads were joined together using FLASH. Sequences were clustered into Operational Taxonomic Units (OTU's) and chimeras removed using USEARCH (Version 7.0-64bit). Taxonomy was assigned to OTUs with BLAST against Silva123.Alpha and beta diversities were calculated within Qiime (v1.9.0). PCoA plots were visualised with Emperor.

2.2.11 BrdU/NeuN Immunohistochemistry

Animals were sacrificed 6 weeks following on from BrdU injections, dual-staining immunohistochemistry was performed to identify co-existence of BrdU, a marker of cell proliferation at the time of injection with the neuronal specific nuclear protein, neuronal nuclei (NeuN) to identify mature neurons in hippocampal slices.This allowed for a snapshot of cells that were proliferating at the time of injection and became fully formed neurons by the end of the study. Sixty micron microtome sections were sequentially washed with phosphate buffered saline, incubated (2M HCl, 37°C, 30min), washed (5min 0.1M borate buffer), incubated for 1h in a 2% blocking solution (goat serum, 1% BSA containing 0.3% Triton-x100) and then primary antibody (rat anti-BrdU, 1:100) overnight at 4^{o}C. The next day sections were washed and incubated with secondary antibody (Alexa Flour 568 goat anti-rat, 1:500 1h room temperature). Following a further wash sections were incubated with Mouse anti-NeuN, (1:100 at 4^{o}C overnight), washed again and incubated with secondary antibody (Alexa Flour 488 rabbit anti-mouse, 1:500 for 1h). Sections were mounted on gelatinised slides, rinsed with distilled water, DAPI applied (1:1000, 30s in

darkness), cover slipped and positive cells quantified by an observer who was unaware of the rat housing condition.

2.2.12 Cytokine and mTOR analysis.

Hippocampal and frontal cortex samples were removed from -80°C and homogenised by sonication in RIPA buffer (1ml per 12mg of tissue containing 1:100 protease inhibitor, P8340, Sigma). Lowry assay was performed to prepare all samples to a common protein concentration. Cytokine (IL-1β, IL-6, IL-10 and TNF-α) and mTOR (total and phosphorylated) levels in brain samples were determined in duplicate using a multiplex kit (MILLIPLEX MAP, Merck Millipore, UK) against standards with quality controls as described previously (Hind et al., 2016).

2.2.13 Statistical analysis

GraphPad Prism v6 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS22 (IBM Analytics, UK) were used for statistical analyses. All data were checked for normality and homogeneity of variance using Shapiro-Wilk's and Levene's tests, respectively. Locomotor activity and NOD data were analysed by Two-way repeated measures analysis of variance (RM ANOVA, with rearing condition as a between-subject factor, and time and object as repeated measures). Measures made without a time course in open field, elevated plus maze and NOD, such as choice trial discrimination ratio and cytokine, mTOR and NeuN were analysed by ANOVA (with rearing condition as a factor). In each case, where appropriate, multiple comparison Sidak post-hoc tests were used where ANOVA suggested statistical significance ($p<0.05$) as this test is more powerful than the Bonferroni method and the data are expressed as mean $\pm$ sem. Microbiota analysis was performed in R (version 3.3.2). The vegan package (version 2.3-4) was used for alpha diversity indices and Adonis test. This package provides tools for descriptive community ecology. It has most basic functions of diversity analysis, community ordination and dissimilarity analysis. Differences in alpha diversity and taxonomic composition were calculated with Mann-Whitney U test. Spearman correlations were performed between relative microbiota abundance and behaviour and cytokine measures. All post-hoc tests and Spearman correlations included correction for multiple comparison.

2.3. Results

2.3.1 Open field behaviour

SI rats travelled significantly further than GH control rats ($p<0.001$, Fig. 2.3.1 A) and at a higher overall velocity ($p<0.001$, Fig. 2.3.1 B) in the whole open field thus demonstrating hyperactivity in a novel arena consistent with development of the isolation-reared behavioural syndrome.

As expected both GH and SI rats spent significantly more time in the outer than the central (more aversive) zone of the open field (ANOVA; main effect of zone $p<0.0001$, Fig. 2.3.1 C) but this was unaffected by rearing condition. Further ethological analysis failed to show any significant difference in either the number of rears or time spent grooming between GH and SI rats (data not shown). In contrast, at the end of the trial SI rats had produced significantly fewer ($p<0.05$) faecal boli than GH littermates (1.6 ± 0.8 and 3.9 ± 0.7, respectively, Fig. 2.3.1 D), consistent with the development of an anxiogenic profile by SI.

2.3.2 Effects of isolation-rearing on exploration and locomotor activity

In accordance with open field data SI rats displayed significantly higher ($p<0.01$, Fig. 2.3.1 E) total horizontal locomotor activity over 1h in the activity boxes compared to GH rats. Temporal analysis of this locomotion (Fig. 2.3.1 E) showed that the change was due to a slower rate of habituation, especially during the first 30min, consistent with SI inducing neophobia. Although there was no significant difference in the overall total number of rears, SI rats also showed significantly more ($p<0.05$) rears during the second 5min in the box and a similar trend for reduced attenuation of rearing behaviour during the first 30min as seen with ambulation (data not shown).

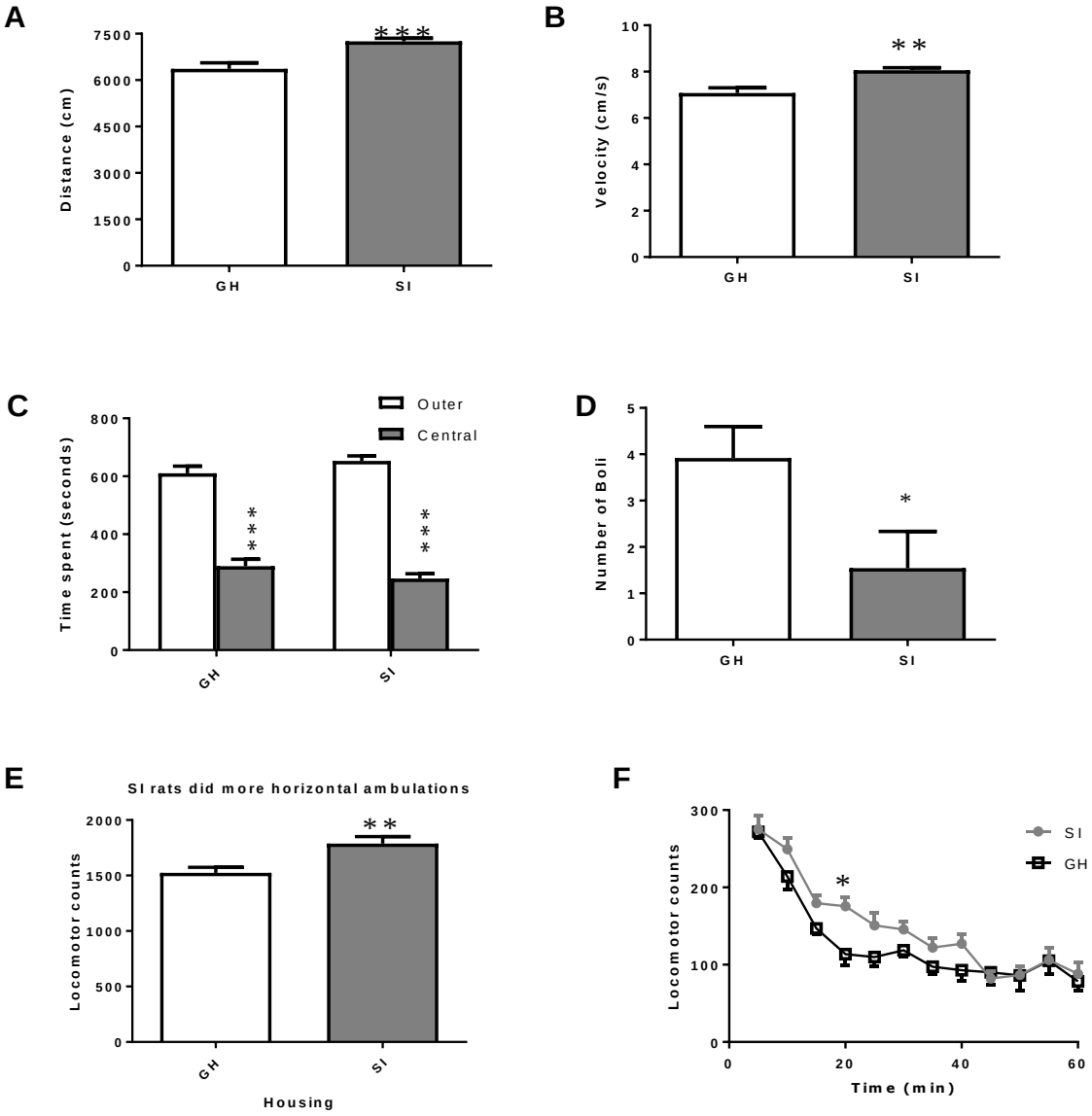


Figure 2.3.1: Behavioural data from Open field and LMA task. GH n=12 SI n=12 Comparison of: (A) distance travelled (cm), (B) average velocity (cm/s), (C) time spent (s) in the central and outer zone and (D) number of faecal boli in grouped-housed (GH) and isolation-reared (SI) rats recorded over 15min in a circular open field arena. (E) and (F) respectively show the total number of beam breaks and the time course (in consecutive 5min epochs) of the same rats recorded in computerised activity boxes over 60min. Data are mean ± sem. Figures A, B, D, E, F * indicates difference from GH, and in C * indicates difference from the outer zone. ***p<0.001, **p<0.01 and *p<0.05; for Figures A, B, D and E Student's unpaired *t*-test, and C and F Sidak's post-hoc test.

2.3.3 Novel Object Discrimination

During the first (familiarisation) trial of the NOD task neither SI nor GH controls showed any preference for either identical object (Table 2.1). Analysis of

differential object exploration in the second (choice) trial (Table 2.1) showed that both SI and GH rats tended to spend more time exploring the novel object, but this did not reach significance (ANOVA showed no main effect of housing ($F_{(1, 21)}$=2.32, p=0.14) or object ($F_{(1, 21)}$=2.35, p=0.14)). Neither did the apparent small reduction in discrimination ratio in the SI rats reach significance.

Object type and Trial	**GH (n=12)**	**SI (n=12)**
Front Object T1	19.9 ± 2.4	23.2 ± 2.6
Back Object T1	24.5 ± 3.5	23.1 ± 2.3
Familiar Object T2	17.3 ± 3.2	13.4 ± 2.1
Novel Object T2	21.5 ±1.7	15.8± 3.4
Discrimination Ratio T2	0.6 ± 0.1	0.5 ± 0.1

Table 2.1: Lack of effect of housing condition (GH = group and SI = individual housing during rearing) on time (s, mean ± SEM) spent exploring two identical objects (front and back) in the first familiarisation (T1), the novel and familiar object in the second (T2) choice trial, and the choice trial discrimination ratio (novel/novel + familiar times) during the novel object discrimination task.

2.3.4 Elevated Plus Maze

The effect of post-weaning SI was examined on a task widely used to assess unconditioned 'anxiety-like' behaviour; the elevated plus maze (Fone et al., 1996; Griebel et al., 1997). There was no effect of housing on time spent exploring the arms of the maze (F(1, 44)=0.00001, p=0.99). Irrespective of housing, rats spent significantly longer (p<0.001) in the closed (GH, 61.5 ± 2.7; SI, 59.4 ± 3.3s) than the open (GH, 38.5 ± 2.7; SI, 40.6 ± 3.3s) arms of the maze. ANOVA showed a main effect of arm type (Open vs Closed; $F_{(1, 44)}$=47.89, p<0.001). Indicating both housing groups showed a preference for the less aversive 'closed arms'. . A very similar profile was seen for both percentage open and closed arm time and entries; ANOVA showed a significant main effect of arm type ($F_{(1, 44)}$=47.89, p<0.001 and $F_{(1, 44)}$=12.57, p<0.001,respectively) but no main effect of housing during development and no significant arm type x housing interaction for either parameter. Thus rats spent significantly more time in, and made more entries into, the closed than the open arms irrespective of housing during development. There was no significant difference in distance travelled by GH or SI rats (2.43 $\pm$ 0.10 and 2.69 $\pm$ 0.10m respectively, not significant Student's *t*-test) on the plus maze, confirming that difference in ambulation did not confound interpretation of ethological measures made as

indices of anxiety in this task. More detailed ethological analysis of behaviours failed to show any significant effect of housing condition on unprotected stretch attend postures (risk assessment) or open arm head-dips (directed exploratory behaviour; data not shown).

2.3.5 Conditioned Freezing Response

To assess the effect of post-weaning SI on associative learning and memory, freezing behaviour was measured as an index of cognition in a cue and context motivated fear conditioning paradigm (Jones et al., 2011; Woods et al., 2012).

During the acquisition phase (Time spent Freezing between the first and second followed by the second and third shock) SI rats froze for significantly less time ($p<0.001$ Sidak's post-hoc test) than GH rats after the first shock, but not after the second shock such that there was a main effect of housing ($F_{(1, 22)}=6.97$, $p<0.05$, Fig. 2.3.2 A). This suggests that although isolates have a different initial response to the first foot shock they then find the conditioning equally aversive (Fig. 2.3.2 A).

At both 24 and 48h after conditioning GH froze for significantly longer ($p<0.05$) than SI rats when returned to the conditioning chamber without exposure to any further shock or cue, consistent with impaired contextual learning and memory. SI froze for significantly less time ($p<0.01$) than GH rats at 48h post-conditioning when the light and tone (but no shock) was re-applied demonstrating impaired cue-mediated learning and memory. ANOVA confirmed there was a significant main effect of housing ($F_{(1, 22)}=12.63$, $p<0.01$) on freezing duration across all three trial periods (Fig. 2.3.2 B).

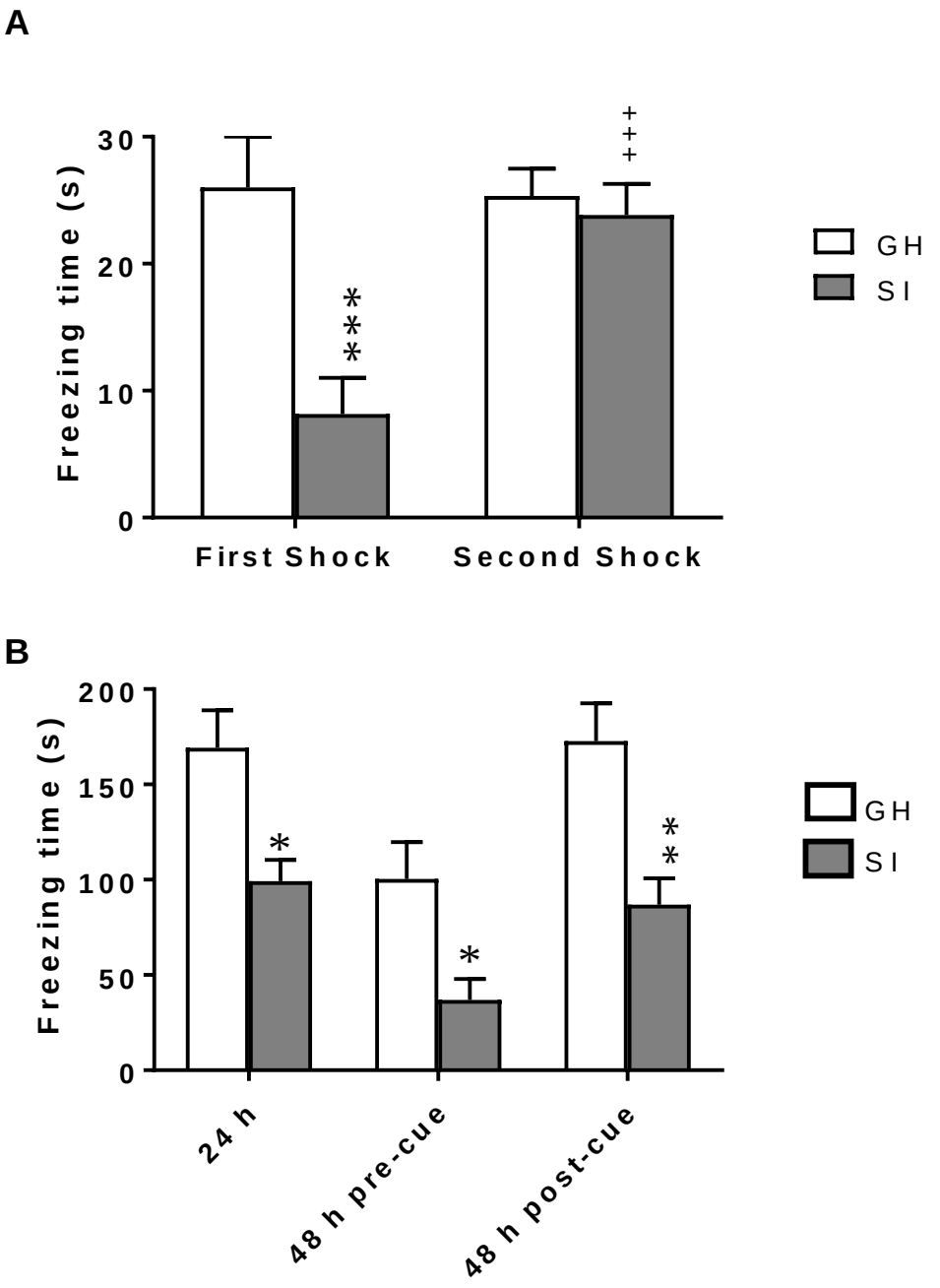


Figure 2.3.2 CFR Task Results: Comparison of the time spent freezing (s, mean ± sem) in group-housed (GH n=12) and isolation reared (SI n=12) rats during: (A) 55s following the first and second foot shocks on the conditioning day in the conditioned freezing paradigm (***p<0.001 from GH, +++p<0.001 from SI First Shock, Sidak post-hoc test) or (B) 5min when rats were returned to the chamber 24 and 48h post-conditioning (as indicated, without further foot shock or cue) and at 48h post-conditioning with re-exposure to the tone and light conditioning stimulus (48h post-cue). *p<0.05, **p<0.01 from GH, Sidak's post-hoc test following ANOVA.

2.3.6 Restraint stress-induced plasma corticosterone response

Restraint caused the expected elevation in plasma corticosterone (from 69.0 ± 3.6 to 92.5 ± 3.6 in GH and 70.4 ± 4.9 to 94.0 ± 7.6ng/ml in SI rats) but this was not modified by housing condition during development. Thus two-way ANOVA showed no main effect of housing ($F_{(1, 20)}$=0.084, p=0.77) but a main effect of restraint ($F_{(1, 20)}$=20.68, p<0.001) on plasma corticosterone.

2.3.7 Hippocampal cell proliferation and neurogenesis

The level of neuronal cell proliferation as an index of neurogenesis in the dentate gyrus of the hippocampus was examined using BrdU/NeuN dual-labelled immunohistochemistry (see representative image Fig. 2.3.3 A). GH Rats had significantly higher numbers in cells in the dentate gyrus dual-labelled for BrdU and NeuN compared to SI rats (243 ± 23 and 172 ± 19, respectively p<0.05, Student's *t*-test) showing that isolation rearing attenuated the number of cells proliferating into a neuronal phenotype during the study (Fig. 2.3.3 B).

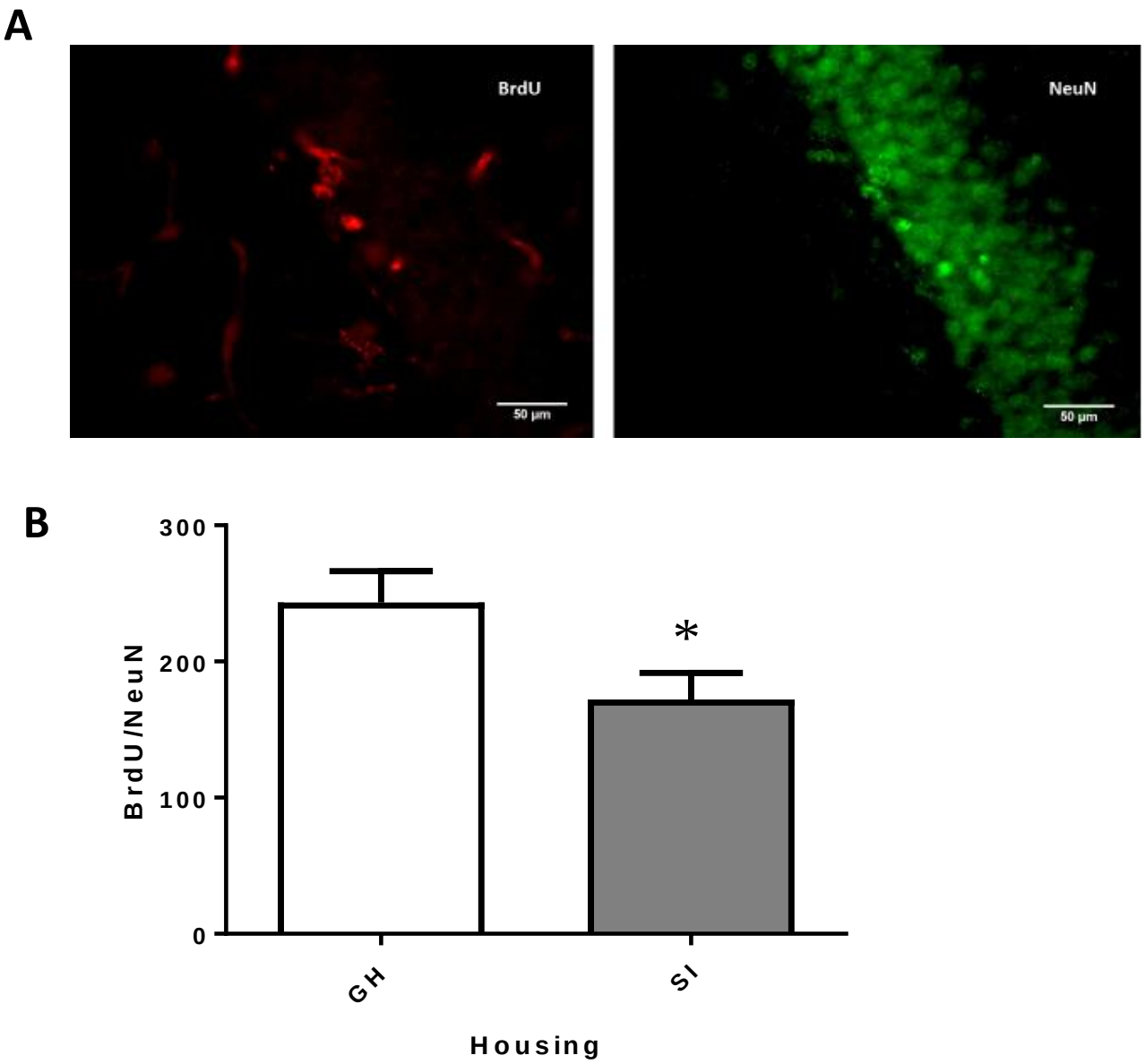


Figure 2.3.3 BrdU/NeuN Cell Count: (A) Representative images of BrdU (red) and NeuN (green) staining; scale bar = 50µm) and (B) Number of cells (mean ±sem) dual-labelled for BrdU/NeuN in the dentate gyrus of GH (n=11) and SI (n=12) rats. *p<0.05 Student's unpaired *t*-test.

2.3.8 Hippocampal and prefrontal cortical (PFC) cytokine and mTOR levels

To determine any change in markers of pro- and anti-inflammatory cytokines, IL-1β, IL-6, IL-10 and TNF-α levels were measured in hippocampal and PFC extracts using multiplex assays. A number of samples for both cytokine and mTOR levels were eliminated from the analysis as significant levels of protein could not be detected for calculation.

Cytokine	GH Hippocampus	SI Hippocampus	GH PFC	SI PFC
IL-1β	30.20 ± 2.38 (n=11)	25.49 ± 1.12 (n=11)	32.14 ± 2.84 (n=11)	32.64 ± 1.73 (n=12)
IL-6	1441± 101.3 (n=11)	1137 ± 98.6 (n=11) *****	491.9 ± 77.7 (n=10)	504.6 ± 30.8 (n=11)
IL-10	9.58 ± 1.98 (n=10)	4.84 ± 0.75 (n=8) *****	14.97 ± 3.66 (n=9)	7.67 ± 1.67 (n=11)
TNF-α	0.31 ± 0.05 (n=10)	0.21 ± 0.04 (n=8)	0.27 ± 0.05 (n=6)	0.18 ± 0.02 (n=7)

Table 2.2 Cytokine Levels: (mean ± sem, ng/ml) in hippocampus and prefrontal cortex (PFC) of GH and SI rats. Any sample whose cytokine value was outside the linear part of the curve was omitted. *$p<0.05$ Unpaired Student's *t*-test from hippocampal GH control.

As rats received either restraint or were housed in the home cage (control) for the hour immediately before tissue collection, and short term physical restraint can alter brain cytokine levels, 2-way ANOVA with housing and restraint as factors was performed on cytokine levels. In the PFC there was no significant main effect of restraint, nor any housing restraint interaction on any of the cytokines measured and only IL-6 in the hippocampus showed a housing x restraint interaction, so results for each cytokine were pooled to determine the impact of housing in Table 2.2. Of particular note for data pooled irrespective of restraint, significant reductions ($p<0.05$) in both IL-6 and IL-10 (Table 2.2) occurred in the hippocampus, but not the PFC of SI compared to control GH littermates. In contrast, no significant alterations in IL-1β or TNF-α occurred in either brain region (Table 2.2). Further analysis of hippocampal IL-6 (Fig. 2.3.4 B) by 2-way ANOVA showed a significant interaction ($F_{(1,19)} = 11$, $p<0.05$) between housing and restraint, such that hippocampal IL-6 was significantly lower in SI rats that had been restrained compared toSI home cage ($p<0.05$) or GH restrained rats ($p<0.05$). In comparison no similar significant interaction was seen with hippocampal IL-1β (Fig. 2.3.4A) or other cytokines (data not shown).

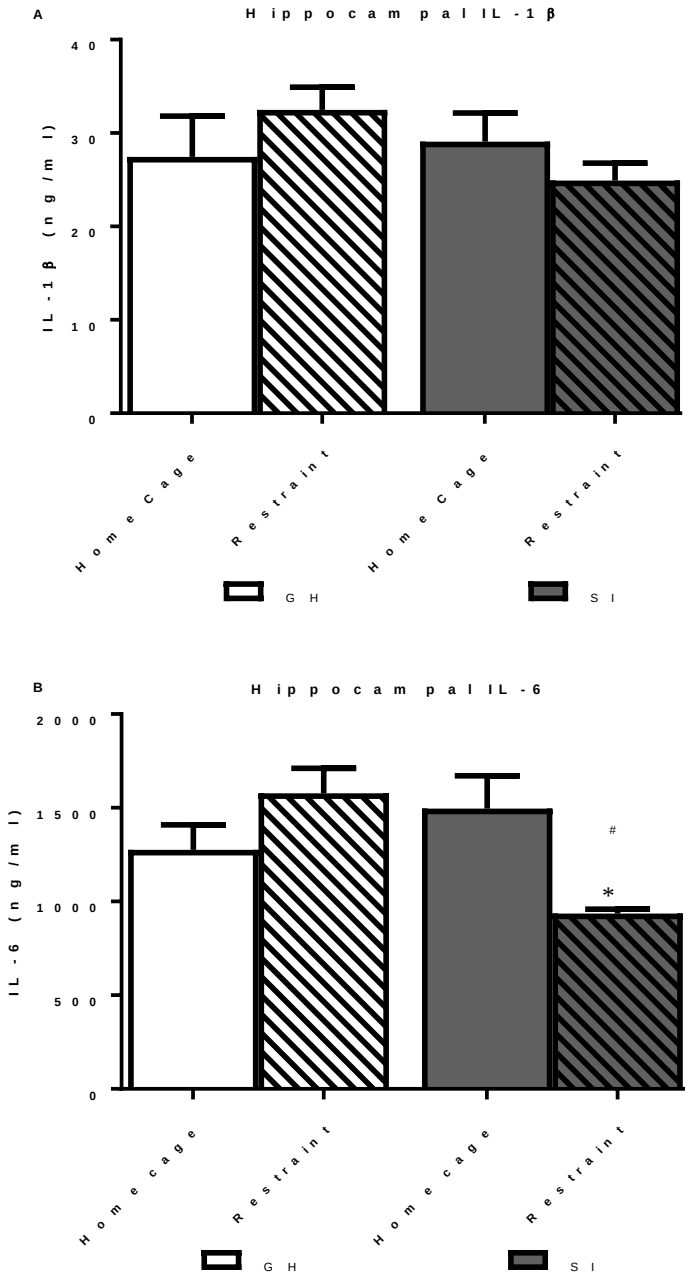


Fig.2.3.4 Hippocampal Cytokines: Comparison of hippocampal (A) IL-1β and (B) IL-6 levels (ng/ml, mean ± SEM, n=5-6 each) in group-housed (GH n=11) and rats housed in isolation from weaning (SI n=12) determined immediately following 1h restraint in a perspex tube or continued housing in the home cage, as indicated. *p<0.05 from SI home cage and #P<0.05 from GH restrained rats Sidak's multiple comparison test following ANOVA.

Although there were no significant differences in levels of phospho-mTOR or total mTOR in either brain region according to housing condition, there was a significant increase in the ratio of phospho-mTOR to total mTOR in the PFC

($p<0.05$) which was not observed in the hippocampus ($p=0.78$). This suggests that selective enhancement of the mTOR signalling pathway might occur in the PFC as a consequence of SI (Table 2.3).

	GH Hippocampus	**SI Hippocampus**	**GH PFC**	**SI PFC**
mTOR	3886 ± 161 (n=10)	3255 ± 281 (n=12)	2409 ± 114 (n=10)	2071 ± 129 (n=12)
Phospho-mTOR	10.60 ± 0.74 (n=10)	9.73 ± 1.36 (n=12)	5.58 ± 0.82 (n=9)	7.79 ± 0.87 (n=11)
Ratio	0.0028 ± 0.0002 (n=11)	0.0029 ± 0.0004 (n=12)	0.0024 ± 0.0003 (n=9)	0.0036 ± 0.0003 (n=11) *****

Table 2.3 Levels of mTOR, phosphor-mTOR and their ratio: (mean ± sem) in the hippocampus and prefrontal cortex (PFC) of rats housed in social groups (GH) or isolation (SI), measured post-mortem using ELISA. No significant differences were found between housing groups for mTOR and phospho-mTOR in either the hippocampus and PFC but there was a significant increase in the ratio of phospho-mTOR to total mTOR in the PFC (*$p<0.05$, Student's unpaired *t*-test).

2.3.9 Microbiota composition and analysis

All analysis was carried out at the APC Microbiome Institute, University College Cork, Ireland by Siobhain O'Mahony and Veronica Peterson. The data can be found in appendix 1 of this thesis. The results from it are discussed in the current chapter.

2.4. Discussion

The current study demonstrates that rearing rats in SI from weaning produced several behavioural changes akin to those seen in disorders such as schizophrenia and depression, including cognitive impairment, reduced neurogenesis (decreased DG BrdU/NeuN positive cells) and changes in hippocampal cytokine levels and of particular note is the first to show accompanying alterations in microbiota composition. As expected rearing rats in SI from the point of weaning altered horizontal activity both in the open field and locomotor activity task; SI rats being hyperactive is a reliable characteristic feature of the resultant 'isolation syndrome' observed in almost all previous studies (Fone and Porkess, 2008; Gentsch et al., 1981b; King et al., 2009; McIntosh et al., 2013; Watson et al., 2016). This increased activity in a novel environment is consistent with SI rats demonstrating neophobia (Gentsch et al., 1988; Sahakian et al., 1977). SI-induced hyperactivity is accompanied by increased spontaneous activity in (Fabricius et al., 2010) and enhanced dopamine release (Robbins et al., 1996) from ventral tegmental dopamine neurones. In contrast, SI rats showed no overt changes compared to GH littermates in the elevated plus maze. This is contrary to several previous studies where SI rats show reduced total arm and percentage open entries respectively (Parker and Morinan, 1986; Weiss et al., 2004). This study also found no differences in anxiety-related behaviours in the open field (rearing and grooming) although both groups spent significantly more time in the less aversive outer zone and more time in the closed arms of the plus maze as expected. Interestingly SI rats left significantly fewer faecal boli in the open field consistent with stress-induced activation of the sympathetic nervous system which reduces defecation (Perhach and Barry, 1970), although studies have found other early-life stressors such as maternal separation increased faecal boli accompanying adult exposure to a novel stress (O'Mahony et al., 2009).

A variety of cognitive deficits frequently accompany psychiatric illness so rats were assessed for visual and associative learning and memory using the NOD and CFR tasks, respectively. In the NOD task, although there was a tendency for SI rats to have a lower discrimination index they did not show the expected impairment in visual discrimination (Bianchi et al., 2006; Gaskin et al., 2016; McIntosh et al., 2013; Watson et al., 2016) possibly because of high inter-individual variation in this cohort. Although SI rats froze less than group-hosed littermates after the first conditioning shock in the CFR chamber they froze for the same duration after the second shock, suggesting both groups found the stimulus aversive and that impaired acquisition did not contribute to altered conditioned freezing behaviour but it is difficult to exclude this possibility. In agreement with several previous studies (McIntosh et al., 2013; Watson et al., 2016) SI rats showed decrease freezing on re-exposure to both the conditioning context and cue in the CFR paradigm indicative of impairment in associative learning and memory following this early-life stress. Consistent with our

previous observation SI rats showed an elevation of the ratio of active (phospho-mTOR) to inactive forms of mTOR in the PFC which we have previously shown to be associated with cognitive impairment (Meffre et al., 2012).

Compared with home-cage housed rats, plasma corticosterone levels were significantly elevated 1h after restraint but to a comparable level in SI and GH littermates, thus irrespective of housing during development. Previous research has shown that exposure to a variety of early-life stressors including maternal separation or SI can modify the HPA axis and amplify the plasma corticosterone response to exposure to an acute stress (O'Mahony et al., 2009; Plotsky et al., 2005). As no time course assessment of the corticosterone response was available in the current study it is possible that plasma corticosterone had reached a ceiling level following this period of restraint which would mask any housing effect. The impact of SI on corticosterone levels is varied and probably depends on the stressor used as well as the temporal relationship of the measure to stress application (Scaccianoce et al., 2006; Serra et al., 2005; Weiss et al., 2004).

Adult neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus in rats (Kuhn et al., 1996) and dysfunction of this process is implicated in several human psychiatric disorders. Of note neurogenesis is reduced by stress and increased by antidepressants (Duman et al., 1999; Levone et al., 2015; Malberg et al., 2000). Consistent with this regulation and previous studies using rodent models of early-life stress (Mirescu & Gould, 2006) SI rats showed a significant reduction in BrdU/NeuN positive dentate gyrus neurons. As changes in neurogenesis have been associated with cognitive deficits (Rola et al., 2004) and proposed to occur in depression (Millan et al., 2012a) their contribution to change in hippocampal dependent cognitive function, such as impaired CFR reported here, is clearly of great interest. Moreover, gut microbiota is associated with changes in hippocampal neurogenesis; a lack of microbiota in germ-free mice being associated with increased neurogenesis (Ogbonnaya et al., 2015). Conversely, antibiotic and probiotic-induced changes in the microbiota are also associated with alterations in neurogenesis (Mohle et al., 2016). Furthermore, adult germ-free mice have enlarged hippocampi and shorter less branched ventral hippocampal pyramidal neurons (consistent with dendritic atrophy) which might contribute to concomitant changes in anxiety and cognition (Jiang et al., 2015), potentially resulting from alteration in gut microbiota during development.

Alterations in the immune system are implicated in the pathophysiology of major depressive disorder and also cardinal to the brain-gut axis interaction with the immune system playing a major role in the communication between the gut microbiota and the brain (Dinan and Cryan, 2017; O'Mahony et al., 2017). Previous research has shown that maternal separation results in an enduring pro-inflammatory phenotype in rats (O'Mahony et al., 2009) but few studies have examined changes following adolescent SI. Ko et al. (2015) found elevated plasma levels of the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) after

four weeks isolation rearing of Sprague-Dawley rats but there are no reports of changes in brain regions as documented herein. Of interest, the primary changes in cytokine levels found in this study were restricted to the hippocampus (significant reductions in IL-10 and IL-6 in SI rats) and not replicated in the PFC, implying brain regional specificity that might contribute to hippocampal-dependent phenotypic changes in cognitive behaviour and/or neurogenesis (Hueston et al., 2017). A reduction in the anti-inflammatory cytokine IL-10 is consistent with findings from the microbiota levels in the current study and previous research (Scarpa et al., 2011). However, the decrease in IL-6 is unexpected since, at least in the periphery, elevated IL-6 has been associated with a depressive-like phenotype and alterations in the microbiome in mice (Zhang et al., 2017). Hippocampal IL-1β has been shown to be elevated by a variety of acute stressors, such as lipopolysaccharide injection or inescapable foot shock, however this rise is prevented by a simultaneous rapid rise in corticosterone (Nguyen et al., 1998) or following repeated exposure to stress (Lovelock and Deak, 2017), consistent with the absence of IL-1β elevation in this study. Interestingly restraint stress of Wistar rats for 1h elevated plasma corticosterone and hippocampal IL-6 mRNA (Chen et al., 2016) analogous to that seen herein in GH rats. In contrast, in this study SI rats that received restraint had significantly lower hippocampal IL-6 than GH-restrained or SI home cage rats suggesting that exposure to chronic early-life stress (SI) may have attenuated the usual brain cytokine response evoked when adult rats are exposed to a severe short-term physical stressor. Further studies on a larger cohort with sufficient statistical power specifically designed to investigate this phenomenon would be worthwhile to establish if this is reproducible, occurs with other cytokines and could represent a maladaptive response.

Analysis of the microbiota revealed a number of differences that may contribute to phenotypic changes in SI rats mediated in part via the gut-brain axis bicommunication. Peptostreptococcaceae was lower in the SI rats and positively correlated to the lower IL-6 level in the hippocampus. Papillibacter which was decreased in SI rats shows preferential occurrence in healthy controls across all populations (Rehman et al., 2016) and may represent a potential indicator of health status. Oscillospira was significantly reduced in SI rats and a similar observation has been made in Crohn's disease (Perez-Brocal et al., 2013), potentially indicating that this bacteria is associated with immune alterations. Interestingly elevation in Actinobacteria found in the current SI rats was also one of a few phyla elevated in patients with active major depressive disorder compared to that in healthy controls (Jiang et al., 2015) but any translational relevance remains to be established. Bacteria in the order Clostridales were negatively correlated with cognitive performance in the CFR task. Of note an elevation in bacteria in the family of Lactinospiraceae and Clostridiaceae was observed to correlate with impairment of NOD produced following 7 days administration of phencyclidine (a pharmacological model for schizophrenia) to Lister hooded rats (Pyndt Jorgensen et al., 2015); an impairment which was

prevented by ampicillin administration, supporting the supposition herein that gut microbiota can modify memory performance. The complex bidirectional interaction between gut microbiota and stress-related behaviour makes it difficult to determine the cause or consequence of changes seen in SI rats, which is clearly imperative to understand the neurobiological sequelae and develop novel preventative therapies. Recently it has been demonstrated that mice exposed to chronic stress develop behavioural despair accompanied by reduced faecal Lactobacillus resulting in increased circulating kynurenine levels and that restoration of intestinal Lactobacillus improved the metabolic alterations and behavioural abnormalities (Marin et al., 2017). Similarly, treatment of mice undergoing chronic social defeat with Lactobacillus rhamnosus reduced the development of 'anxiety-related' behaviour, deficits in social interaction and increase in number of T cells producing IL-10 (Bharwani et al., 2017). Furthermore, elevating caecal levels of Bifidobacteria by 3 weeks administration of galacto-oligosaccharides prevented the lipopolysaccharide-induced elevation in cortical IL-1β and 5-HT_{2A} receptors and anxiety behaviour in a light-dark box (Sarkar et al., 2016; Savignac et al., 2016). Such integrated studies are vital to provide mechanistic insight into the interaction of microbiota and their metabolites with the immune system and brain development and resultant long-term behavioural consequences. SI in juvenile rats may alter the microbiome (perhaps because of associated stress) and with contemporaneous or subsequent changes in HPA axis (Weiss et al., 2004) and inflammatory cytokines, produce microglial activation (Schiavone et al., 2009), reduction in BDNF, loss of parvalbumin-containing GABA interneurons and synaptic pruning in PFC and hippocampus (Bianchi et al., 2006; Gaskin et al., 2016; Harte et al., 2007; Schubert et al., 2009; Silva-Gomez et al., 2003), alter glutamatergic corticostriatal neurotransmission (Napolitano et al., 2014a) and ventral tegmental dopaminergic firing (Fabricius et al., 2010); ultimately resulting in development of the isolation syndrome. Careful temporal analysis of the changes in microbiota, cytokines and behaviour during development of the isolation syndrome might reveal the cascade of events.

2.5 Conclusion

In conclusion, the current study established that rearing rats in SI from weaning induces changes to behaviour, neurogenesis and the gut microbiome that can have potential implications on the gut-brain axis. Differences in cytokines in the hippocampus correlated to gut microbiota changes, opening the possibility that the gut microbiota influences immune changes in the central nervous system and behaviour that may contribute to the pathophysiology of psychiatric disorders. This provides opportunities to target and modify the gut microbiota and potentially improve outcome in these life-changing disorders.

Chapter 3:

Characterising physiological and behavioural changes in isolation rearing model of depression and the effects of treatment with chronic fluoxetine

3.1. Introduction

In the current chapter the isolation rearing rat model was further validated. The battery of behavioural measures previously adopted in Chapter 2 were employed to assess the depressive-like phenotype and to further characterise the model by chronically administering the SSRI fluoxetine.

3.1.1 Fluoxetine

Fluoxetine is the most commonly prescribed SSRI and a first line treatment for MDD (Preskorn et al., 2004). Its clinical use is not strictly reserved for depression and it is also prescribed for anxiety disorders, obsessive compulsive disorder and post-traumatic stress disorder among other conditions involving serotonergic abnormalities (Wong et al., 2005). Fluoxetine was approved by the FDA to treat depression in 1987 and was quickly favoured for use in clinical populations over tri-cyclic antidepressants (TCAs) with which it displayed similar efficacy but reduced side effects, reflecting higher affinity to the 5-HT transporter and lower affinity to several receptor types (D_1, D_2 dopaminiergic, muscarinic, α- β-adrenoreceptors) (Owens et al., 1997). None the less, like other monoamine acting antidepressants there are common problems. The delay in onset of action of the drug, which can take several weeks to begin to show clinical response, creates time for patients to often experience other side-effects, including sexual dysfunction, loss of appetite, insomnia and most importantly increased suicidal ideation and behaviour, particularly in younger populations.

3.1.2 Fluoxetine's Mechanism of Action

As an SSRI and its primary pharmacological mechanism of action is inhibition of 5-HT reuptake. Typically, 5-HT is released pre-synaptically and binds to pre- and post-synaptic receptors. Any remaining 5-HT is removed from the synaptic cleft via serotonin reuptake transporters (SERT). SSRIs bind to SERT and compete with 5-HT, ultimately resulting in increased synaptic levels of 5-HT (Stahl, 1998). As described later, because of presynaptic 5-HT regulation of 5-HT release this elevation may be delayed. Fluoxetine is metabolised by demethylation to norfluoxetine which importantly is an active metabolite being an even more potent SSRI with a longer half-life than the former (Hyttel, 1994). Fluoxetine has a long half-life compared to other SSRIs (fluvoxamine, paroxetine, sertraline and citalopram), with plasma elimination taking 1-3 days in humans, and the half-life of the active metabolite norfluoxetine being between 7–15 days. In rats there is a plasma half-life of 1-3 days for fluoxetine and 4-16 days for norfluoxetine (Mandrioli, Forti, & Raggi, 2006). This long half-life has advantages and disadvantages for although it avoids the induction of a withdrawal syndrome, it also delays the ability for a patient to switch to another antidepressant medication if required due to potential drug interactions. Acute treatment with fluoxetine *in vitro* and after 10mg/kg i.p. administration in male Wistar rats has demonstrated up to a 57% reduction in 5-HT uptake in rat brain synaptosomes but not that of noradrenaline or dopamine (Wong et al., 1975),

with maximal inhibition occurring after 4 hours and a return to normal levels after 48 hours. There is also an effect of 5-HT binding to post-synaptic receptors. As discussed in Chapter 1 the neurotrophic hypothesis of depression states that individuals with low levels of BDNF are more susceptible to depression and antidepressants increase levels of BDNF. As shown in Figure 3.1.1, 5-HT binding to the post-synaptic receptors (5-$HT_{4,6,7}$) causes upregulation of intracellular BDNF through activation of cAMP signalling which in turn activates protein kinase A and CREB pathways leading to stimulation of BDNF gene expression (Duman et al., 2001b)

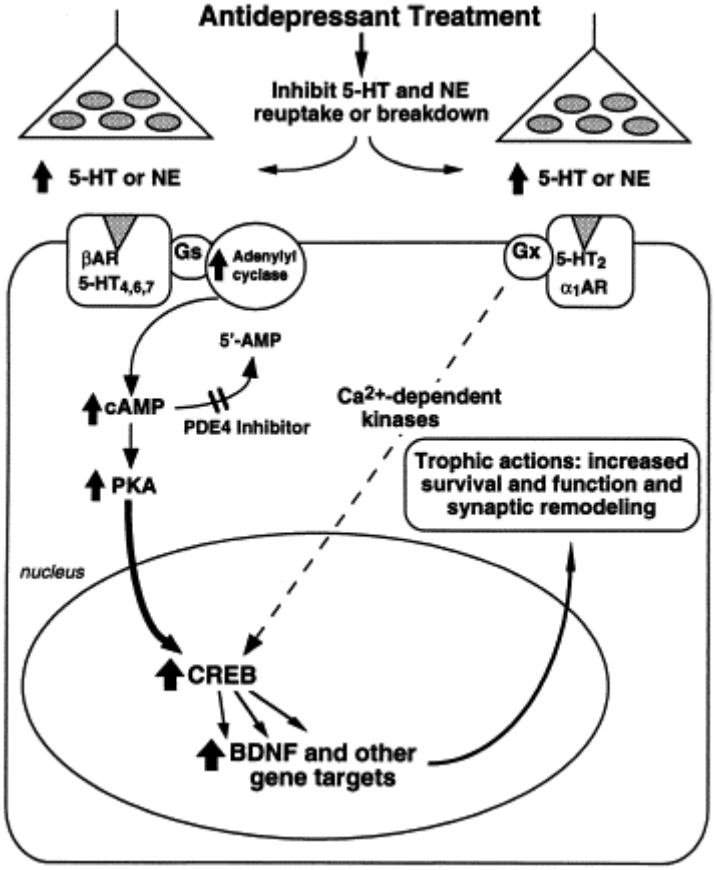


Figure 3.1.1: Diagram illustrating SSRI action on signalling through post-synaptic 5-$HT_{4,6,7}$ receptors and its subsequent effects on BDNF (Duman et al., 2001b).

There is also evidence to suggest that fluoxetine's effect on BDNF is biphasic; inducing downregulation at 4 hours post-treatment (chronic and acute) followed by an increase after 24 hours after chronic treatment (Coppell et al., 2003).

3.1.3 Fluoxetine effects in pre-clinical models

Fluoxetine has frequently been used to assess antidepressant effects and mechanisms in preclinical models of psychiatric disorders. Fluoxetine can

reverse many indices thought to reflect 'depression' in animal models, whether they be pharmacological, stress-induced or surgical. It has been widely demonstrated to increase mobility in the forced swim test (FST) (Detke et al., 1995) a validated measure of 'depression-like' behaviour in rodents and more recently in the modified FST (Cryan, Valentino, & Lucki, 2005). Interestingly, fluoxetine reduces immobility (a depressive-like behaviour) with both acute and chronic treatments, while it only works clinically after chronic dosing questioning the translational validity of this test. Studies also suggest that genetics may influence fluoxetine's efficacy in rodent behavioural paradigms. While Sprague Dawley rats respond to fluoxetine, the mutant strain Wistar Kyoto (WKY) rats display a resistance in the FST (Griebel et al., 1999). Chronic fluoxetine treatment reversed anhedonic behaviour in the CMS model of depression (Grippo et al., 2006). Rats exposed to the CMS paradigm for 4 weeks displayed a lower preference for sucrose (a validated anhedonia behaviour) which was prevented with chronic fluoxetine treatment.

As mentioned at the beginning of this chapter fluoxetine is often prescribed for anxiety disorders in clinical populations and shows anxiolytic properties in pre-clinical testing, (Lapmanee, Charoenphandhu, & Charoenphandhu, 2013) demonstrated 4 weeks fluoxetine (10mg/kg/day) was effective at improving restraint stress induced anxiety-like behaviours in the elevated T-maze and the FST. This is in line with the central role of 5-HT in the modulation of anxiety related behaviours (Griebel, 1995). The high comorbidity of anxiety disorders with MDD is also well documented. In the past, studies of SSRI's for anxiety have yielded inconsistent results for both acute and chronic treatments (Griebel, 1995). Many things account for this: variations in the model used, the behaviours used to assess anxiety, the strain of animal used and route of administration of fluoxetine (Durand et al., 1999). Although results on anxiolytic efficacy of fluoxetine are contradictory there have been very promising studies, a review by (Mohammad et al., 2016a) noted that chronic treatment with fluoxetine in the CMS model of depression attenuated anxiogenic behaviours in locomotor, open field, light-dark box and elevated plus maze tests. They also observed that anxiolytic changes to behaviours were greater in non-stressed groups compared to the CMS groups.

Major depression is often associated with memory dysfunction and another role in the treatment of depression is to alleviate this dysfunction. Although fluoxetine is not thought to have a cognitive effect on healthy subjects (Monleón et al., 2008), some studies provide evidence that fluoxetine improves cognition when this has been impaired. In clinical studies, fluoxetine has been reported to improve deficits in memory of patients with moderate to severe depression (Gallassi et al.; Levkovitz et al., 2002; Vythilingam et al., 2004), mild cognitive impairment (Mowla et al., 2007), traumatic brain injury (Horsfield et al., 2002) and post-traumatic stress disorder (Vermetten et al., 2003). Whereas previous

classes of antidepressant were thought to enhance cognitive dysfunction (through side effects such as dizziness), fluoxetine has been shown to improve cognition and reverse cognitive deficits in animal models and in humans, reviewed by (Monleón et al., 2008; Mostert et al., 2008). In support of its role in cognition, fluoxetine has shown to increase levels of hippocampal neurogenesis in the dentate gyrus (David et al., 2010; Dranovsky and Hen, 2006; Malberg, 2004; Malberg et al., 2000; Santarelli et al., 2003a) and to decrease reductions in experimentally induced neurogenesis depletion (Lyons, ELBeltagy, Bennett, & Wigmore, 2012). This effect may be mediated through upregulation of BDNF through the CREB pathway briefly discussed above (See figure 1(Duman et al., 2001b). Santarelli et al. (2003a) observed that blocking neurogenesis with irradiation also blocked the effect of fluoxetine in reducing anxiolytic behaviour although Holick et al. (2008) also ablated neurogenesis with radiation and found no difference in Fluoxetine's effect on the forced swim and novelty-induced hypophagia tests. Therefore although it has been demonstrated that neurogenesis is downregulated by stress and upregulated by fluoxetine treatment this doesn't necessarily established a causal relationship between hippocampal neurogenesis and symptoms of major depression.

3.1.4 Aims and Objectives

The aim of the current study was to evaluate the use of chronic fluoxetine on the behavioural changes seen in rats that have undergone 5 weeks of post-weaning isolation rearing. Depressive-like behaviours in the resultant adult rat were assessed by a battery of behavioural tasks. Anxiety-related behaviour, known to have high levels of comorbidity with depression were assessed by monitoring exploration and neophobia in the open field and elevated plus maze. Two separate tasks were used to examine cognitive functioning, the NOD task and the CFR task (described in Chapter 2). Physiological response to a stressful situation was also assessed and several physiological factors post-mortem were examined including levels of cell proliferation and neurogenesis in the dentate gyrus and analysis of plasma levels of corticosterone. The principle objectives of the current studies were:

1. Investigate the effects of isolation-rearing on behavioural/cognitive/physiological measures.

2. To investigate if the changes induced by isolation-rearing can be prevented or reversed by the chronic administration of fluoxetine.

3.2. Methods

3.2.1 Animals

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986), with approval of the University of Nottingham Local Ethical Review Committee, and conform to the ARRIVE guidelines. The animals used for the study (n=60) were male Lister-hooded rats obtained from Charles River UK. For the study rats arrived at post-natal day (PND) 21 in five litters of 12, with their dam.

3.2.2 Isolation Rearing

Each litter of twelve was split equally into groups of three (GH) or one per cage (SI) in this gave a total of n=30 single housed and n=30 group housed. The rats were housed for four weeks before receiving an injection and implanting of a temperature transponder (see below) and were housed for a total of five weeks before behavioural testing began. Drug administration began three weeks after the animals arrived and were housed.

3.2.3 BrdU Administration/Transponder Implantation

During week four the rats were injected i.p. with BrdU (5-bromo-2'-deoxyuridine; Sigma Aldrich, 150mg/kg). BrdU is a thymidine analogue commonly used as a biomarker of cell proliferation (Gratzner, 1982). Rats also received a subcutaneous implant with a temperature transponder (Animalcare Ltd) between the shoulder blades using a sterile injector to enable temperature measurement.

3.2.4 Oral Fluoxetine Administration

Fluoxetine hydrochloride was donated from the National Institute for Mental Health (NIMH). Three weeks after arrival half of each housing group (n=15 per group) began receiving fluoxetine administered to the drinking water at 10mg/kg/day. Dosing was informed on body weight (recorded once per week) and the daily water consumption of each rat, as fluoxetine can alter fluid intake. For group housed animals an average intake as there was no way to accurately measure individual intake in these cages. The dose of 10mg/kg was calculated through the intake of water each day to ensure that each cage was receiving as close as possible to the correct dose. Increases in weight throughout the week were also taken into account and this wasbased on previous studies on weight gain in male Lister hooded rats at this age in GH versus SI conditions to modify the fluoxetine concentration. This continued throughout the experiment until the final behavioural task was concluded.

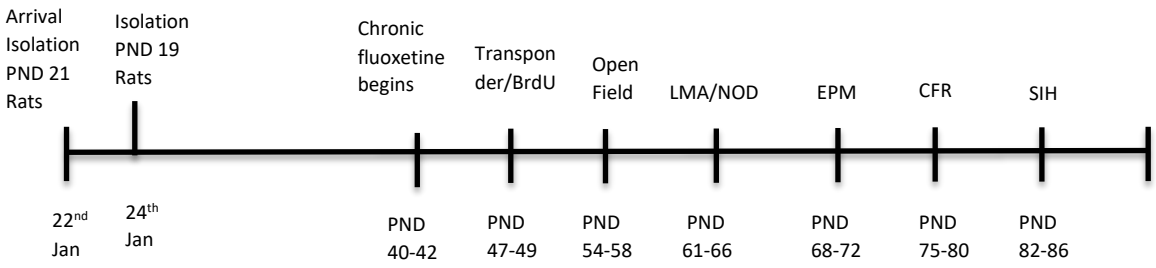


Figure 3.2.1: Timeline of the protocol from the arrival of the animals until the end of the behavioural testing. PND: Post-natal day (age of the rat) Behavioural paradigms are OF: Open Field, Loco: Locomotor Activity, NOD: Novel object discrimination, EPM: Elevated plus maze, CFR: Conditioned freezing response and SIH: Stress-induced hyperthermia.

3.2.5 Behaviour Assessments & Post-Mortem

Behavioural test's and their protocols were the same as previously described as were all post-mortem protocols for plasma and tissue collection (see Chapter 2 methodology).

3.2.6 Doublecortin (DCX) Immunohistochemistry

Sixty micron microtome sections were first removed from anti-freeze and washed for 3 x 5 minute washes in phosphate buffer saline (PBS). Blocking solution was made (1:10, wash buffer (PBS + BSA (1.5percentage) + Triton-x(0.5percentage) + Normal Donkey Serum, PBS-T) with primary antibody goat anti-DCX (1:100). Sections were incubated in blocking solution and placed in a fridge overnight at 4°C. The next day sections were washed 3 x 5 min in PBS-T (0.3percentage), and incubated in secondary antibody (Alexa Flour 594 Donkey anti-goat, 1:400) in PBS-T for 60 min at RT in darkness. Sections were sequentially washed 2 x 5 minute washes in PBS-T and 1 x 5 min wash in PBS, Mounted on gelatinised slides and allow to dry, rinsed with distilled water and DAPI (1:1000) was applied for 30 sec in darkness. After rinsing with distilled water. DABCO mounting medium was applied and sections were cover slipped. The number of DCX positive cells were counted and calculated as described in Chapter 2.

3.2.7 Statistical analysis

All data was analysed by two-way ANOVA (with housing (GH vs SI) and treatment (CON vs FLX) as between factors) and (percentage time and amount of locomotor activity in central/peripheral zones, rears and grooming behaviour and the number of faecal boli, in open field. Time and rearing in LMA, object in NOD, Number of entries to open/closed arms, distance moved, number of head dips and stretch attends postures in EPM, freezing time in CFR and core temperature change will be used as repeated measures in the SIH) with Sidak multiple comparisons post-hoc where appropriate. All analyses were performed

using GraphPad Prism 6 and SPSS 22 software. All data are presented as mean ± sem, and $p<0.05$ was considered significant.

3.3. Results

3.3.1 The Effects of Isolation-Rearing and Fluoxetine on Behaviour in the Open Field Task

Distance and Velocity

There was a significant change in the distance travelled (cm, mean ± sem, n=15 per group) in the open field, SI rats travelled further than GH rats ($F_{(1, 56)} = 6.486$, $p<0.05$, Fig 3.3.1 A) and there was also a significant effect of treatment with FLX rats travelling further than CON ($F_{(1, 56)} = 5.032$, $p< 0.05$, Fig 3.3.1 A); Sidak post-hoc analysis showing there was a significant increase in distance travelled in the SI/FLX compared to GH/CON rats. Overall average velocity (cm/s, mean ± sem, n=15 per group) was also examine, there was a significant main effect of housing, SI rats had a higher velocity than GH rats ($F_{(1, 56)} = 6.489$, $p=0.01$, Fig 3.3.1 B). A significant main effect of treatment was also observed, FLX rats had a higher average velocity than CON rats ($F_{(1, 56)} = 5.042$, $p<0.05$, Fig 3.3.1 B); and sidak post-hoc test showed that there was a significant increase in velocity in the SI/FLX compared to GH/CON rats ($p<0.01$, Fig 3.3.1 B). This is consistent with development of hyperactivity a component of the behavioural syndrome typically seen in isolation-reared rats.

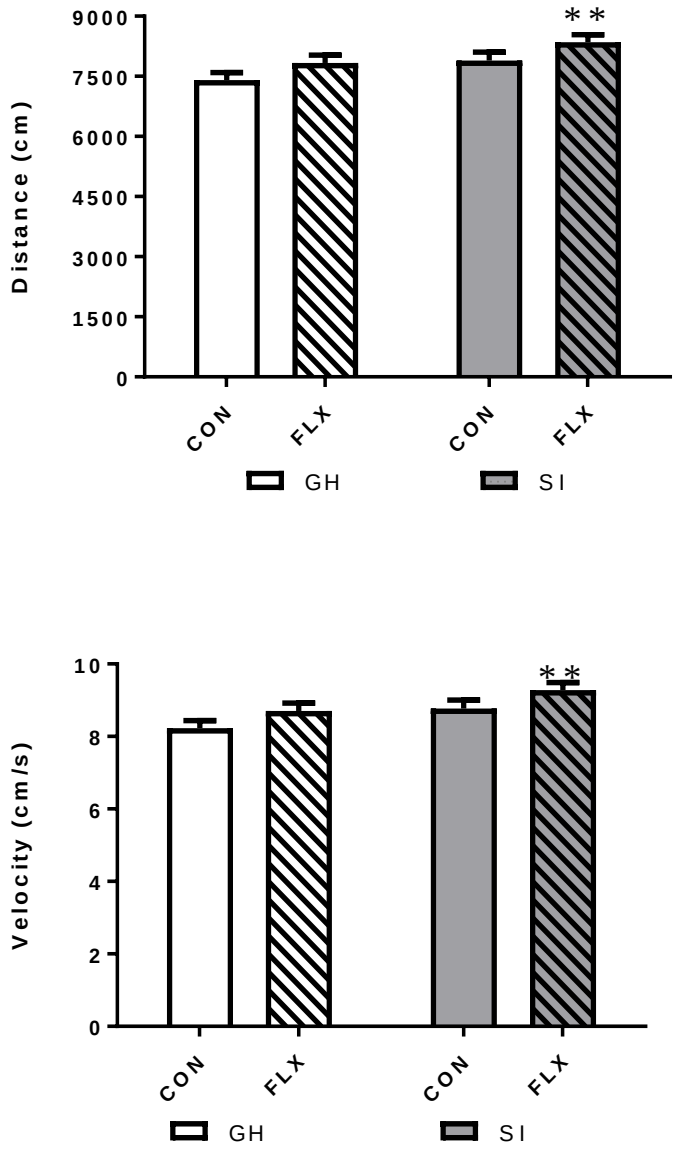


Figure 3.3.1 Distance and Velocity: Comparison of (A) distance travelled (cm, mean ± sem, n=15 per group) and (B) average velocity (cm/s) by GH/CON, GH/FLX, SI/CON and SI/FLX rats recorded in the open field. (A) Two-way ANOVA showed a significant main effect of housing ($p<0.01$) and treatment ($p<0.05$). ++$p<0.01$ Sidak post-hoc test between GH/CON and SI/FLX. (B) Two-way ANOVA showed a significant effect of housing ($p<0.01$) and treatment ($p<0.05$). ++$p<0.01$ Sidak post-hoc test between SI/FLX and GH/CON.

Time in Central and Outer Zone

In the more aversive central zone there was no significant main effect of housing ($F_{(1, 56)} = 1.562$, $p=0.22$, Fig 3.3.2 A), there was a significant effect of treatment with FLX rats spending more time there compared to CON rats ($F_{(1, 56)} = 4.670$, $p<0.05$, Fig 3.3.2 A). A significant effect of treatment in time spent in the outer zone was established with FLX rats spending less time in the outer zone compared to CON rats ($F_{(1, 56)} = 4.670$, $p<0.05$ Fig 3.3.2 B). Again there was no effect of housing ($F_{(1, 56)} = 1.562$, $p=0.22$ Fig 3.3.2 B). Post-hoc tests indicated there were no significant differences between groups in time spent in either central or outer zone.

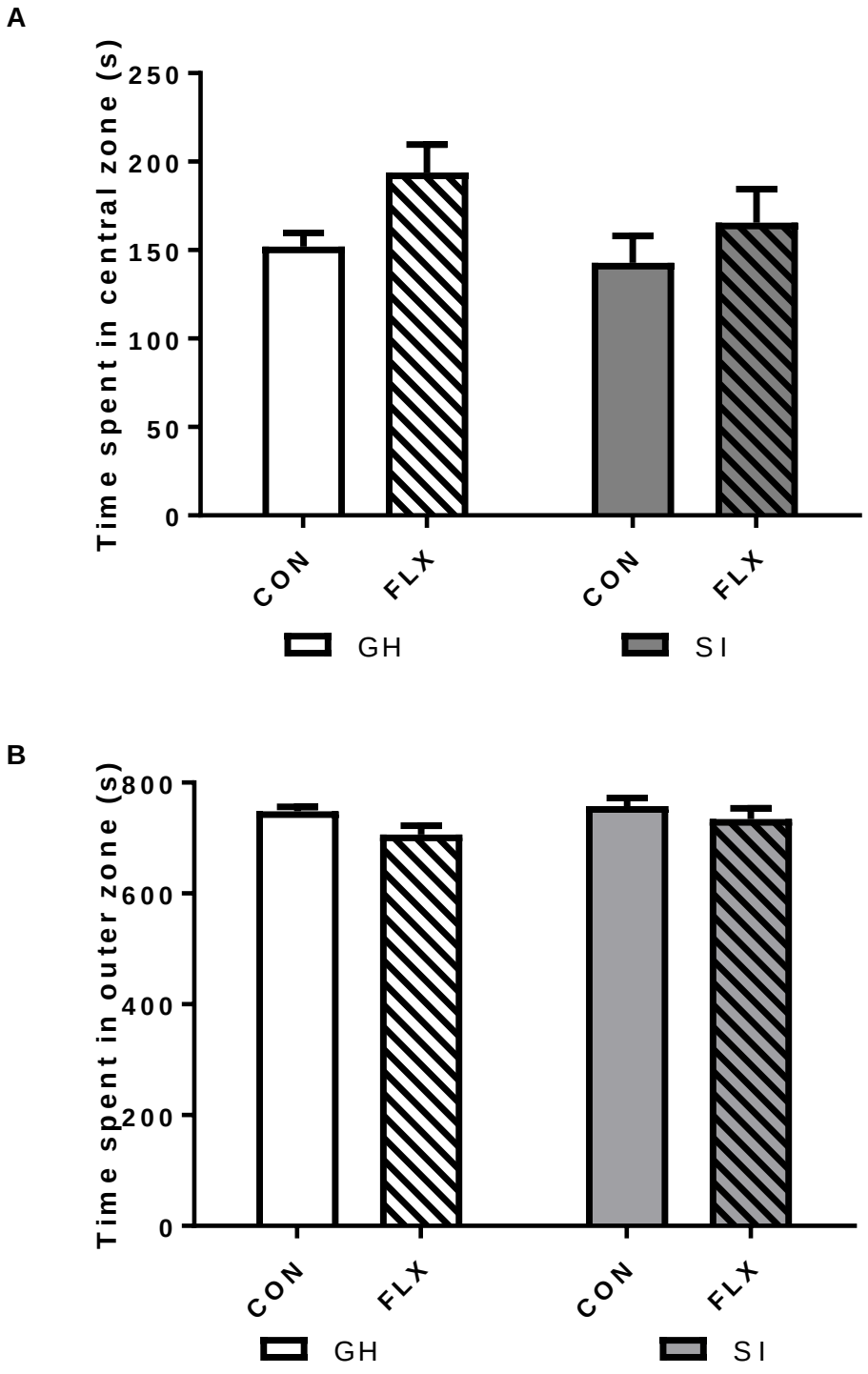


Figure 3.3.2 Time in Zones in Open Field: (A) Time spent in the central zone of the open field (s, mean ± sem, n=15 per group). A two-way ANOVA showed a significant effect of treatment ($p<0.05$). (B) Time spent in the outer zone of the open field. A two-way ANOVA showed a significant effect of treatment ($p<0.05$).

Grooming in Central and Outer Zone

The first of two ethological behavioural measures scored in the open field task was time spent grooming (s). In the central zone there was a significant effect of treatment, FLX rats spent longer grooming than CON rats ($F_{(1, 56)} = 5.015$, $p<0.05$, Fig 3.3.3 A), there was no significant main effect of housing ($F_{(1, 56)} = 0.4107$, $p=0.52$ Fig 3.3.3 A). Sidak post-hoc test revealed that a significant difference with GH/FLX rats grooming more than GH/CON ($p<0.05$ Fig 3.3.3 A). In the outer zone there was a significant effect of housing with GH spent longer grooming compared to SI rats ($F_{(1, 56)} = 13.38$, $p<0.001$ Fig 3.3.3 B), although there was no effect of treatment ($F_{(1, 56)} = 1.943$, $p=0.1$ Fig 4 B). Sidak post-hoc test did reveal a significant difference with SI/CON grooming less than GH/FLX ($p<0.01$ Fig 3.3.3 B).

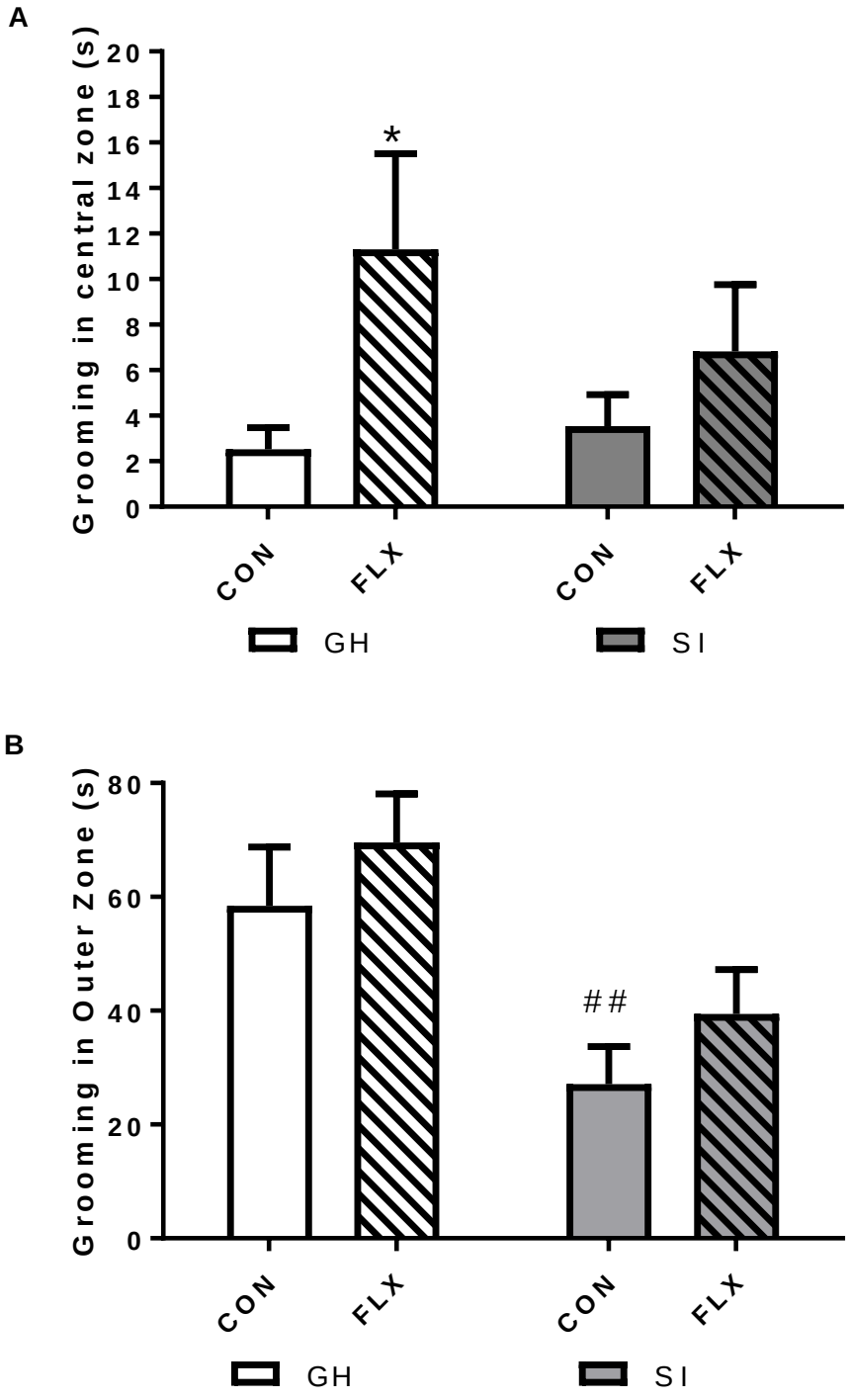


Figure 3.3.3 Grooming: (A) Time spent (s, mean ± sem, n=15 per group) grooming in the central zone. A two-way ANOVA revealed a significant effect of treatment ($p<0.05$). *$p<0.05$ Sidak post-hoc tests GH/CON grooming less than GH/FLX. (B) Time spent grooming in the outer zone(s). A two-way ANOVA revealed a significant effect of housing ($p<0.001$). ##$p<0.01$ Sidak post-hoc tests between SI/CON and GH/FLX.

Rearing in Central and Outer Zone

There was a significant main effect of treatment on the number of rears in the central zone such that FLX treated rats rearing more than CON ($F_{(1, 56)} = 6.144$, $p<0.05$, Fig 3.3.4 A) there was no effect of housing ($F_{(1, 56)} = 1.605$, $p=0.21$ Fig 3.3.4 A). In the outer zone there was no effect housing ($F_{(1, 56)} = 3.300$, $p=0.07$ Fig 3.3.4 B) nor treatment ($F_{(1, 56)} = 2.678$, $p=0.1$ Fig 3.3.4 B), there was also no interaction effect. Sidak post-hoc test demonstrated no differences between groups.

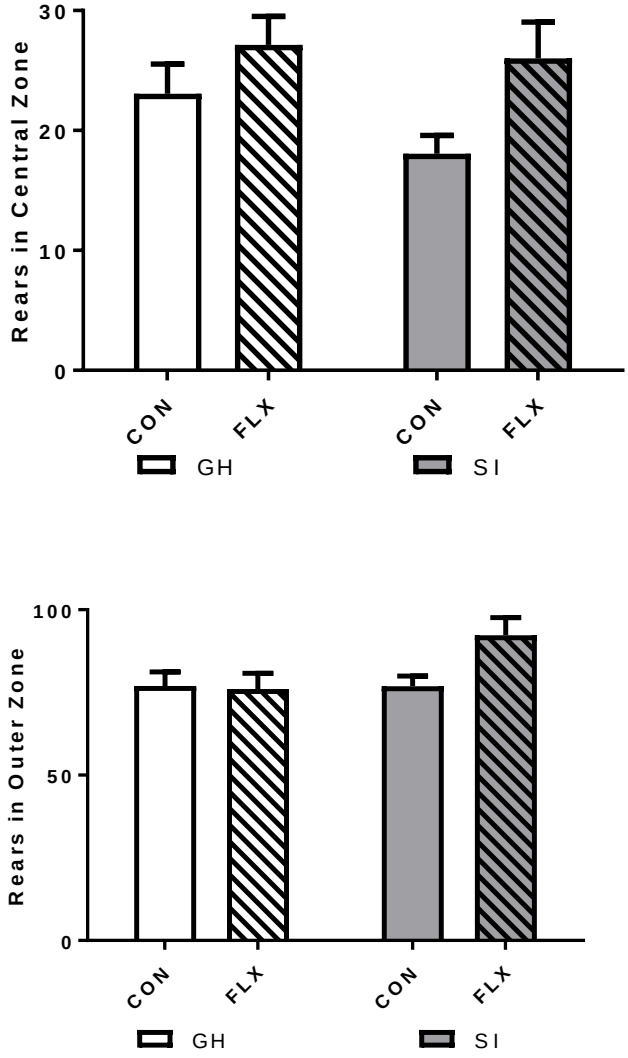


Fig 3.3.4 Rears in central/outer zone: (number of rears, mean ± sem, n=15 per group) (A) Number of rears in the central zone. A two-way ANOVA revealed a significant effect of treatment ($p<0.05$). (B) Number of rears in the outer zone. No significant differences between groups.

3.3.2 The Effects of Isolation-rearing and Fluoxetine on Habituation and Neophobia in the Locomotor Activity Task

Locomotor activity was measured as a common behavioural change often observed in isolates.

Ambulations

Ambulations (infrared beam breaks) were recorded as measure of locomotor activity. A three-way ANOVA measuring the time course of activity across the trial revealed a main effect of housing such that overall SI rats had a higher number of horizontal beam breaks compared to GH rats ($F_{(1, 336)} = 18.9$, $P<0.001$ Fig 3.3.5 A). There was no effect of treatment ($F_{(1, 336)} = 3.28$, $P=0.07$ Fig 3.3.5 A). Sidak post-hoc test showed that only at the 10 minute interval GH/CON displayed a significantly lower beam breaks compared to SI/FLX rats; ($P<0.05$ Fig 3.3.5 A). When the total number of beam breaks across the trial were examined there was a significant effect of housing again showing that SI rats were more active than GH ($F_{(1, 56)} = 4.57$, $P<0.05$ Fig 3.3.5 B), there was no effect of treatment ($F_{(1, 56)} = 3.09$, $P=0.08$ Fig B). Sidak post-hoc test showed no differences between groups.

Beam Breaks	GH/CON	GH/FLX	SI/CON	SI/FLX
Over 30 mins	177 ± 23	182 ± 23	195 ± 28	209 ± 28
Total	1590 ± 87	1767 ± 79	1800 ± 47	1926 ± 101

Table 3.3.1 : Number of beam breaks for each group in both measures of the locomotor activity task (LMA, mean ± SEM.).

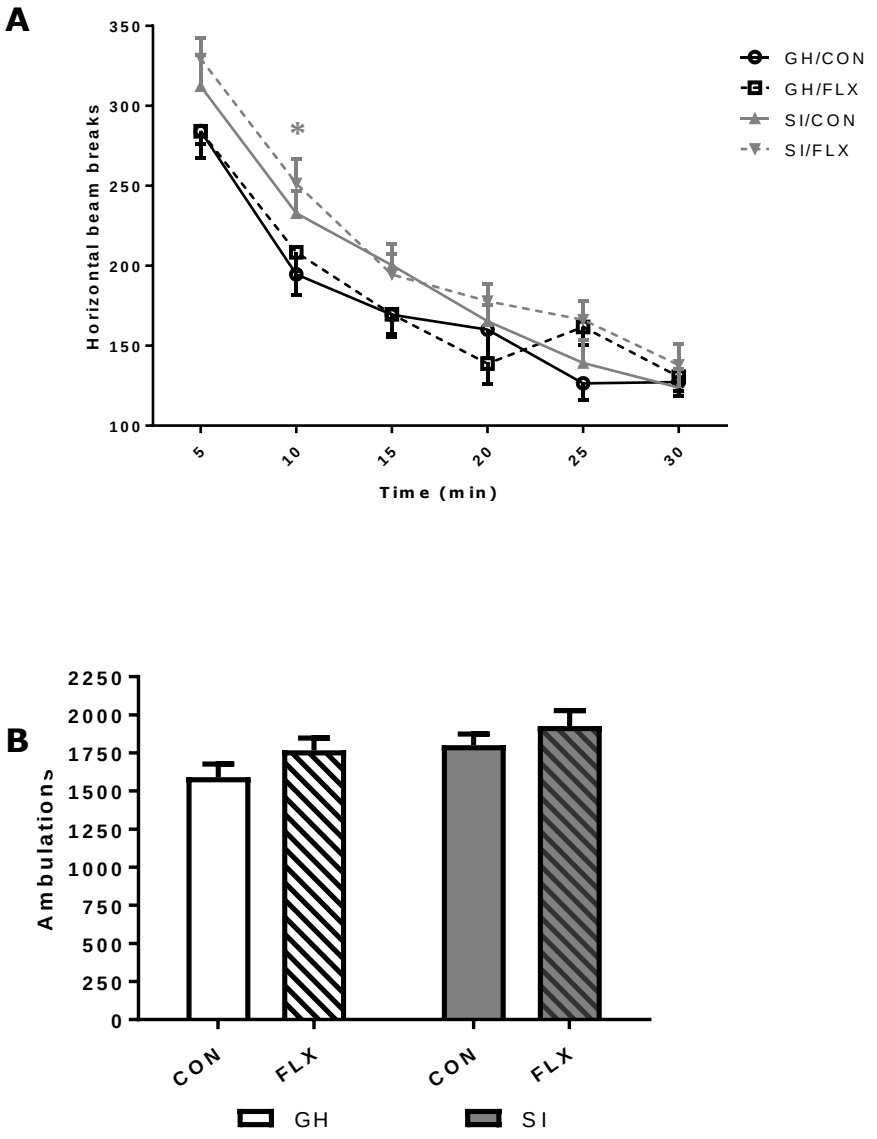


Fig 3.3.5 Locomotor Activity Task: Comparison of the change in horizontal locomotor activity (number of ambulations, mean ± sem, n=15 per group) in a novel chamber recorded as: (A) cumulative beam breaks in consecutive 5 min epochs over 30 min. A three-way ANOVA revealed a significant effect of housing ($p<0.001$). +$p<0.05$ Sidak post-hoc tests showed GH/CON lower ambulations compared to SI/FLX at the 10 minute time point. (B) Total beam breaks in the trial. Two-way ANOVA revealed a significant effect of housing ($p<0.05$). #$p<0.05$ Sidak post-hoc tests significant difference between GH/CON and SI/FLX.

Rears

Rears were measured as the number of vertical beam breaks throughout the trial. Again, there was no effect of treatment across the trial or housing. For the total number of rears, there was no significant effect of housing or treatment.

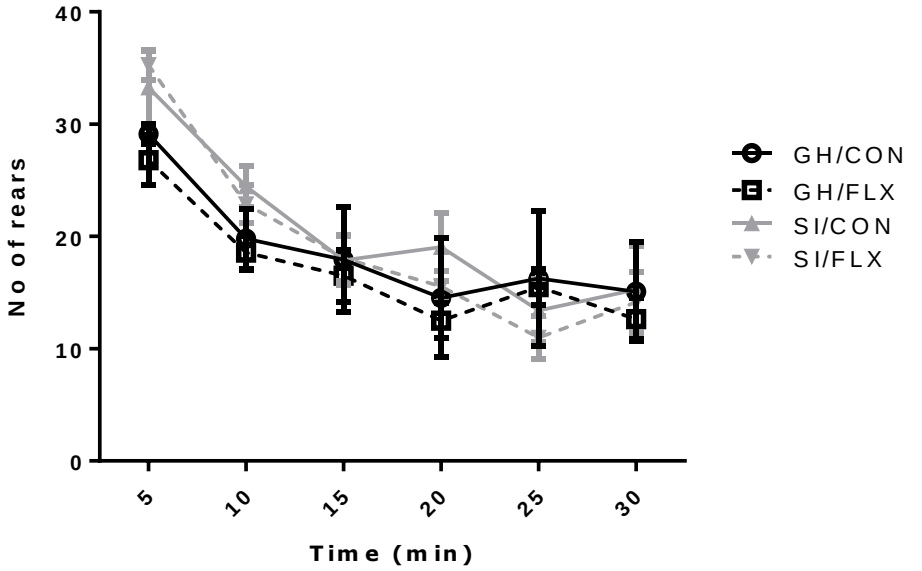


Fig 3.3.6Rearing in LMA Task: Comparison of the change in vertical locomotor activity (number of ambulations, n= 15 per group, mean ± sem.) in a novel chamber recorded as cumulative beam breaks in consecutive 5 min epochs over 30 min. A three-way ANOVA revealed no significant differences between groups.

3.3.3 Isolation-Rearing and Fluoxetine's Effect on Visual Recognition Memory in the Novel Object Discrimination Task

The novel object discrimination task was employed to assess visual memory. A two-way repeated measures ANOVA revealed there was no significant effect of housing ($F_{(1, 112)} = 3.34$, $p= 0.07$ Fig 3.3.7 A) or treatment ($F_{(1, 112)} = .301$, $p=0.58$ Fig 3.3.7 A) and there was no difference between time spent exploring each object ($F_{(1, 112)} = 0.164$, $p=0.68$ Fig 3.3.7 A) in the familiarisation trial.

Choice Trial

During the second choice trial there was no significant effect of housing ($F_{(1, 112)} = 3.16$, $p= 0.08$ Fig 3.3.7 B) or treatment ($F_{(1, 112)} = 0.6$, $p=0.44$ Fig 3.3.7 B) but there was a significant effect of object (Familiar vs Novel) ($F_{(1, 112)} = 39.1$, $p<0.001$ Fig 3.3.7 B) indicating exploration of the familiar object was less than the novel object. Sidak post-hoc tests showed GH/CON group spent more time exploring the novel than the familiar object (***$p<0.001$ Fig 3.3.7 B). This was also observed with the GH/FLX group (*$p<0.05$ Fig 3.3.7 B) and the SI/FLX group, (**$p<0.01$ Fig 3.3.7 B). The only group not to have a significant difference was the SI/CON group ($p=0.07$ Fig 3.3.7B).

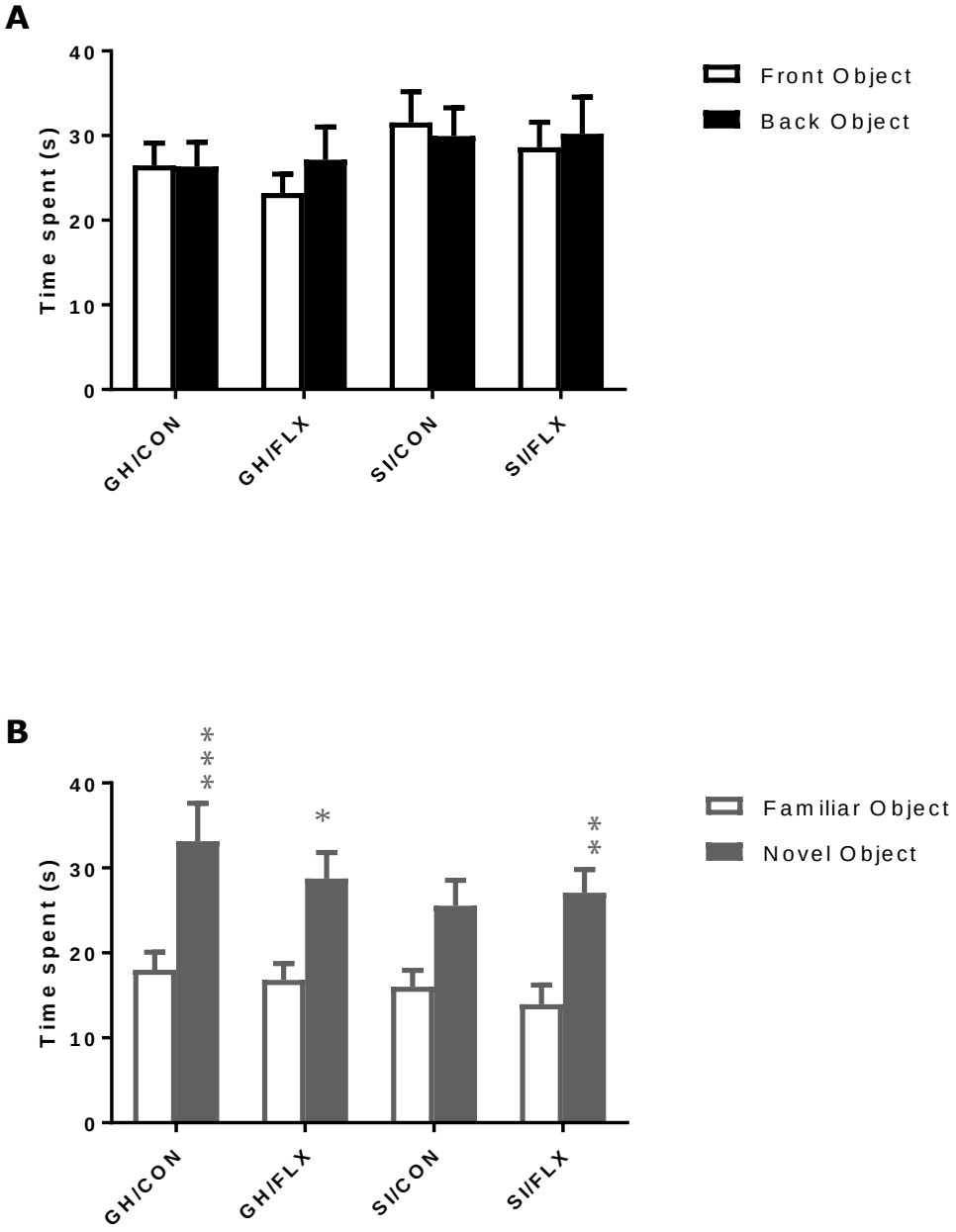


Fig 3.3.7 NOD Task: Time spent exploring in familiarisation (A) and choice (B) trials (s, mean ± sem. n=15 per group,). (A); No significant differences were observed between groups. (B)***p<0.001 (GH/CON) *p<0.05 (GH/FLX), **p<0.01 (SI/FLX) Sidak post-hoc tests demonstrating longer times exploring the novel object.

Discrimination Ratio

The discrimination ratio (the time spent exploring each object divided by the total time exploring) was also measured as a derived index of any preferential object exploration. There was no effect of housing ($F_{(1, 56)} = 0.1307$, p=0.71 Fig 3.3.8), or treatment ($F_{(1, 56)} = 0.4612$, p=0.49 Fig 3.3.8).

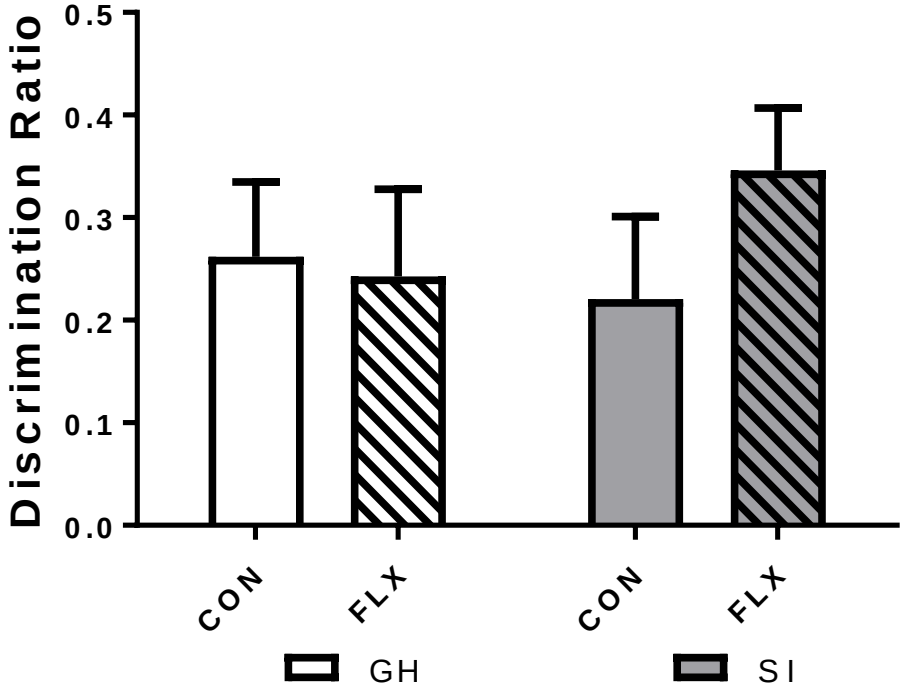


Fig 3.3.8 D2 Discrimination Ratio: (n= 15 per group, mean ± sem.). There were no significant differences between groups.

3.3.4 Isolation-Rearing and Fluoxetine effects on anxiety behaviour assessed by the Elevated Plus Maze

Distance and Velocity

A two-way ANOVA showing a significant main effect of treatment withFLX animals travelling significantly less compared to CON animals, ($F_{(1, 5)} = 10.01$, $P < 0.01$, Fig 3.3.9 A). There was no effect of housing on distance travelled ($F_{(1, 54)} = 2.385$, $P= 0.12$, Fig 3.3.9 A). There was no interaction effect between groups. Sidak post-hoc analysis revealed a significant decrease in distance travelled in the SI/FLX compared to GH/CON and SI/CON ($p<0.05$, Fig 3.3.9 A).

Average velocity (cm/s) was also examined. A significant effect of treatment was observed, FLX animals had a significantly lower average velocity compared to CON animals($F_{(1, 54)} = 9.94$, $P<0.01$ Fig, 3.3.9 B). There was no effect of housing ($F_{(1, 54)} = 2.039$, $P=0.15$, Fig 3.3.9 B). Sidak post-hoc test showed that there was a significant decrease in velocity in the SI/FLX rats compared to GH/CON ($P<0.01$, Fig 3.3.9 B) and a significant decrease between SI/FLX compared to SI/CON ($P<0.05$, Fig 3.3.9 B).

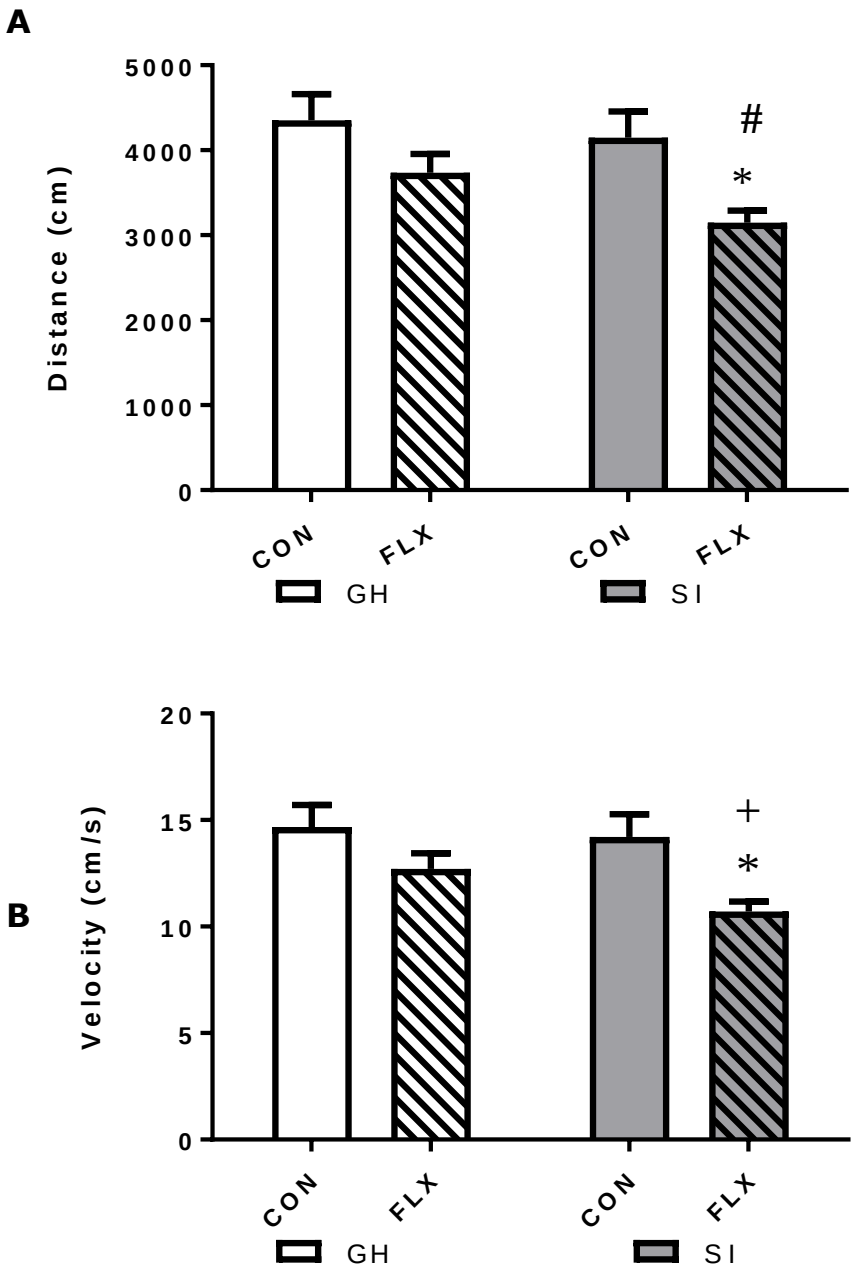
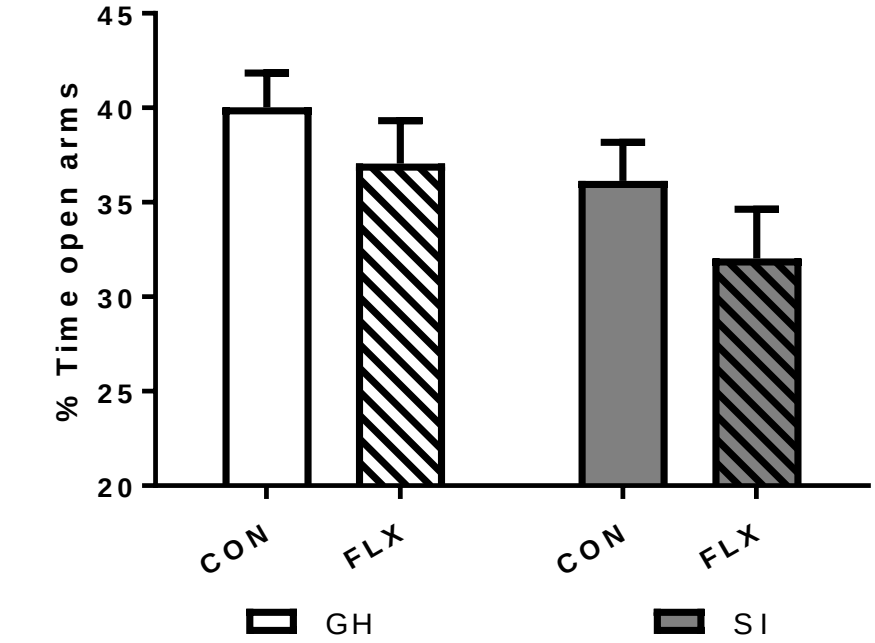


Figure 3.3.9 Distance and Velocity in the EPM: One rat from GH/CON and SI/FLX were eliminated from the data due to falling from the maze twice during the trial (n= 14-15 per group, mean ± sem.) (A) Distance (cm) in EPM. A two-way ANOVA showed significant effect of treatment ($p<0.01$). +$p<0.05$ Sidak post-hoc test between GH/CON & SI/FLX. *$p<0.05$ Sidak post-hoc test between SI/CON and SI/FLX. (B) The average velocity (cm/s). A two-way ANOVA showed a significant effect of treatment ($p<0.01$). +$p<0.05$ Sidak post-hoc test between GH/CON & SI/FLX. *$p<0.05$ Sidak post-hoc test between SI/CON and SI/FLX.

Percentage Time in Open and Closed Arms

For percentage of time spent in the open arms there was a significant effect of housing, GH rats spent longer in the open arms compared to SI rats ($F_{(1, 54)} = 4.100$, $p<0.05$, Fig 3.3.10 A). There was no significant effect of treatment ($F_{(1, 54)} = 2.593$, $p=0.11$, Fig 3.3.10 A) and no interaction effect. Sidak post-hoc analysis showed no difference between any of the groups. For percentage time spent in the closed arms again there was an effect of housing ($F_{(1, 54)} = 4.100$, $p<0.05$, Fig 3.3.10 B) SI rats spent longer in the closed arms compared to GH rats. There was no effect of treatment ($F_{(1, 54)} = 2.593$, $p=0.11$, Fig 3.3.10 B) and

A hoc tests revealed there were no differences between the groups.



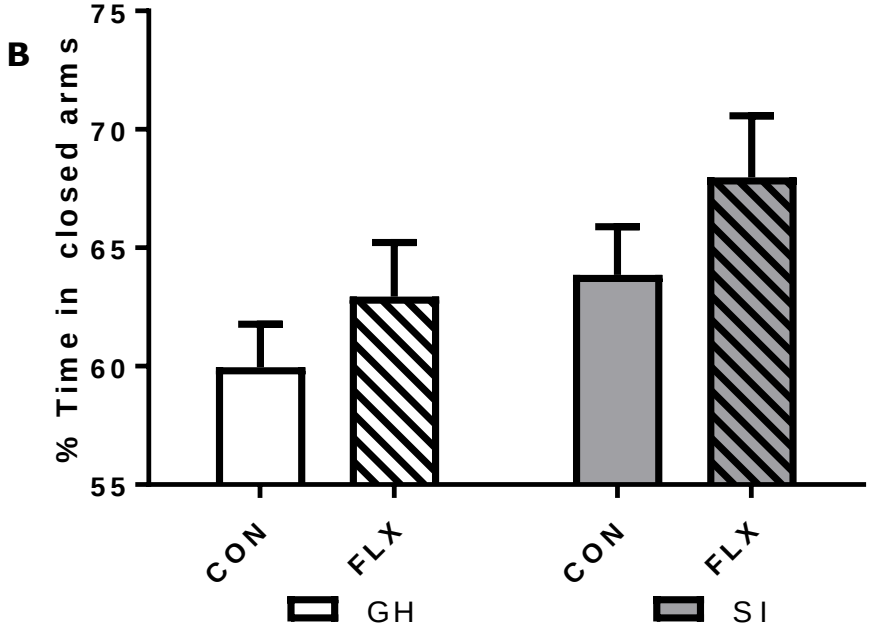


Figure 3.3.10 % Time in Arms: One rat from GH/CON and one from SI/FLX were eliminated from the data due to falling from the maze twice during the trial (n= 14-15 per group, mean ± sem.) (A) A two-way ANOVA showed a significant effect of housing ($p<0.05$). (B) A two-way ANOVA showed a significant effect of housing ($p<0.05$).

Head Dips & Stretch Attends

Two ethological behaviours were analysed to further assess any change in anxiety-like behaviour, exploratory head dips and risk assessment stretch attend postures (Blanchard et al., 1990; Blanchard et al., 1991). GH rats engaged in more head dips than their SI counterparts, a two-way ANOVA demonstrated a main effect of housing ($F_{(1, 53)} = 8.148$, $p<0.01$, Fig 3.3.11 A) There was also a main effect of treatment, FLX rats engaged in fewer head dips compared to CON rats($F_{(1, 53)} = 5.125$, $p<0.05$, Fig 3.3.11 A). Sidak post-hoc tests revealed SI/FLX had fewer head dips than GH/CON ($p<0.01$, Fig 3.3.11 A).

For risk assessment stretch attends, there was a significant effect of housing ($F_{(1, 54)} = 7.59$, $p<0.01$, Fig 3.3.11 B); GH rats engaging in fewer stretch attends than SI rats, a main effect of treatment was also observed with CON rats showing fewer stretch attends than FLX treated rats ($F_{(1, 54)} = 4.05$, $p<0.05$, Fig 3.3.11 B). Sidak post-hoc tests showed a significant difference with GH/CON displaying lower stretch attends compared to SI/FLX ($p<0.01$, Fig 3.3.11 B).

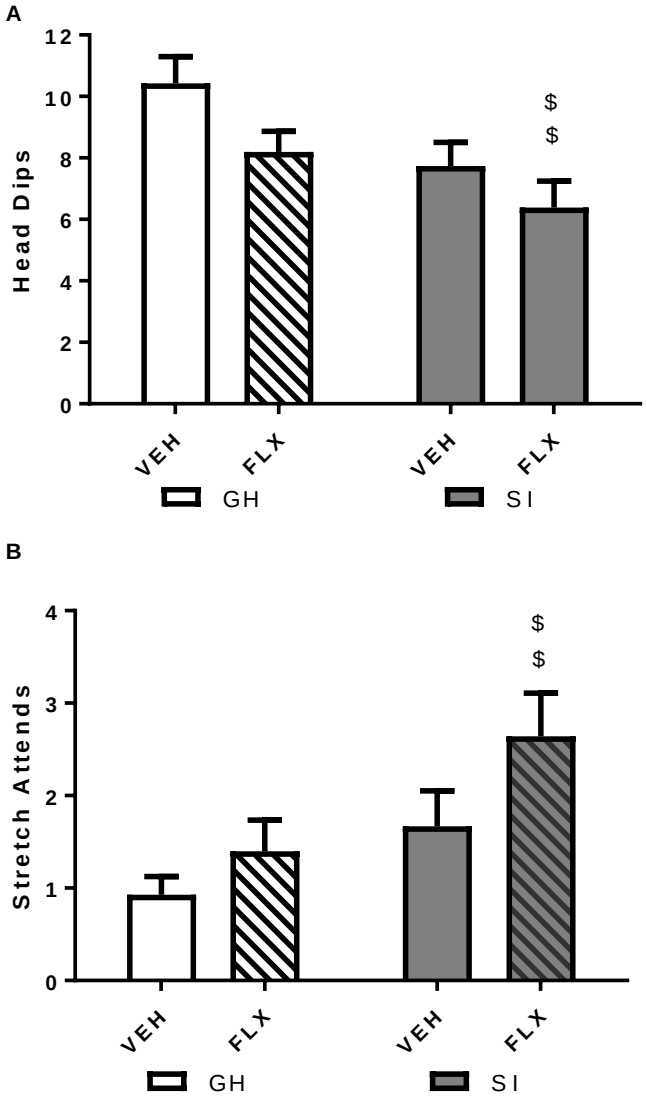


Figure 3.3.11: One rat from GH/CON and one from SI/FLX were eliminated from the data due to falling from the maze twice during the trial (n= 14-15 per group, mean ± sem.) (A) Number of exploratory head dips in the plus maze. A two-way ANOVA showed a significant effect of housing ($p<0.05$) and treatment ($p<0.05$). \$\$$p<0.01$ Sidak post-hoc between GH/CON & SI/FLX. (B) Number of risk assessment stretch attends in the plus maze. A two-way ANOVA showed a significant effect of housing ($p<0.01$) & treatment ($p<0.05$). \$\$$p<0.01$ Sidak post-hoc between GH/CON & SI/FLX.

3.3.5 The Conditioned Freezing Response Task: Effects of Isolation-rearing and Fluoxetine on Associative Memory.

Rats were tested in the conditioned freezing response task where the total time freezing (s) recorded as previously validated (Woods et al, 2012).

Acquisition

During the acquisition phase a two-way repeated measures ANOVA demonstrated that there was a main effect of housing; GH rats scored significantly higher than SI rats ($F_{(1, 55)} = 10.48$, $p<0.01$, Fig 3.3.12). There was no main effect treatment ($F_{(1, 55)} = 2.59$, $p=0.113$, Fig 3.3.12) and there was a main effect of shock demonstrating freezing time significantly increased after the second shock ($F_{(1, 55)} = 43.41$, $p<0.001$, Fig 3.3.12). Sidak post-hoc tests showed that after the second shock GH/CON froze significantly more than SI/FLX ($p<0.01$, Fig 3.3.12). There was also a significant increase in freezing time after the second shock compared to the first in GH/CON ($p<0.001$, Fig 3.3.12) and SI/CON ($p<0.05$, Fig 3.3.12).

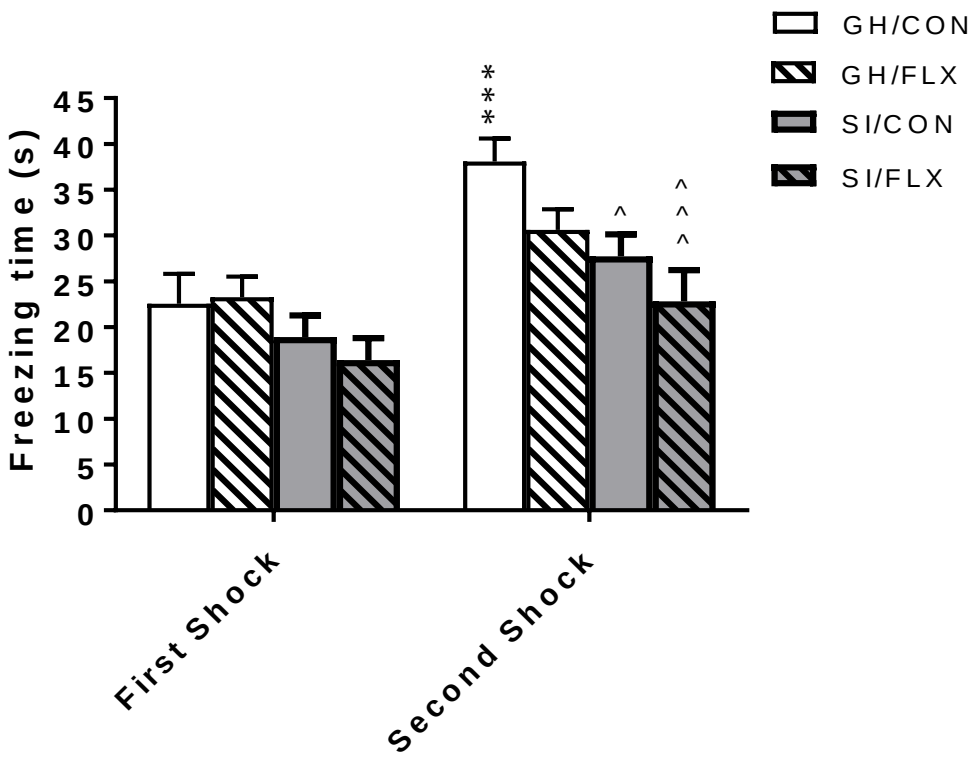


Figure 3.3.12 Acquisition Phase of CFR: One rat was eliminated from the analysis due to an error with the equipment (s, n=14-15 per group, mean ± sem.). A two-way repeated measures ANOVA showed a significant effect of housing ($p<0.05$) and shock ($p<0.001$). ^^^ $p<0.001$ Sidak post-hoc tests showed a difference between GH/CON & SI/FLX after the second shock. ***P<0.001 Sidak post-hoc test between GH/CON first shock vs second shock. ^p<0.05 Sidak post-hoc test between SI/CON and GH/CON after the second shock

Retention

24 hours after conditioning, a two-way repeated measures ANOVA demonstrated an effect of housing with GH rats freezing for longer compared to SI rats ($F_{(1,56)} = 22.5$, $p<0.001$, Fig 3.3.13). There was no effect of treatment ($F_{(1, 56)} = 1.30$, $P=0.25$, Fig 3.3.13). A significant housing x treatment interaction effect was observed ($F_{(1, 56)} = 15.47$, $p<0.001$, Fig 3.3.13). Sidak post-hoc tests showed a significant decrease in freezing in SI/CON compared to GH/CON ($p<0.001$), GH/FLX ($p<0.01$) and SI/FLX, ($p<0.01$) (Fig 3.3.13)

48 hours post-conditioning, a main effect of housing was observed with GH rats freezing more than SI rats ($F_{(1,56)} = 4.61$, $p<0.05$ Fig 3.3.13) an effect of treatment was also observed, CON rats freezing less than FLX rats ($F_{(1, 56)} = 6.07$, $p<0.05$ Fig 3.3.13). A significant housing x treatment interaction effect was observed ($F_{(1, 56)} = 10.13$, $p<0.01$, Fig 3.3.13). Sidak post-hoc tests showed a significant decrease in freezing in SI/CON compared to GH/CON ($p<0.01$), GH/FLX ($p<0.05$) and SI/FLX, ($p<0.01$) (Fig 3.3.13).

Following this, rats were presented with the conditioned cue and again freezing behaviour was scored. There was an effect of housing ($F_{(1, 56)} = 7.65$, $p<0.01$ Fig 3.3.13), there was no effect of treatment ($F_{(1, 56)} = 0.49$, $p=0.49$ Fig 3.3.13). A significant housing x treatment interaction effect was observed ($F_{(1, 56)} = 20.92$, $p<0.001$, Fig 3.3.13). Sidak post-hoc tests showed a significant decrease in freezing in SI/CON compared to GH/CON ($p<0.001$) and SI/FLX, ($p<0.01$) and a decrease in freezing time in GH/FLX compared to GH/CON ($p<0.05$) (Fig 3.3.13). Sidak post-hoc tests also revealed a significant decrease in freezing time of all groups at 48 h pre-tone compared to the 24 h time point ($p<0.001$, Fig 3.3.13) and a significant increase of freezing time again after presentation of the cue ($p<0.001$, Fig 3.3.13).

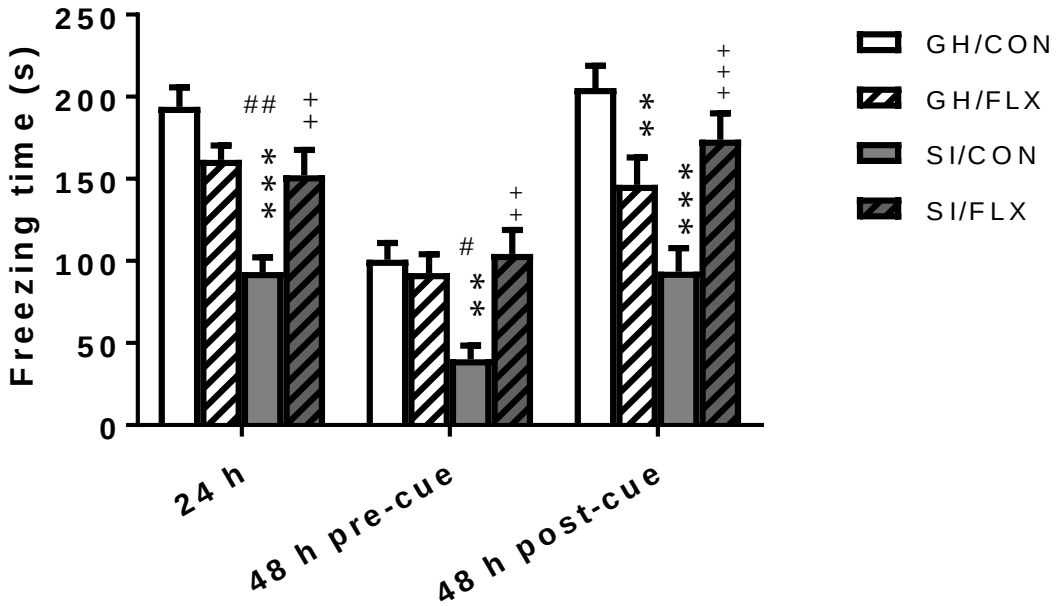


Figure 3.3.13 Retention Phase CFR: Time spent engaged in freezing behaviour (s, n=14-15 per group, mean ± sem.). A two-way repeated measures ANOVA showed a significant effect of housing ($p<0.05$). A two-way ANOVA showed a significant effect of housing ($p<0.05$) and treatment ($p<0.05$). ** =$p<0.01$ from GH/CON, ***=$p<0.01$ from GH/CON, ++ =$p<0.01$ from SI/CON, +++=$p<0.001$ from SI/CON, #=$p<0.05$ from GH/FLX.

3.3.6 The effects of isolation and fluoxetine on temperature changes and corticosterone induced by restraint stress.

Temperature was measured at 5 min intervals for 30 min as a change from baseline temperature. A two-way repeated measures ANOVA demonstrated no significant effect of housing ($F_{(1, 52)}$ =0.47, p=0.49, Fig 3.3.14) or treatment ($F_{(1, 52)}$ =0.047, p=0.82, Fig 3.3.14) on temperature levels during the task. Restraint did have a significant effect with restrained animals presenting with a higher temperature compared to non-restrained animals ($F_{(1, 52)}$ =13.01, p<0.001, Fig 3.3.14). There were no interaction effects between any groups.

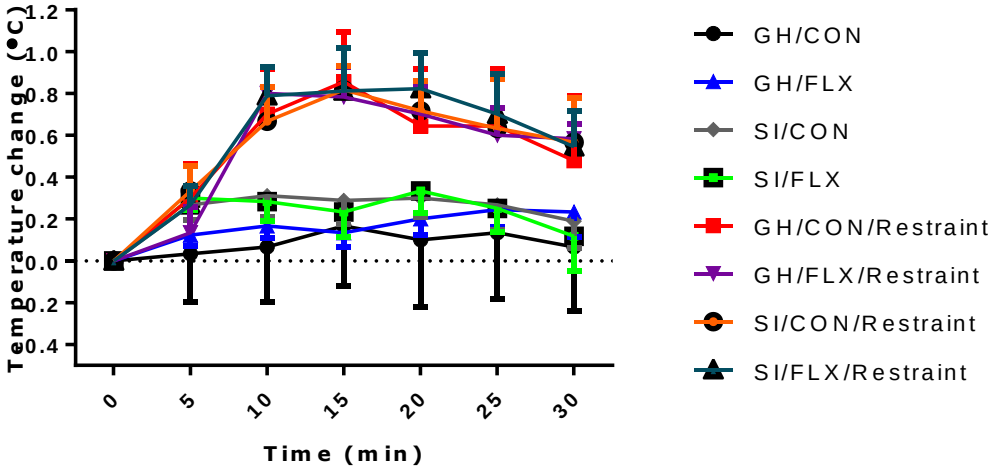


Figure 3.3.14 Temperature Change in SIH: Temperature change from baseline (°C, n=7-8 per group, mean ± sem.): A two-way repeated measures ANOVA showed a significant effect of 'Restraint' (p<0.001).

3.3.7 The effects of isolation and fluoxetine on corticosterone changes induced by restraint stress.

Plasma corticosterone was measured by ELISA, a main effect of restraint was observed ($F_{(1, 52)} = 104.2$ $p<0.001$, Fig 3.3.15), although there was no effect of housing ($F_{(1, 52)} = 0.64$, $p=0.80$, Fig 3.3.15) or treatment ($F_{(1, 52)} = 0.35$, $p= 0.56$, Fig 3.3.15). Sidak post-hoc results showed that all 4 treatment groups were significantly different from their respective restrained group. GH/CON ($p<0.001$), GH/FLX ($p<0.01$), SI/CON ($p<0.001$) and SI/FLX ($p<0.001$).

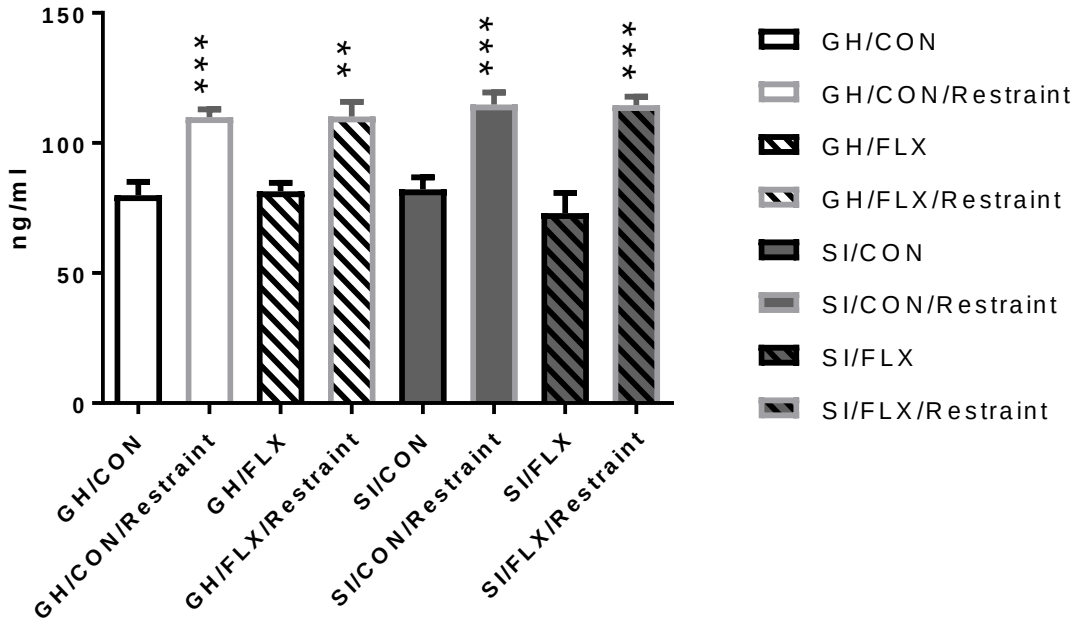


Figure 3.3.15 Plasma Corticosterone Levels: Levels of plasma corticosterone (ng/ml, n=7-8 per group, mean ± sem.). A two-way repeated measures ANOVA showed a main effect of restraint ($P<0.001$). ***$P<0.001$, **$P<0.01$ Sidak post-hoc tests compared to non-restraint counterparts.

3.3.8 Hippocampal cell proliferation and neurogenesis

The level of cell proliferation and neurogenesis in the dentate gyrus of the hippocampus was examined using DCX immunohistochemistry. There was a main effect of housing with SI rats having significantly fewer positively stained cells ($F_{(1, 50)} = 16.81$, $p<0.001$, Fig 3.3.16) in the dentate gyrus, there was also a main effect of treatment with FLX treated animals having higher levels of positive stained DCX cells compared to CON ($F_{(1, 50)} = 10.12$, $p<0.01$, Fig 3.3.16). Sidak post-hoc tests revealed an increase in positive cells in GH/FLX compared to GH/CON ($p<0.05$). SI/CON had fewer positive cells compared to GH/FLX ($p<0.001$) as did SI/FLX ($p<0.01$) (Fig 3.3.16).

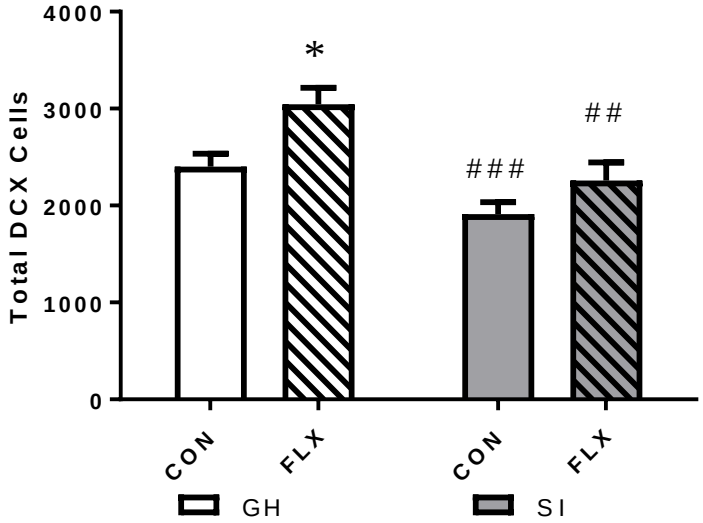


Figure 3.3.16: Six samples removed from the analysis due to problems with the staining. One from the GH/CON, SI/CON and SI/FLX respectively and three from GH/FLX (Number of cells, n=7-8 per group, mean ± sem.). Two-way ANOVA showed a main effect for housing ($p<0.001$) and treatment ($P<0.01$). Sidak post-hoc tests *$p<0.05$ compared to GH/CON, ##$p<0.01$, ###$p<0.001$ compared to GH/FLX.

3.4. Discussion

The current study aimed to assess the ability of the SSRI fluoxetine to restore behavioural alterations induced by the isolation-rearing paradigm and therefore assess the potential for this neurodevelopmental model to produce 'depressive-like' behaviours in rodents. The behaviours examined included increases in anxiety-like behaviour, cognitive dysfunction and, the stress response both behaviourally and through levels of plasma corticosterone post-mortem, and alterations in neurogenesis.

Isolated rats were hyperactive compared to group-housed rats, consistent with development of the isolation syndrome (Sahakian and Robbins, 1977). Fluoxetine treated rats appeared to be more active than their control treated counterparts, although only the isolated fluoxetine group was significantly more active than the group-housed control group. Hyperactivity and delayed habituation to a novel environment are robust and long established characteristics in isolation-reared rats (Gentsch, Lichtsteiner, Frischknecht, Feer, & Siegfried, 1988; Sahakian, Robbins, & Iversen, 1977) thought to reflect neophobia (Fone & Porkess, 2008). Typically, rats become progressively less active during exploration of a novel arena, however isolation-reared rats exhibit increased activity through a deficit in this normal habituation, supported in previous research (Lapiz et al., 2003). Bianchi et al, (2009) examined chronic fluoxetine treatment (10 mg/kg/day i.p., 28 days) in socially isolated rats and found that it had no effect on the hyperactivity of the isolated rats which was present.

Fluoxetine treated rats spent longer in the more aversive central zone of the open field in comparison to control rats indicating a potential anxiolytic effect of fluoxetine. There were no differences in time spent in either zone between group housed and isolated rats suggesting that isolation-rearing was not causing anxiety-like behaviour. This is consistent with previous studies of anxious behaviour in a rodent restraint stress-model of depression (Lapmanee et al., 2013). Treatment with chronic fluoxetine (10 mg/kg/day p.o., 28 days) had an anxiolytic effect in the elevated T-maze and reduced depressive-like behaviour in the FST. Other studies have shown contradictory results to what was observed in the present study, chronic fluoxetine treatment has been found to be anxiogenic in the open field test (Gray & Hughes, 2015). The current study also examined two behaviours in the open field, grooming and rearing. For grooming in the central zone, fluoxetine treated rats showed an increase compared to control rats, in contrast in the outer zone isolation rearing decreased levels of grooming. Housing did have a significant effect on activity in the LMA task; with SI rats showing more activity than GH rats in 5 min bins across the trial. (There was also a significant increase in total number of beam breaks in the SI compared to GH rats (Fig 3.3.5)). Consistent with the open field, SI rats were hyperactive compared to GH in the activity boxes but fluoxetine appeared to

enhance total ambulations further compared to that in GH/CON, suggesting both isolation and fluoxetine increased locomotor activity, reducing habituation to a novel environment although the effect of fluoxetine alone did not reach significance. This supports the open field data suggesting SI rats acquired the isolation syndrome, although there was no major change in concomitant rears. Increases in locomotor activity may be partly due to hyperactivity of the mesolimbic dopamine (DA) system, as it has been demonstrated in previous studies that isolation-reared rats have elevated basal extracellular DA levels and a more rapid DA turnover in the nucleus accumbens (Fone & Porkess 2008; Jones et al. 1990; Lapiz et al. 2003). The firing rate of VTA DA neurons also elevated by SI (Fabricius et al, 2010) and the SI hyperactivity is attenuated by antipsychotics (McIntosh et al 2013) which are potent D2 receptor antagonists. This evidence suggests that post-weaning social isolation causes an increase in presynaptic dopamine activity within the nucleus accumbens which may contribute to the hyperactivity observed. There has also been some investigation particularly into the role of the ventral tegmental area (VTA) and the nucleus accumbens (NAc) pathway (Espejo & Miñano, 1999; Roth & Horger, 1996). Studies like these have shown that in animal models of depression, increased stress activates VTA dopamine neurons increasing dopaminergic transmission to the NAc. Experimental manipulation of dopaminergic transmission in the VTA-NAc pathway could regulate depression-like behaviour in animal models (Nestler & Carlezon, 2006). Research into depression has focused largely on serotonergic and noradrenergic mechanisms in other regions (e.g. hippocampus and cortex), while research on dopaminergic mechanisms and the VTA-NAc pathway has largely focused on addiction or schizophrenia. Due to this, there is no consensus in the field about the potential role of the VTA-NAc pathway in depression.

In the novel object discrimination task to assess visual recognition memory, there was no difference in object exploration in the familiarisation trial showing neither housing nor fluoxetine altered basal object preference. However, there was also very little impact of either isolation housing or fluoxetine on discrimination in the choice trial. Examination of the discrimination ratio showed SI/CON rats were the only group unable to discriminate the novel from the familiar object suggesting the mild NOD deficit was reversed with chronic fluoxetine administration but this would need confirmation, this could potentially be donewith another study and using a larger cohort of rats. Evidence from previous studies in our laboratory support the findings of the current study, that isolation rearing induces deficits in the novel object discrimination task (Bianchi et al., 2006; Watson, Marsden, Millan, & Fone, 2012b) and Bianchi et al (2009) observed no change in performance on the novel object task in rats treated with fluoxetine for 4 weeks.

Previous research in patients has shown fluoxetine can improve memory performance during major depression (Levkovitz, Caftori, Avital, & Richter-Levin, 2002). Furthermore, in a rat paradigm looking at NOD deficits induced by chemotherapy, fluoxetine improved memory (ElBeltagy et al., 2010). El Hage et al. (2004) found that predatory stress caused a deficit in mice in the novel object recognition task and animals returned to control levels of performance after treatment with fluoxetine. Although there is some evidence contrary to this, Valluzzi and Chan (2007) state that fluoxetine had no effect on a hippocampal dependent cognition, while causing a deficit in hippocampal-independent tasks. Some of the mechanisms responsible for the pro-cognitive effect of fluoxetine are the down-regulation of the MAPK/ERK pathway (Carlini et al., 2012) but not with other antidepressants. There is also evidence for changes to the 5-HT_{2A} receptor in isolation-reared rats (Preece, Dalley, Theobald, Robbins, & Reynolds, 2004) and the Castañé et al. (2015) study, which states that 5-HT_{2A} receptors are involved in the pro-cognitive but not the antidepressant effects of fluoxetine. The primary index of anxiety measured in the plus maze is the time spent in the open arms versus the closed (Cruz et al, 1994). On the plus maze, rats tend to avoid the open arms and prefer to stay in the closed arms and this is because they prevent the rat from engaging in thigmotactic behaviour (Treit, Menard, & Royan, 1993). Anxiolytic compounds, such as benzodiazepines, increase the number of entries into, and the time spent in the open arms, whereas anxiogenic agents such as the 5-HT_{2C} agonist mCPP do the opposite (Handley & Mithani, 1984; Pellow & File, 1986). Results from the current study showed a decrease in the time spent in the open arms suggesting an increase in the levels of anxiety in the SI relative to GH rats although overall all groups spent more time in the closed arms in the trial. There was no effect of fluoxetine treatment on time spent in the open arms. A study conducted by Silva and Brandao (2000) observed similar findings that treatment with chronic fluoxetine had no effect on time spent in the open arms of the plus maze, but acute treatment actually decreased time spent and entries into the open arms, suggesting an anxiogenic effect of acute fluoxetine only. As well as monitoring time in the aversive open arms, two ethological behaviours were examined in the current study. Exploratory head dips and risk-assessment stretch attend postures both of which are well established measures in the elevated plus maze (Blanchard et al., 1990; Blanchard et al., 1991). In the current study, isolation rearing reduced the number of head dips in the trial indicating increased anxiety in the SI rats compared to GH, interestingly fluoxetine treatment also reduced the number of head dips which would suggest that it was having an anxiogenic effect. With the opposite effect for stretch attend postures, isolation rearing and fluoxetine treatment causing an increase, again indicating an anxiogenic effect. In the Silva and Brandao study (2000) chronic fluoxetine treatment had no effect on ethological behaviours but acute treatment caused some anxiogenic behaviour profiles similar to the current study.

In the conditioned freezing response task, to assess fear motivated associative learning and memory, as expected isolation rearing decreased both contextual and cue-dependent fear conditioning 24 and 48 h after conditioning. Freezing behaviour increased with all groups following exposure to the conditioned cue (light/tone) at 48 h after training. Contextual fear conditioning behaviour has been reported to involve the hippocampus (Richmond et al., 1999) and the results of the current study are consistent with a study showing 13 weeks social isolation produced deficits in associative learning and memory in a contextual fear conditioning task (Weiss et al., 2004b). The current study demonstrated isolation rearing caused a deficit in associative learning that was both context and cue dependent. It also showed that treatment with fluoxetine reversed the deficit indicating chronic fluoxetine treatment was having an anxiolytic and pro-cognitive effect. Previous studies support the current findings, Zhang et al (2000) showed chronic treatment with fluoxetine had an anxiolytic effect on freezing behaviour in the conditioned emotional response task as well as other indices of anxiety (e.g. defecation). Studies using citalopram have shown comparable results to that seen in the clinic, that treatment with acute doses increases anxiety behaviours but chronic treatment with SSRIs is anxiolytic (Burghardt, Sullivan, McEwen, Gorman, & Ledoux, 2004). Although there is some evidence that intensity of the fear conditioning can affect SSRI treatment (Santos, Martinez, & Brandão, 2006).

In the SIH task, physical restraint induced a significant hyperthermia by about 0.8 ^{o}C irrespective of housing or drug treatment. Consistent with a previous report, the stress-induced hyperthermia appeared to reach a plateau after 10 min and continue for 30 min or more (Olivier et al., 2003). The current study demonstrated that chronic treatment with fluoxetine did not have an effect on the stress response induced by restraint. In contrast to this, Hesketh et al (2005) demonstrated the different actions of acute vs chronic treatment with the SSRI citalopram, they found that acute treatment prolonged the increase in plasma ACTH and corticosterone induced by restraint stress. Chronic FLX treatment had no effect on basal levels of either ACTH or corticosterone but the ACTH response to restraint was attenuated. Corticosterone analysis demonstrated restraint significantly increased plasma corticosterone although there was no significant difference between housing or treatment conditions at the time point measured (1 h post-stress). This supports observations in Chapter 2 that the restraint paradigm employed in the current study significantly increased plasma corticosterone levels, regardless of housing condition. Stress response can be utilised as a measure as it has been shown to be altered in depression both clinically and in pre-clinical models (Biegler, 2008; Risch et al., 2009; Binder and Nemeroff, 2010; Young and Korszun, 2010; Pizzagalli, 2014) as the SI rats are not displaying this feature this may have little translational relevance for this aspect of MDD in the current model.

By using dual-labelled IHC to count DCX/NeuN positive cells in the hippocampal dentate gyrus as a marker for newly synthesised neurons isolation rearing was found to reduce hippocampal neurogenesis. Interestingly, chronic fluoxetine significantly increased levels of neurogenesis in the dentate gyrus herein in both GH and SI rats, consistent with several previous studies (David et al., 2010; Dranovsky and Hen, 2006; Malberg, 2004; Malberg et al., 2000; Santarelli et al., 2003a) although it cannot be stated that there was a reversal of the SI induced deficit. The study demonstrated that repeated SSRI treatment (and in particular fluoxetine) enhances hippocampal neurogenesis. Of further interest, this enhancement of neurogenesis may relate to the observed impairment in NOD and CFR following isolation rearing which was partially restored by chronic fluoxetine. This could provide some evidence towards the idea that the mechanism by which fluoxetine has a pro-cognitive effect is through neurogenesis.

3.4.2 Conclusions

The current study demonstrated that isolation-rearing from weaning largely produced the expected behavioural phenotype including novelty-induced hyperactivity in the open field and locomotor activity tasks, increased anxiety-related behaviours in the open field (although there was little change in elevated plus maze exploration), deficits in visual recognition memory and associative memory in the novel object discrimination and conditioned emotional response task, respectively. It also demonstrated that chronic treatment with the SSRI fluoxetine had differential effects on anxiety behaviours which appeared to be task dependent; but it partially reversed some of the cognitive deficits induced by isolation-rearing. The following chapter will examine the effects of acute ketamine on SI rats in the same battery of behavioural tasks as ketamine has been demonstrated to be an effective and rapid-acting treatment for both MDD and TRD.

Chapter 4:

Characterising physiological and behavioural changes in isolation rearing model of depression and the effects of treatment with ketamine.

4.1. Introduction

This chapter examines the effect of acute ketamine treatment on behavioural and neurochemical changes produced by isolation rearing of Lister hooded rats. The same rearing paradigm was employed as in previous chapters (See chapter 2 section 2.2 for details) and the battery of behavioural tasks were replicated for consistency, so any changes observed could be compared across pharmacological interventions. Post-mortem analysis consisted of plasma corticosterone levels taken immediately following restraint stress and measurement of DCX positive cells in the hippocampal dentate gyrus.

4.1.1 Ketamine in depression

Ketamine is a glutamate N-Methyl-D-Aspartate receptor (NMDA-R) antagonist and is a U.S. Food and Drug Administration (FDA) approved sedative medication originally developed for the induction and maintenance of anaesthesia in adults. It has a short plasma half-life of 4 min and a 2.5 h plasma terminal half-life. It can be administered intravenously (most common), intramuscularly (93% bioavailability (BA)), intranasally (50% BA), intrarectally (25% BA), and orally (20% BA). During a randomised control trial (RCT), Berman et al. (2000) demonstrated an i.v. infusion of ketamine at a sub-anaesthetic dose (0.5 mg/kg, for 40 min) significantly and rapidly reduced symptoms of depression (measured as decrease in score on the Hamilton Depression Rating Scale), with the effects lasting for up to a week later. This sparked a series of RCT's into ketamine as a potential rapid-acting antidepressant for the treatment of TRD (DiazGranados et al., 2010; Murrough et al., 2013; Valentine et al., 2011; Zarate et al., 2012; Zarate et al., 2006). The overall response rate at 72 h was 29 % compared to 7 % with saline placebo (Aan Het Rot et al., 2012). A problem identified in many of the RCTs was a large range in the duration of the antidepressant response from hours to weeks. Ketamine also has a potential for abuse and psychomimetic effects often accompany its administration. These psychoactive effects threaten any chance of a double-blind design, as there is a lack of a psychoactive placebo control; allowing patients to realise whether they have been given the active compound, which can confound results. One of the trials (Murrough et al., 2013) tried to combat this by adopting midazalom (a benzodiazepine) as an active placebo control. Both agents were administered with an i.v. infusion and the results demonstrated that at the 24 h time point patients had a greater response rate with ketamine (64%) than midazalom (28%). However, depression scores were not significantly different after 7 days. Another disadvantage of the psychoactive effects of ketamine highlighted in this study was the dissociative effects experienced, which include confusion, perceptual disturbances, elevated blood pressure and dizziness. In this particular case, 17 % of the patients (8/42) reported severe dissociative symptoms. Although these are transient and usually last no more than two hours, 2 participants required their infusions be stopped due to haemodynamic reasons (Murrough et al, 2013). Studies have continued

to examine some of the problems that arose with these RCTs; some of these include trialling ketamine alongside ECT or using different routes of administration, with promising results observed with intranasal administration.

4.1.2 Preclinical evidence of ketamine efficacy of MDD

Preclinical studies into the efficacy of ketamine as an antidepressant have been widely available for a number of years. Many studies have attempted to replicate findings in the clinic by using acute doses and measuring behavioural effects over a longer period. The efficacy of ketamine is often assessed in models of CMS as these have been shown to induce 'depressive-like' symptoms in rodents. The behavioural paradigms most commonly associated with assessment of these symptoms are the forced swim test (FST), tail suspension test (TST), sucrose preference test, novelty-suppressed feeding (NSF), EPM, LMA and learned helplessness (LH).

Standard antidepressants administered acutely before the FST have been shown to be effective, these include SSRIs, TCAs & MAOIs (Cryan et al., 2005). By comparison, some groups have reported acute administration of ketamine also causes a decrease in immobility in the FST (Burgdorf et al., 2013; Gigliucci et al., 2013; Koike et al., 2013; Muller et al., 2013) although this is not always replicable (Bechtholt-Gompf et al., 2011; Popik et al., 2008). The majority of studies indicate that the FST remains sensitive to the protracted effects of ketamine up to 1 week after a single injection (Browne and Lucki, 2013a), however some studies demonstrate this effect lasting up to 2 weeks (Maeng et al., 2008). Other investigators have examined the use of a 10 or 12 day dosing regimen to establish longer-lasting effects of chronic ketamine on depressive-like activity in the FST (Akinfiresoye and Tizabi, 2013; Tizabi et al., 2012). Similar effects have been observed in the TST; ketamine reduced immobility 30 min after administration (Cruz et al., 2009; Koike et al., 2011). For the NSF task (where latency to eat in a novel environment is used as a measure of anxiety, a higher latency indicating a more anxious phenotype), ketamine reduced the latency to eat, with the effective dose varying: 30 min and 24 h following 5–10 mg/kg (Carrier and Kabbaj, 2013; Li et al., 2010) and 30 mg/kg (Iijima et al., 2012). All tests resulted in a reduction in the latency to feed in the novel environment.

In the sucrose preference test, a standard test of anhedonia, administration of a low dose of ketamine (0.5 mg/kg) for 10 days significantly increased sucrose consumption in WKY rats (Akinfiresoye and Tizabi, 2013). The increases in sucrose consumption in rats persisted at 1, 3, 5, and 7 days after a single treatment with ketamine (10 mg/kg) (Li et al., 2011b), indicating significant protracted effects of ketamine on this behaviour.

Ketamine induced an anxiolytic phenotype in rats during exposure to the EPM 30 min after a single ketamine injection (Engin et al., 2009). A similar effect was observed in mice 1 and 2 h following treatment (Hayase et al.,

2006).However, these effects were only observed at higher doses of ketamine (30 mg/kg), while lower doses were ineffective in stress naïve mice (Autry et al., 2011). Taking into account the injection time and the high doses it cannot be ruled out that there is a locomotor effect from ketamine causing the anxiolytic phenotype observed.

Locomotor activity is often monitored alongside antidepressant and anxiolytic effects due to a potential confounding of results of behavioural tasks, as increases in activity may lead to false positives. However, ketamine is more likely to have no effect or reduce locomotor activity. Engin et al. (2009) observed a reduction in open field activity in rats 30 mins following ketamine injection (50 mg/kg). Gigliucci et al. (2013) found similar results at 1 h post-injection (10 and 25 mg/kg). A bolus injection of ketamine had no effect on locomotor activity after 30 min post-injection in rats (Akinfiresoye and Tizabi, 2013; Reus et al., 2011; Tizabi et al., 2012; Yang et al., 2012). At 24 h post-injection, ketamine had no effect on locomotor activity in mice; neither did the NMDA antagonists, CPP and MK-801 (Autry et al., 2011).

Ketamine has also been tested in models of depression; in particular the stress-induced CMS paradigm. Ketamine reversed the behavioural and physiological alterations induced by CMS in rats following acute administration and the effects were maintained following chronic treatment (Browne and Lucki, 2013b). Acute and chronic treatment with ketamine reversed the increase in adrenal weight and normalized circulating corticosterone and ACTH levels (Garcia et al., 2009). Physiological alterations induced by CMS were reversed by acute ketamine treatment in a similar study but failed to reverse CMS-induced anhedonia in the SPT (Rezin et al., 2009). Rezin et al. (2009) observed similar results with acute ketamine reversing physiological alterations induced by CMS but there were no changes to depressive-like behaviours. Adolescent rats that underwent CMS displayed decreased immobility, increased sucrose consumption and latency to feed following acute ketamine treatment (Parise et al., 2013).

As mentioned previously, the CMS model is an accepted rodent model of depression and demonstrates how stress-induced models are a suitable method with which to screen the antidepressant-like effects of novel therapeutics like ketamine. Few studies have examined the impact of ketamine treatment on the changes induced by isolation rearing of rats, which is the focus of this chapter.

4.1.3 Aims and Objectives

The aims of the current chapter are to evaluate ketamine's ability to attenuate or reverse the physiological and behavioural changes produced by 5 weeks of post-weaning isolation rearing. As with previous chapters, a battery of behavioural tasks was used to assess cognitive, depressive and anxiety-like behaviours known to be altered in isolation-reared rats. Physiological analysis was also conducted on plasma corticosterone levels following an acute stressor (restraint) and post-mortem tissue was taken to quantify neurogenesis by counting DCX positive cells in the hippocampal dentate gyrus. Specific objectives were:

- To investigate the effects of isolation-rearing on behavioural/cognitive/physiological measures.
- To investigate if the changes induced by isolation-rearing can be prevented or reversed by repeated administration of ketamine.

4.2. Methods

4.2.1 Animals

The animals used for the study were male Lister-hooded rats obtained from Charles River UK. For this study, 66 rats arrived at post-natal day (PND) 19-22 in 10 litters of 6-8, with their dam.

4.2.3 Isolation Rearing

The rearing paradigm employed was the same as adopted in previous chapters. Each litter was split equally into groups of three (GH) or one per cage (SI) in this gave a total of n=33 single housed and n=33 group housed. Animals were housed for a total of five weeks before behavioural testing began.

4.2.4 BrdU Administration/Transponder Implantation

During week four, rats were injected i.p. with BrdU (5-bromo-2'-deoxyuridine; Sigma Aldrich, 150 mg/kg). Rats also received a subcutaneous implant with a temperature transponder (Animalcare ltd) between the shoulder blades using an injector to enable temperature measurement.

4.2.5 Ketamine Administration

Ketamine hydrochloride was obtained from Sigma Aldrich. Each housing group of rats (n=33) was divided into 'Control' (CON) and 'Ketamine' (KET) (n=16-17). Ketamine was administered 24 h before each behavioural task at a dose of 5 mg/kg i.p. This dose was selected based on advice from collaborators working with ketamine that suggested the dose had behavioural effects for up to seven days and previous studies examining ketamine as a novel therapeutic for depression (Garcia et al., 2008). Based on this evidence the current dosing regimen of one injection per week would be sufficient without becoming chronic administration.

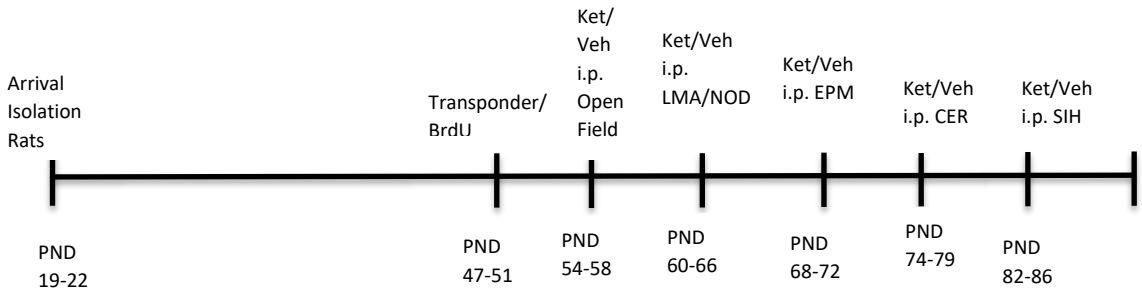


Figure 4.2.1 Timeline of the Protocol: from the arrival of the animals until the end of the behavioural testing. PND: Post-natal day (age of the rat) Behavioural paradigms are OF: Open Field, LMA: Locomotor Activity, NOR: Novel object recognition, EPM: Elevated plus maze, CFR: Conditioned Freezing response and SIH: Stress-induced hyperthermia.

4.2.6 Behaviour Assessments

Behavioural tests and their protocols were the same as previously adopted (see Chapter 2 methodology).

4.2.7 Post-Mortem

All post-mortem analysis was conducted as described in Chapter 3 (section 3.2.).

4.2.8 Statistical analysis

All data were analysed as previously described (section 3.2.12). All analyses were performed using GraphPad Prism 6 and SPSS 22 software, values presented are mean $\pm$ sem. and $p<0.05$ was considered significant.

4.3 Results

4.3.1 The Effects of Isolation-Rearing and Ketamine on Behaviour in the Open Field Task

The open field task was used to measure both locomotor activity and anxiety.

Distance and Velocity

Distance travelled (cm) in the open field was measured. It was found there was a significant main effect of housing; SI rats travelled further than GH rats (GH or SI, n=33 per group) ($F_{(1, 62)} = 4.791$, P=0.03, Fig 4.3.1 A). There was no main effect of treatment ($F_{(1, 62)} =0.59$, P=0.45, 4.3.1 A). A Sidak post-hoc analysis showed there was a significant increase in distance travelled (P<0.05, Fig 4.3.1 A) in the SI/KET group compared to GH/KET. Average velocity was also assessed in the open field, once again there was a significant main effect of housing such that SI rats had a higher average velocity than GH rats ($F_{(1, 62)} = 4.639$, P=0.04, Fig 4.3.1 B). There was no main effect of treatment ($F_{(1, 62)} =0.59$, P=0.45, Fig 4.3.1 B) and a Sidak post-hoc test showed there was a significant increase in velocity (P<0.05, Fig 4.3.1) in the SI/KET group compared to GH/KET.

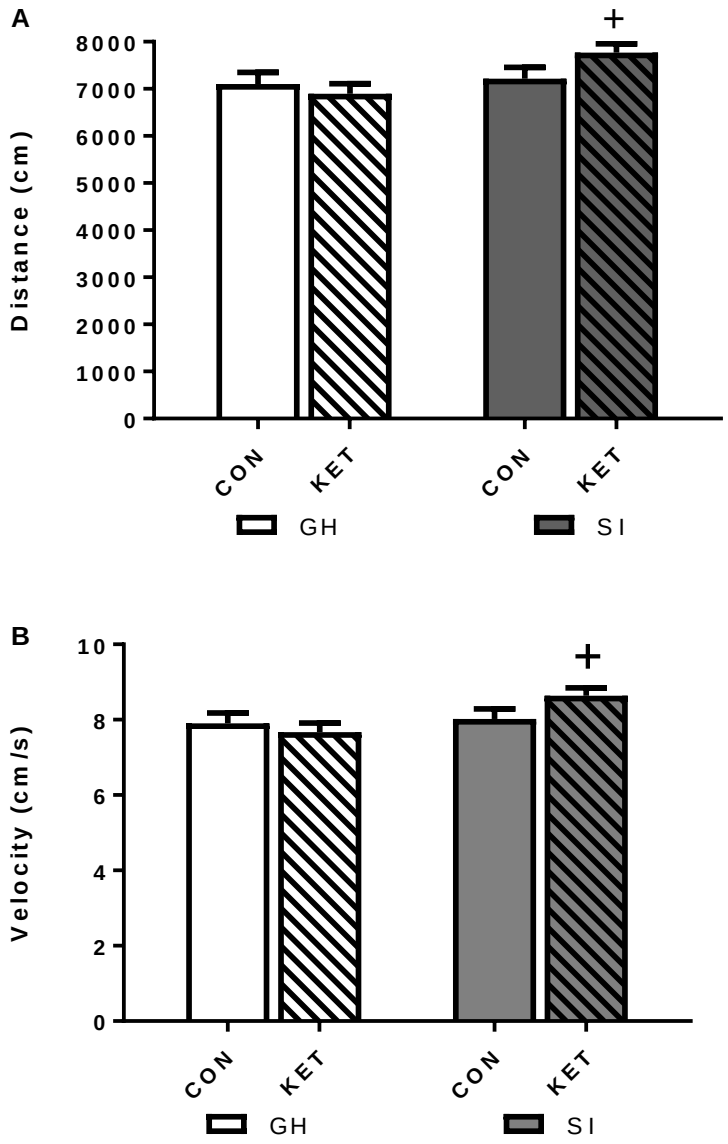


Figure 4.3.1 Distance and Velocity in Open Field: Comparison of (A) distance travelled (cm, mean ± sem, n=16-17 per group) and (B) average velocity (cm/s) by GH/CON, GH/KET, SI/CON and SI/KET rats recorded in the open field. (A) Two-way ANOVA showed a significant main effect of housing ($P<0.05$). +$P<0.05$ Sidak post-hoc test between GH/KET and SI/KET. (B) Two-way ANOVA showed a significant effect main effect of housing ($P<0.05$). +$P<0.05$ Sidak post-hoc test between GH/KET and SI/KET.

Time Spent in Central Zone

Time spent in the central zone of the open field was examined as an index of anxiety. There was a significant main effect of housing ($F_{(1, 62)} = 8.24$, $P<0.01$, Fig 4.3.2) with GH rats spending more time than SI rats in the central zone of

the open field consistent with an anxiogenic profile in the isolation reared rats. There was no significant effect of treatment following ketamine ($F_{(1, 62)} = 0.66$, P=0.42, Fig 4.3.2) and no post-hoc differences between any groups.

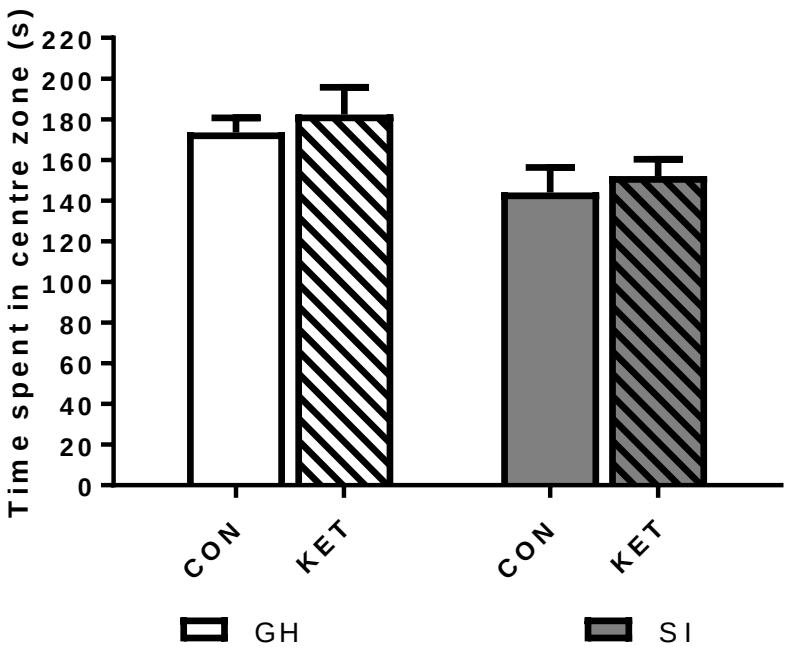


Figure 4.3.2: Comparison of time spent in centre zone: (s, mean ± sem, n=16-17 per group) in the open field task. A two-way ANOVA showed a significant main effect of housing (P<0.01). Sidak post-hoc test revealed no differences between groups.

Grooming and Rearing

Two behaviours were measured in the open field task, grooming and rearing as further ethological markers relevant to an anxious phenotype. For grooming behaviour there was no significant main effect of housing ($F_{(1, 62)} = 0.64$, P=0.43, Fig 4.3.3 A) and no main effect of treatment ($F_{(1, 62)} = 0.21$, P=0.65, Fig 4.3.3 A) .Similarly, there was no main effect of housing ($F_{(1, 62)} = 0.46$, P= 0.49, Fig 4.3.3 B) or treatment ($F_{(1, 62)} = 0.01$, P= 0.92, Fig 4.3.3 B) on the number of rears.

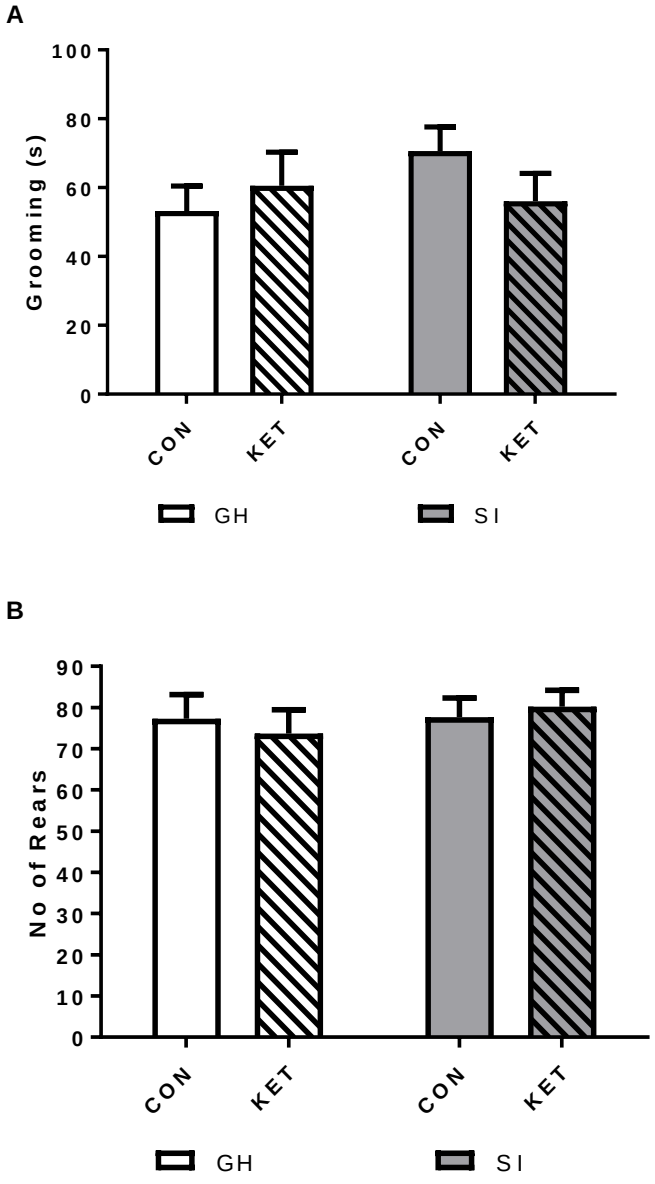


Figure 4.3.3Grooming and Rearing in the Open Field : Comparison of (A) time spent grooming (s mean ± sem, n=16-17) and (B) number of rears in the open field task. A two-way ANOVA revealed no significant differences between groups in either time spent grooming (A) or number of rears (B).

Faecal Boli

The number of faecal boli remaining after each trial in the open field was counted and there was a significant effect of housing ($F_{(1, 62)}$= 9.96, P<0.01, Fig 4.3.4) such that GH produced significantly more than SI rats. However, Sidak post-hoc test showed there was no significant difference between any of the groups, so the number of boli was reduced by isolation irrespective of ketamine treatment.

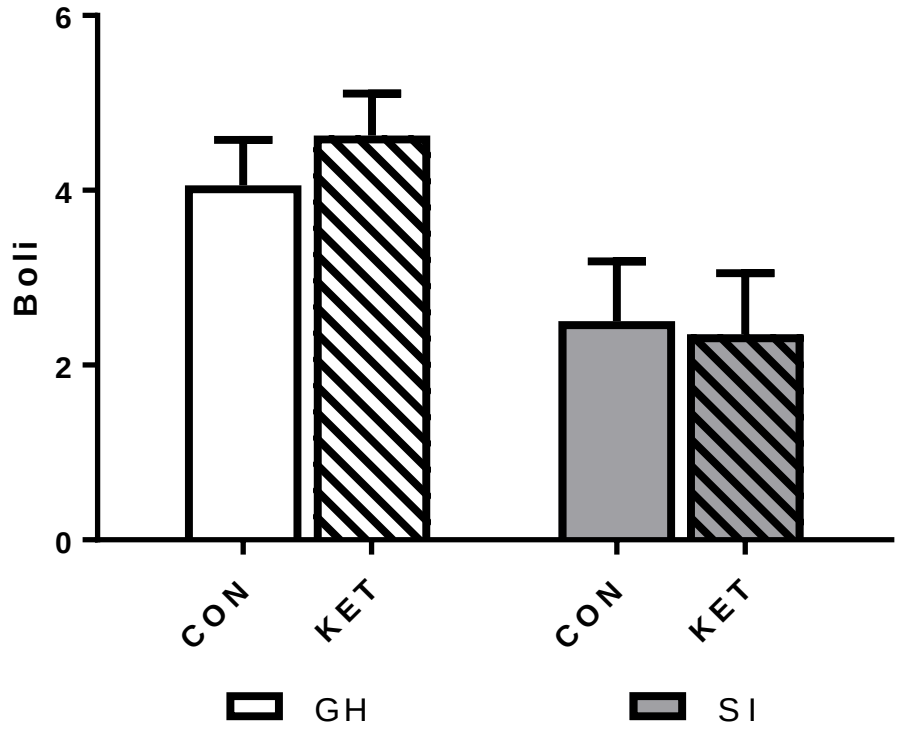


Figure 4.3.4 No of Boli: Comparison of number of fecal boli (mean ± sem, n=16-17) recorded after the open field test was complete. A two-way ANOVA showed a significant main effect of housing (P<0.01).

4.3.2 The Effect of Isolation-rearing and Ketamine on the Locomotor Activity Task

Ambulations

Across the trial, there was a significant main effect of housing GH having significantly less horizontal activity than SI rats ($F_{(1,62)}$ =18.15, P<0.001, Fig 4.3.5 A). However, there was no main effect of treatment ($F_{(1, 62)}$ =0.74, P=0.39, Fig 4.3.5 A) and there was no housing x treatment interaction ($F_{(1, 62)}$ = 2.82, P=0.09, Fig 4.3.5 A). Sidak post-hoc tests revealed a significant increase in ambulations in SI/CON compared to GH/KET at 15 min time point (P<0.05), 25 min time point (P<0.05) and 60 min time point (P<0.05). There was a significant increase in ambulations in SI/KET in comparison with GH/KET at the 15 min time point (P<0.05), 20 min time point (P<0.01), 25 min time point (P<0.001), 30 min time point (P<0.05) and 45 min time point (P<0.05) (Fig 4.3.5 A). For the total number of ambulations, there was a significant main effect of housing on ambulation in the activity box; GH having significantly less horizontal activity than SI rats ($F_{(1,62)}$ =18.57, P<0.001, Fig 4.3.5 B). However, there was no main effect of treatment ($F_{(1, 62)}$ =0.95, P=0.33, Fig 4.3.5 B) and there was no housing x treatment interaction ($F_{(1, 62)}$ = 2.82, P=0.09, Fig 4.3.5 B) so the hyperactivity in isolates was unaltered by ketamine. Sidak post-hoc test showed there was a significant increase in SI/CON rats compared to GH/KET rats (P<0.01, Fig 4.3.5 B) and also a significant increase in SI/KET compared to GH/KET rats ($F_{(1, 62)}$ = 4.29, P<0.001, Fig 4.3.5).

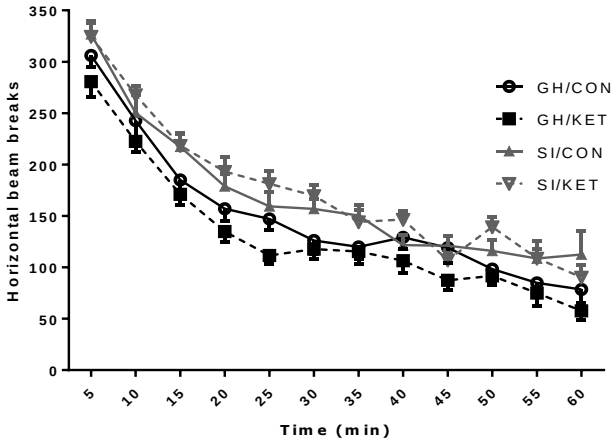


Figure 4.3.5: Comparison of the number of ambulations (A) across a 1 hour period (B) total (mean ± sem, n=16-17). A two-way ANOVA revealed a significant main effect of housing (P<0.001).

Rearing

The number of rears was also measured in the chamber although this behaviour was excluded for one rat where there was a problem with the recording. There was no significant main effect of housing ($F_{(1, 61)} = 2.56$, P=0.11 Fig 4.3.6) or treatment ($F_{(1, 61)} = 2.09$, P=0.11, Fig 4.3.6) and no interaction ($F_{(1, 61)} = 1.08$, P=0.3, Fig 4.3.6).

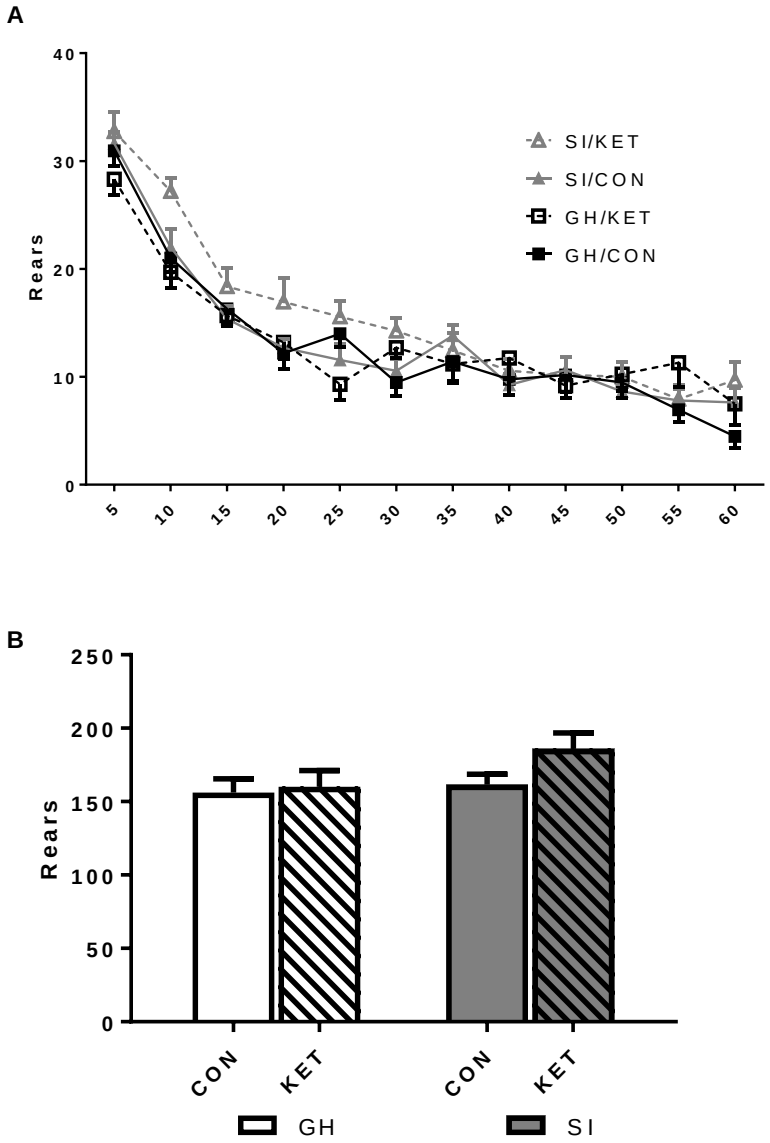


Figure 4.3.6 Rearing in LMA Task: Comparison of number of rears in the locomotor activity task. A two way ANOVA revealed no differences between groups.

4.3.3 The Effect of Isolation-rearing and Ketamine in the Novel Object Discrimination Task.

The novel object discrimination task was employed to assess changes in visual recognition memory.

Familiarisation Trial

During the first familiarisation trial with two identical objects there was no significant main effect of housing ($F_{(1, 124)} = 3.79$, $P = 0.054$, Fig 4.3.7) treatment ($F_{(1, 124)} = 0.18$, $P = 0.67$, Fig 4.3.7) or object ($F_{(1, 124)} = 0.89$, $P = 0.77$ Fig 4.3.7) on exploration. Thus, none of the groups showed any preference for either of the identical objects in the trial and all investigate both objects equally.

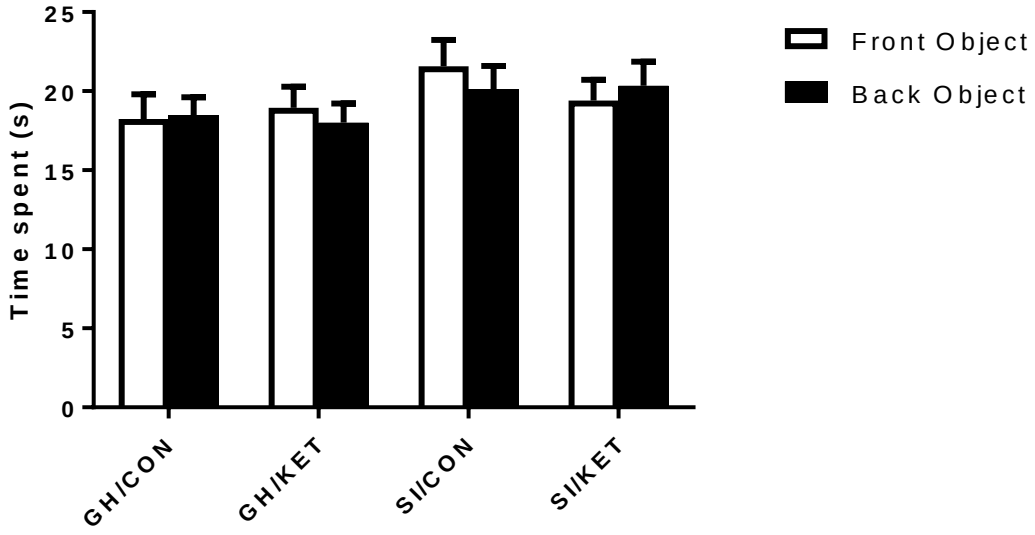


Figure 4.3.7: Comparison of time spent exploring (s, mean ± sem, n=16-17) in the familiarisation trial of the NOD task. There were no significant differences between groups.

Choice Trial

Although there was the expected significant main effect of object ($F_{(1, 124)} = 86.95$, $P<0.001$, Fig 4.3.8) during the second choice trial, such that the familiar object was explored significantly less than the novel in every group, there was no main effect of housing ($F_{(1, 124)} = 0.29$, $P= 0.59$, Fig 4.3.8) or treatment ($F_{(1, 124)} = 0.95$, $P= 0.33$ Fig 4.3.8). Sidak post-hoc tests showed there was a significant increase in time spent exploring the novel compared to the familiar object in all 4 groups (GH/CON $P<0.001$; GH/KET $P<0.001$; SI/CON $P<0.01$; and SI/KET $P<0.001$).

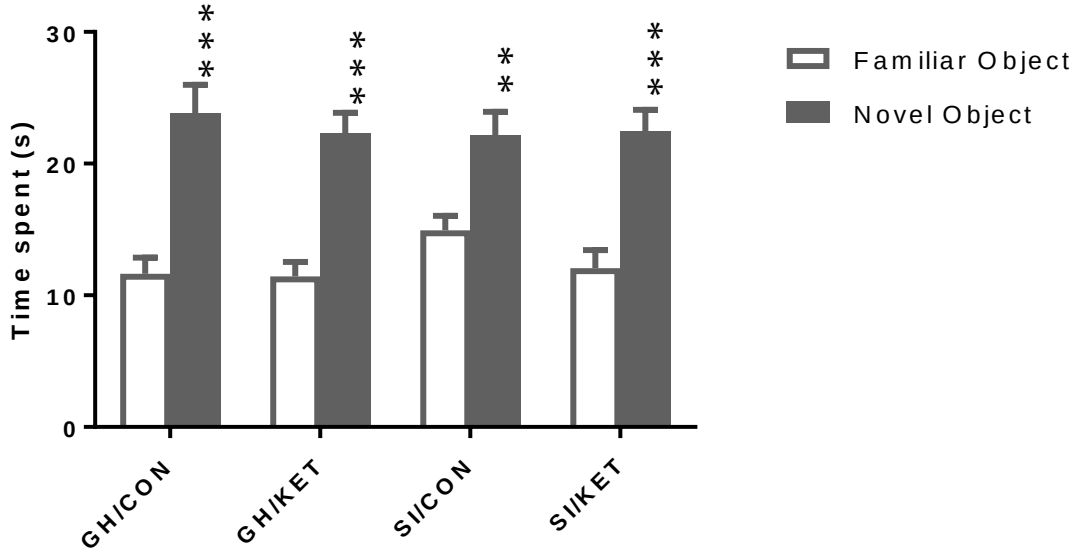


Figure 4.3.8: Comparison of time spent exploring (s, mean ± sem, n=16-17) in the choice trial of the NOD task. A two-way ANOVA revealed there were no significant differences between groups, but there was a main effect of object ($P<0.001$). Thus, all rats preferentially explored the novel object in the choice trial irrespective of housing or ketamine treatment. ** $P<0.01$ Sidak post-hoc test between familiar and novel object. *** $P<0.001$ Sidak post-hoc test between familiar and novel object

Discrimination Ratio

The discrimination ratio was also assessed to provide a further derived index of visual recognition memory. However, there was no significant main effect of housing ($F_{(1, 62)} =2.29$, $P=0.27$, Fig 4.3.9) or treatment ($F_{(1, 62)} =1.2$, $P=0.28$, Fig 4.3.9).

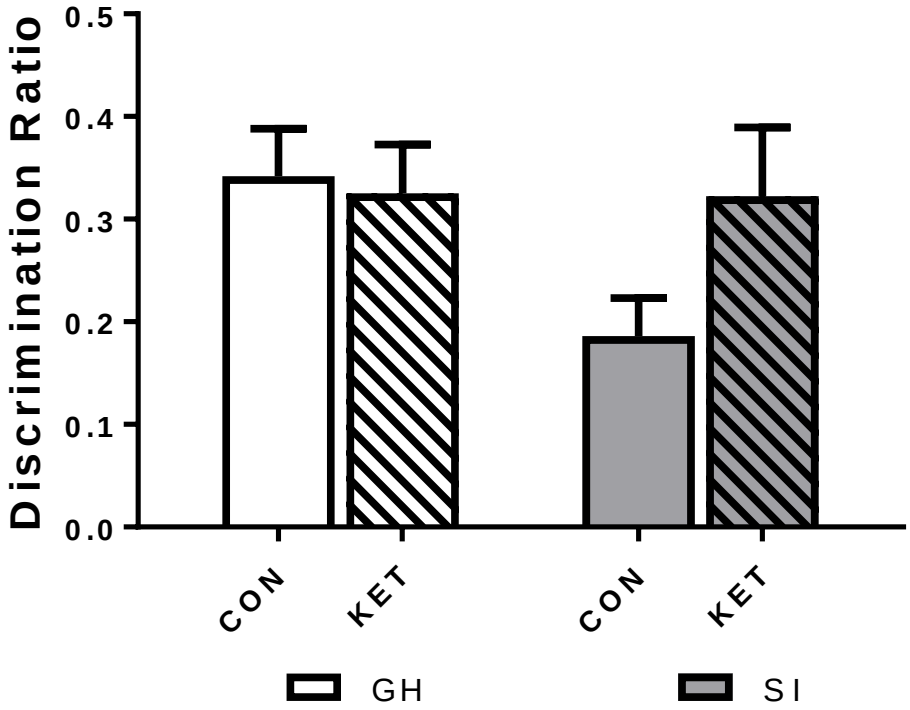


Figure 4.3.9 Discrimination Ratio: Comparison of the discrimination ratio (mean ± sem, n=16-17) in the familiarisation trial of the NOD task.

4.3.4 The Effects of Isolation-rearing and Ketamine Treatment on Anxiety Related Behaviour in the Elevated Plus Maze.

% Time Spent in Open Arms

The amount of time spent in the open arms of the plus maze were measured and expressed as a % and then analysed. There was no main effect of housing ($F_{(1, 60)} = 0.03$, P=0.86, Fig 4.3.10) or treatment ($F_{(1, 60)} = 0.16$, P=0.69, Fig 4.3.10) and no interaction effect ($F_{(1, 60)} = 0.57$, P= 0.45, Fig 4.3.10).

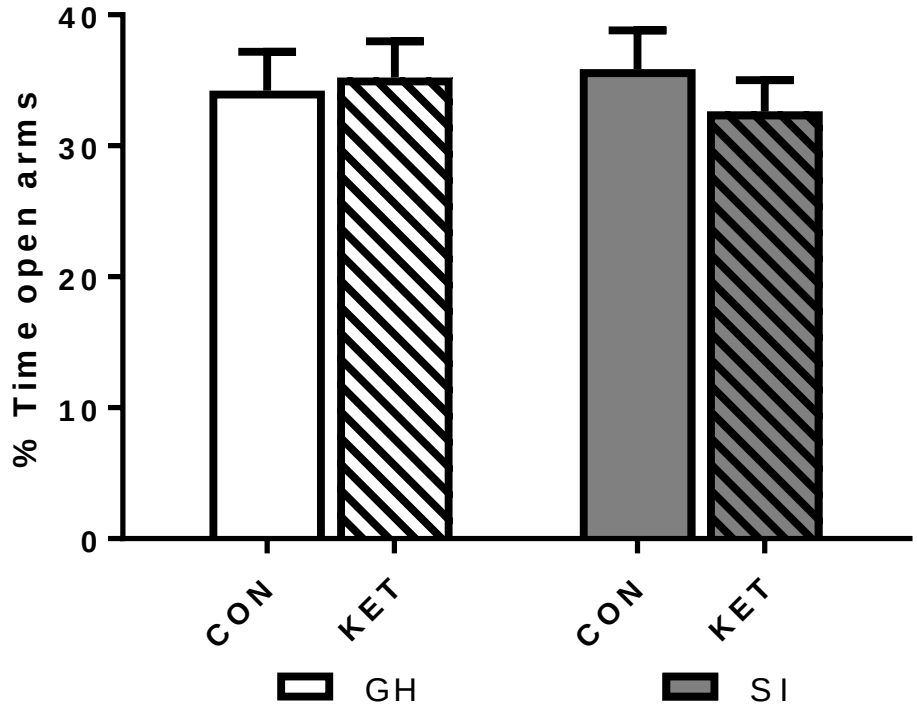


Figure 4.3.10 Percentage of time spent in the open arms of the plus maze: Two animals were eliminated from the data after falling from the maze twice (%, mean ± sem, n=15-17). No significant differences were found between groups.

Head Dips and Stretch Attends

There was no main effect of housing ($F_{(1, 60)} = 0.8103$, P=0.37, Fig 4.3.11 A) or treatment on the number of head dips ($F_{(1, 60)} = 3.111$, P=0.08, Fig 4.3.11 A) and no interaction ($F_{(1, 60)} = 0.00191$, P = 0.96, Fig 4.3.11 A). Again for stretch attend posture, there was no main effect of housing ($F_{(1, 60)} = 2.028$, P= 0.16, Fig) or treatment ($F_{(1, 60)} = 0.4479$, P= 0.51, Fig 4.3.11 B) but a significant interaction was observed ($F_{(1, 60)} = 4.557$, P<0.05, Fig 4.3.11 B) indicating that

ketamine might have reduced stretch attends in the GH but increased them in the SI rats, although this failed to reach post-hoc significance.

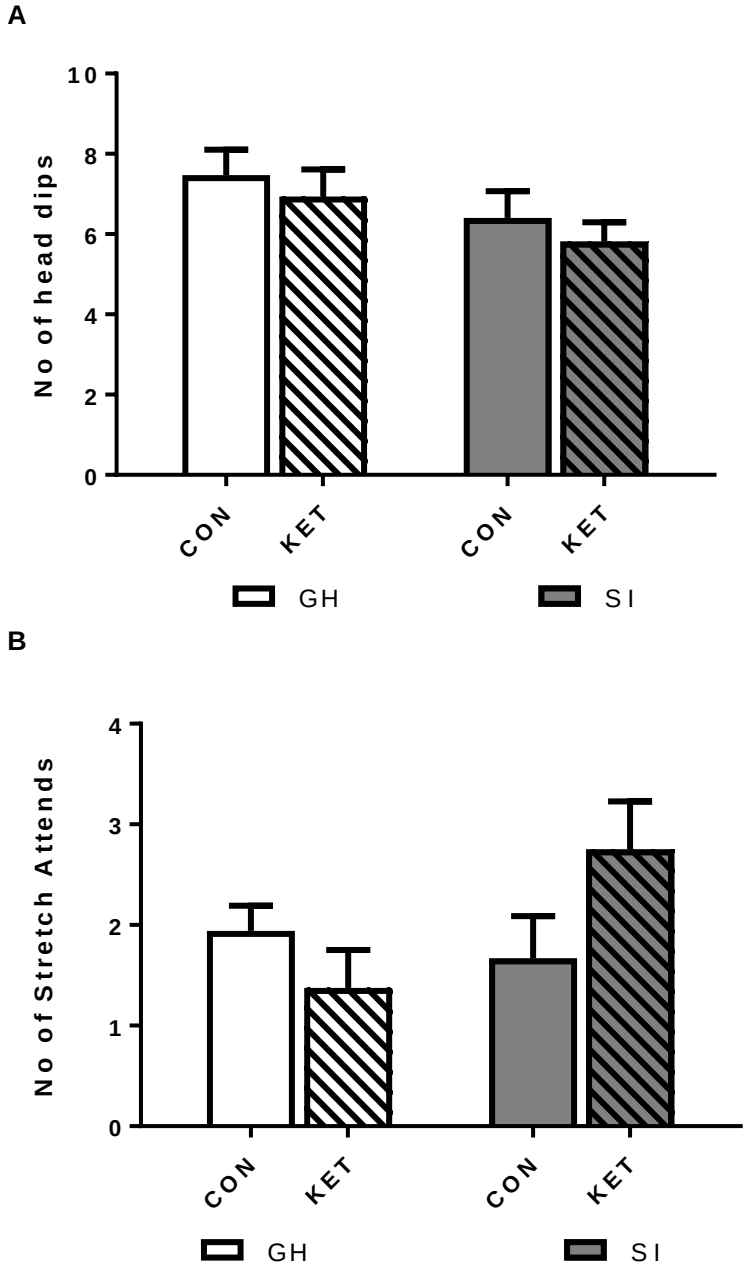


Figure 4.3.11 Head Dips & Stretch Attends: Two animals were eliminated from the data after falling from the maze twice (mean ± sem, n=15-17). (A) No of head dips, no significant differences found between groups. (B) No of stretch attends no significant differences found between groups. Housing x treatment interaction effect.

4.3.5 The Conditioned Freezing Response Task: Effects of Isolation-rearing and Ketamine on Associative Memory.

Acquisition

During the acquisition phase, rats were conditioned to associate foot shocks with a light and tone cue. A two-way ANOVA demonstrated a main effect of housing; with GH freezing more than SI ($F_{(1, 62)}$ =30.88, P<0.001 Fig 4.3.12 A), but no main effect of treatment ($F_{(1, 62)}$ =2.55, P=0.12 Fig 4.3.12 A). A main effect of shock was observed on the time spent freezing post shock 1 and shock 2. All rats also froze significantly more after the second than the first shock. Sidak post-hoc tests showed that there was a significant difference after the first shock between GH/CON freezing more than SI/CON (P<0.01) and SI/KET (P<0.05), GH/KET froze more than SI/CON (P<0.001) and GH/KET more than SI/KET (P<0.001). Following the second shock there was a significant difference between GH/KET which froze longer than SI/CON (P<0.05) and GH/KET froze for longer than SI/KET (P<0.01). Post-hoc tests also showed an increase in freezing time after the second shock in comparison with the first shock in GH/CON (P<0.05), SI/CON (P<0.001) and SI/KET (P<0.01).

Retention

The retention phase measured freezing behaviour in response to return to the conditioned cue/context as an index of associative memory at 24 and 48 h after exposure to the stimulus paring. A two-way repeated measures ANOVA revealed a main effect of trial (24 h, 48 h pre-tone and 48 h post-tone; $F_{(2, 124)}$ = 80.36, P<0.001, Fig 4.3.12 B), indicating that all groups showed a reduction in freezing time during the 48 h pre-tone trial in comparison with the 24 h trial but on presentation on the light/tone cue, all groups showed an increase in freezing time again. Across the three retention trials there was no main effect of housing ($F_{(1, 62)}$ = 2.78, P=0.1, Fig 4.3.12. B), there was a main effect of treatment indicating that treatment with ketamine lead to increased freezing time across all trials compared to control groups ($F_{(1,62)}$ = 11.9, P<0.001, Fig 4.3.12 B). When examining each trial individually, the 24 h post-conditioning trial displayed a main effect of treatment with the KET groups freezing for longer than CON groups ($F_{(1, 62)}$ = 12.03, P<0.001, Fig 4.3.12 B) and Sidak post-hoc showed GH/KET freezing more than and SI/CON (P<0.05). The 48 hour pre-tone trial demonstrated no main effects of housing or treatment. The 48 h post-tone trial showed there was a main effect of housing such that GH froze longer than SI rats ($F_{(1, 62)}$ = 4.13, P<0.05). A main effect of treatment was also observed with KET animals freezing more than CON animals ($F_{(1, 62)}$ = 9.286, P<0.01). Sidak post-hoc tests showed significant differences, GH/CON froze more than SI/CON (P<0.05), GH/KET froze more than SI/CON (P<0.01), SI/CON froze less than SI/KET (P<0.01) (Fig 4.3.12 B).

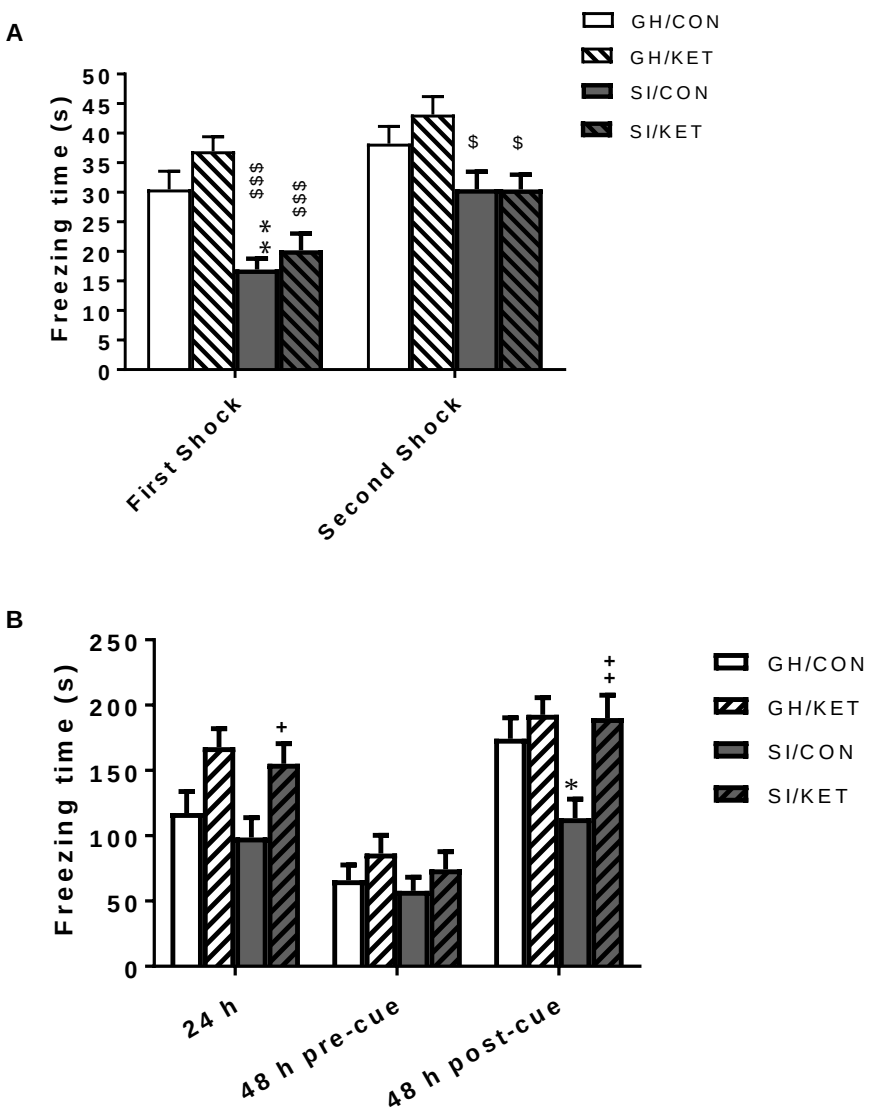


Figure: 4.3.12 (A) Comparison of freezing time (s, mean ± sem, n=16-17) post first and second shock in the acquisition phase of the task. Two-way ANOVA showed a main effect of housing (P<0.001) and shock (P<0.001). **P<0.01 from GH/CON $P<0.05 from GH/KET, $$$P<0.001 from GH/KET, Sidak post-hoc test. (B) Comparison of time spent freezing (s mean ± sem, n=16-17) in the three retention trials post-shock. Two-way repeated measures ANOVA revealed a main effect of trial (P<0.001) and treatment (P<0.001). At the 24 h trial, there was a main effect of treatment (P<0.001), at the 48 h post-tone trial there was a main effect of housing (P<0.05) and treatment (P<0.01). *P<0.05 from GH/CON, +P<0.05 from SI/CON, ++P<0.01 from SI/CON, Sidak post-hoc test.

4.3.6 Temperature and Corticosterone Changes during Stress-Induced Hyperthermia

10 minute temperature changes

Following restraint in a tube, there was no main effect of housing ($F_{(1, 58)} = 3.05$, P=0.08, Fig 4.3.13 A), treatment ($F_{(1, 58)} = 0.01$, P=0.93, Fig 4.3.13 A), or restraint ($F_{(1, 58)} = 0.13$, P=0.72, Fig 4.3.13 A) on the body temperature change measured from the telemetry implant. There was a significant interaction effect between housing and restraint ($F_{(1, 58)} = 13.27$ P<0.001, Fig 4.3.13 A) but no post-hoc differences between any treatment groups (Fig 4.3.13 A).

30 min temperature changes

Changes in temperature was also assessed at 5 min intervals for 30 min. There was no main effect of housing ($F_{(1, 58)} = 3.79$, P=0.06, Fig 4.3.13 B) or treatment ($F_{(1, 58)} = 0.53$, P=0.47, Fig 4.3.13 B) there was a significant main effect of restraint indicating that restraint significantly increased temperature compared to the non-restraint group ($F_{(1, 58)} = 4.47$, P<0.05, Fig 4.3.13 B) and there was also an interaction effect between housing and restraint ($F_{(1, 58)} = 9.67$, P<0.01, Fig 4.3.14 B). Post-hoc analysis revealed a significant difference with GH/CON/Non-restraint rats whose temperature was significantly lower than SI/VEH/Non-restraint (P<0.05), and GH/VEH/Non-restraint was lower than GH/KET/Restraint (P<0.05) (Fig 4.3.13 B).

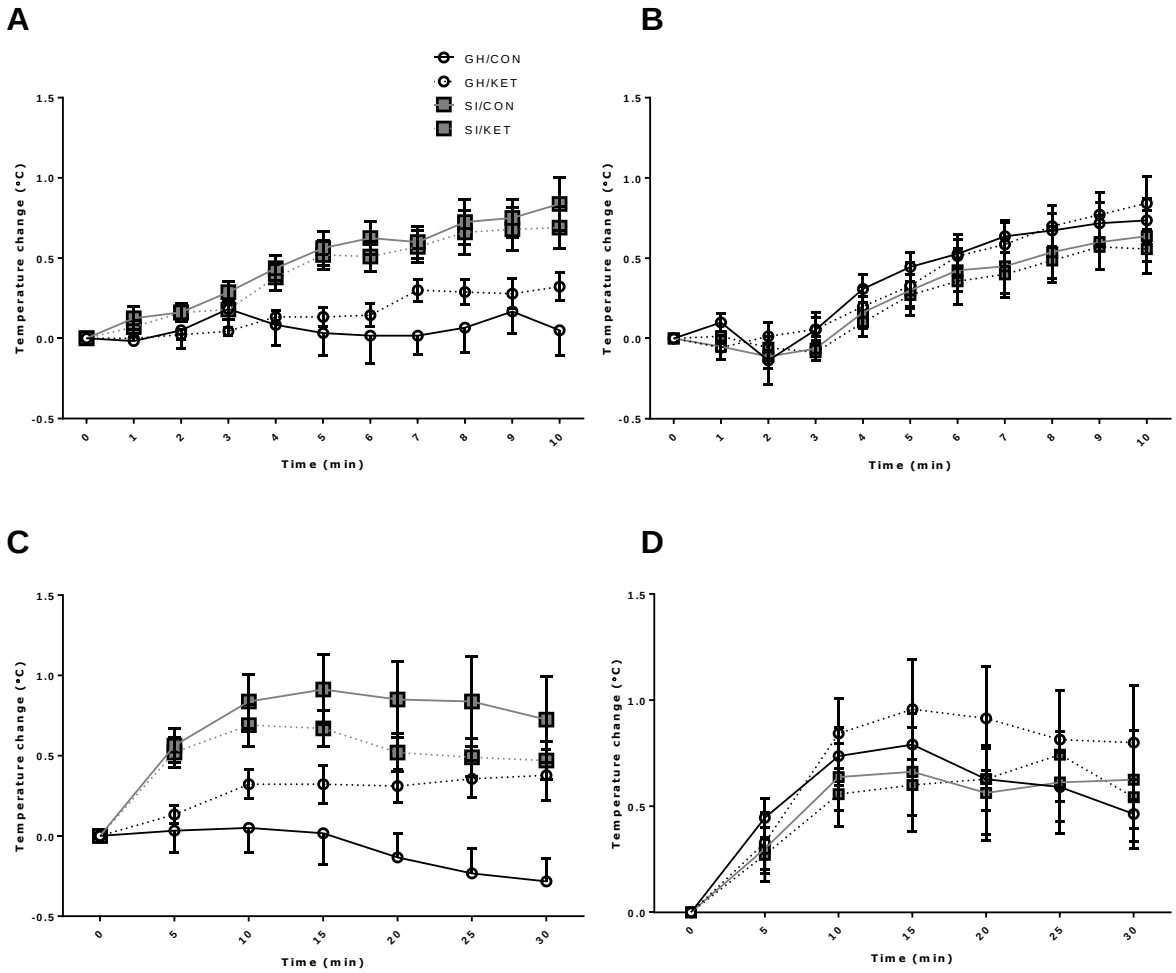


Figure 4.3.13 Temperature Change in SIH: Comparison of temperature change (°C, mean ± sem, n=6-11) (A) The first 10 min of restraint stress. (B)First 10 minutes of home cage animals. Two-way repeated measures ANOVA revealed a significant interaction effect between housing and restraint in the first 10 minutes ($P<0.001$) there were no statistical differences between groups post-hoc. (C) Temperature change in 5 min time bins across 30 min period with restraint (D) Temperature change in 5 min time bins across 30 min period while in the home cage. Two-way ANOVA showed a main effect of restraint ($P<0.05$) and a main interaction effect between housing and restraint ($P<0.01$) over the 30 minute period. Post-hoc tests revealed a significant difference with GH/CON/Non-restraint rat temperature being significantly lower than SI/VEH/Non-restraint ($P<0.05$), and GH/VEH/Non-restraint was lower than GH/KET/Restraint ($P<0.05$) across the full trial.

Corticosterone Changes

Immediately following 30 min restraint and body temperature measurement, the rats were removed from restraint tubes or their home cage and immediately killed, plasma was collected for corticosterone analysis. There was a main effect of restraint ($F_{(1, 58)} = 53.82$, $P<0.001$, Fig 4.3.14) which demonstrated that restraint stress significantly increased plasma corticosterone as would be expected. However, there was no main effect of treatment ($F_{(1, 58)} = 0.05$, $P=0.83$, Fig 4.3.14) on plasma corticosterone irrespective of restraint condition. Post-hoc analysis showed that each treatment group's respective restraint group had significantly higher levels of plasma corticosterone than those in the home cage, (GH/CON, $P<0.01$) (GH/KET, $P<0.01$) (SI/KET, $P<0.001$) excluding SI/CON ($P=0.78$) except for the SI/CON where this apparent difference was not significant (Fig 4.3.14).

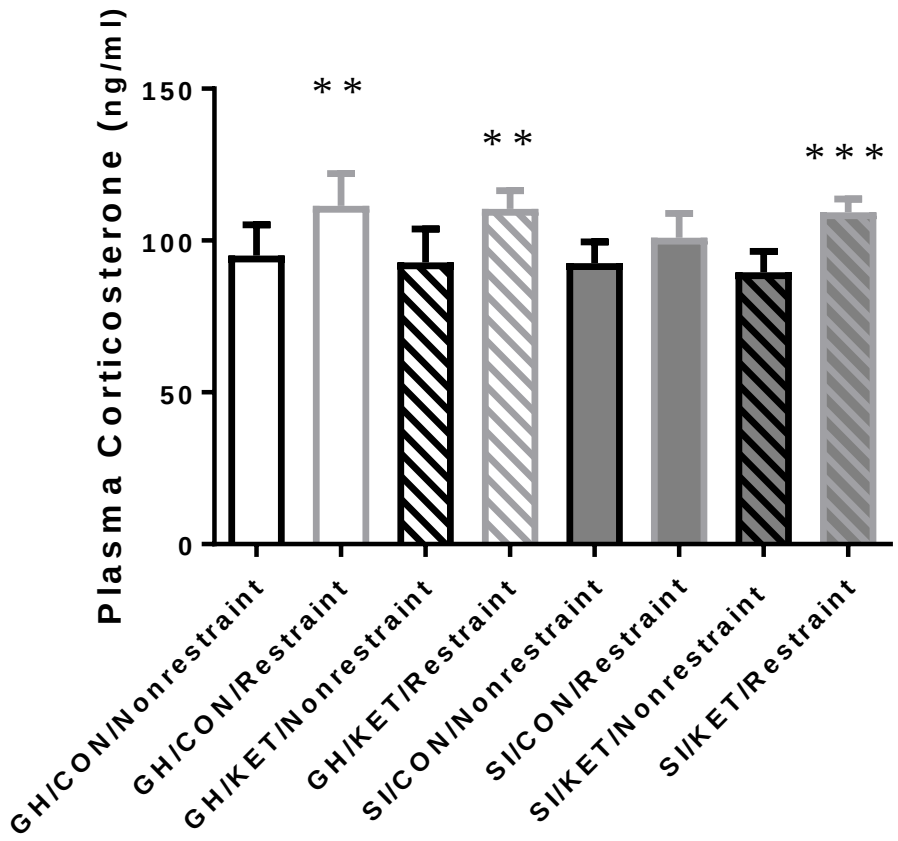


Figure 4.3.14 Corticosterone Changes: Comparison of changes in plasma corticosterone (ng/ml, mean ± sem, n=6-11) after 30 min of restraint stress. Two-way ANOVA showed a main effect of restraint (P<0.001). **=P<0.01 from non-restraint, ***=P<0.001 from non-restraint, Sidak post-hoc test.

4.3.7 Hippocampal Neurogenesis

There was no main effect of housing ($F_{(1, 58)} = 0.002$ P=0.96, Fig 4.3.15) or treatment ($F_{(1, 58)} = 2.61$, P=0.11, Fig 4.3.15) indicating that neither isolation rearing nor treatment with ketamine had any effect on the number of DCX positive neurones in the dentate gyrus as an index of levels of hippocampal neurogenesis.

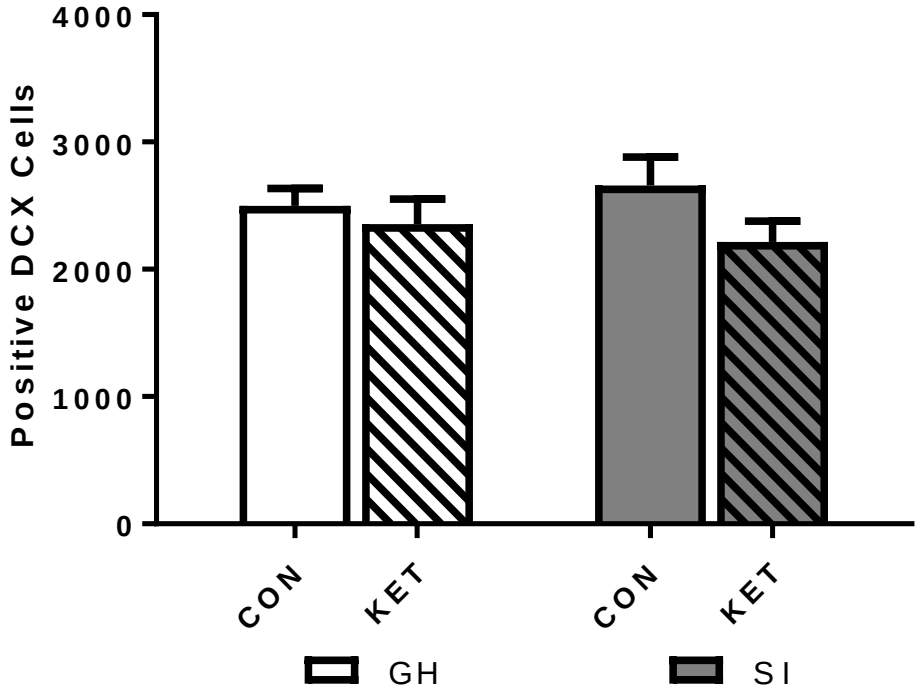


Figure 4.3.15 Levels of DCX Positive Cells: Comparison of the total number of cells positive for DCX (mean ± sem, n=16-17) in the hippocampal dentate gyrus. There were no main effects or significant differences between any of the four groups.

4.4. Discussion

The current study aimed to assess the ability of acute ketamine injection to alter the behavioural effects of the isolation-rearing paradigm and to provide potential preclinical data evaluating a possible mechanism for its reported use as a fast-acting treatment for depression. The behaviours examined included anxiety-like behaviour, cognitive dysfunction and alterations in neurogenesis, and the stress response, both behaviourally and through measurement of plasma corticosterone post-mortem.

There was a significant effect of housing conditions in the open field on both distance travelled and average velocity. For housing, SI rats were hyperactive compared to GH rats, which supports findings already presented in Chapters 2 and 3 and in many published reports. As mentioned previously, hyperactivity to a novel environment are common and long established characteristics in isolation-reared rats (Sahakian et al., 1977 Gentsch et al., 1981, Gentsch et al., 1982, Bianchi et al, 2006, Watson et al, 2012). This is thought to result, at least in part, from increased dopaminergic neuronal activity in the ventral tegmental area neurones projecting to the nucleus accumbens (Fabricius et al., 2010), which can be attenuated by antipsychotics with dopamine D2 receptor antagonist activity (Watson et al., 2016) .

Time spent in the central zone was also examined in the open field and, as expected, SI rats did not spend as much time in this aversive central area as GH rats, which is indicative of a potential anxiogenic effect of isolation rearing. This is in contrast with what was observed in the previous study, where there were no differences in time spent in either zone between GH and SI rats. Irrespective of this, treatment with ketamine had no effect on the zonal pattern of exploration. Grooming and rearing were also examined but, in both cases, there were no differences in either behaviour as a consequence of either isolation or drug treatment. There was a significant change in number of faecal boli in the arena after the task. SI rats produce fewer boli, which has been proposed to indicate a heightened state of anxiety, reflecting activation of the parasympathetic nervous system and leading to decreased levels of defecation (Perhach and Barry, 1970). However, other studies report increases in faecal boli following increased stress (Saito et al., 2005).

In the LMA task, SI rats showed more horizontal ambulatory activity than GH rats in 5 min epochs across the 1 h trial such that there was a significant increase in total number of beam breaks in the SI compared to GH rats, irrespective of ketamine. Both vehicle and ketamine treated isolation-reared groups displayed significantly higher levels of activity than the GH/KET group. It could be argued that treatment with ketamine in the GH animals caused a decrease in activity, which could be expected in acute treatment with ketamine due to a sedative effect reducing activity. However, our choice of dose (5 mg/kg) and time point

of behavioural measurement (24 h post ketamine injection) would suggest this is unlikely to be a confounding factor, as ketamine's half-life is around 3 h. When number of rears was examined in the activity boxes, neither housing nor treatment had an overall significant effect.

In the novel object discrimination task of visual recognition memory, there was no difference in object exploration in the familiarisation trial for any of the groups. This demonstrates that there was no preference for object position (front or back) and that any differences observed in the subsequent choice trial was due to cognitive impairment and not to a difference in acquisition or fear of exploring the objects. In the choice trial, all groups spent significantly more time exploring the novel object, regardless of treatment or housing. The discrimination (D2) ratio was also examined and there appeared to a deficit in SI/CON, compared to the other treatment groups, but this difference was not significant. As no group had a deficit in recognition memory it was not possible to explore the impact of ketamine on performance in this task. Evidence from previous chapters and studies in our lab contradict the findings of the current study that isolation rearing has no effect on performance in the novel object discrimination task (Bianchi et al., 2006; McIntosh et al., 2013; Watson et al., 2016); this may have resulted from larger than normal inter-individual variation in the data from this cohort of rats.

Previous research into clinical populations has shown that treatment with ketamine can have negative effects on cognition associated with long-term use, in particular visual-spatial disruption and dissociative symptoms related to decreased activation in the right hippocampus and left parahippocampal gyrus (Morgan et al., 2014). Such negative cognitive effects could lead to a lower quality of life. Other groups have proposed that ketamine has a pro-cognitive effect in patients; Lara et al. (2013) showed 77% of patients demonstrated not only improvement in their mood but also their cognition with a sublingual low-dose of ketamine. A review conducted by Lee et al. (2016) found that acute administration of low dose ketamine helped improve visual memory, simple working memory, and complex working memory in individuals with treatment-resistant depression. It has been proposed that acute ketamine may mediate this effect through activation of intracellular proteins BDNF and mTOR (Cavalleri et al., 2017). In contrast to the current findings, acute (Goulart et al., 2010) and chronic low doses (Venâncio et al., 2011) of ketamine have been reported to impair NOR memory and other NMDAR antagonists, such as phencyclidine (PCP) and dizocilpine (MK-801), have been used to induce cognitive deficits in NOR to model symptoms of schizophrenia (Rajagopal et al., 2014).

In the elevated plus maze, neither isolation nor ketamine altered distance or velocity in the current study. The main index of anxiety measured in the plus maze is the percentage time spent in the open arms. Once again, in the current study, there was no difference between housing groups, contrary to what was

observed in previous studies (Parker and Morinan, 1986; Weiss et al., 2004). Thus, this cohort of SI rats failed to exhibit an anxiogenic profile in the EPM compared to their GH counterparts. There was no effect of ketamine treatment on time spent in the open arms. A study conducted by (Engin, Treit, & Dickson, 2009) demonstrated an anxiolytic effect of a single injection of ketamine in the EPM when administered 30 min before testing and this was replicated in mice at 1 and 2 h time points (Hayase et al., 2006).

As well as monitoring time in the aversive open arms, two ethological behaviours were examined in the current study; exploratory head dips and risk-assessment stretch attend postures, both of which are established indices of anxiety in the EPM (Blanchard et al., 1990; Blanchard et al., 1991). In the current study, neither exploratory head dips nor risk-assessment stretch attend postures were altered by housing or treatment although there was an interaction effect with stretch attends, suggesting ketamine treatment reduced them in GH but increased them in SI rats. It should also be noted that in another behavioural measure of anxiety, the NSF task, ketamine successfully reduced the latency to eat at 30 min and 24 h following 5–10 mg/kg administration (Carrier and Kabbaj, 2013; Li et al., 2010) and 30 mg/kg (Iijima et al., 2012) consistent with it mediating a reduction in anxiety-like behaviour.

In order to assess any impact of ketamine on associative learning and memory freezing, behaviour was compared in a conditioned freezing response that has been validated by our group to provide an index of learning and memory (Woods et al., 2012). The results from the current study support findings found in previous chapters that isolation rearing decreased both contextual and cue-dependent associative memory 24 and 48 h post-conditioning. Freezing duration increased with all groups following exposure to the conditioned cue (light/tone) at 48 h after training. The current data are also consistent with another social isolation study that produced deficits in associative learning and memory in a contextual fear conditioning task (Weiss et al., 2004b). From the current study, we can see that isolation rearing caused a deficit in associative learning and memory indicated by reduced freezing times that were both context and cue dependent. Furthermore, treatment with ketamine increased freezing times compared to vehicle at 24 h post-shock and at 48 h post-shock in response to presentation of the light/tone cue. There were no significant differences between the groups at the 48 h post-conditioning but extinction of the fear associated memory from the 24 h group may have been sufficient to attenuate any further effect of ketamine or could indicate that it preferentially restores retention and not extinction processes. Previous research has shown that ketamine neutralised fear-conditioned increases in freezing behaviour (Pietersen et al., 2006), although this is not a consistent finding. Amann et al. (2009) demonstrated that chronic administration of ketamine leads to an impairment in fear-conditioning in mice.

We assessed restraint stress-induced hyperthermia in isolation reared rats, as a final potential index of depressive-like phenotype. By using indwelling telemetry probes, this study was able to monitor core body temperature change repeatedly (as often as every minute), without the need of handling or insertion of a rectal probe, which would itself produce a temperature change as a result of stress. The restraint-induced temperature response was compared to that in the home cage, which reduced the group size from that in other tasks but enabled accurate determination of the change. There was no effect of housing or treatment on temperature change but a highly significant hyperthermia was observed in response to restraint, indicating restraint was an effective stressor, increasing core body temperature compared to the control 'non-restraint' home cage rats as observed in previous chapters. Plasma corticosterone was also significantly elevated by restraint, irrespective of ketamine treatment in agreement data presented in previous chapters. This also supports previous studies undertaken as part of this PhD, demonstrating that the restraint paradigm employed significantly increased plasma corticosterone, regardless of housing condition. The current study also demonstrated that repeated acute treatment with ketamine did not affect the stress response (neither hyperthermia nor corticosterone) induced by restraint.

Neurogenesis was measured by post-mortem analysis of the number of DCX positive cells in the hippocampal dentate gyrus. Surprisingly, results found no significant effect of housing or treatment on the number of hippocampal DCX positive cells in contrast to the decrease following isolation rearing documented in previous chapters. As discussed previously, ketamine treatment has been associated with increases in hippocampal BDNF, which has been shown to upregulate neurogenesis. Ketamine has been shown to have negative effects on hippocampal neurogenesis (Huang et al., 2016). It has also been shown to increase the proportion of functionally mature young granule neurons counted within 2 h and increase cell proliferation and functional maturation, and decrease depressive-like behaviour, for at least 4 weeks in rats given long-term corticosterone administration (to model depression) (Soumier et al., 2016). However, in that study after ablation of neurogenesis, the antidepressant effects remained suggesting ketamines effects on depressive-like behaviour may be independent of neurogenesis.

4.5 Conclusions

The current study demonstrated that isolation rearing from weaning failed to replicate all the normal syndrome of behavioural phenotypes in the tasks examined, both compared to previous chapters presented here and many published studies. It also demonstrated that acute treatment with the NMDA-R antagonist ketamine had limited ability to restore behavioural deficits induced by isolation rearing. One possibility is that the dosing regimen may not be ideal to demonstrate efficacy, but administration was chosen to be given once weekly 24 h before behavioural observation to eliminate any possible confounding acute pharmacological effect of ketamine. It is possible that the sub-chronic dosing regimen may have resulted in compensatory changes, such as NMDA receptor internalisation which could modify the response. As an alternative novel mechanism to induce rapid onset changes in behaviour by alteration of intracellular signalling pathways, the following chapter will examine the effects of a JNK-1 inhibitor peptide administered into the lateral ventricles on SI rats using a similar battery of behavioural tasks.

Chapter 5:

Characterising physiological and behavioural changes in isolation rearing model of depression and the effects of treatment with DJNKI

5.1 Introduction

Chapter 5 characterised anxiety and depressive-like behavioural changes resulting from isolation rearing of rat pups assessed using a battery of behavioural tasks. The current chapter assessed whether a novel potential treatment with a JNK-1 inhibitor could modify some of these behavioural changes. Due to poor blood brain barrier penetration, the selective JNK-1 inhibitor, DJNKI, was administered by I.C.V. injection. The dose selection for DJNKI was based on work conducted by collaborators within the r'BIRTH network (Mohammad et al., 2016b).

One theory of the aetiology of depression is altered immune activation. Raison et al. (2006) described how stress-immune interactions can occur, NF-κB being activated through Toll-like receptors during an immune challenge or through an environmental stressor, which increases pro-inflammatory cytokines (including IL-1β, IL-6 and TNF-α) (O'Connor et al., 2003) these gain access to the brain via leaky regions within the BBB. Once in the brain, these cytokines may activate pathways related to the development of depression. They also cause activation of CRH, an upregulation of ACTH and the HPA axis resulting in increased cortisol levels, often seen in severely depressed patients. This activation can lead to disruption of synaptic plasticity via nerve growth factors (BDNF, NGF) providing a mechanistic link between HPA axis activation and the immunological system.

5.1.2 Clinical Evidence support cytokine role in depression

It was observed by Valentine et al. (1998) that patients undergoing treatment with IFN-α displayed a set of symptoms analogous to that seen in depressive disorders. This effect was so profound that it led investigators to adopt IFN-α as a model of cytokine-induced depression in rodents (Capuron and Miller, 2004; Musselman et al., 2001). Therapeutic administration of IFN-α was associated with depression developing in 30-50 % of patients, which could be treated successfully using the SSRI, paroxetine (Musselman et al 2001). Furthermore depressed patients show increased concentrations of IL-6, IL-1 β, IL-2 and IFN-γ (Maes et al., 1997; Song et al., 1994) and there has also been reports of increased serum IL-1β levels (Griffiths J, 1996), which returned to normal after treatment with the antidepressant sertraline. Increased IL-1β was only observed in patients with typical depression and absent from those suffering from atypical depression. There is strong evidence for increased cytokine levels (IL-1 β, TNF-α and IL-6) in depressed patients and hypercortisolemia is also regularly a symptom in these patients, although a causal relationship has yet to be definitively established. It is well documented that cytokine activation can be triggered acutely through an immunological insult (e.g. LPS administration) or through an environmental stressor (O'Connor et al, 2003); causing upregulation of IL-1β and TNF-α, which in turn triggers IL-6. IL-6 has an inhibitory effect

on IL-1β and TNF-α while at the same time stimulating CRH release (Hirano et al., 1990).

5.1.3 Preclinical evidence supporting role of cytokines in depression

Analogous to evidence shown in clinical populations administration of LPS has been used to examine immunological response in rodents, both post-mortem and *in vivo*. Again administration of LPS challenge elevates IL-1β, IL-6 and TNF-α (Dunn, 1992) which in turn affects CNS monoamine systems. Several studies using cytokine administration, have shown ICV administration of IL-1β, IL-2 TNF-α altered dopaminergic (Connor et al., 1998) and serotonergic (Connor et al., 1999) activity in the rat. Of all the behavioural effects observed after cytokine administration *in vivo*, the most relevant is the increase in anhedonic and anxiety-like behaviours as this is analogous to what is observed when using stress models of depression such as CMS or UCMS. It is well documented that stress releases of cytokines in rats (LeMay et al., 1990; Takaki et al., 1994; Zhou et al., 1993) and increase in IL-6 from psychical and psychological stressors. Connor et al. (2005) also demonstrated that exposing animals to an acute stressor (15 min swimming) reduced cytokine release after an immunological insult, suggesting a modulatory interaction between stress and the cytokine response.

There is evidence to suggest that pro-inflammatory cytokines (IL-1β, TNF-α) can stimulate the uptake of serotonin in preclinical models and that these mechanisms are activated through MAP kinase (Zhu et al., 2006). These pro-inflammatory cytokines lead to activation of the HPA axis through the release of CRH, as mentioned above. The molecular mechanisms behind this increase in CRH in chronic inflammation is through glucocorticoid receptor resistance in immunocytes through induction of MAP kinases, JNK and p38 (Irwin and Miller, 2007). A paper recently published from our colleagues (Mohammed et al, 2016) investigated the role of JNK1 in the change in anxiety and depressive-like behaviours in mice, suggesting that hippocampal neurogenesis may play a role in modifying these behaviours. They used JNK1 knockout and mice treated ICV with a JNK1 inhibitor 'DJNKI'. JNK1 knockout mice displayed decreased anxiety-like behaviour in the EPM, light/dark box and the open field as well as significant decreases in immobility in the forced swim test and a trend towards decreased anhedonia in a sucrose preference test, together with significant increase in neurogenesis. In addition, acute treatment with DJNKI was unsuccessful at attenuating anxiety-like behaviour, chronic treatment both lowered anxiety behaviour (in the EPM) and significantly increased neurogenesis, measured using DCX and BrdU/NeuN co-staining. This suggested that the anxiolytic effect of the JNK 1 inhibitor was dependent on adult neurogenesis. Working in collaboration with our colleagues the aim of the current study was to observe if similar changes occur in post-weaning social isolation reared rats.

5.2. Methods

5.2.1 Animals

48 male Lister-hooded pups arrived in eight litters of six and were weaned and isolated immediately. Rats were housed individually (n=24) or in groups of three (n=24). The animals were housed as previously described in Chapter 2.

5.2.2 Surgical Procedure

On week 6, the animals underwent anaesthesia to allow intra cerebral ventricular I.C.V. injection of D-JNKI peptide or a DTAT control peptide (an inactive form of the D-JNKI peptide Figure 5.2.1). Rats were anaesthesia using 5 % isoflurane and maintained with 2/3 % isoflurane anaesthesia. The surgical area was shaved and swabbed with 10 % chlorhexidine and s.c injection of carprofen (200 µl) used for post-surgical analgesia. The rat was then placed in a stereotaxic frame and, under aseptic conditions, two burr holes were drilled through the skull using an electric drill to enable bilateral injections (2.4 mm apart, coordinates from Bregma: ML: 1.2, AP: -0.5, DV: 3.7) of either vehicle control (D-TAT peptide, 200 µM) or drug (D-JNKI peptide, 200 µM), (2 µl infused by Hamilton syringe and an infusion pump over 2 min and left in place for a further 2 min). After injections, the incision was sewn up, the wound area was treated with lignocaine and 1 ml of saline injected i.p. before placing rats in a recovery cage on a heating pad until fully recovered and returned to their home cage.

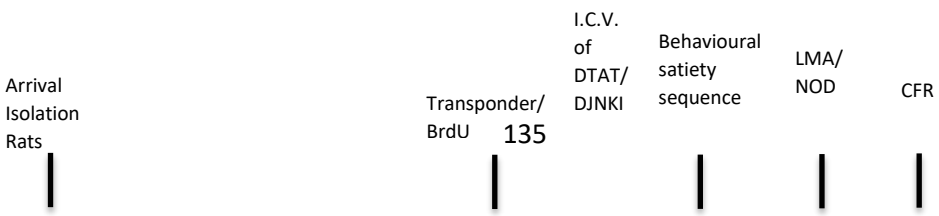
D-TAT Peptide Sequence:

H-(D-Arg)-(D-Arg)-(D-Arg)-(D-Gln)-(D-Arg)-(D-Arg)-(D-Lys)-(D-Lys)-(D-Lys)-(D-Arg)-(Gly)-NH2

D-JNKI-1 Peptide Sequence:

H-(D-Asp)-D-Gln)-(D-Ser)-(D-Arg)-(D-Pro)-(D-Val)-(D-Gln)-(D-Pro)-(D-Phe)-(D-Leu)-(D-Asn)-(D-Leu)-(D-Thr)-(D-Thr)-(D-Pro)-(D-Arg)-(D-Lys)-(D-Pro)-(D-Arg)-(D-Pro)-(D-Pro) -(D-Arg)-(D-Arg)-(D-Arg)-(D-Gln)-(D-Arg)-(D-Arg)-(D-Lys)-(D-Lys)-(D-Lys)-(D-Arg)-(Gly)-NH2

Following seven days recovery, testing began with a sequential battery of behavioural tasks; behavioural satiety sequence (novelty-supressed feeding), locomotor activity, novel object discrimination and conditioned freezing response. Details of all the behavioural tasks are as described in Chapter 2, section 2 on the test days shown in the protocol stick below (Figure 5.2.2) and behavioural satiety sequence is detailed below.



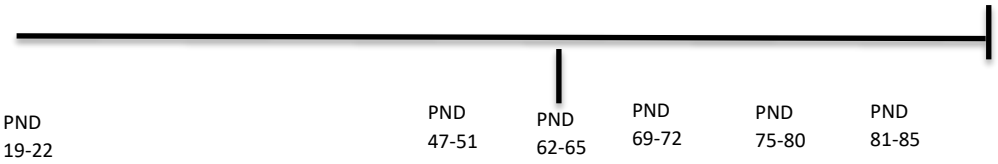


Figure 5.2.2: Timeline of the protocol from the arrival of the animals until the end of the behavioural testing. PND: Post-natal day (age of the rat) Behavioural paradigms are OF: Open Field, LMA: Locomotor Activity, NOD: Novel Object Discrimination, CFR: Conditioned Freezing Response, I.C.V.: Intracerebroventricular.

5.2.4 Behavioural Satiety Sequence

This test assesses stress-induced anxiety by measuring the latency of an animal to approach and eat a familiar food in an aversive environment and was selected due to its previous use as an animal model of anxiety-like behaviour and the subsequent testing of acute and chronic antidepressant treatments and behavioural effects of neurogenesis (Bodnoff et al, 1988; Santarelli et al, 2003). Rats were restricted from eating for 24 h before completing the task. Behaviour was recorded in an unfamiliar circular (diameter 75 cm, walls 45 cm tall) open field arena using a computerised tracking system (Noldus, Ethovision XT ver7.1) for 15 min. A single food pellet (600 mg) was placed in the centre of the arena and the central zone (15 cm from the wall) was set using Ethovision to differentiate behaviours occurring in central or peripheral zones. Behaviours monitored included latency to eat the food, locomotor activity (speed and distance travelled in peripheral and central zones), rears and grooming behaviour and the number of faecal boli as a further index of anxiety. Following reintroduction into the home cage, latency to eat was again recorded. The arena was cleaned with 20 % ethanol between runs.

5.2.5 Statistical analysis

All data were analysed by repeated measures ANOVA (with housing or drug as between factor) and (percentage time and amount of locomotor activity in central/peripheral zones, rears and grooming behaviour and the number of faecal boli, in behavioural satiety sequence. Time and rearing in LMA, object in NOD, freezing time in CFR were used as repeated measures) with Bonferroni multiple comparisons post-hoc where appropriate. All analyses were performed using GraphPad Prism 7 or SPSS 23 software. Data are expressed as mean $\pm$ sem and the observer was unaware of drug treatment or housing during data analysis.

5.3 Results

5.3.1 Behaviour Satiety Sequence (Novelty-supressed Feeding)

The latency to eat the food pellet placed in the centre of the arena showed a main effect of housing such that SI rats took significantly longer to begin eating than their GH counterparts ($F_{(1, 43)} = 20.64$, $P<0.0001$, Fig 5.3.1 A). There was no main effect of treatment ($F_{(1, 43)} = 0.32$, $P=0.57$, Fig 5.3.1 A). Sidak post-hoc tests revealed GH/CON demonstrated a lower latency to eat compared to SI/DJNKI ($P<0.05$), GH/DJNKI demonstrated a lower latency to eat compared to SI/CON ($P<0.01$), and SI/DJNKI ($P<0.001$) (Fig 5.3.1 A). In contrast, analysis of the latency to eat food on return to the home cage showed no significant difference in housing ($F_{(1, 43)} = 0.7352$, $P=0.39$, Fig 5.3.1 B) or treatment groups ($F_{(1, 43)} = 0.4138$, $P=0.52$, Fig 5.3.1 B) and no interaction ($F_{(1, 43)} = 0.003144$, $P=0.95$, Fig 5.3.1 B) Thus SI rats specifically took longer to initiate eating in the open field and not the home cage but this was not reversed by DJNKI treatment.

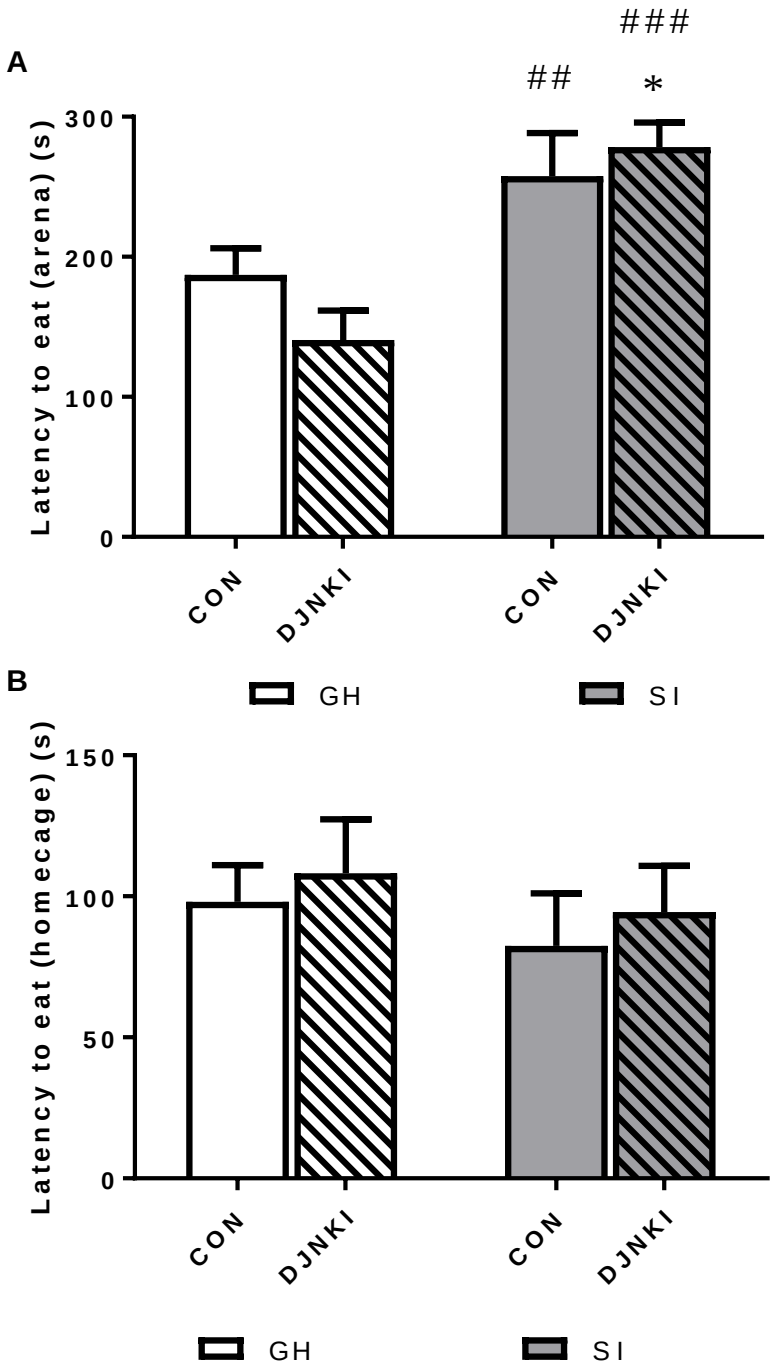


Figure 5.3.1 Latency to eat in NSF: (s, mean ± sem., n=12 per group) in both the arena during the NSF paradigm and on returning to the home cage. (A) There was a main effect of housing ($P<0.05$) for latency to eat in the arena. *$P<0.05$ Sidak post-hoc tests compared to GH/CON ##$P<0.01$, ###$P<0.001$ Sidak post-hoc test compared to GH/DJNKI.

5.3.2 Locomotor activity Task

Ambulations

To assess any change in isolation-induce hyper-reactivity to a novel arena as an index of anxiety, exploratory activity was recorded in a novel arena. A two-way repeated measures ANOVA measuring beam breaks across the time course of the trial revealed a main effect of housing such that SI exhibited more horizontal beam breaks compared to GH rats ($F_{(1, 44)} = 16.84$, $P<0.001$ Fig 5.3.2 A). There was no main effect of treatment ($F_{(1, 44)} = 0.38$, $P=0.543$ Fig 5.3.2 A). Sidak post-hoc tests showed that at the 10 min interval GH/DJNKI displayed significantly fewer beam breaks compared to SI/CON rats ($P<0.05$) and SI/DJNKI rats ($P<0.05$). At the 15 min interval, GH/CON displayed significantly fewer beam breaks than SI/CON ($P<0.05$), and again GH/DJNKI were significantly different to SI/CON ($P<0.001$) and ($P<0.05$) respectively. When the total number of beam breaks across the trial were examined, there was a significant main effect of housing with SI rats being more active than GH rats ($F_{(1, 44)} = 16.84$, $P<0.001$ Fig 5.3.2 B) with no effect of treatment ($F_{(1, 44)} = 0.37$, $P=0.54$ Fig 5.3.2 B). Sidak post-hoc test showed a significant increase in SI/CON compared to GH/CON and GH/DJNK ($P<0.05$) and an SI/DJNKI increase compared to GH/DJNKI ($P<0.05$).

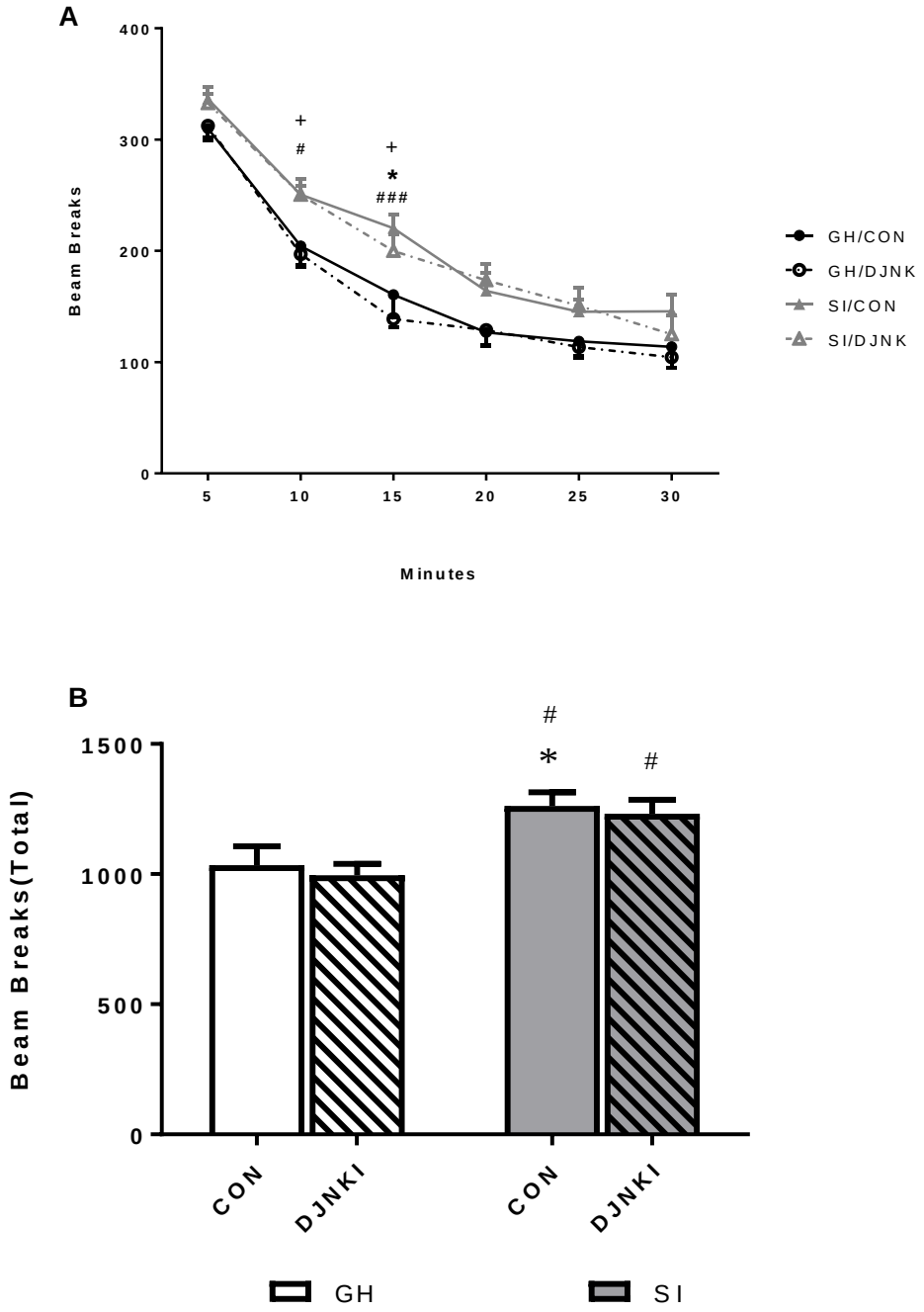


Figure 5.3.2 Locomotor Activity: (Beam breaks, mean ± semsem n=12 per group) (A) Across 30 min. A two-way repeated measures ANOVA revealed a significant effect of housing (P<0.001). Sidak post-hoc tests showed a significant difference between groups at the 10 min and 15 min interval (#P<0.05, ###P<0.001 SI/CON from GH/DJNKI, *P<0.05 SI/CON from GH/CON, +P<0.05 SI/DJNK from GH/DJNK). (B) Total beam breaks in the trial. A two-way ANOVA revealed a significant effect of housing (P<0.001) *P<0.05 Sidak post-hoc compared to GH/CON, #P<0.05 compared to GH/DJNKI

Rears

A two-way repeated measures ANOVA revealed no significant main effect for housing ($F_{(1, 44)} = 3.57$, P=0.06) or treatment ($F_{(1, 44)} = 0.02$, P=0.90) across the trial and no interaction. The total number of rears was also not significantly altered by housing or treatment (data not shown).

5.3.3 Novel Object Discrimination

Familiarisation Trial

To assess any alteration in visual recognition memory induced by social isolation, behaviour was recorded in a non-spatial novel object discrimination task. There was no significant effect of housing ($F_{(1, 88)} = 1.39$, P= 0.242 Fig 5.3.3) or treatment ($F_{(1, 88)} = 0.582$, P=0.45 Fig 5.3.3) and there was no difference between time spent exploring each object ($F_{(1, 88)} = 0.20$, P=0.88 Fig 5.3.3) during the first familiarisation access to two identical objects. There was a significant interaction effect between housing and treatment ($F_{(1, 88)} = 4.63$, P<0.05) but no difference between any two groups on post-hoc analysis

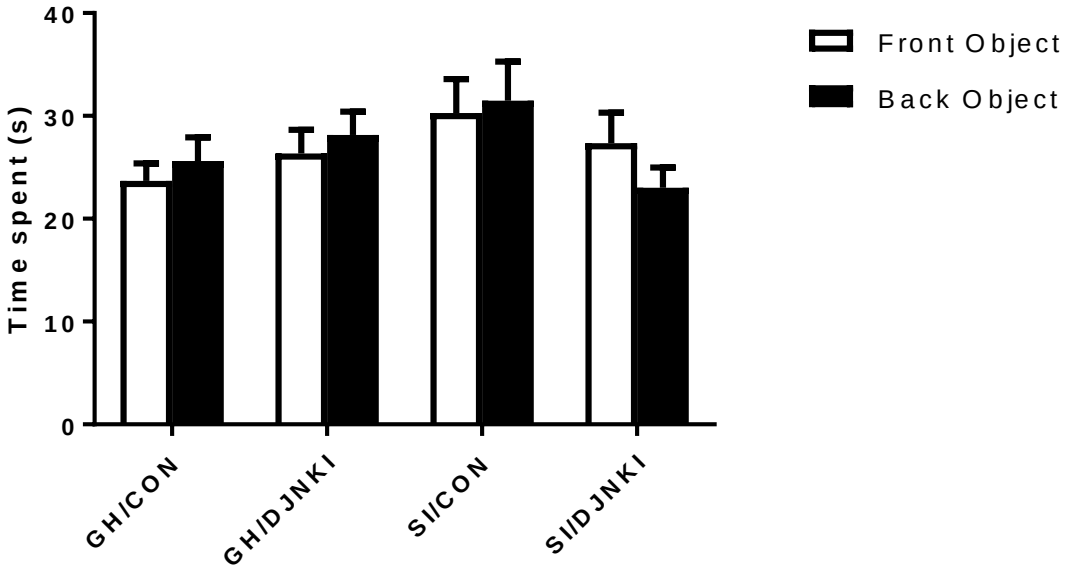


Figure 5.3.3 Familiarisation Trial in NOD: Time spent (s, mean ± semsem, n=12 per group) exploring both identical objects in the familiarisation trial of the novel object task. No significant differences between groups. Significant 'housing x treatment' interaction (P<0.05).

Choice Trial

In the second choice trial, there was a significant effect of housing such that GH rats explored the objects more than their SI counterparts ($F_{(1, 88)} = 4.66$, $P < 0.05$ Fig 5.3.4). There was no main effect of treatment ($F_{(1, 88)} = 0.45$, $P=0.5$ Fig 5.3.4) but there was a significant main effect of object such that rats spent less time exploring the familiar compared to the novel object ($F_{(1, 88)} = 20.46$, $P<0.001$ Fig 5.3.4). Sidak post-hoc tests showed that only the GH/DJNKI ($P<0.001$ Fig 5.3.4) and SI/DJNKI group, ($P<0.05$ Fig 5.3.4) spent more time exploring the familiar than the novel object.

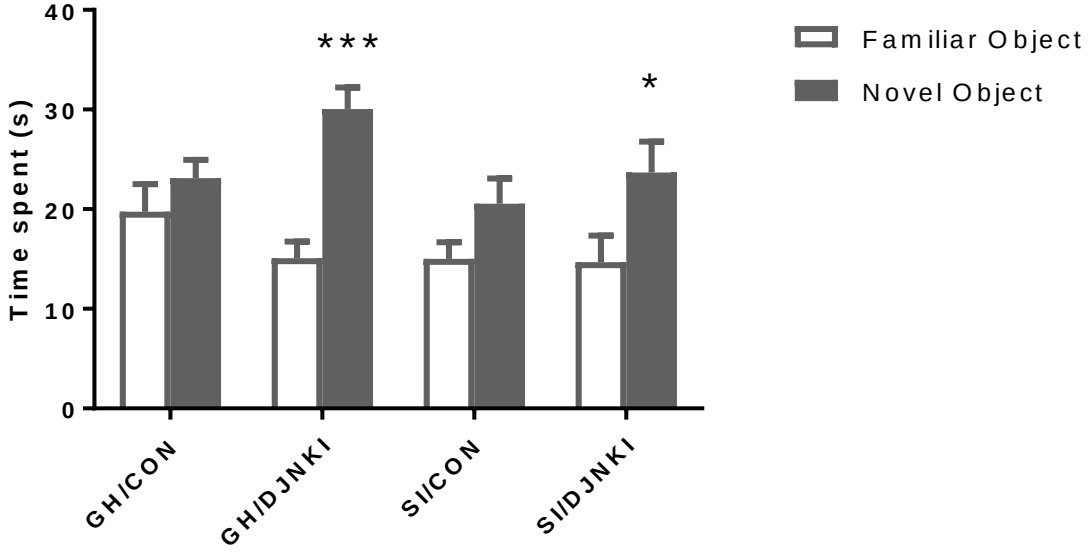


Figure 5.3.4 Choice Trial in NOD: Time spent (s, mean ± sem, n=12 per group) exploring the familiar and novel objects. Two way ANOVA revealed a main effect of housing ($P<0.05$). ***$P<0.001$, *$P<0.05$ Sidak post-hoc tests compared to familiar object.

Discrimination Ratio

Consistent with the raw data analysis of the derived discrimination ratio during the choice trial showed there was no effect of housing ($F_{(1, 44)} = 0.22$, $P=0.64$ Fig 5.3.5), but a main effect of treatment with DJNKI treated rats appearing to have a significantly higher ratio than either CON group but this failed to reach post-hoc significance. ($F_{(1, 44)} = 5.21$, $P=<0.05$ Fig 5.3.5). There was no interaction effect and no post-hoc tests revealed no differences between groups.

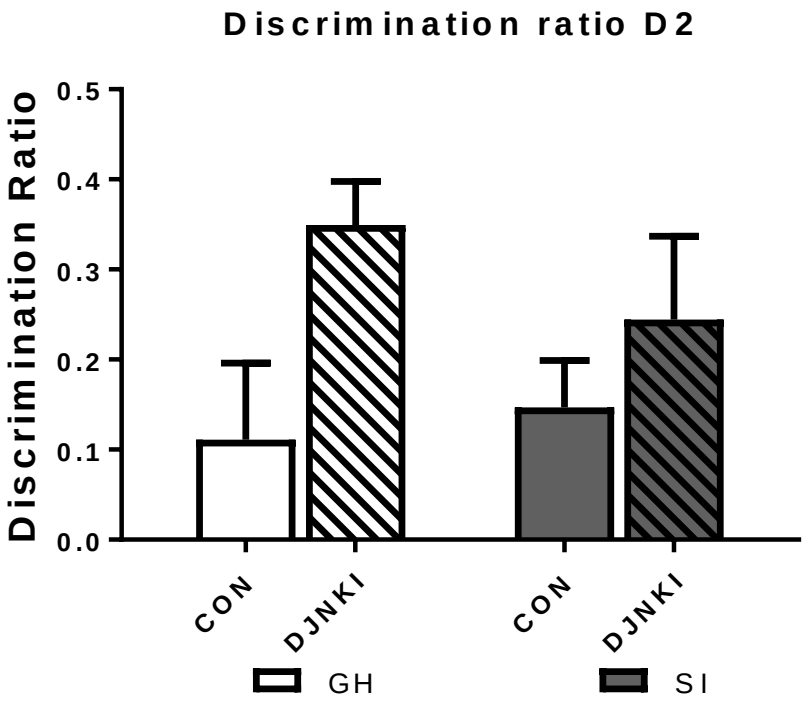


Figure 5.3.5 D2 Discrimination ratio: (mean ± sem, n=12 per group). Two way ANOVA demonstrated a main effect of treatment (P<0.05), post-hoc tests revealed there were no significant differences between groups.

5.3.4 The Conditioned Freezing Response Task: Effects of Isolation-rearing and DJNKI on Associative Memory.

Acquisition

To assess any specific effect of isolation rearing and/or DJNKI treatment on associative learning and memory, both were evaluated on a three step conditioned emotional freezing response task.

The duration of freezing in response to the conditioning was determined to see any impact on acquisition of the task. A two-way repeated measures ANOVA demonstrated that there was no main effect of housing ($F_{(1, 92)} = 2.74$, P=0.1 Fig 5.3.6) nor any main effect treatment ($F_{(1, 92)} = 0.01$, P=0.91 Fig 5.3.6). There was a main effect of shock showing that freezing time significantly increased from the first to the second shock ($F_{(1, 92)} = 289.87$, P<0.001, Fig 5.3.6). Sidak post-hoc tests showed that there was a significant increase in freezing times after the second shock compared to the first for every group GH/CON (P<0.01), GH/DJNKI (P<0.05), SI/CON (P<0.01) & SI/DJNKI (P<0.001) (Fig 5.3.6).

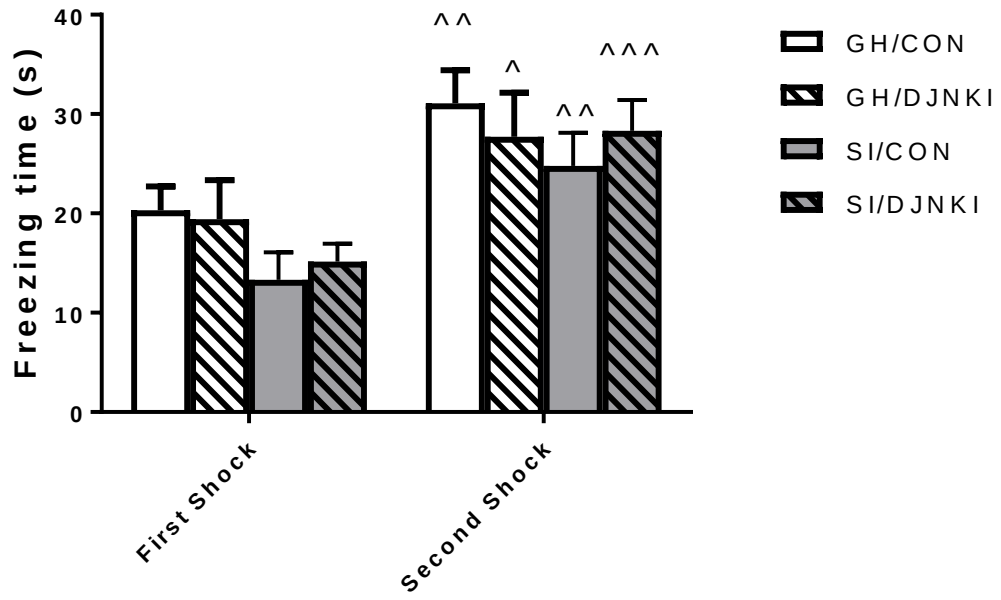


Figure 5.3.6 Acquisition Trial CFR: Time spent engaged in freezing behaviour (s, mean ± semsem, n=11-12 per group). One rat was eliminated from the analysis due to an error with the equipment. A two-way repeated measures ANOVA showed a significant effect of shock ($P<0.001$) and no main effect of housing or treatment. ^$P<0.05$, ^^$P<0.01$, ^^^$P<0.001$ Sidak post-hoc test increase compared to corresponding group first shock.

Retention

At 24 hours post-conditioning when placed back in the conditioning chamber, there was no main effect of housing ($F_{(1,44)}=2.46$, $P=0.12$ Fig 5.3.7), or treatment ($F_{(1, 44)}=0.51$, $P=0.47$ Fig 5.3.7) on freezing duration, although there was a significant housing x treatment interaction ($F_{(1,44)}=4.13$, $P<0.05$, Fig 5.3.7) on freezing duration. Sidak post-hoc tests showed no significant differences between the groups. At 48 h post-conditioning without re-exposure to the tone, there was no main effect of housing ($F_{(1,44)}= 1.49$, ,$P=0.23$ Fig 5.3.7) or treatment ($F_{(1, 44)}=0.22$, $P= 0.64$ Fig, 5.3.7) on freezing duration. Sidak post-hoc tests showed no significant differences between groups. At 48 h post-conditioning there was a main effect of housing ($F_{(1, 44)} = 5.73$, $P<0.05$ Fig 5.3.7) but no effect of treatment ($F_{(1, 44)} = 0.42$, $P=0.51$ Fig 5.3.7) and Sidak post-hoc tests showed no significant differences between groups. Sidak post-hoc tests also revealed a significant decrease in freezing time of all groups at 48 h pre-tone compared to the 24 h time point ($P<0.05$) and a significant increase of freezing time again after presentation of the cue ($P<0.001$).

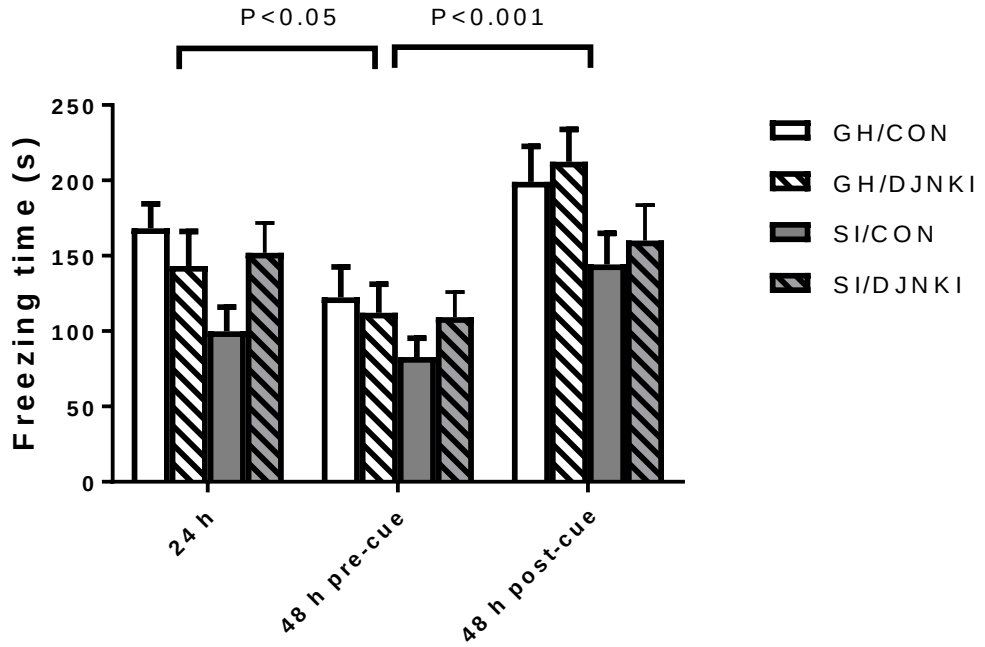


Figure 5.3.7 Retention Trial CFR: Time spent engaged in freezing behaviour (s, mean ± sem, n=11-12 per group). A two-way ANOVA showed a significant interaction effect at 24 h ($P<0.05$) and a main effect of housing at 48 h post-cue ($P<0.05$). Sidak post-hoc tests show a significant decrease in freezing time at the 48 h pre-cue time point compared to both 24 h ($P<0.05$) and 48 h post-cue ($P<0.001$).

5.3.5 Hippocampal cell proliferation and neurogenesis

The number of DCX positive cells in hippocampal sections was counted as an index of neurogenesis. There was no main effect of housing ($F_{(1, 42)} = 1.42$, $P=0.24$, Fig) or treatment ($F_{(1, 42)} = 0.821$, $P=0.37$, Fig) and no interaction. Sidak post-hoc tests revealed no differences between groups.

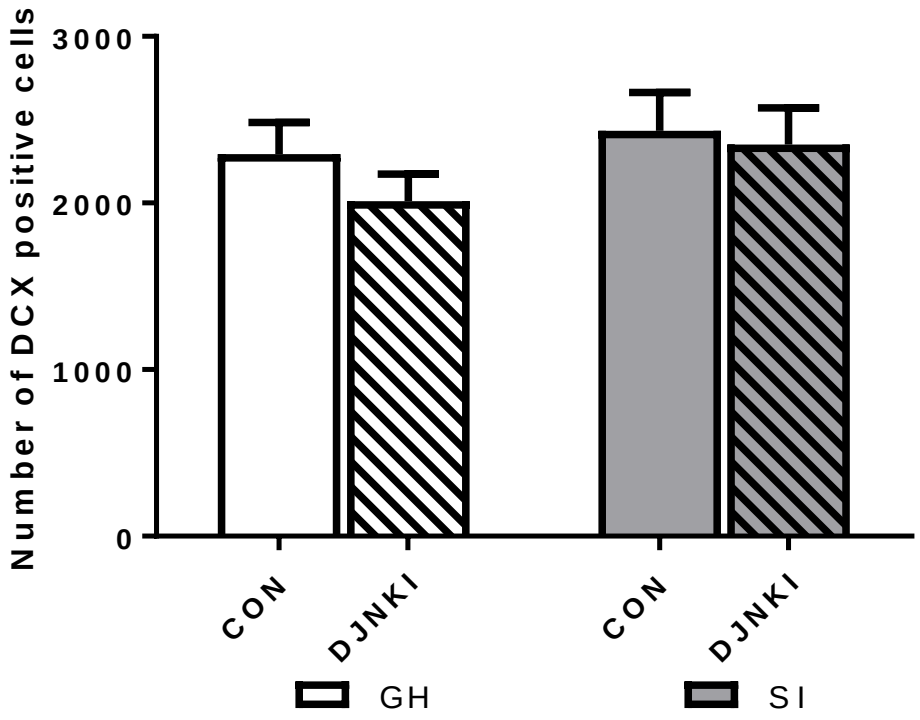


Figure 5.3.8 Postive DCX Cells: Number of positive DCX cells (mean ± sem, n=12 per group). in the dentate gyrus. Two-way ANOVA showed no main effect of housing or DJNKI treatment. Sidak post-hoc tests showed no significant differences between groups.

5.4 Discussion

The current study aimed to assess DJNKI's ability to alter the behavioural effects of the post-weaning social isolation of rat pups. The behaviours selected for examination included those thought to reflect 'anxiety-like' behaviour, cognitive dysfunction and a neurochemical marker of neurogenesis selected from previous studies conducted as part of this study as being altered following social isolation. As a further index of anxiety related behaviour for the first time our group also examined the impact of isolation rearing on novelty suppressed feeding which is thought to be a useful index of anxiety-like behaviour (Bodnoff et al., 1988; Hermes et al., 2011; Santarelli et al., 2003b). Of great interest in the novelty-suppressed feeding task was that isolation rearing increased the latency to commence eating the food pellet placed in the most aversive central position of the open arena compared to GH control littermates. This suggests SI rats have a higher level of anxiety as previously observed by ourselves (Fone & Porkess, 2008) and others which can suppress the proponent stimulus to eat following 24h absence of food. Importantly on return to their home cage, the latency to eat food was equivalent in the same SI and GH rats eliminating the possibility that SI rats were not hungry or motivated to eat the food for a non-specific reason. All animals ate the food pellet during the task and all animals also ate after returning to the home cage, suggesting their latency to eat in the task was due to the aversive environment and neophagia. There was no main effect of treatment in the task, suggesting that administration of DJNKI (7 days previous) had no effect on the anxious phenotype displayed by SI rats in this task. This is consistent with reports in mice (Mohammed et al, 2016), which found acute DJNKI administration had no anxiolytic effect in the EPM and light/dark box. In contrast, they did find significant anxiolytic effects following repeated systemic DJNKI administration. It was hoped that a single icv administration of DJNKI in the current study would produce long-term inhibition of JNK1.

In the locomotor activity task again, socially isolated rats were more active than their group housed littermates as expected but this was unaltered by DJNKI administration, similar to the observation by Mohammed et al (2016) in mice. As demonstrated in previous chapters, there were no changes to rearing behaviour regardless of housing or treatment group. In the novel object discrimination task, as expected, there were no significant differences in object exploration during the familiarisation trial while during the choice trial, rats tended to spend more time exploring the novel than the familiar object. However only GH/DJNKI and SI/DJNKI rats spent significant more time on the novel object. Typically, it would be expected for the GH/CON rats would also exhibit object discrimination but perhaps due to large inter-individual variation this did not occur. Therefore, it cannot be stated that the socially isolated rats had a deficit in visual memory compared to the group-housed controls as previously repeated demonstrated with socially isolated rats in our group (Bianchi et al, 2006, Watson et al, 2012). It is noteworthy while SI/CON rats, did not

successfully discriminate the novel from the familiar object the SI/DJNKI rats did. This suggestsa potential pro-cognitive effect of JNK inhibition worthy of further investigation. The mechanism by which this could occur is speculative (Mohammed et al, 2016) but could be mediated by the increase in hippocampal neurogenesis observed after JNK inhibition. The overall patter of change of the discrimination ratio in the four groups lends support to the potential impact of treatment with DJNKI on visual recognition memory but did not reach significance. In the CFR task (another cognitive measure), isolates performed as well as group housed at 24 h and 48 h post-shock, as with the NOD task this is inconsistent with the robust impairment (reduction in conditioned freezing) expected from social isolation. However, there was a significant effect of housing on freezing behaviour after presentation of the cue 48 h post-conditioning. Previous chapters have demonstrated a significant effect of housing at all time points measured in the CFR paradigm and, in the current study, the isolate control group did freeze the least compared to the other groups, however, this was not significant. The cue for the shock may be the strongest association related to the memory and this could potentially explain why it was the only measure to reach significance. The lack of any treatment effect would suggest DJNKI is not having the 'pro-cognitive' effect in the NOD task, or any enzyme inhibition may have have diminished since the icv injection as the CFR task occurred one week after the NOD and three weeks after the DJNKI administration. Potentially any downstream effects it caused may have faded by that time as well. Consistent with the lack of impact on cognitive behaviour, DJNKI also failed to show any alteration in hippocampal neurogenesis measured by DCX immunohistochemistry. Once again, this is inconsistent with previous isolation reared rat cohorts which have shown reduced hippocampal neurogenesis and decrease in hippocampal synaptophysin (Varty et al., 1999), microtubular proteins (Bianchi et al., 2009) suggesting this is a brain region particularly vulnerable to early-life stress and which may mediate some of the cognitive impairments usually accompanying this paradigm.

The overall goal of the study was to assess the DJNKI peptide to restore behavioural changes in the post-weaning social isolation model of depression. There were no strong effects of treatment with DJNKI in the behaviours observed even though socially isolated animals did exhibit some changes. The lack of efficacy of DJNKI could be to a number of reasons. In comparison with the Mohammed (2016) paper, firstly the models they used were in mice. A genetic model with a JNK deletion and for the pharmacological intervention they used (male C57BL/6J wild-type). Although we attempted to adapt the treatment, it may not have been possible to see the same effects in rats. The paper also demonstrated that acute administration was not very successful at modifying anxious behaviours or neurogenesis. However due to practical limitations (insolubility of the compound), it was not possible for us to implant osmotic mini-pumps to enable chronic administration of the peptide. As discussed above,

the single administration of the peptide may have been insufficient to produce sufficient or long-term enzyme inhibition to affect the behaviours recorded.

Chapter 6:

General Discussion

6.1 Aims

The aim of this thesis was to evaluate the suitability of post-weaning social isolation as a neurodevelopmental model to reproduce symptoms in resultant adult rats that resemble 'depressive-like' behaviour and accompanying underlying neurochemical alterations. With the rate of depression increasing globally, the need for improved treatment outcomes together with a reduction in therapeutic side-effects and a more rapid speed of onset are required. In recent years, one of the major constraints on the successful development of new drugs has been recognised to be the lack of translational reliance of existing animal models for a large array of common CNS disorders. Further characterisation of neurodevelopmental models together with the production of two-hit strategies is currently a topical consideration.

Current preclinical models of depression include neurosurgical, pharmacological, genetic/strain inbred, stress-induced or developmental models, all of which have varying degrees of validity. No single model can encompass the heterogeneous symptomology of MDD, so each model must look to replicate specific phenotypic elements of the illness with as much accuracy, validity and translation as possible. This thesis examined the post-weaning social isolation model and its potential to replicate some of the composite core symptoms of depression; including anxiety, cognitive deficits and an altered stress response and alteration in neurogenesis. In addition changes in the gut microbiota were established for the first time, in this model to investigate any causal relationship with the resultant isolation syndrome. Finally, conventional (SSRI, fluoxetine) and novel (ketamine and DJNK inhibitor) treatments were compared in their ability to reverse changes induced.

6.2 Main Findings

Isolation rearing was selected as the model for this thesis as it is well-characterised and validated within the laboratory at Nottingham. In addition, isolation rearing has been shown to produce reliable changes in several behaviours in previous publications and again throughout the experiments documented herein, although it failed to elicit deficits in NOD in some cohorts. One of the more reliable behavioural changes was neophobia characterised by a hyperactive locomotor and rearing response in the LMA and open field tasks. Isolates reliably demonstrated hyperactivity compared to their group housed counterparts when placed into a novel mildly aversive arena, taking longer to habituate especially during the first 30 minutes. At least in part, this hyperactivity is thought to reflect increased firing rate in VTA neurones innervating the nACc (Fabricius et al, 2010) and can be attenuated by dopamine D2 receptor antagonists consistent with hyperactivity of the mesolimbic dopaminergic neurons (Watson et al, 2016). A consistent deficit in associative

memory was also observed in isolates in the conditioned fear response task indicative of the expected impairment in associative learning and memory. In the novelty-suppressed feeding task, isolation had an effect increasing the latency to eat following an overnight food restriction period (a behavioural readout thought to reflect increased anxiety). This was the first time that novelty-suppressed feeding had been demonstrated in isolation reared rats using our Nottingham paradigm but is consistent with one previous report in mice (Linge et al, 2013). It would be important to establish if this suppression of feeding were reproducible as it was only conducted in the final study. Other behavioural measures were less reliable. Previous studies within our laboratory group demonstrated that isolates displayed an inability to discriminate a novel from a familiar object in the novel object discrimination task (Bianchi et al., 2006; McIntosh et al., 2013; Watson et al., 2016). This was replicated in some chapters of this thesis, however, in others, isolates successfully discriminated the novel from the familiar object. This may have been due to larger inter-individual variation in the task and it was notable that there was often a tendency for the discrimination index to be lower in isolates without reaching significance. In the EPM, there were variable results in the time spent in the open arms between housing groups. The neuroendocrine and thermogenic response to restraint stress was consistently unaffected by housing condition, being greater in both isolates and group-housed littermates at the time measured. Although, this could be due to other factors such as reaching a ceiling effect of stress. The measurement of corticosterone at several time points during the stress would have been useful if this had been permitted under the project licence. As the SI rats had undergone 5 weeks of isolation before behavioural tasks began, all animals took part in a total of 5 behavioural measures (in Chapters 2,3,4) over a period of 4 weeks designed so that the least aversive paradigm was performed first. As both cohorts had been repeatedly exposed to stressors, this may also account for both groups responding to restraint stress equally, there is the possibility of a habituation effect following on from cumulative behaviour exposures. There were variations in isolation rearing's effect on neurogenesis, in chapter 2 & 3 it was significantly reduced in the dentate gyrus consistent with literature using chronic stress models (Mirescu and Gould, 2006). However, isolation appeared to have no effect on the number of DCX positive cells in chapters 4 and 5 and the reason for this is unclear. The second aim of this thesis was to assess novel potential antidepressant treatments on isolation reared rats against a 'gold-standard' existing antidepressant, fluoxetine. To avoid any confounding impact of repeated handling, fluoxetine was delivered by the drinking water, and although no assay was available to determine plasma levels, several changes were observed suggesting appropriate dosage had been provided which was based on previous reports (Bianchi et al., 2009; Silva and Brandao, 2000). In the open field, fluoxetine increased locomotor activity, one of the key features of isolation reared animals (Gentsch et al., 1981a) while having no effect in the LMA task and reduced horizontal activity in the EPM. Fluoxetine

is prescribed as an anxiolytic drug in clinical populations, but its effects on anxiety-like behaviours in the current study were mixed. It increased time spent grooming and rearing in the more aversive central zone of the open field. In the EPM, it had no effect on time spent in the open arms, it reduced the number of head dips and increased stretch attends consistent with a modest anxiogenic effect. Increased anxiety is a side-effect of SSRI treatment in man, but this is often an transient observation which attenuates with time, while in the current study, rats had received fluoxetine chronically for two weeks. For cognitive measures, findings were again inconsistent. Fluoxetine had no effect in NOD task and significantly increased freezing at the 48 h pre-tone time point in the CFR task and, interestingly, significantly increased hippocampal neurogenesis consistent with the literature.

Ketamine had no impact in the open field, LMA, NOD or SIH tasks. It had no effect on corticosterone levels in response to a stressor or hippocampal neurogenesis. The only behavioural assay affected by ketamine was CFR. Ketamine treated animals displayed an increase in freezing time at 24 and 48 h post-conditioning after presentation of the light/tone cue. One interpretation of this finding is that ketamine partially reversed the decrease in associative memory induced by isolation rearing which is worthy of further investigation. The final treatment examined was the JNK-1 inhibitor, DJNKI. As with ketamine, this treatment had limited effects in the measures employed in the study. In the NOD task, it enhanced the D2 discrimination ratio, suggesting a restorative effect on visual learning and memory, but only when the information was expressed as a ratio of exploration. In the CFR task there was an interaction effect between housing and treatment 24 h post-conditioning. As discussed previously (section 5.4.), the lack of efficacy of DJNKI could be due to a number of factors but the most likely is insufficient attenuation of enzyme activity following the single ICV injection protocol used. For the first time, a change in gut microbiome was observed in isolation-reared rats (Doherty et al., 2017). Analysis of the microbiota revealed a number of differences that may contribute to phenotypic changes in SI rats mediated in part via the gut-brain axis bicommunication. Although, the complex bidirectional interaction between gut microbiota and stress-related behaviour makes it difficult to determine the cause or consequence of changes seen in SI rats, which is clearly imperative to understand the neurobiological sequelae and develop novel preventative therapies. Temporal analysis of the changes in microbiota, cytokines and behaviour during development of the isolation syndrome may reveal a cascade of events. Table 6.1 below displays a summary of the findings of the chapters of the current thesis.

	Open Field	LMA	NOD	EPM	CFR	SIH/Cort	NSF	Neurogenesis
SI	↑Distance/velocity, ↓Time spent in central zone	↑locomotor activity	↓ SI induced an impairment in the NOD in chapters	↓ SI decreased % time spent in open arms in chapter 2 only.	↓SI decreased freezing times post conditioning	No effect of housing on change in temp or plasma corticosterone levels	↓Reduced latency to eat in task	↓Reduced in chapters 2 & 3 but had no effect in chapters 4 & 5
FLX	↑ Time in central zone in both groups ↑ distance/velocity ↑ grooming/rearing	No effect	No main effect of treatment. SI/FLX rats discriminated novel object, CON animals did not	↓Reduced activity in the task. Reduced head dips, increased stretch attends	↑ Freezing at 48hrs pre-tone. Significant increase compared to SI/CON at all time points	No effect	n/a	↑Neurogenesis
KET	No effect	No effect	No effect	No effect	↑ freezing at 24hr and post-tone. ↑freezing time overall.	No effect	n/a	No effect
DJNKI	No effect	No effect	↑ Discrimination compared to controls. Both in time explored (s) and D2 ratio	No effect	Housing x treatment interaction at 24hr time point	No effect	No effect	No effect

Table 6.1: Summary of effects of interventions and treatments on behaviours within the thesis. (SI=Social Isolation, FLX= Fluoxetine, KET= Ketamine, LMA = Locomotor Activity, NOD= Novel Object Discrimination, EPM= Elevated Plus Maze, CFR= Conditioned Freezing Response, SIH= Stress-induced Hyperthermia, Cort=Corticosterone, NSF= Novelty Suppressed Feeding, CON= Control

6.3 Future studies/Considerations

The proposed future studies and amendments to current protocols work towards the aim of this thesis by attempting to improve the model and better understand the mechanisms underlying the behavioural deficits induced and changes observed from post-treatment intervention.

The first avenue for further investigation is the alteration of the isolation rearing procedure itself. Despite efforts to ensure that the isolation procedure matched as closely as possible between studies, there is a possibility that minor changes to handling, cage changing, weighing or drug administration may prevent the development of the isolation syndrome (Gentsch et al., 1988; Geyer et al., 2001).

The use of more severe housing conditions has been shown to produce a stronger isolation syndrome (Greco et al. 1989; Holson et al. 1991) and reducing the amount of time animals are handled or allowed interact with the experimenter could reduce variation and enhance the magnitude of effect. However, alterations like this may raise ethical questions regarding the animals' welfare which, under the 3 Rs, it is paramount to use minimal aversive effects required to establish significant effects. Another strain may be successfully adopted for the model. Lister-Hooded rats were selected for the current studies based on their reproducible record of isolation rearing induced deficits. Much of the literature published on models of MDD favour other strains, such as Sprague-Dawley or Wistar, the use of these strains could result in a stronger depressive-like phenotype. One potential example is the endogenous depression model, the Wistar-Kyoto rat; recent work has demonstrated the potential efficacy of this 'dual-hit' model. Shetty & Sadananda (2017) demonstrated post-weaning social isolation of WKY rats lead to changes in anxiety-related behaviours, decreases in 50kHz ultrasonic vocalisations, longer lasting effects on social interaction measures and anhedonia. They also showed that rehousing did not reverse the phenotype. Another study (Malkesman et al., 2006) found significant alterations in the levels of HPA hormones after social isolation in "depressed-like" strains (WKY and FSL), and WKY rats exhibited less frequent aggressive-like and social behaviour compared to Wistar controls.

The battery of the behavioural tasks could also be altered as some yielded inconsistent results and replacing them may be beneficial. Detection of any anxiety phenotype in the EPM task, for example, was unreliable in this thesis. As a measure of anxiety, it failed to highlight any differences in exploration. There are a number of potential confounding factors with the EPM as a measure of anxiety. Several aspects can alter behaviours in the task, ranging from the light levels, to the height of the maze from the floor, the height of the walls, differences in animal strains, circadian rhythms, animal habituation and stress levels before testing (Hogg, 1996; Walf and Frye, 2007). The use of the NSF test in the final study may be a viable alternative as isolates showed a strong deficit latency to retrieve food. As cognitive deficits are observed in clinical

populations of MDD (Bailey et al., 2017; Millan et al., 2012b), it is necessary to account for this in a translatable rodent model. The NOD task was selected due to its robust efficacy in our laboratory group (Bianchi et al., 2006; McIntosh et al., 2013; Watson et al., 2016), although results in the current rat cohorts were not reproducible. A refinement of the protocol may need to be employed to ensure reproducibility across studies, such as a longer inter-trial interval to increase task difficulty or alternating the procedure to novel object location which may have a stronger hippocampal dependent component. In contrast, in the CFR paradigm isolates showed a consistent cognitive deficit across all chapters of this thesis which is also consistent with our previous findings (McIntosh et al., 2013; Watson et al., 2016). As mentioned above, results from the Morris water maze are not reliable in isolation reared rats and in some cases isolated rats have been shown to show enhanced acquisition (Wongwithecha & Marsden, 1996). Other cognitive measures could be employed to investigate potential deficits such as the delayed non-match to sample task. As discussed above, (Malkesman, Maayan, Weizman, & Weller, 2006) found a change in corticosterone levels in isolation reared WKY rats, however the SIH task which was employed across all chapters in the current thesis failed to demonstrate any changes in the stress response between isolates and group-housed animals in either temperature change or corticosterone levels. Alternative stressors may achieve a greater differentiation than the restraint method employed in this thesis. The lack of efficacy could be due to a ceiling effect, even though the isolated animals have gone through isolation stress for several weeks before behaviours began. Both sets of rats had undergone several behavioural tests and administration of drugs over several weeks before this task was conducted, so it could be that both cohorts were exposed to significant stressors over a prolonged albeit intermittent period. Future studies could assess the stress response in animals immediately following isolation rearing, before any other behavioural tests are performed.

The battery of behavioural tasks chosen for the current thesis does not contain a task that directly assesses one of the 'core' depressive symptoms in a pre-clinical model such as the sucrose consumption task for anhedonia or the FST/TST for behavioural despair. This was done for a number of reasons. First, although the FST is regularly selected to assess antidepressant efficacy in preclinical models there is some debate as to its validity as a measure of depressive-like behaviour (Borsini and Meli, 1988; Kara et al., 2017; Lucki, 1997). Also, the robust hyperactivity that is observed in isolation-reared rats may confound any measure of immobility in the FST. For the sucrose consumption task, rats are required to be singly housed to accurately measure intake, as having singly housed animals is the basis of the model, it was decided to not adopt this behaviour.

The use of female animals must also be addressed. The rate of MDD in the population is higher for females than males, so ideally preclinical testing should apply to both sexes to ensure a truly translational model. However, the majority

of studies in rodents including those in the present thesis were carried out in males. This was done as the behavioural phenotype of the isolation syndrome is more pronounced in male Lister-hooded rats than female rats (Fone and Porkess, 2008) and also to avoid any influence of the oestrogen cycle, which can affect response and cognitive behaviour in humans and rodents (Galea et al., 2008; Maki and Dumas, 2009; Sundermann et al., 2010) and neurogenesis in rodents (Galea et al., 2008; Li et al., 2011a; Tanapat et al., 1999). A recent meta-analysis (Becker, Prendergast, & Liang, 2016) examined variability in data of male versus female rats in over 300 articles and found no differences in variability between males and females in behavioural, histological neurochemical and electrophysiological measures, it also demonstrated no influence from the oestrus cycle. Further analyses like this may allow for a move in research towards the use of female animals equally to males.

Alteration of the dose of pharmacological compounds selected and/or the administration routes adopted may also be beneficial for future studies. In chapter three, fluoxetine was chosen as it is a first line treatment for MDD, although it may be worth comparing it to another commonly used treatment such as a TCA, SNRI or another SSRI, to establish a clearer picture of effects of standard treatments in this preclinical model. Fluoxetine was administered in the drinking water, which was chosen due to its translational relevance but produces some practical limitations as it's difficult to control how much drug is received. Future studies could investigate use of oral gavage to administer drugs or accept that i.p. or s.c. routes are sufficiently translatable. For the ketamine treatment, our administration regimen was given 24 hours before each behavioural measure. As this amounted to 5 injections over a period of 5 weeks, this may be regarded as being a sub-chronic treatment regimen rather than acute and may possibly affect the behaviours in the later tasks. Ketamine may not be the best choice for these studies due to its abuse potential in humans, so several other compounds are under investigation such as R-ketamine (Yang et al., 2015). There has also been research into other NMDAR antagonists, most notably MK-801 which has previously been combined with isolation rearing to attempt to produce a greater effect (Ashby et al., 2010; Gilabert-Juan et al., 2013; Hawken et al., 2013; Hickey et al., 2012; Lim et al., 2012). Lim et al. (2012) were able to demonstrate that NMDA receptor antagonism in the early post-natal period could produce PPI and NOD deficits in isolation reared animals that were more robust than when saline was administered instead. For the final and most novel treatment, the JNK-1 inhibitor DJNKI, there are several alterations which could be made. As discussed in chapter 5, the choice of dose was speculative as previous studies had examined its effects in mice not rats (Mohammad et al., 2016b). They had also employed a mini-pump to allow for repeated administration of the peptide, which, due to practical issues, could not be done in this study. Future studies should address this and allow for repeated dosing of the peptide. The results of the levels of hippocampal neurogenesis in the dentate

gyrus were quite variable from study to study. This may be due to changes and alterations in the staining protocol and targets that were measured. In chapter 2, neurogenesis was measured assessing levels of cells co-stained BrdU/NeuN, which was selected as a method for examining both cells that were dividing at the time of injection (post-isolation, pre-behavioural testing) and would allow quantification of the number of those cells which became mature neurons (NeuN). Unfortunately, due to difficulties with immunohistochemistry this had to be altered in chapters 3, 4 and 5 to counting the number of cells positive for DCX a marker of immature neurons. Ideally, future studies would all examine both BrdU/NeuN positive cells and DCX positive cells.

In chapter 2, it was demonstrated that isolation rearing altered both cytokine levels and, for the first time, demonstrated changes gut microbiota in isolation-reared rats(Doherty et al., 2017) (Doherty et al, 2017). Future studies should look to investigate any potential changes to cytokine levels and gut microbiota in isolates treated with SSRI's, NMDAR antagonists and JNK inhibitors. Alternatively, the potential for use of probiotics to reverse isolation induced changes in rats should be examined.

6.4 Conclusions

The aim of this thesis was to evaluate the isolation-rearing paradigm as a model of depressive-like behaviour and to assist in the development of potential novel therapeutics. From the results of the current thesis, the isolation-rearing model has some worth as a model for depressive-like behaviour. It is an early-life stress, developmental model which lends to its translational relevance. It holds advantages over similar models (such as CMS) due to it being practically simpler, and deficits appear with a developmental trajectory akin to depression. The model did not consistently produce the behavioural changes expected from previous reports and in that it may need to change which behaviours are examined. Improvements in analysis of neurogenesis would help with the value of this marker although the findings from the post-mortem analysis of cytokine levels coupled with the changes observed in the gut microbiota open an interesting and novel pathway for future studies. Overall, the model presented here shows some promising features but requires greater refinement before it will could be a valuable model for depression research.

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Appendix 1: Microbiota composition and analysis

At the phylum level, Mann-Whitney test revealed significant differences in low relative abundance (~1%) *Actinobacteria* (p=0.04) with an increase in SI compared to GH rats (Fig. 1 and Table 4). Increases in phylum *Actinobacteria* were attributed to increases in genus *Rhodococcus* (p=0.03; Fig. 2). SI significantly decreased *Clostridia* (p=0.03) at the class level; this was reflected in decreases in order *Clostridiales* (p=0.03), family *Clostridiaceae* group 1 (p=0.01) and *Peptostreptococcaceae* (p=0.02; Fig. 3). However, at the genus level, both increases and decreases were observed within the *Clostridia* class, with increases in *Defluvitaleaceae* UCG-011 (p=0.03), *Eubacterium oxidoreducens* group (p=0.02), *Marvinbryantia* (p=0.04), and decreases in *Lachnospiraceae* UCG-009 (p=0.02), *Ocillospira* (p=0.03) and *Papillibacter* (p=0.02). Mann-Whitney test revealed significant increases in other genra of bacteria in SI compared to GH, including *Bacillus* (p=0.02), *Prevotellaceae* UCG-001 (p=0.04), and *Veillonella* (p=0.03; Table 1). Alpha diversity analysis showed a trend toward decreased diversity in the SI compared to GH rats, but this was not significant (Shannon index, p=0.28). Beta diversity analysis by group showed a trend toward clustering by group, however this trend was also not significant (Adonis, p=0.12).

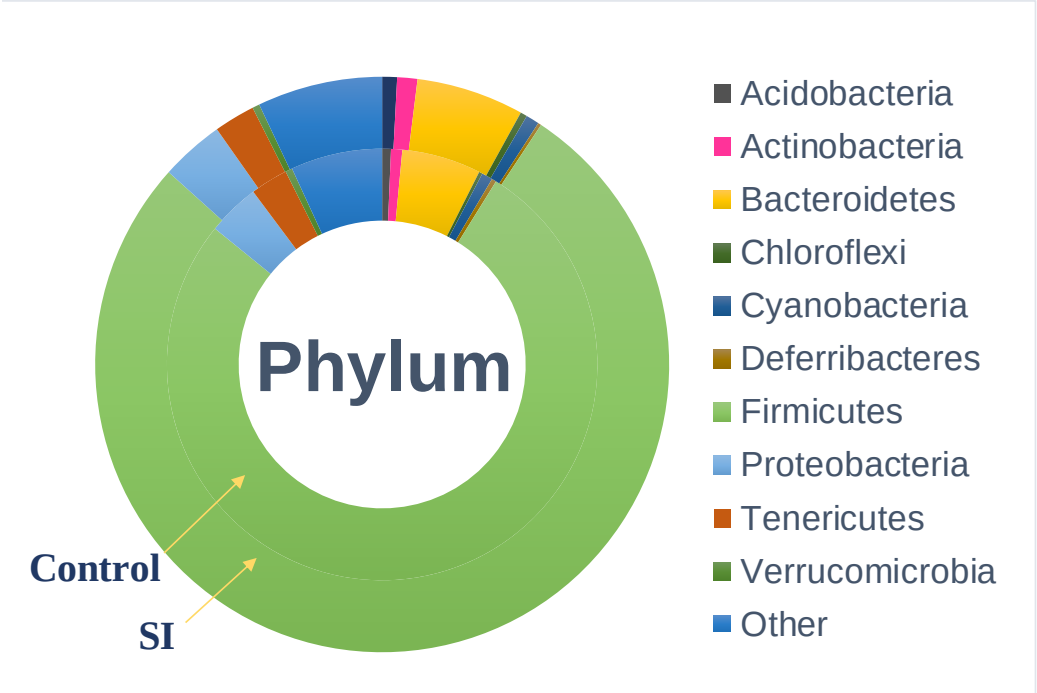


Figure 1: Relative abundance of bacteria at phylum level in group-housed (GH, Control) and isolation reared (SI) littermates. The main phyla are *Firmicutes*.

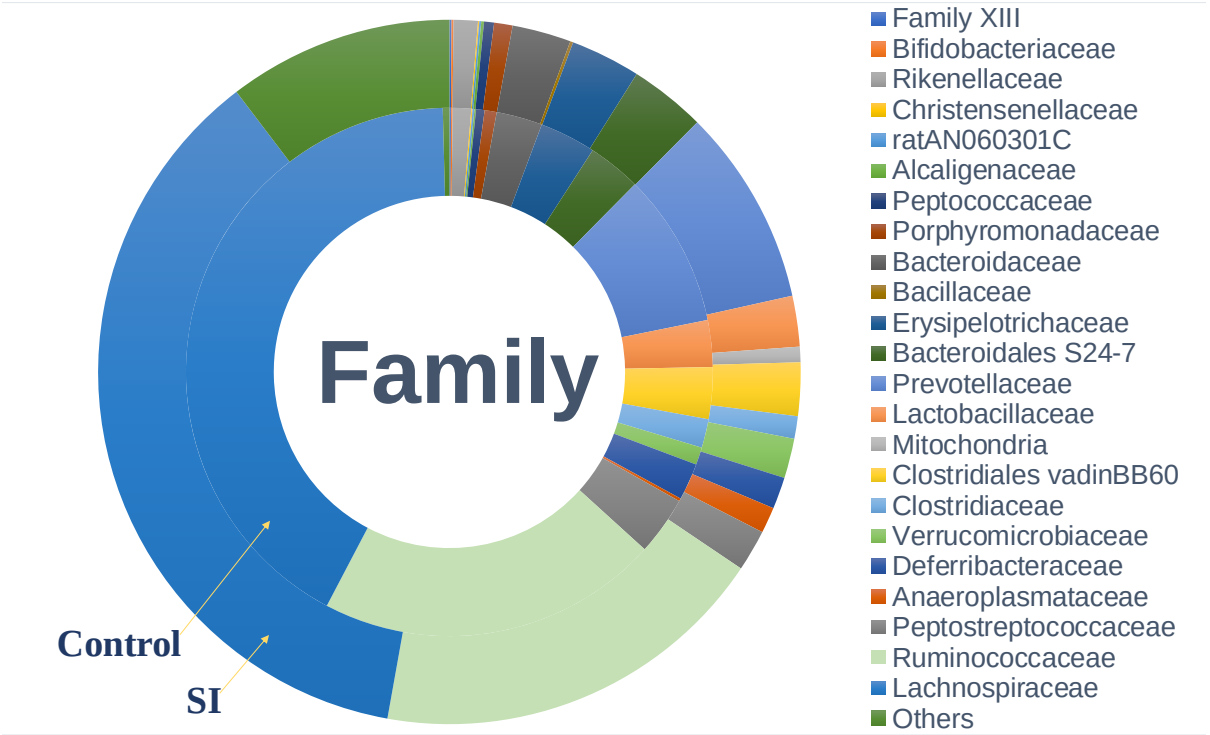


Figure 2: Relative abundance of bacteria at family level in group-housed (GH, Control) and isolation reared (SI) rats. The main families are *Lachnospiraceae* and *Ruminococcaceae*.

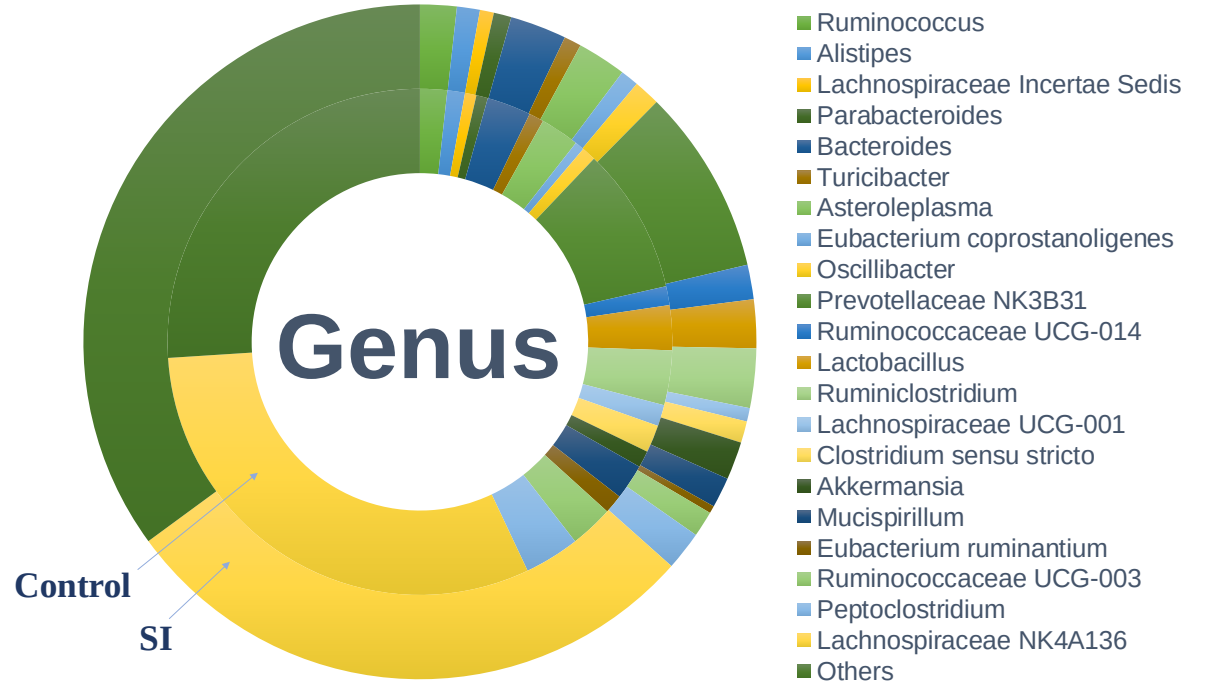


Figure 3: Relative abundance of bacteria at genus level in isolation reared (SI) compared with group-housed (GH, Control) rats.

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	↑ **Actinobacteria**	Actinobacteria	↑ **Corynebacteriales**	↑ **Nocardiaceae**	↑ ***Rhodococcus***
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	↑ ***Prevotellaceae UCG-001***
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	ratAN060301C	↑ ***uncultured bacterium***
Bacteria	Firmicutes	Bacilli	↑ **Bacillales**	↑ **Bacillaceae**	↑ ***Bacillus***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	↓ **Clostridiaceae 1**	*Clostridium sensu stricto 1*
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Defluviitaleaceae	↑ ***Defluviitaleaceae UCG-011***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Lachnospiraceae	↑ ***Eubacterium oxidoreducens group***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Lachnospiraceae	↑ ***Marvinbryantia***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Lachnospiraceae	↓ ***Lachnospiraceae UCG-009***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Ruminococcaceae	↓ ***Oscillospira***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	Ruminococcaceae	↓ ***Papillibacter***
Bacteria	Firmicutes	↓ **Clostridia**	↓ **Clostridiales**	↓ **Peptostreptococcaceae**	*Peptoclostridium*
Bacteria	Firmicutes	↑ **Negativicutes**	↑ **Selenomonadales**	↑ **Veillonellaceae**	↑ ***Veillonella***

↓ Decrease, ↑ Increase in SI relative to GH | **Significant at taxanomic level (p<0.05)**

Table 1: Summary of the significant changes in bacterial composition in isolation reared (SI) relative to group-housed (GH) rats. Yellow highlighting of Taxa indicates significance ($p<0.05$), arrows indicate increases or decrease in SI relative to GH rats.

Correlations were performed between salient individual representative values of specific behaviours and neurochemical measures that had been significantly altered by housing and significant changes in microbiota found in the same rat, thus focussing on those parameters that were significantly altered by early-life housing. Microbiota that significantly differed between groups were highly correlated to hippocampal IL-6 and faecal boli in the open field test (Fig. 3). Taxa in the order *Clostridales* were positively correlated to faecal boli ($\rho=0.54$, $p=0.01$) in all groups. This positive correlation continued at the family level (*Ruminococcaceae*, $\rho=0.57$, $p=0.01$), and genus level (*Papillibacter*, $\rho=0.57$, $p=0.01$; *Oscillospira*, $\rho=0.47$, $p=0.02$). Many of the same taxa in the order of *Clostridales* were negatively correlated to distance travelled ($\rho=-0.52$, $p=0.02$) and velocity ($\rho=-0.52$, $p=0.02$) in the open field test. Relative abundance of bacteria in the order *Bacillales* was negatively correlated to faecal boli ($\rho=-0.44$, $p=0.03$) and hippocampal IL-6 ($\rho=-0.53$, $p=0.01$), with significance remaining at the Family and Genus levels. Bacteria in the order *Bacillales* was also positively correlated to distance travelled ($\rho=0.59$, $p=0.01$) and velocity ($\rho=0.59$, $p=0.01$) in the open field, significant correlations remained at the family and genus level. Further correlations could be seen in microbiota and other measures significantly different between groups. Hippocampal IL-10 ($\rho=0.56$, $p=0.02$) and IL-6 ($\rho=0.48$, $p=0.02$) was positively correlated to *Peptostreptococcaceae*. Negative correlations to 48 h pre-cue measures from the CFR task were seen in genra *Veillonella* ($\rho=-0.45$, $p=0.03$) from order *Selenomonadales* and *Eubacterium oxidoreducens* group ($\rho=-0.41$, $p=0.05$) belonging to the order *Clostridales*. Negative correlations were also seen in genus *Defluviitaleaceae* UGC-001 of the order *Clostridales* for CFR freezing time at 24 h ($\rho=-0.50$, $p=0.02$) and 48 h post-conditioning ($\rho=-0.54$, $p=0.01$).

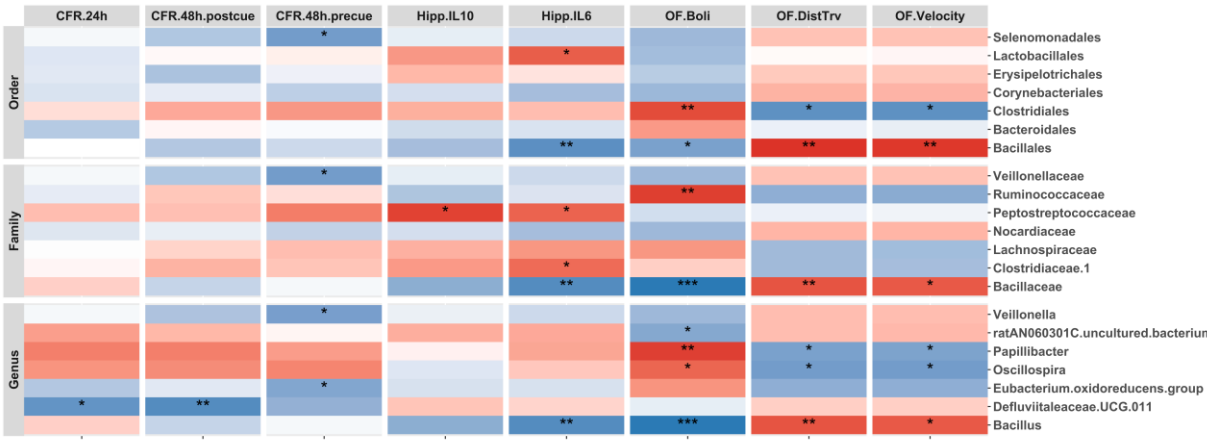


Figure 4: Spearman heatmap focussing on correlation between microbiota and behavioural and cytokine measures found to be significantly different as a consequence of housing during development. Top x-axis columns: Conditioned Freezing Response (CFR) task, hippocampal (Hipp) cytokines, and open field (OF) left to right, respectively. Y-axis groups taxa by phylogeny (right y-axis) and corresponding taxonomic names (left y-axis). Scale (right legend) indicates level of positive (red) or negative (blue) correlation, asterisk indicates significance (*P<0.05, **P<0.01 and ***P<0.00