Development and characterisation of a novel preclinical test battery for detecting anxiolytic and antidepressant properties of drugs

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Development and characterisation of a novel preclinical test battery for detecting anxiolytic and antidepressant properties of drugs

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Abstract

Depression is a chronic, recurring and potentially life-threatening disorder that is highly comorbid with anxiety. The aetiology of depression, anxiety and their comorbidity is still largely unknown. Despite the development of several different neurochemical theories of these disorders, all current first-line treatments work by the same fundamental mechanism – by altering monoamine neurotransmission. However, these drugs have several limitations, including a therapeutic lag, numerous adverse side effects and low remission rates. Therefore, there is a need for novel acting drugs, and thus, a need for improved preclinical screening for detection of these drugs. The present work aimed to develop and characterise a novel preclinical test battery for assessing acute and chronic anxiolytic and antidepressant properties of drugs in rats. Behavioural tests of anxiety (elevated plus maze; EPM) and depression (forced swim test; FST) were first characterised to determine optimal experimental parameters for detecting drug efficacy. Following this, several behavioural tests were incorporated into two battery study designs to provide a more time-efficient and detailed screening of a drug’s pharmacological properties, with subsequent assessment of hippocampal brain derived neurotrophic factor (BDNF) and its receptor, tyrosine receptor kinase B (TrkB) as potential biomarkers of antidepressant response. Briefly, the first battery study design involved the assessment of acute anxiolytic properties in the EPM and open field test or the resident-intruder paradigm. Animals from both subsets received further chronic dosing, after which they were tested in the FST or by assessment of anhedonia in the saccharin preference test (SPT) after acute systemic lipopolysaccharide (LPS) administration. The second battery study involved acute/subacute drug assessment in the FST or novelty-induced hypophagia test (NIH), followed by assessment of chronic drug effects in the SPT, NIH testing and FST. Standard antidepressant and anxiolytic drugs were used to validate the first (desipramine, fluoxetine and diazepam) and second (desipramine, fluoxetine, venlafaxine, ketamine and diazepam) test battery. In the initial characterisation studies, whilst some experimental parameters did not affect behavioural testing (breeding source), the route of drug administration and the subject’s age and housing environment differentially affected baseline and/or drug-induced effects in the EPM and FST. In addition, an improved method for screening false positive drugs prior to the FST was developed, whereby home cage locomotor assessment was successfully incorporated into the FST study design. The first battery study design provided important verification that EPM and open field testing can be reliably incorporated prior to assessment of drug effects in the FST, validating a more efficacious screening of compounds. Whilst the resident-intruder protocol used within subset 2 reliably detected antidepressant and anxiolytic drug effects, the systemic LPS model of depression did not induce an anhedonia effect, and thus, chronic drug effects could not be assessed in this test. In the second battery study, whilst the FST successfully detected subacute drug effects, chronic drug effects in the FST, systemic LPS model and NIH test, as well acute effects in the NIH test were not detected, due to several methodological factors. Nocturnal home cage locomotor activity was reduced in the desipramine group on days 7 and 14, and in the venlafaxine group on day 14 of drug treatment. As expected, due to the lack of chronic drug effects, hippocampal BDNF and
TrkB mRNA levels were not altered. This research has provided important methodological information for the improved implementation of behavioural tests for detecting anxiolytic and antidepressant drug efficacy, and emphasised the importance of standardisation between studies. Moreover, it has provided a basis for the future development of an improved battery study design, having provided insight into important methodological complications that can arise when incorporating several behavioural tests into one study design.
Author’s Declaration

I hereby declare that the work presented in this thesis was carried out in accordance with the regulations of the National University of Ireland, Galway. The research is original and entirely my own with the following assistance:

- Characterisation studies in Chapter 3 were assisted by Kiah McCabe, Ann Cronin and Silke Kleefeld.
- Battery Studies in Chapter 4 were assisted by Rebecca Culkin and Fatma Giumaa.

The thesis or any part thereof has not been submitted to the National University of Ireland, Galway, or any other institution in connection with any other academic award. Any views expressed herein are those of the author.

Signed: ____________________________ Date: __________
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<tr>
<td>%OAE</td>
<td>Percentage open arm entries</td>
</tr>
<tr>
<td>%OAT</td>
<td>Percentage open arm time</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<tr>
<td>AMP</td>
<td>Amphetamine</td>
</tr>
<tr>
<td>APA</td>
<td>American Psychiatric Association</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>CMS</td>
<td>Chronic mild stress</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotrophin releasing factor</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin releasing hormone</td>
</tr>
<tr>
<td>CUMS</td>
<td>Chronic unpredictable mild stress</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DALYs</td>
<td>Disability-adjusted life years</td>
</tr>
<tr>
<td>DMI</td>
<td>Desipramine</td>
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<tr>
<td>DSM</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
</tr>
<tr>
<td>DZP</td>
<td>Diazepam</td>
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<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
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<tr>
<td>EE</td>
<td>Environmental enrichment</td>
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<tr>
<td>EPM</td>
<td>Elevated plus maze</td>
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<tr>
<td>FDA</td>
<td>Food and Drugs Administration</td>
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<tr>
<td>FLX</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>FST</td>
<td>Forced swim test</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-ammino butyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Generalized anxiety disorder</td>
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<tr>
<td>HAM-A</td>
<td>Hamilton rating scale for anxiety</td>
</tr>
<tr>
<td>HAM-D</td>
<td>Hamilton rating scale for depression</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>KET</td>
<td>Ketamine</td>
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</table>
LPS  Lipopolysaccharide
MAOI  Monamine oxidase inhibitor
MASSA  Melatonin agonist and specific serotonin antagonist
mRNA  Messenger ribonucleic acid
MDD  Major depressive disorder
mRNA  Messenger RNA
NA  Noradrenaline
NaSSA  Noradrenergic and specific serotonergic antidepressant
NDRI  Noradrenaline and dopamine reuptake inhibitor
NE  Norepinephrine
NGF  Nerve growth factor
NIH  Novelty-induced hypophagia
N.S.  Not specified
OAE  Open arm entries
OAT  Open arm time
p.o.  Oral gavage
qRT-PCR  Quantitative Real-Time Polymerase Chain Reaction
RIMA  Reversible monoamine oxidase inhibitor
s.c.  subcutaneous
SAD  Social anxiety disorder
SDRI  Selective dopamine reuptake inhibitor
SERT  Serotonin transporter
SNRI  Selective norepinephrine reuptake inhibitor
SPT  Saccharin preference test
SSRE  Selective serotonin reuptake enhancer
SSRI  Selective serotonin re-uptake inhibitor
TCA  Tricyclic antidepressant
TNF  Tumor necrosis factor
TrkB  Tyrosine receptor kinase B
V/A  Videotaped from above
VLX  Venlafaxine
WHO  World Health Organisation
List of Publications and Conference Proceedings

Manuscripts in preparation


Published Abstracts from Conference Proceedings


**Unpublished Abstracts from Conference Proceedings**


Bannerton, K., McCabe, K, McAleavey, Z and Kelly, J.P. May 2013. A comparison of behavioural profiles between commercially obtained and in-house bred rats. College of Medicine, Nursing & Health Sciences postgraduate research day, NUI, Galway. Poster presentation.
Major depressive disorder (MDD), or depression, is a chronic, recurring and potentially life-threatening disorder that affects approximately 350 million people worldwide (www.who.int.). It is a highly debilitating disease that is ranked by the World Health Organisation (WHO) as among the most burdensome diseases to society. In fact, the Global Burden of Disease study 2010 reported that depression ranked fifth among all non-communicable diseases in terms of its disabling impact on the world population (Figure 1.1) (Murray et al., 2012). Indeed the disabling effect of the disease has increased in the past decade, with the disability-adjusted life years (DALYs) associated with depression increasing by 37.5 % between the years of 1997 and 2010, to reach a figure of over 63 million DALYs (Murray et al., 2012). Furthermore, individuals with major depression are faced with significant personal and societal costs due to decreased work productivity (Adler et al., 2006, Plaisier et al., 2010), increased absenteeism (Plaisier et al., 2010, Stewart et al., 2003) and increased utilization of health care services (Simon et al., 2000, Unutzer et al., 1997). The extent of such indirect costs of depression was reported by Ekman et al. (2013) in which the largest cost item for depressed patients in specialized psychiatric care in Sweden was indirect costs due to productivity loss. These findings illustrated that the highest severity of depressive symptoms induced indirect costs of over €25,000 annually. Furthermore, the annual cost of depression in Europe in 2004 was estimated at €118 billion, with 36% and 64% of this due to direct and indirect costs, respectively (Sobocki et al., 2006). Depression is also associated with high rates of complicating comorbidities and mortalities. For example, in a study carried out by Demyttenaere et al. (2006), physical pain symptoms were reported in 21% more people with depression, compared to respondents with no depression. Furthermore, Ferketich et al. (2000) found that men who suffered from depression were not only 71% more likely to develop heart disease, but that they were 2.34 times more likely to die from the condition compared to non-depressed men.
Chapter 1: General Introduction

Figure 1.1: Global DALYs for the top 10 most disabling non-communicable diseases. Data obtained from Murray et al. (2012). COPD = chronic obstructive pulmonary disease.

1.1. Co-morbidity of depression and anxiety
As mentioned, individuals suffering from major depression often have high rates of complicating comorbidities, with anxiety disorders ranking as one of the most common of these. Studies show that the presence of an anxiety disorder is the single strongest risk factor for the development of depression (Wittchen et al., 2000), whilst Kessler et al. (1996) reported that among patients suffering from major depression, over 50% also suffer from an anxiety disorder. In a more recent study examining comorbid patterns, Lamers et al. (2011) reported that in patients with a depressive disorder, 67% had a current and 75% had a lifetime comorbid anxiety disorder. Moreover, in patients that had a current anxiety disorder, 63% had a current and 81% had a lifetime depressive disorder. The comorbidity of major depression and anxiety disorders typically presents itself as one of four clinical combinations:

1) anxiety disorder with subsyndromal levels of depression symptoms
2) major depression with subsyndromal anxiety symptoms
3) full-fledged major depression and anxiety disorder
4) symptoms of both major depression and anxiety disorder that are not severe enough to classify the patients as having major depression or anxiety disorder (known as mixed anxiety depression) (Hirschfeld, 2001).
In the case of the latter, studies have reported that 1 in 5 of these patients will go on to develop full blown major depression within a year (Roy-Byrne et al., 1994). Anxiety in itself is extremely debilitating, with Murray et al. (2012) reporting that anxiety disorders caused more than 26 million DALYs globally in 2010. Furthermore, generalized anxiety disorder (GAD) is associated with increased impairments in psychosocial functioning and work productivity (Revicki et al., 2012). For example, Plaisier et al. (2010) found that individuals with anxiety reported decreased work productivity and increased absenteeism compared to individuals with no diagnosis. The high incidence of depression and anxiety comorbidity is extremely detrimental as it results in a significant increase in personal and societal costs, as well as much greater impairment and mortality in patients. For example, patients with this comorbidity endure increased total health care costs, with an increased cost of 62% compared to patients suffering from GAD alone (Figure 1.2) (Zhu et al., 2009). Moreover, Doering et al. (2010) reported an increased number of deaths in heart disease patients that suffered from comorbid anxiety and depression compared to those suffering from just one of these disorders. Overall, Hranov (2007) reported that comorbid depression and anxiety is associated with greater symptom severity, greater impairment in everyday functioning, greater physical and mental disability, a reduction in quality of life, delayed and reduced treatment response and a greater likelihood of suicide.

![Figure 1.2](image)

**Figure 1.2**: Total health care cost of patients suffering from GAD or comorbid GAD/Depression. Data obtained from Zhu et al. (2009).

1.2. **Diagnostic Criteria for Psychiatric Disease**
Currently, the two main classification systems for mental disorders include the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the mental-disorders
sections in the International Classification of Diseases (ICD). The first nationally acceptable psychiatric classification system for diagnosing in-patients with severe psychiatric and neurological disorders was developed in 1921, although a much broader classification system was developed after the end of World War II in 1945. At a similar time, the WHO published the sixth edition of the ICD (ICD-6), which for the first time, included a section for mental disorders. The American Psychiatric Association published the first edition of the DSM in 1952, which consisted of a variant of the ICD-6. This DSM was the first official manual of mental disorders to focus on clinical use. The listings and explanations of mental disorders in both the DSM and ICD systems slowly advanced throughout the years, with important innovations emerging in 1980 with the publication of DSM-III, which for the first time included explicit diagnostic criteria, a multiaxial diagnostic assessment system, and an approach that attempted to be neutral with respect to the causes of mental disorders. While the function of the DSM was primarily to provide precise definitions of mental disorders for clinicians and researchers, the main function of the ICD was to merely outline categories for the collection of basic health statistics. Thus, due to this lack of specificity, the ICD was modified for use after the publishing of ICD-9, resulting in ICD-9-CM (for clinical modification). DSM-IV was published in 1994 and consisted of numerous changes to the classification system (including addition, deletion, and reorganization). Developers of DSM-IV and ICD-10 (published in 1992) worked closely to coordinate their efforts, resulting in increased congruence between the two systems for the publishing of DSM-V in 2013.

1.2.1. Diagnosis criteria for major depression

According to DSM, the essential feature of a Major Depressive Episode is the presence of either depressed mood or the loss of interest or pleasure in nearly all activities for at least two weeks. In addition, patients must suffer from at least four additional symptoms of those outlined below:

- Alterations in appetite or weight
- Changes in sleep patterns
- Psychomotor agitation or retardation
- Fatigue or loss of energy
- Feelings of worthlessness or excessive guilt
- Diminished ability to think or concentrate
Chapter 1: General Introduction

• Recurrent thoughts of death or suicide

Importantly, a symptom must be either newly present, have worsened compared to the pre-episode status, and be present throughout most of the day, every day for two consecutive weeks. The state of depression can be characterized as mild, moderate or severe, depending on the level of impairment ensued in daily life (American Psychiatric Association, 2013). Given that depression is diagnosed based on having 5 out of 9 symptoms, the disease is extremely heterogeneous. For instance, a patient who suffers from decreased appetite, insomnia and psychomotor agitation is scored as having identical symptoms as another patient who suffers from fatigue, excessive guilt and diminished ability to concentrate. Thus, to claim that all those meeting the DSM criteria for the diagnosis of major depression are suffering from the same disorder seems erroneous. This heterogeneity is one of the factors contributing to the non-definitive pathophysiology and aetiology of the disease, with numerous theories of depression having been developed over the past 70 years. The severity of depression is rated using the Hamilton rating scale for depression (HAM-D) (Hamilton, 1960). This consists of a 21 item questionnaire (originally 17 items), with each item being scored on a 3 or 5 point scale. The severity of depression is related to the total score – the higher the score, the more severe the depression.

1.2.2. Diagnosis criteria for Anxiety

Several distinct anxiety disorders are defined in the DSM. This section will focus on just one of these disorders, GAD, as an example of the type of criteria that defines an anxiety disorder. For GAD, the essential feature is excessive anxiety or worry about a number of events or activities, which appears more days than not for at least 6 months. The anxiety or worry is difficult to control and must be accompanied by at least three additional symptoms:

• Restlessness
• Being easily fatigued
• Difficulty concentrating
• Irritability
• Muscle tension
• Disturbed sleep
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The focus of the anxiety or worry is not confined to features of another Axis 1 disorder and although individuals may not always recognize the symptoms as excessive, subjective distress may be reported due to inability to control the worry, or the individual may experience associated impairment in other important areas of functioning. The intensity, duration or frequency of the anxiety and worry is far out of proportion to the actual likelihood or impact of the feared event (American Psychiatric Association, 2013). The person is unable to keep worrisome thoughts from interfering in their daily tasks. The Hamilton Anxiety Scale (HAM-A) (Hamilton, 1959) is a rating scale that is widely used in the clinical and research settings to quantify the severity of anxiety symptoms. It consists of 14 items, each of which is defined by a series of symptoms. Each of the 14 items is rated on a 5 point scale, with 0 being not present and 4 being severe.

1.3. Neurochemical theories of depression and anxiety

It is clear that depression is among the most burdensome and debilitating diseases to society. Despite the prevalence of the disease, there is still a severe lack of understanding of its aetiology. Much of the current understanding of the neurobiology of depression has evolved from serendipitous discoveries, such as the finding that monoamine-altering pharmacological compounds improve mood. This discovery has led to the development of numerous monoamine altering compounds for treating depression, which ultimately led to the formation of a ‘monoamine’ theory of depression (Elhwuegi, 2004). Indeed, all current pharmacological treatments for depression work by this same fundamental mechanism. However, due to the limited efficacy and the side effects associated with these distinct compounds, efforts have been made to develop novel, non-monoamine acting drugs that may provide increased efficacy and safety profiles. The search for these novel acting compounds has resulted in the development of several other theories of depression, including the Neuroinflammatory Theory (Dantzer et al., 2011), the Neurotrophic Theory (Bjorkholm and Monteggia, 2016) and the Glucocorticoid Theory (Pariante and Miller, 2001).

Anxiety disorders are also among the most prevalent class of psychiatric disorders (Wittchen et al., 2011, Kessler et al., 2005, Hajos-Korcsok et al., 2003). There is considerable variation in the types of anxiety that can be experienced, resulting in the development of several distinct anxiety disorders. Included under the umbrella of anxiety disorders are panic attacks, agoraphobia, panic disorder, specific phobias, social
phobia, obsessive compulsive disorder, post traumatic stress disorder, acute stress
disorder, GAD, anxiety disorder due to a medical condition, and substance-induced
anxiety disorders (American Psychiatric Association, 2013). Due to the variety of
anxiety disorders that exist, and the high comorbidity that occurs with each of them, the
distinct neurochemical substrates of anxiety are also difficult to define. However, two
of the main neural systems that have been reported to be predominantly involved in the
aetiology of anxiety include the GABA and the serotonin or 5-hydroxytryptamine (5-HT)
system. Evidence for each of these theories of depression and anxiety will be
discussed in detail below.

1.3.1. The Monoamine theory of depression
The monoamine theory of depression suggests that depression arises due to a deficiency
in brain monoaminergic (5-HT, noradrenaline (NA) and dopamine (DA)) activity and
that symptoms are relieved by treatment with drugs that restore monoaminergic
functioning. The first evidence for this theory was reported in 1952 with the
serendipitous discovery that iproniazid, a drug which was capable of inhibiting
monoamine oxidase (Zeller et al., 1952) and producing a rapid increase in 5-HT brain
levels (Udenfriend et al., 1957), had mood elevating effects in patients with
tuberculosis and depression (Lopez-Munoz and Alamo, 2009). Further clinical (Crane,
1957, Ayd, 1957, Loomer et al., 1957) and preclinical (Chessin et al., 1957) studies
confirmed these antidepressant effects. The monoamine hypothesis was strengthened in
1958, when the first tricyclic antidepressant (TCA), imipramine showed efficacy in
treating depression (Kuhn, 1958), which led to the development of a whole series of
TCAs throughout the 1960s. Toward the end of the 1960s, the ‘serotonin hypothesis of
depression’ gained momentum, as several studies reported the ability of TCAs to inhibit
cerebral reuptake of 5-HT. The year 1974 saw the development of another class of
antidepressants (Wong et al., 1974), the selective serotonin reuptake inhibitors (SSRIs).
Feighner (1983) reported the first positive results for the treatment of depression with
the SSRI fluoxetine (FLX), whereby FLX was just as effective as TCA drugs, and also
showed far fewer side effects. Consequently, in 1984 FLX was approved by the Food
and Drugs Administration (FDA) for the treatment of depression. Thereafter, from the
mid 1990s, several antidepressant drugs with different pharmacodynamic properties
were developed for the treatment of depression, although they each shared the function
of altering monoaminergic functioning (explained in detail in Table 1.13). The fact that
all currently effective antidepressant drug treatments alter monoamine function provides
the strongest clinical evidence for the role of monoamines in depression. However, other findings such as decreased 5-HT transporter (SERT) and receptor levels in post mortem brain of depressed patients further verifies this theory (Table 1.1). Preclinical investigations have reported similar findings, with these monoamine altering antidepressants attenuating depressive-like behaviour in rodents. Furthermore, preclinical genetic and post mortem studies have illustrated the undeniable correlation between depressive-like behaviour and altered monoamine levels (Table 1.2).

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Imipramine had antidepressant effects</td>
<td>Kuhn (1958)</td>
</tr>
<tr>
<td>Fluoxetine (SSRI) had antidepressant effects</td>
<td>Feighner (1983)</td>
</tr>
<tr>
<td>Several monoamine altering drugs developed for the treatment of depression</td>
<td>Lopez-Munoz and Alamo (2009)</td>
</tr>
<tr>
<td>Increase in 5-HT\textsubscript{1A} receptor levels in post mortem brains of suicide victims compared to control</td>
<td>Stockmeier et al. (1998)</td>
</tr>
<tr>
<td>Alterations in 5-HT\textsubscript{2A} receptor levels in post mortem brains of suicide victims compared to control</td>
<td>Cheetham et al. (1988)</td>
</tr>
<tr>
<td>Increase in SERT levels in post mortem brains of depressed patients compared to control</td>
<td>Dahlstrom et al. (2000); Gross-Isseroff et al. (1989)</td>
</tr>
</tbody>
</table>

Table 1.1: Clinical evidence for the monoamine theory of depression.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Antidepressants reduce behavioural despair</td>
<td>Porsolt et al. (1978); Yamada et al. (2013)</td>
</tr>
<tr>
<td>Serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET) knockout mice showed decreased behavioural despair compared to control</td>
<td>Perona et al. (2008)</td>
</tr>
<tr>
<td>Chronic antidepressant treatment attenuated inflammation-induced anhedonia</td>
<td>Yirmiya (1996); Shen et al. (1999)</td>
</tr>
<tr>
<td>Chronic mild stress induced anhedonia, which was associated with an increased turnover rate of 5-HT and DA in several brain regions</td>
<td>Lin et al. (2016)</td>
</tr>
<tr>
<td>Chronic mild stress induced behavioural despair and reduced 5-HT levels in the hippocampus, which was prevented by antidepressant treatment</td>
<td>Xu et al. (2016)</td>
</tr>
</tbody>
</table>

Table 1.2: Preclinical evidence for the monoamine theory of depression.
1.3.2. The Neuroinflammatory theory of depression

The immune system consists of biological structures and processes that facilitate an organism’s adaptation to physiological or psychological stressors (Haapakoski et al., 2016). Inflammation refers to a process of the immune system which involves a biological host defence mechanism. This process is characterized by increased blood flow and recruitment of innate immune cells to the site of injury (Haapakoski et al., 2016). The principal features of inflammation include elevations in relevant inflammatory cytokines (signalling molecules that regulate immunity and inflammation) and their soluble receptors in peripheral blood and cerebrospinal fluid. In addition, there are elevations in acute phase proteins, chemokines, adhesion molecules and inflammatory mediators such as prostaglandins (Miller et al., 2009). Interactions between the central nervous system (CNS) and the immune system are receiving growing interest as playing a role in several psychiatric disorders, a field which is referred to as psychoneuroimmunology. In particular, a role for the immune system in depression has been uncovered, with considerable experimental and clinical evidence illustrating a link between the rise in pro-inflammatory cytokines and depressive symptoms (Dantzer et al., 2011).

The idea of inflammation-associated depression first arose when Smith (1991) proposed ‘The macrophage theory of depression’, based on findings that cytokines produced by macrophages induce symptoms of depression in healthy volunteers. This theory suggested that depression is associated with excessive production of inflammatory mediators by macrophages and findings thereafter supported this, with Maes (1999) illustrating biochemical evidence for the activation of the innate immune system in depressed patients. Furthermore, treatment of cancer patients with cytokine immunotherapy showed rapid development of a series of sickness-like symptoms that were followed weeks later by depressive-like symptoms (Capuron et al., 2002). Interestingly, antidepressant treatment alleviated the depressive symptoms induced by the cytokine immunotherapy, whilst the sickness-like symptoms were less responsive (Capuron et al., 2002). These results, as well as other clinical findings (Table 1.3) provide considerable evidence for a potential role of the immune system in depressive-like symptoms. As a result of these findings, the inflammation theory of depression was further investigated in rodents by the administration of cytokines (Kentner et al., 2006) or lipopolysaccharide (LPS) (Yirmiya, 1996), an endotoxin that stimulates the production of the pro-inflammatory cytokines interleukin (IL)-1, IL-6, tumor necrosis
factor alpha (TNFα) and interferon gamma (IFNγ). Remarkably, the time course of cytokine-induced effects in rodents was similar to humans, inducing a sickness-like behaviour in the first days after treatment (Kentner et al., 2006), followed later by depressive-like symptoms (O’Connor et al., 2009, Frenois et al., 2007). Moreover, chronic pre-treatment of rodents with antidepressants has been shown to reduce LPS-induced up-regulation of pro-inflammatory cytokines (Molteni et al., 2013), as well as LPS-induced anhedonia (Yirmiya, 1996, Shen et al., 1999) and sickness-like behaviour (Castanon et al., 2001). These findings, along with the depressive-like and antidepressant-like effects of pro- and anti-inflammatory cytokines in rodents (Table 1.4), emphasise the importance of the immune system in depression.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines induced depressive-like symptoms in healthy volunteers</td>
<td>Smith (1991)</td>
</tr>
<tr>
<td>Innate immune system is activated in depressed patients</td>
<td>Maes et al. (1995); Maes (1999)</td>
</tr>
<tr>
<td>Mood and cognitive disorders occurred in cancer patients receiving cytokine therapy</td>
<td>Meyers (1999)</td>
</tr>
<tr>
<td>IFNα induced delayed onset of depressed mood and suicidal ideation that were reversed by antidepressant treatment</td>
<td>Capuron et al. (2002)</td>
</tr>
<tr>
<td>TNFα antagonist reduced symptoms of depression</td>
<td>Tyring et al. (2006)</td>
</tr>
</tbody>
</table>

Table 1.3: Clinical evidence for the neuroinflammatory theory of depression.
**Findings** | **Reference**
--- | ---
Systemic LPS induced anhedonia and behavioural despair | Frenois *et al.* (2007)
Systemic LPS induced symptoms of behavioural despair which correlated to increased pro-inflammatory cytokines | O’Connor *et al.* (2009)
Anti-inflammatory cytokine IL-10 alleviated LPS-induced depressive-like behaviour | Bluthe *et al.* (1999)
IL-10 prevented stress-induced depressive-like behaviour | Pan *et al.* (2013)
IL-6 knockout mice exhibited resistance to stress-induced development of depressive-like behaviour | Chourbaji *et al.* (2006)
Chronically pre-treatment with an antidepressant attenuated LPS-induced anhedonia and sickness behaviour | Yirmiya (1996); Shen *et al.* (1999); Castanon *et al.* (2001)
Chronic pre-treatment with an antidepressant attenuated chronic unpredictable mild stress (CUMS)-induced anhedonia and increases in pro-inflammatory cytokines | Lu *et al.* (2016)
CUMS induced depressive-like behaviour that was associated with increased pro-inflammatory cytokines IL-1β, IL-6 and TNFα and decreased IL-10 | You *et al.* (2011)

**Table 1.4:** Preclinical evidence for the neuroinflammatory theory of depression.

**1.3.3. The Neurotrophic theory of depression**

The neurotrophin family of neurotrophic factors is a family of structurally and functionally related peptides responsible for mediating potent survival and differentiation effects on numerous neuronal populations in the central and peripheral nervous systems (Boyd and Gordon, 2003). Neurons are constantly forming, eliminating and modulating connections in response to the continuous flow of information. Importantly, in order for neurons to sufficiently mediate and respond to activity, they must be plastic. Thus, neural plasticity refers to the ability of neurons to adapt in response to intrinsic and extrinsic signals (Wainwright and Galea, 2013). Our behaviour depends on our ability to process and synthesize this information, and therefore, is dependent on this neural plasticity. Hence, it is no surprise that dysregulation of this neural plasticity may be associated with neuropsychiatric disorders such as depression (Wainwright and Galea, 2013). Nerve growth factor (NGF) was the first prototypical member of the neurotrophin family to be identified, and was found to promote the survival and neurite outgrowth of sympathetic and sensory neurons (Levimontalcini and Hamburger, 1953). Brain derived neurotrophic factor (BDNF) was later purified (Barde *et al*., 1982), whilst currently the neurotrophin family includes four
members in mammals, nerve growth factor (NGF), BDNF, neurotrophin-3 and neurotrophin-4/5 (Boyd and Gordon, 2003).

In terms of neurotrophic alterations in depression, specific attention has been given to BDNF and its receptor, tyrosine receptor kinase B (TrkB). The neurotrophic theory of depression primarily proposes that depressive symptoms emerge due to a reduction in neuronal connections, or neuroplasticity, that are caused by a deficiency in brain BDNF and TrkB signalling, and thus, normalising BDNF and TrkB signalling should ultimately restore plasticity and produce antidepressant-like effects. An association between BDNF and antidepressant treatment was first implicated in the 1990’s, when chronic, but not acute administration of several antidepressant drugs increased BDNF messenger RNA (mRNA) as well as mRNA for its receptor, TrkB in rat hippocampus (Nibuya et al., 1995, Nibuya et al., 1996). Soon after, infusion of BDNF into particular brain regions (Siuciak et al., 1997, Shirayama et al., 2002) produced antidepressant-like effects, illustrating the causal involvement of BDNF in antidepressant responses. Saarelainen et al. (2003) also reported that heterozygous BDNF null mice were resistant to antidepressant effects. Moreover, Liu et al. (2014) reported an association between depressive-like symptoms induced by CUMS and decreases in BDNF in the brain, effects that were attenuated by antidepressant treatment. This preclinical evidence, along with other findings (Table 1.5), is in line with clinical evidence in which BDNF has also been implicated in depression and antidepressant treatment. For example, several studies have reported down-regulation of BDNF and/or TrkB in the post mortem brains of depressed or suicide patients, compared to matched controls (Pandey et al., 2008, Dwivedi et al., 2003, Karege et al., 2005, Guilloux et al., 2012). Moreover, increased BDNF expression has been found in several areas of the hippocampus in depressed patients that were being treated with antidepressants at the time of death, compared to untreated patients (Chen et al., 2001) (Table 1.6).
Findings | Reference
---|---
Chronic, but not acute antidepressant treatment increased BDNF and trkB mRNA | Nibuya et al. (1995); Xu et al. (2003)
Infusion of BDNF into the midbrain or hippocampus has antidepressant-like effects | Siuciak et al. (1997); Shirayama et al. (2002)
Heterozygous BDNF null mice were resistant to antidepressant effects | Saarelainen et al. (2003)
Loss of BDNF in the dentate gyrus of the hippocampus reduced antidepressant actions on behavioural despair | Adachi et al. (2008)
CUMS induced symptoms of behavioural despair and anhedonia, and decreased BDNF in the amygdala and hippocampus | Liu et al. (2016); Sun et al. (2016)
Antidepressant treatment normalised CUMS-induced symptoms of behavioural despair and anhedonia, as well as decreases in BDNF in the amygdala and hippocampus | Liu et al. (2014)

Table 1.5: Preclinical evidence for the neurotrophin theory of depression.

Findings | Reference
---|---
Post mortem brains of depressed patients showed down-regulation of BDNF in amygdala | Guilloux et al. (2012)
BDNF (prefrontal cortex) and TrkB (prefrontal cortex and hippocampus) levels were decreased in suicide victims compared to matched controls. | Pandey et al. (2008)
BDNF and TrkB levels were decreased in prefrontal cortex and hippocampus of suicide victims compared to matched controls | Dwivedi et al. (2003)
BDNF levels were decreased in the hippocampus of suicide victims compared to matched controls | Karege et al. (2005)
BDNF levels in the hilus region of the hippocampus were increased in depressed patients treated with antidepressants compared to those who were not treated | Chen et al. (2001)

Table 1.6: Clinical evidence for the neurotrophin theory of depression.

1.3.4. The Glucocorticoid theory of depression
One of the characteristic features of depression is the disturbance of the hypothalamic-pituitary-adrenal (HPA) functionality (Pariante, 2003, Pariante, 2006). Activity of the
HPA axis is controlled by the secretion of corticotrophin releasing hormone (CRH), as well as arginine-vasopressin from the hypothalamus, which ultimately activates the secretion of adrenocorticotrophin hormone (ACTH) from the pituitary. ACTH in turn stimulates the release of glucocorticoids (cortisol in humans and corticosterone in rodents), which interact with their receptors (Zunszain et al., 2011). There is a substantial amount of both clinical and preclinical evidence to support the role of the HPA axis in depressive symptomatology. For example, depressed patients have been reported to have reduced glucocorticoid receptor binding (Sallee et al., 1995), as well as elevated levels of CRH (Raadsheer et al., 1994, Nemeroff et al., 1988), whilst antidepressant treatment has been found to normalise this dysregulation (Binder et al., 2009) (Table 1.7). In terms of preclinical evidence, depressive-like behaviour has been found to be increased in glucocorticoid receptor heterozygous mutant mice (Ridder et al., 2005) and after chronic corticosterone injections (Zhao et al., 2008), whilst chronic antidepressant treatment has been found to normalize CUMS-induced depressive-like behaviour, which was correlated with a normalisation of CUMS-induced alterations in HPA axis functioning (Xing et al., 2015) (Table 1.8).

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid receptor binding was decreased in depressed patients</td>
<td>Yehuda et al. (1993); Sallee et al. (1995)</td>
</tr>
<tr>
<td>Depressed patients showed elevated concentrations of corticotropin-releasing factor-like immunoreactivity in the cerebrospinal fluid</td>
<td>Nemeroff et al. (1984)</td>
</tr>
<tr>
<td>Depressed patients had increased numbers of CRH expressing neurons in the hypothalamic paraventricular nucleus region of the brain</td>
<td>Raadsheer et al. (1994)</td>
</tr>
<tr>
<td>Suicide victims showed a marked reduction in the number of CRH binding sites in the frontal cortex</td>
<td>Nemeroff et al. (1988)</td>
</tr>
<tr>
<td>Antidepressant treatment normalised HPA-axis dysregulation in remitter, but not non-remitter patients</td>
<td>Binder et al. (2009)</td>
</tr>
</tbody>
</table>

**Table 1.7:** Clinical evidence for the glucocorticoid theory of depression.
Chapter 1: General Introduction

### Findings

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucocorticoid receptor heterozygous mutant mice demonstrate increased helplessness after stress exposure compared to wild type mice</td>
<td>Ridder et al. (2005)</td>
</tr>
<tr>
<td>Repeated corticosterone injections increased behavioural despair in the FST and tail suspension test</td>
<td>Zhao et al. (2008); Gregus et al. (2005)</td>
</tr>
<tr>
<td>Antidepressants increase glucocorticoid receptor expression</td>
<td>Rossby et al. (1995); Reul et al. (1994)</td>
</tr>
<tr>
<td>Antidepressants enhance HPA axis function inhibition</td>
<td>Lopez et al. (1998); Montkowski et al. (1995)</td>
</tr>
<tr>
<td>Fluoxetine normalised CUMS-induced anhedonia and behavioural despair, and decreased CUMS-induced increases in ACTH and corticosterone</td>
<td>Xing et al. (2015); Cai et al. (2015)</td>
</tr>
</tbody>
</table>

**Table 1.8:** Preclinical evidence for the glucocorticoid theory of depression.

1.3.5. **An integrated view of the molecular neurobiology of depression**

In a review which assessed the neurobiology of depression, Maletic et al. (2007) discussed an integrated view of key findings, whereby the role of monoamines, inflammatory cytokines, brain derived growth factors and glucocorticoids (i.e. all of the theories) are combined into an integrated model of depression. Within this integrated model, Maletic et al. (2007) proposes the following processes in the development of depression:

1. Molecular processes are impacted by stress and depression
2. Stress in turn, causes an increase in glucocorticoids, CRH and pro-inflammatory cytokines
3. In depression, disruption of monoamine transmission (5-HT, NE and DA) impairs the feedback loop that ‘switches off’ the stress response
4. Sympathetic overactivity contributes to activation of the immune system and subsequent release of inflammatory cytokines
5. These cytokines then further interfere with monoamine and neurotrophic signalling, whilst they may also diminish central corticosteroid receptor sensitivity, which could disrupt feedback control (Maletic et al., 2007).

A diagrammatic depiction of this proposed model is illustrated below in Figure 1.3.
1.3.6. The Gamma-ammino butyric acid (GABA) theory of anxiety

GABA is the main inhibitory neurotransmitter in the CNS (Cryan and Kaupmann, 2005). GABA-mediated neurotransmission regulates many physiological and psychological processes, and has been shown to induce a calming effect on the brain. There are three GABA receptors in the brain, A, B and C. Benzodiazepine drugs, the first major breakthrough drugs for the treatment of anxiety, interact with GABA_A receptors, and this highlighted the role that GABA neurotransmission plays in anxiety disorders. Several preclinical studies have been carried out to uncover the role of this neurotransmitter system in anxiety disorders. For example, numerous benzodiazepine drugs have been reported to decrease anxiety-like behaviour, whilst anxiety/stress have been found to be associated with altered GABA neurotransmission in several brain regions. Several key preclinical findings are outlined in Table 1.9, which emphasise the functional and important role that GABA neurotransmission plays in anxiety. Considerable clinical evidence also exists which highlights the role that GABA plays in anxiety disorders. For example, not only were benzodiazepine drugs the mainstay treatment for anxiety disorders for a significant amount of time, but patients suffering from anxiety disorders also show altered GABA neurotransmitter and receptor levels in
several brain regions (Table 1.10). Taken together, these preclinical and clinical findings emphasise the vital role of the GABA system in anxiety disorders.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzodiazepine drugs decrease anxiety-like behaviour</td>
<td>Cosquer et al. (2005b); Haller et al. (2010)</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor antagonists produce anxiogenic-like effects, while GABA&lt;sub&gt;A&lt;/sub&gt; agonists produce anxiolytic-like effects</td>
<td>Sanders and Shekhar (1995)</td>
</tr>
<tr>
<td>Anxious rats have lower benzodiazepine receptors in the frontal cortex compared to non-anxious rats</td>
<td>Harro et al. (1990)</td>
</tr>
<tr>
<td>Chronic stress caused presynaptic functional alterations in GABAergic input in the paraventricular nucleus</td>
<td>Verkuyl et al. (2004)</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptor positive modulator has anxiolytic effects</td>
<td>Cryan et al. (2004); Dalvi and Rodgers (1996)</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt; receptor agonist has anxiolytic effects</td>
<td>Shephard et al. (1992); Ketelaars et al. (1988)</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;B(1)&lt;/sub&gt; receptor deficient mice illustrated increased anxiety and reduced sensitivity to anxiolytics</td>
<td>Cryan et al. (2004); Mombereau et al. (2004)</td>
</tr>
</tbody>
</table>

**Table 1.9:** Preclinical evidence for the involvement of GABA in anxiety.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzodiazepines attenuate symptoms of anxiety</td>
<td>Ravindran and Stein (2010)</td>
</tr>
<tr>
<td>Patients with social anxiety disorder (SAD) had lower GABA levels in the thalamus compared to healthy controls</td>
<td>Pollack et al. (2008)</td>
</tr>
<tr>
<td>Frontocortical and temporocortical GABA&lt;sub&gt;A&lt;/sub&gt; receptors were decreased in patients with panic disorder (PD) and GAD, respectively</td>
<td>Nikolaus et al. (2010)</td>
</tr>
<tr>
<td>Patients with panic disorder had reduced occipital cortex GABA levels</td>
<td>Goddard et al. (2001)</td>
</tr>
</tbody>
</table>

**Table 1.10:** Clinical evidence for the involvement of GABA in anxiety.

1.3.7. **The Serotonin theory of anxiety**

The fact that SSRIs are now considered first line pharmacological treatment for anxiety disorders (Outhoff et al., 2013) makes it clear that the 5-HT system plays a pivotal role
in mediating anxiety. Numerous preclinical studies have supported a role for the serotonin system in anxiety. For example, genetic studies have illustrated that mice lacking SERT or the 5-HT$_{1A}$ receptor elicit increased anxiety-like behaviour (Olivier et al., 2008, Heisler et al., 1998). Moreover, direct correlations have been made between brain 5-HT levels and anxious behaviour (Jahng et al., 2007). These findings, along with further evidence that is outlined in Table 1.11 prove the importance of 5-HT in anxiety. There is also considerable clinical evidence to suggest a role for 5-HT in anxiety disorders. In addition to SSRIs being the first line treatment for anxiety disorders, alterations in 5-HT receptor levels have also been reported in patients suffering from anxiety disorders (Table 1.12). The substantial evidence that can be seen in Table 1.11 and Table 1.12 highlight the key role of the 5-HT system in the modulation of anxiety.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT knockout mice displayed increased anxiety-like behaviour</td>
<td>Olivier et al. (2008)</td>
</tr>
<tr>
<td>5-HT$_{1A}$ receptor knockout mice displayed increased anxiety-like behaviour</td>
<td>Heisler et al. (1998)</td>
</tr>
<tr>
<td>Animals with serotonergic lesions displayed decreased anxiety-like behaviour</td>
<td>Briley et al. (1990)</td>
</tr>
<tr>
<td>Increase in anxiety behaviour was associated with increased 5-HT in the hippocampus in long-term food deprived animals</td>
<td>Jahng et al. (2007)</td>
</tr>
<tr>
<td>Footshock stress increased extracellular levels of 5-HT in the hippocampus</td>
<td>Hajos-Korcsok et al. (2003)</td>
</tr>
<tr>
<td>Chronic SSRI treatment prevented CUMS-induced anxiety-like behaviour</td>
<td>Bondi et al. (2008)</td>
</tr>
</tbody>
</table>

**Table 1.11:** Preclinical evidence for the serotonin theory of anxiety.
5-HT1A receptor binding was reduced in several brain regions in patients with SAD and panic disorder

SSRIs are effective at treating GAD

SSRIs are effective at treating panic disorder

SSRIs are effective at treating social phobia

SSRIs are effective at treating obsessive compulsive disorder

<table>
<thead>
<tr>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A receptor binding was reduced in several brain regions in patients with SAD and panic disorder</td>
<td>Lanzenberger et al. (2007); Neumeister et al. (2004); Nash et al. (2008)</td>
</tr>
<tr>
<td>SSRIs are effective at treating GAD</td>
<td>Baldwin et al. (2011a);</td>
</tr>
<tr>
<td></td>
<td>Baldwin et al. (2011b)</td>
</tr>
<tr>
<td>SSRIs are effective at treating panic disorder</td>
<td>Ballenger et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Lepola et al. (1998)</td>
</tr>
<tr>
<td>SSRIs are effective at treating social phobia</td>
<td>Stein et al. (1998);</td>
</tr>
<tr>
<td></td>
<td>Katzelnick et al. (1995)</td>
</tr>
<tr>
<td>SSRIs are effective at treating obsessive compulsive disorder</td>
<td>Greist et al. (1995)</td>
</tr>
</tbody>
</table>

Table 1.12: Clinical evidence for the serotonin theory of anxiety.

1.4. Treatment of depression and anxiety

1.4.1. Treatment of depression

Current treatments for depression include electroconvulsive therapy (ECT), pharmacological intervention and psychotherapy. ECT was the first effective method for the treatment of depression and was introduced in 1937. ECT involves applying electricity to the scalp to induce seizure activity, resulting in acute attenuation of depressive symptoms. Psychopharmacological treatments were later developed in the 1950’s with the serendipitous discovery that the compounds iproniazid and imipramine induced antidepressant effects. These discoveries resulted in the development of several other pharmacological compounds that were successfully used for the treatment of depression over the decades that followed. In the late 1970’s, psychotherapies were shown for the first time to effectively attenuate symptoms of depression, either alone or in combination with antidepressant drugs. Currently, pharmacological treatments are typically the first line of treatment for major depression, followed by psychotherapy, or a combination of both, while ECT is most often used only when these other treatment methods are not effective. The focus of this thesis is primarily on the pharmacological treatment of depression, and thus, only the pharmacological treatments are described further.

As mentioned earlier, both preclinical and clinical depressive symptoms are associated with deficient monoamine levels (Elhwuegi, 2004). This deficiency in monoamine functioning is the primary target of all currently effective antidepressants. Although
antidepressant drugs with different pharmacodynamic properties have been developed, they each share the function of altering monoaminergic functioning in some way. Since the development of TCA and monoamine oxidase inhibitor (MAOI) drugs in the 1950s, an extensive amount of similar but distinct monoamine altering drug classes have emerged for the successful treatment of depression (Table 1.13).

Of these drug classes, the most commonly prescribed for the treatment of depression include the TCAs, SSRIs, SNRIs and atypical antidepressants such as bupropion and agomelatine. The primary target of TCAs is NA, and to a lesser extent, 5-HT. TCAs inhibit the reuptake of the neurotransmitters NA and 5-HT, and thus, the levels of these neurotransmitters are increased in the synapse. Examples of TCAs include imipramine, desipramine (DMI), and amitriptyline. TCAs are associated with several adverse effects (described in detail later), and thus the development of the safer SSRI drugs, resulted in a decrease in TCA prescription, and a concomitant increase in SSRI prescription for the treatment of depression. As the name suggests, SSRIs selectively block the reuptake of 5-HT into synaptic neurons. The neuronal transporter for 5-HT is inhibited, thus resulting in an increase in synaptic 5-HT. Examples of SSRIs include citalopram, escitalopram, paroxetine and sertraline. Discovered in the 1970’s, the SNRI drug class inhibit the reuptake of both 5-HT and NA, resulting in synaptic increases of both of these neurotransmitters. Examples of SNRIs include duloxetine, venlafaxine (VLX) and milnacipran. Atypical antidepressants refer to those drugs that do not fit into any of the other defined classes of antidepressants. Within this category, each drug has a distinct mechanism of action, although the alteration of 5-HT, NA and DA are still the prime targets. Examples of atypical antidepressants include bupropion, nefazodone, mirtazapine and agomelatine. Although TCAs, SSRIs, SNRIs and atypical antidepressants are among the most commonly prescribed antidepressants, there are several other classes, which are outlined in Table 1.13.
<table>
<thead>
<tr>
<th>Classification</th>
<th>Acronym</th>
<th>Prototype Substance</th>
<th>Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT and NA reuptake inhibitors with blocking action of diverse receptors</td>
<td>TCA</td>
<td>Imipramine</td>
<td>1957-1980</td>
</tr>
<tr>
<td>Irreversible MAO inhibitors</td>
<td>MAOI</td>
<td>Phenelzine</td>
<td>1960-1965</td>
</tr>
<tr>
<td>NA reuptake inhibitors with blocking action of diverse receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antagonists of α&lt;sub&gt;2&lt;/sub&gt; auto-receptors</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Selective DA reuptake inhibitors</td>
<td>SDRI</td>
<td>Bupropion</td>
<td>1980-1990</td>
</tr>
<tr>
<td>Selective 5-HT reuptake inhibitors</td>
<td>SSRI</td>
<td>Fluoxetine</td>
<td>1980-1990</td>
</tr>
<tr>
<td>Reversible MAOIs</td>
<td>RIMA</td>
<td>Moclobemide</td>
<td>1980-1995</td>
</tr>
<tr>
<td>5-HT reuptake inhibitors and blockers of 5-HT&lt;sub&gt;2&lt;/sub&gt; receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antagonists of α&lt;sub&gt;2&lt;/sub&gt; auto- and hetero-receptors and 5-HT&lt;sub&gt;2&lt;/sub&gt; and 5-HT&lt;sub&gt;3&lt;/sub&gt; receptors</td>
<td>NaSSA</td>
<td>Mirtazapine</td>
<td>1975-2000</td>
</tr>
<tr>
<td>NA and 5-HT reuptake inhibitors</td>
<td>SNRI</td>
<td>Venlafaxine</td>
<td>1975-2000</td>
</tr>
<tr>
<td>Selective NA reuptake inhibitors</td>
<td>NRI</td>
<td>Reboxetine</td>
<td>1980-2000</td>
</tr>
<tr>
<td>NA and DA reuptake inhibitor</td>
<td>NDRI</td>
<td>Nomifensine</td>
<td>1975</td>
</tr>
<tr>
<td>Selective 5-HT reuptake enhancer, and µ opioid receptor agonist</td>
<td>SSRE</td>
<td>Tianeptine</td>
<td>1988</td>
</tr>
<tr>
<td>Agonist at melatonin receptors and selective antagonist at 5-HT&lt;sub&gt;3c&lt;/sub&gt; receptors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT reuptake inhibitor and 5-HT&lt;sub&gt;1A&lt;/sub&gt; receptor partial agonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;3&lt;/sub&gt; and 5-HT&lt;sub&gt;7&lt;/sub&gt; receptor antagonism, 5-HT&lt;sub&gt;1B&lt;/sub&gt; receptor partial agonism, 5-HT&lt;sub&gt;1A&lt;/sub&gt; receptor agonism, SERT inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.13:** List of various classes of antidepressant drugs that have been developed. Information adapted from Lopez-Munoz and Alamo (2009), Guardiola-Lemaître *et al.* (2014), Wagstaff *et al.* (2001), Gassaway *et al.* (2014), Defrance *et al.* (1988), Hanks (1977), Reed *et al.* (2012) and Katona and Katona (2014). TCA= tricyclic antidepressant; MAOI= monoamine oxidase inhibitor; SDRI= selective dopamine reuptake inhibitor; SSRI= selective serotonin reuptake inhibitor; RIMA= reversible monoamine oxidase inhibitor; NaSSA= noradrenergic and specific serotonergic antidepressant; SNRI= noradrenaline and serotonin reuptake inhibitor; NRI= noradrenaline reuptake inhibitor; NDRI= noradrenaline and dopamine reuptake inhibitor; SSRE= selective serotonin reuptake enhancer.
1.4.2. Prescription patterns for depressive disorders

After the initial discovery of pharmacological compounds for the treatment of depression in the late 1950s, TCAs became the first line treatment choice for the disorder. However, these drugs were associated with several adverse side effects (discussed in detail later), and thus, the discovery of SSRI antidepressants as safer alternatives in the 1980s resulted in a decrease in TCA prescription patterns, and a concomitant increase in SSRI prescriptions. In a study that assessed antidepressant prescribing patterns in 12 European countries, TCAs were among the least commonly prescribed antidepressants, whilst SSRIs were found to be the most commonly prescribed, being prescribed to 63.6% of depressed patients (Bauer et al., 2008). Selective NA and 5-HT reuptake inhibitors (SNRIs) followed in popularity, with 13.6% of patients being prescribed with this antidepressant class. Moreover, in a study that examined prescription trends in England from 1998 to 2010, Ilyas and Moncrieff (2012) found the two most prescribed drugs in 1998 to be the TCAs amitriptyline and dothiepin, whilst in 2010 the most prescribed antidepressant was the SSRI citalopram, followed by amitriptyline. Interestingly, the prescription of atypical antidepressants increased greatly throughout these years, with mirtazapine going from the least commonly prescribed antidepressant in 1998 to the fourth most prescribed antidepressant in 2010 (Ilyas and Moncrieff, 2012). More specifically, in a recent literature review, the prescribing pattern of antidepressants in Ireland between the years 2004 and 2013 was assessed (Garvey and Kelly, 2015). This data showed that although the prescription rates changed for each drug in the 10 years that elapsed, the ten most commonly prescribed antidepressants in 2004 remained almost the exact same as those prescribed in 2013, except for the replacement of trazodone (2004) with duloxetine (2013) (Figure 1.4 and Table 1.14). It is evident from Figure 1.4 that there has been considerable growth in antidepressant prescription rates, suggesting the growing demand for these drugs, yet the choice of antidepressant drugs remains predominantly static.
Figure 1. 4: The 10 most commonly prescribed antidepressants (in the thousands) in Ireland in 2004 and 2013. The drugs prescribed were the same for both years, except with the replacement of trazodone (2004) with duloxetine (2013). Data provided by Garvey and Kelly, 2015.

<table>
<thead>
<tr>
<th>Drug</th>
<th>2004</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Escitalopram</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Sertraline</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Dosulepin</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Mirtazapine</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Trazodone</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>N/A</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1. 14: Ranking of the 10 most commonly prescribed antidepressant drugs in Ireland in 2004 and 2013. N/A = Not applicable (Trazodone was replaced with Duloxetine in 2013).

The almost identical list of the 10 most commonly prescribed drugs in 2004 and 2013 signifies the standstill in antidepressant development, with just one different drug being
prescribed in 2013. This standstill in antidepressant development is a major problem, considering the several limitations to these currently prescribed antidepressants, including several adverse side effects, a delay in onset of action and a high percentage of non-responder patients (discussed in detail later).

1.4.3. Treatment of anxiety

As with depression, pharmacological intervention and psychotherapy are the two main treatments for anxiety disorders. These treatments are effective when carried out in isolation, or when combined, depending on the patient. As previously mentioned, the focus of this thesis is primarily on the pharmacological treatment for the disorders of anxiety and depression, and thus, only these will be described further. Before the 1950s, barbiturates were essentially the only pharmacological tools used in the treatment of anxiety, although they were not ideal due to the vast side effects elicited by these drugs. The first major breakthrough in the treatment of anxiety was the clinical introduction of meprobamate which exerted pharmacological properties that included muscle relaxation, anticonvulsant effects and a calming action (Berger, 1952) and this became the first medicine specifically marketed as an anxiolytic. However, this drug was subsequently withdrawn due to adverse side effects. Benzodiazepines later became the first line treatment for anxiety disorders. These drugs act as positive allosteric modulators on the GABA<sub>A</sub> receptor. They were developed in the 1960’s with the synthesis of chlordiazepoxide, which was approved by the US FDA for the treatment of anxiety in 1960 (Sternbach et al., 1963). Diazepam (DZP), a much more potent benzodiazepine was later synthesized in 1963, whilst the third generation of benzodiazepines was introduced in the 1970s, including alprazolam (used as an anxiolytic) and triazolam (used as a hypnotic). As mentioned, these drugs became the first-line treatment for anxiety disorders, with prescriptions for these drugs increasing by 110% between the years 1965 and 1970 (Parish, 1971). Although dependence became a problem for these drugs (Serfaty and Masterton, 1993), the implementation of various guides for the rational use of benzodiazepines saw this drug group enter a period of scientific and commercial stability from the 1990s onwards. Whilst several benzodiazepines are still commonly prescribed for the clinical treatment of anxiety disorders (Griffin et al., 2013), benzodiazepines have since become a second-line treatment, with the British National Institute for Health and Clinical Excellence concluding that benzodiazepines have fewer favourable outcomes than other treatment options such as SSRIs (National Institute for Health and Clinical Excellence, 2011).
A wide range of other psychotropic drugs have been investigated for the treatment of anxiety, and of these, antidepressants are among the most common. For example, TCAs, MAOIs, SSRIs, SNRIs and atypical antidepressants have shown efficacy in the treatment of several anxiety disorders (for review see Ravindran and Stein (2010)). Of all of these drugs, SSRIs, as well as some SNRIs are currently the first line treatment for anxiety disorders (Baldwin et al., 2011a) (Figure 1.5). The dominant role that the benzodiazepine and antidepressant drug classes have played in the treatment of anxiety is no surprise, considering that alterations in GABA (benzodiazepine target) and 5-HT neurotransmission are two of the main theories for the development of anxiety disorders. Thus, the deficiency in GABA and 5-HT functioning is the primary target of all currently effective anxiety-relieving drugs, which is illustrated by the list of evidence-based drug treatment options for anxiety disorders in Figure 1.5.
1.4.4. Prescription patterns for anxiety disorders

As described above, whilst benzodiazepines are still used for the treatment of anxiety, antidepressants have become the first line treatment for these disorders. The trends of drug prescription patterns for anxiety disorders can be seen in a study carried out by Benson et al. (2015). This study reported the 12 month prevalence rates for several types of psychotropic medications in the treatment of various different mental disorders within Northern Ireland (2004-2008). The results illustrated that antidepressants were the most common form of pharmacological treatment for ‘any anxiety disorder’, with 31.2% taking these types of drugs; a figure that is quite similar to the amount of people taking antidepressants for major depressive disorder (36.7%) (Figure 1.6). In comparison, anxiolytic drugs were taken as treatment in only 16.9% of patients.
suffering from ‘any anxiety disorder’. In terms of individual anxiety disorder, panic disorder was the anxiety disorder in which the most patients were taking antidepressants (48.9%). Interestingly, only 28.3% of those with panic disorder were being treated with anxiolytic drugs. This data (Figure 1.6) illustrates the diversity of treatments that are prescribed for patients suffering from anxiety, and emphasises the important role that both antidepressant and anxiolytic drugs play in alleviating these symptoms, compared to other drugs such as antipsychotics and mood stabilisers. As will be discussed in detail below, although antidepressants and anxiolytics are the most efficacious pharmacological compounds for relieving anxiety symptoms, these drugs are also associated with several adverse side effects that impact on patient safety, health and daily quality of life. Thus, there is high demand for safer and faster acting drugs to treat anxiety.
Figure 1.6: 12-month prevalence rates for each type of psychotropic medication by 12-month mental disorder. Figure adapted from Benson et al. (2015).
1.4.5. Treatment of comorbid depression and anxiety

As previously mentioned, depression and anxiety symptoms commonly co-exist. One choice for pharmacological treatment in patients suffering from this comorbidity is the combination of a benzodiazepine with an antidepressant (Furukawa et al., 2001). The main reason for this combination treatment is due to the delay in onset of antidepressant action (discussed under section ‘Limitations of current antidepressant/anxiolytic drugs’). Whilst antidepressants can take up to two weeks for efficacy to be observed, benzodiazepines work within hours (Altamura et al., 2013) to alleviate symptoms of anxiety. Thus, benzodiazepines provide rapid anxiety symptom relief at the beginning of pharmacological therapy, whilst the patient can be tapered off of the benzodiazepine once the antidepressant drug starts to become efficacious at relieving anxiety and depression symptoms. Moreover, due to the high rates of SSRI discontinuation that can occur with initial therapy, the rapid improvement of anxiety symptoms may provide a stable start for future adherence to antidepressant treatment, and thus improve overall results (Weisberg et al., 2007).

On the other hand, some comorbid depression and anxiety patients are treated with antidepressants alone. As previously mentioned, SSRIs and SNRIs are the first line treatment for anxiety disorders when they occur in isolation, whilst several studies have been carried out to assess the potential of SSRIs, SNRIs, and atypical antidepressants for the treatment of both depressive and anxiety symptoms in depressed patients. For example SSRIs such as paroxetine, citalopram, FLX and sertraline are among the SSRIs that have been found to be effective in the treatment of depression and anxiety symptoms (Schneier et al., 2003, Ravindran et al., 1997, Fava et al., 2000), whilst the SNRI VLX (Khan et al., 1998, Feighner et al., 1998) and the atypical antidepressant mirtazapine (Fawcett and Barkin, 1998) are among other antidepressant classes that have proven effective at treating this comorbidity.

While the administration of benzodiazepines + antidepressants, or antidepressants alone provide the best treatment for comorbid depression and anxiety symptoms, there are still several limitations of these drugs. As mentioned earlier, benzodiazepines and antidepressants are associated with several adverse side effects, as well as increased mortality. While the benzodiazepine + antidepressant treatment provides the added benefit of acutely treating the anxiety symptoms and potentially increasing the overall
adherence to the treatment, the potential risks of combination of these drug classes such as the increased side effect profile, drug-drug interaction profile and the increased risk of abuse of medication make this treatment method problematic. However, the alternative method of treatment with an antidepressant means that symptoms may not be alleviated for up to two weeks after treatment begins, increasing the health risk of depressed patients during these initial stages. It is clear therefore, that current treatments for comorbid depression and anxiety symptoms are far from optimal, and that there is a need for drugs with an improved safety profile as well as improved efficacy, that may ideally be derived from one pharmacological agent.

1.5. Limitations of current antidepressants/anxiolytic drugs

As mentioned, antidepressants and anxiolytics are commonly prescribed for the treatment of depression and anxiety, as well as their comorbidity. Although these compounds are the most effective at alleviating symptoms of these disorders, there are several limitations to these drugs, such as adverse side effects, a delay in onset of action, insufficient remission rates and a considerable amount of non-responder patients. Thus, these drugs are far from ‘ideal’. In an effort to define the ideal antidepressant, Richelson (1994) listed the following characteristics: rapid onset of action, intermediate half-life, defined therapeutic blood level, no side effects, minimal drug interaction, low toxicity associated with overdose, and a broad spectrum of efficacy. The addition of ‘efficacy in 100% of patients’ provides a complete description of the ideal antidepressant, while the same characteristics apply in defining the ideal anxiolytic drug. Although compounds comprising of these characteristics have been sought after for many decades, current antidepressant and anxiolytic drugs are still inadequate. Thus, the possibility of developing such an ideal drug for the treatment of comorbid depressive and anxiety symptoms is even more challenging. The various limitations associated with both antidepressant and anxiolytic drugs are listed below.

1.5.1. Safety and side effects of antidepressants and benzodiazepines

The side effects associated with depression vary in extremity, from the most severe adversity of death due to toxicity, to milder side effects such as restlessness or nausea. After the discovery of SSRIs, a series of studies showed that this drug class elicited a milder side-effect profile and was more tolerable compared to the TCAs (Benfield et al., 1986, Benfield and Ward, 1986, Dechant and Clissold, 1991, Murdoch and McTavish,
1992). Thus, as previously mentioned, SSRIs became the agents of first choice for the treatment of depression, and remain so today (Chee et al., 2015)(Garvey and Kelly, 2015). Despite the improved safety profile of the newer drug classes, all current antidepressants are still associated with side effects, and several studies have been carried out to compare both the fatal toxicity index and adverse drug reactions of certain antidepressant classes, which will each be dealt with in the next paragraphs.

In terms of fatal toxicity index, Ojanpera et al. (2016) reported that out of 70 medicinal drugs in Finland, the antidepressants doxepin, amitriptyline, trimipramine and bupropion were among the drugs with the highest individual fatal toxicity indices. More specifically, in a study that compared the relative toxicity of several TCAs (amitriptyline, clomipramine, dosulepin (dothiepin), doxepin, imipramine, nortriptyline, trimipramine), a SNRI (VLX), a noradrenergic and specific serotonergic antidepressant (mirtazapine), and several SSRIs (citalopram, FLX, fluvoxamine, paroxetine and sertraline) in the UK, case fatality rate ratios showed greater toxicity for TCAs than the SNRI VLX and the NaSSA mirtazapine, both of which had greater toxicity than the SSRIs (Figure 1.7). Moreover, even within the distinct drug classes of TCAs and SSRIs, there were wide differences in toxicity between specific drugs (Hawton et al., 2010).

![Table of Rates and Toxicity Indices](image)

**Figure 1.7:** Case fatality: rate ratios and relative toxicity indices for individual antidepressants based on rates of death (suicide and undetermined intent) in England and Wales (adapted from Hawton et al. (2010)).
In relation to the fatal toxicity index for benzodiazepines, Ojanpera et al. (2016) reported that out of 70 medicinal drugs in Finland, the benzodiazepine alprazolam was among the top 30 drugs with the highest individual fatal toxicity indices. Furthermore, Reith et al. (2003) reported that DZP and clonazepam were among the top 12 drug substances involved in poisoning deaths in New Zealand in 2001. Moreover, the total number of deaths in which benzodiazepines are involved in is accelerating, with this number increasing by almost five told from 2001 to 2014 in the U.S.A. (Figure 1.8) (www.drugabuse.gov).

Figure 1.8: Total number of U.S. overdose deaths involving benzodiazepines from 2001 to 2014 (adapted from www.drugabuse.gov).

As mentioned, antidepressants are also associated with non-fatal adverse side effects. These side effects, as well as the likelihood of their occurrence, vary between drug classes, with TCAs having been commonly reported as the most deleterious drug class. For example, in a controlled trials systematic review of the adverse event profiles of different antidepressant drug classes, the largest variety of side effects occurred in
TCAs compared to other drug classes (Watanabe et al., 2010). For instance, 29 different side effects occurred with TCAs compared to 24, 13 and 18 for SSRIs, SNRIs and ‘other’ ADs, respectively, suggesting that SNRIs were the safest antidepressant drugs. Side effects such as dry mouth, nausea/vomiting, increased appetite/body weight, sleep alterations and headache were more commonly reported in SSRI trials than TCA trials. However, the TCAs were associated with the highest number of severe side effects. For example, 5 trials reported suicide attempt in the TCA drug group, compared to 1, 0 and 1 trial for SSRIs, SNRIs and Other ADs, respectively, whilst a total of 6 TCA trials reported abnormal heart rate, compared to 1, 1 and 2 trials for the other drug groups, respectively (Watanabe et al., 2010). Moreover, Bet et al. (2013) assessed the side effects of different antidepressant classes during long-term use in a more naturalistic setting. In this study, it was reported that TCAs were associated with more side effects than other drug classes. In particular, TCAs produced more anticholinergic effects such as dry mouth and constipation. These results, along with results from several other studies (Anderson, 2000, Peretti et al., 2000) highlight the extent of the side effect profiles of different antidepressant drug classes. Although SSRIs provide a safer pharmacological alternative than the original TCA drugs, these drugs still produce several adverse side effects, emphasising the need for safer pharmacological alternatives.

Benzodiazepine drugs for the treatment of anxiety are also associated with several adverse side effects. Some of the most commonly reported side effects of benzodiazepine drugs include drowsiness, lethargy, fatigue, impairment in motor coordination and behavioural tasks, dizziness, vertigo, slurred speech, blurry vision, mood swings, euphoria, hostile or erratic behaviour, memory impairment, drug dependence, abuse and withdrawal symptoms (Griffin et al., 2013, Lader, 2014, Longo and Johnson, 2000). It is due to the extent of these side effects and the fewer favourable outcomes compared to SSRIs, that the SSRIs have replaced benzodiazepines as the first line drug treatment for anxiety disorders.

1.5.2. Remission rates with antidepressants and benzodiazepines

As mentioned, another limitation of current antidepressants is the high incidence of patients that do not respond to antidepressant drug treatment. It has been reported that at least 30% of depressed patients fail to achieve a satisfactory response, while less than 50% achieve remission (Frank et al., 1991, Kennedy et al., 2001). Several studies have
been carried out to specifically investigate the response and/or remission rate with antidepressants. Studies assessing SSRI efficacy have shown that remission rate varies from as low as 28% to 45.4%, while response rates with the atypical antidepressants mirtazapine and agomelatine are 64% and 49.1%, respectively (Table 1.15).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Response rate</th>
<th>Remission rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citalopram (SSRI)</td>
<td>46%</td>
<td>28%</td>
<td>Trivedi et al. (2006)</td>
</tr>
<tr>
<td>Duloxetine (SNRI)</td>
<td>N.S.</td>
<td>40.3%</td>
<td>Thase et al. (2007)</td>
</tr>
<tr>
<td>Paroxetine (SSRI)</td>
<td>N.S.</td>
<td>38.3%</td>
<td>Thase et al. (2007)</td>
</tr>
<tr>
<td>FLX (SSRI)</td>
<td>N.S.</td>
<td>28.4%</td>
<td>Thase et al. (2007)</td>
</tr>
<tr>
<td>Mirtazapine (atypical)</td>
<td>64%</td>
<td>45.4%</td>
<td>Carpenter et al. (2002)</td>
</tr>
<tr>
<td>Agomelatine (atypical)</td>
<td>49.1%</td>
<td>N.S.</td>
<td>Kennedy and Emsley (2006)</td>
</tr>
<tr>
<td>Imipramine (TCA)</td>
<td>60%</td>
<td>34.3%</td>
<td>Beasley et al. (1993)</td>
</tr>
</tbody>
</table>

Table 1.15: Response and remission rates of different classes of antidepressants. N.S.=Not specified.

This lack of efficacy of antidepressants is not only detrimental to the patient's psychological well-being, but it is also associated with an increase in health costs and resource utilization. For example, Lepine et al. (2012) reported that treatment resistant depressed patients used significantly more psychiatric service resources, and were more likely to require hospitalizations, compared to non-treatment resistant patients. Moreover, the annual costs of treatment resistant patients were significantly higher than those in which treatment was effective. The lack of efficacy of antidepressants, and the secondary effects that this causes highlights the need for antidepressants with improved responder rates.

Response and remission rates for benzodiazepine drugs are generally higher than those observed with antidepressants, although these figures are still not optimal. Several studies have reported the response and remission rates for different benzodiazepines, and figures for some of these drugs are reported in Table 1.16. As can be seen from this table, response rates vary from as low as 43% to as high as 78.3%. Remission rates also vary, depending on the specific drug. It should be noted however, that due to the diversity of anxiety disorders that there are, every benzodiazepine has a different response and remission rate, depending on the specific anxiety disorder.
Table 1. 16: Response and remission rates of different benzodiazepines in anxiety disorders. N.S. = Not specified.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Response rate</th>
<th>Remission rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZP</td>
<td>N.S.</td>
<td>66%</td>
<td>Rickels et al. (1993)</td>
</tr>
<tr>
<td>Bromazepam</td>
<td>N.S.</td>
<td>60%</td>
<td>Llorca et al. (2002)</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>61%;54.7%</td>
<td>57%;N.S.</td>
<td>Pande et al. (2003); Feltner et al. (2003)</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>43%</td>
<td>N.S.</td>
<td>Rickels et al. (2005)</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>78.3%</td>
<td>N.S.</td>
<td>Davidson et al. (1993)</td>
</tr>
</tbody>
</table>

Importantly, whilst benzodiazepines initially provide better response and remission rates for anxiety symptoms compared to antidepressants, in the long term, antidepressants have been found to surpass these benzodiazepine response figures when they eventually start to elicit their effects. For example, several studies have shown that although benzodiazepines induce marked improvement in symptoms within the first two weeks, when effects of antidepressants are not evident, response and remission rates are the same or even surpassed by antidepressants after approximately 6-12 weeks of treatment (Rickels et al., 1993, Rickels et al., 2003). Therefore a disadvantage of benzodiazepines is that although they are superior to antidepressants for the initial acute treatment of anxiety symptoms, their effects become inferior to those of antidepressants after long-term treatment. In addition to the low remission rates observed, the delay in onset of antidepressant action mentioned earlier is another important limitation, and is discussed further in the next section.

1.5.3. Onset of antidepressants and anxiolytic action

Standard antidepressants typically require several weeks, if not months, for effects to emerge. Numerous studies have been carried out to determine the time course of antidepressant action, with initial studies reporting that several weeks emerged before there was an improved response in patients (Hekimian et al., 1983, Hekimian et al., 1978). Stassen et al. (2007) reported that the mean time to onset of improvement with antidepressants was almost two weeks. Furthermore, in one of the largest effectiveness studies conducted in patients with unipolar depression, after 10-14 weeks treatment with a standard antidepressant, remission was achieved in only 28% of patients, which also eludes to the previously mentioned problem of low remission rates. This delay in onset
is seen with all classes of antidepressants, and the table below gives an insight into the extent of the delay in response rate in several antidepressant classes (Table 1.17).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time effective</th>
<th>Response rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>Within the first 2 weeks</td>
<td>N.S.</td>
<td>Stassen et al. (1999)</td>
</tr>
<tr>
<td>Moclobemide</td>
<td>Within the first 2 weeks</td>
<td>N.S.</td>
<td>Stassen et al. (1999)</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>2, 4 &amp; 6 weeks</td>
<td>56, 80 &amp; 90%</td>
<td>Nierenberg et al. (2000)</td>
</tr>
<tr>
<td>Escitalopram</td>
<td>1 &amp; 2 weeks</td>
<td>22% &amp; 35%</td>
<td>Nierenberg et al. (2007)</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>1 &amp; 2 weeks</td>
<td>27% &amp; 43%</td>
<td>Nierenberg et al. (2007)</td>
</tr>
<tr>
<td>Desipramine</td>
<td>3-13 days</td>
<td>N.S.</td>
<td>Katz et al. (2004)</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>13 days</td>
<td>N.S.</td>
<td>Katz et al. (2004)</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>35 days</td>
<td>30%</td>
<td>Brannan et al. (2005)</td>
</tr>
</tbody>
</table>

Table 1.17: Onset of action and response rate of different classes of antidepressants. N.S.= Not specified.

This delay in onset of antidepressants is interesting, considering that antidepressant drugs are active at their molecular and cellular sites almost immediately. This discrepancy between immediate action of antidepressants molecularly and delayed clinical effects, poses a major limitation of the monoamine hypothesis described earlier, and several efforts have been made to explain this discrepancy by investigation of numerous other theories. For example, the neurotrophin theory has been proposed as an explanation of the discrepancy between these effects (Lee and Kim, 2010), as changes in neurotrophins such as BDNF occur at a similar time point in which antidepressant efficacy is evident, with chronic but not acute antidepressant treatment increasing BDNF preclinically (Nibuya et al., 1995, Xu et al., 2003). Additionally, chronic antidepressant treatment has been found to attenuate CUMS-induced depressive-like behaviour in a manner that is associated with normalisation of the decreased BDNF levels that were also induced from CUMS (Liu et al., 2014). Thus, among several others, BDNF has been suggested as a potential biomarker of antidepressant efficacy. The development of such a biomarker of antidepressant efficacy may improve the diagnosis of such disorders, as well as aid in the development of more effective medications. Unfortunately, although investigations are ongoing into the potential systems involved in the delayed onset of action, no progress has been made in developing faster acting antidepressant drugs. This delay in efficacy is extremely detrimental, with patients remaining symptomatic, functionally impaired and having to endure reduced quality of life during this period.
Contrastingly, while a delay in onset of action is a major limitation of antidepressants, benzodiazepines are widely used due to their rapid onset of action. Benzodiazepines vary in their onset of action and are divided into rapid, intermediate and slow onset of action. However, the time scale of this onset of action is much shorter when compared to antidepressants, with peak plasma concentration being reached as rapidly as 0.5 hours to 6 hours (Griffin et al., 2013), and effects being induced as quickly as 15, 15-30 or 30-60 minutes for rapid, intermediate and slow acting benzodiazepines, respectively. Thus, this property is a major advantage in the treatment of acute anxiety symptoms, considering that antidepressants do not alleviate these symptoms for several weeks. However, as mentioned, the response and remission rates of benzodiazepines are the same or even surpassed by antidepressants after approximately 6 weeks (Rickels et al., 1993). It is clear therefore, that in order for symptoms of anxiety and depression to be adequately treated continuously (acutely and chronically), combination therapy is required. Thus, the development of novel drugs that would treat comorbid anxiety and depressive symptoms acutely is essential for improved as well as time and cost-effective treatment. The first phase in the development of such improved drugs is the examination of potential compounds in preclinical animal models.

1.6. Preclinical testing for assessing antidepressant and anxiolytic drug properties
Since the mid 1960’s, several animal models of depression have been developed that have been crucial in the discovery and development of effective antidepressant drugs. Due to the fact that the defining core symptoms of depression, such as low mood, feelings of worthlessness, guilt and suicidal ideation are inherently human, it was clear from the beginning that it was not possible to model the complete host of symptoms observed with clinical depression. Instead, the modelling of one or few distinct behavioural symptoms of depression in a particular model became the objective, with the emphasis placed on symptoms that were objectively identifiable, that had high inter-rater reliability and that were responsive to clinical antidepressant treatments. These models of depression were developed over the years based on three criteria, first elaborated by Willner; face, predictive and construct validity (Willner, 1984). Face validity refers to the degree to which an animal model covers the entire concept of clinical depression. Therefore, if an animal model of depression is to have face validity
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the model must resemble depression in a number of respects which are specific to
depression, and that actually coexist in a specific sub-group of depressions.
Furthermore, chronic antidepressants must be effective in the model, and the model
must show no features that are not seen in clinical depression (Willner, 1984).
Construct validity refers to the degree of similarity between what is being measured in
the animals and the clinical symptom of depression. Thus, both the behaviour in the
model and the features of depression being modelled should be unambiguously
interpreted and homologous (Willner, 1984). Finally, the degree to which drug effects
in an animal model predict whether the drug will be effective in humans is referred to as
predictive validity. Thus, this criteria is only concerned with pharmacological effects
and proposes that the model should correctly identify antidepressants of diverse
pharmacology with similar potency in animals and humans, and without errors of
omission or commission (Willner, 1984).

The first animal models of depression consisted of neurochemical models. The findings
that depletion of monoamines by reserpine induced depression in humans that were
treated for hypertension formed the basis of the first of these models. Monoamine-depleting
compounds such as reserpine, tetrabenazine, Ro-4-1284, parachloramphetamine and 6-hydroxydopamine
were used to test whether antidepressants could reverse the behavioural effects induced by these agents, such as
ptosis, hypomotility, diarrhoea and bradycardia (Costa et al., 1960, Colpaert et al.,
1975). Indeed agents such as TCAs and MAOIs potently reversed these effects. In
keeping with this theory were findings that other direct and indirect monoamine
agonists such as amphetamine and levodopa were potentiated by antidepressants
(Morpurgo and Theobald, 1965, Silvestrini, 1982). These models proved helpful in the
decades that followed to investigate the relative contribution of one monoamine system
over another, as well as receptors thought to be involved in the aetiology of depression.
However, although these models were simple and efficient methods for screening novel
enhancers of monoamine activity, a major downfall of such models was their low
likelihood of uncovering new chemical entities with novel mechanisms of action.

Therefore, it became clear that there was a need for non-mechanistic models of
depression that did not rely on neurochemical manipulation. Instead, models began to
focus on replicating core symptoms of depression that were induced ethologically.
With stress being considered an important risk factor in the development of clinical
depression, these next generation models assessed the behavioural changes induced by
either social stress such as maternal separation (Hinde et al., 1966), prolonged social isolation (Kostowski et al., 1984), social hierarchy and subordination (Malatynska and Kostowski, 1984) and the resident-intruder test (Mitchell, 2005), or environmental stress such as the learned helplessness model (Anisman and Waller, 1973), the forced swim test (FST) (Porsolt et al., 1978), restraint-induced depression (Kennett et al., 1985) and CUMS (Katz, 1982, Willner, 1997). The symptoms induced by these models varied, and included despair, aggression, submission and flight behaviours, decreased motivation and reduced locomotor activity, all of which were successfully attenuated by antidepressant treatment (Degraaf et al., 1985, Garzon et al., 1979, Mitchell and Redfern, 1992b, Mitchell and Redfern, 1992a, Besson et al., 1999, Borsini et al., 1981, Kennett et al., 1987). The CUMS was recognised as a superior model to the other environmental stress models, in that it more closely resembled the clinical situation of exposure to mild, frequent stress over a long period of time, as opposed to a brief but intense stress that occurred in all of the other models. Overall, the behaviours assessed in these ethological models, the way in which they are induced (CUMS), and the findings that chronic antidepressant treatment can reverse these symptoms, vastly improved the face validity of animal models of depression.

Another model of depression, the olfactory bulbectomy model (Cairncross et al., 1977) involves inducing lesions in the olfactory bulbs of rats, resulting in symptoms that resemble clinical depression, such as increased sensitivity to stress, agitation, alterations in sleep patterns, weight loss and anhedonia. The most prominent behaviour induced by the olfactory bulbectomy is hyperactivity in an open field environment, which is attenuated after chronic antidepressant treatment (Broekkamp et al., 1980, Cairncross et al., 1979). Other models that were later developed and utilised include operant response models and natural genetic models, in which a wide range of antidepressants were also found to be effective (O'Donnell and Seiden, 1982, Seiden et al., 1985) (Overstreet et al., 2005, Lopez-Rubalcava and Lucki, 2000).

The stress-induced models have improved the validity of animal models of depression due to the ethological means by which they are derived, as well as the similar molecular, biochemical and physiological symptoms to those observed in depressed patients. However, there is still a severe lack of understanding of the aetiology of depression, due to the fact that the defining symptoms of the disease are inherently human. As mentioned, instead of modelling the disease in its entirety, only distinct symptoms or groups of symptoms can be modelled and assessed for antidepressant
efficacy, whilst the optimal models will show effects only after chronic antidepressant administration, to more closely resemble the clinical situation. Importantly, however, the majority of drug effects in these animal models are detected after acute or subacute administration, which limits the translatability of these tests to the clinical situation, in which chronic administration is required before any symptoms are alleviated. Figure 1.9 represents the most commonly used behavioural tests for screening antidepressant efficacy – results derived from the PubMed search engine using the words ‘antidepressant properties and rat’, within the previous 5 years. The criteria for inclusion within the pie chart consisted of the administration of a drug, followed by behavioural analysis for antidepressant properties. It is clear from the pie chart that the FST (symptom of behavioural despair) was by far the most popular behavioural test, having been carried out 49% of the time to assess potential antidepressant properties of compounds. This was followed by the open field test (locomotor alterations; 20%), sucrose preference (anhedonia; 13%), the CUMS (which ultimately assessed depressive-like behaviour in one of the other graphed tests; 10%) and object recognition tests (memory; 6%).
Anxiety disorders are extremely heterogeneous due to the numerous symptoms that define the disorder. Moreover, with the core symptoms of anxiety being primarily cognitive and psychological, such as excessive worry and guilt, it is impossible to create an animal model that represents an anxiety disorder in its entirety. Thus, similar to depression models, the focus of animal models of anxiety is largely on measuring a distinct symptom or groups of symptoms that can be objectively measured in a replicable manner and that can be attenuated by anxiolytic treatment. Animal models of anxiety fall under distinct categories of tests – pharmacological, approach-avoidance, conflict based, hyponeophagia, separation-induced ultrasonic vocalizations, stress-induced hypothermia and fear conditioning (Cryan and Sweeney, 2011).

The first animal models of anxiety came with the discovery of benzodiazepine drugs for the treatment of anxiety. These original tests involved pharmacological approaches to screening drugs, and included the mouse-inclined screen test which was indicative of...
muscle relaxation and sedation, a foot shock test which showed taming effects, the anaesthetized cat model of muscle relaxation, electroshock and seizure-based pentylenetetrazol tests (Randall, 1960, Cryan and Sweeney, 2011). An improvement of the models came with the development of the Skinner box-based Sidman avoidance task (Sidman, 1953), a more sensitive test in which measurement of depressant action on behaviours was more reliable. There were several limitations to these original tests, including their lack of distinct specificity for anxiety. Later in the 1980s, more ethologically based models were developed, such as approach-avoidance tests. These tests were derived based on the conflict that arises in a rodent between exploring a novel environment, and its aversion to a brightly lit arena. Some of these approach-avoidance models included the elevated plus maze (EPM), elevated zero maze (Shepherd et al., 1994), the light-dark box (Crawley and Goodwin, 1980), the open field (Hall et al. 1934), mirrored arena (Rodgers, 1997), the staircase test (Simiand et al., 1984) and the modified hole-board test (Ohl et al., 2001), each of which are sensitive to anxiolytic drugs. In these tests, the rat’s avoidance of the open, elevated and/or a brightly lit area is measured, and the alteration of these behaviours by anxiolytic drugs is observed. A limitation of these models is the large differences that exist between strains (Schmitt and Hiemke, 1998), making it hard to determine the optimal model.

Conflict-based anxiety tests involve the induction of two opposing impulses in the subject (Rodgers, 1997). A subject is trained to press a lever for a food reward, and when a punishment is imposed on the subjects (a mild electric shock), a suppression of this preconditioned response for reinforcement occurs, an effect that anxiolytics can attenuate (Cryan and Sweeney, 2011). Vogel et al. (1971) developed a novel, more efficient method that addressed the initial limitation of the intensive labour associated with these tests. With this development, much less training was required and the model was still sensitive to anxiolytic effects (Millan and Brocco, 2003). Defensive burying and shock probe tests are also included in this category of models, along with other models such as novelty-induced hypophagia (the suppression of eating due to anxious states), separation-induced ultrasonic vocalizations, and stress-induced hypothermia, all of which have proved valuable in anxiolytic drug screening (Broekkamp et al., 1986, Sluyter et al., 1996). Alterations in conditioned fear learning and cognitive defects that commonly occur in anxiety are also modelled in animals through fear-conditioning based models, including the Pavlovian fear conditioning (Ledgerwood et al., 2005, Delgado et al., 2006) and the conditioned taste aversion paradigm, each of which are
sensitive to anxiolytic treatment. In contrast to the antidepressant models, the treatment regime utilised prior to these anxiolytic screening models quite closely resembles effects in the clinical scenario. For example, in most of these tests, drugs are administered acutely prior to testing (approximately 30-60 minutes), while anxiolytic drugs are fast acting in humans also, inducing effects within hours of administration.

The next sections will explain in greater detail the behavioural models that were used within this thesis for assessing antidepressant and anxiolytic drug effects.

1.6.1. Models for screening antidepressant efficacy:

1.6.1.1. FST
The rat FST, originally developed by Porsolt and colleagues (Porsolt et al., 1978), is currently the most widely used preclinical behavioural test for assessing antidepressant efficacy of compounds. The most common protocol consists of a 15 minute preswim, followed 24 hours later by a 5 min test swim. When the FST was first developed, the only behaviour of interest was ‘immobility’, a posture in which a rat exerts the minimum amount of bodily movements to keep itself afloat (Figure 1.10 (A)). This immobile posture is thought to reflect a sense of “behavioural despair” in the rat, whereby the rat is no longer motivated to attempt to escape from the water-filled cylinder. For the traditional FST, drugs are administered subacutely, with 3 injections received over the 24 hours that elapse between the preswim and the test swim (three treatment administrations provide more stable pharmacological effects (Porsolt et al., 1977) and are typically administered at 24, 5 and 1 hour prior to the test swim (Figure 1.10 (B)), although some more recent short-term studies have deviated from this 3 dose regime (Kawahara et al., 2013, Wasik et al., 2014). Drugs are assessed for antidepressant efficacy by their ability to reduce the time the rat spends immobile. Indeed, the FST is also often used to illustrate a depressive-like phenotype in other models of depression, such as the Wistar-Kyoto model of depression and the CUMS paradigm, with Wistar-Kyoto rats showing increased ‘depressive-like’ or immobile behaviour compared to other rat strains, and chronic mild stress increasing immobility compared to rats that are not stressed (Marti and Armario, 1996, Tejani-Butt et al., 2003, Xu et al., 2016).
Figure 1.10: The behaviour of immobility in the FST (A) and the effects of different dosing regimes of imipramine in the original FST study by Porsolt and colleagues (B). ((A) and (B) adapted from Cryan et al. (2002) and Porsolt et al. (1978), respectively).

The FST was originally assessed using a continuous scoring method in which the total amount of time the animal spends immobile in the 5 minute test is scored (Porsolt et al., 1978). However, Detke et al. (1995) developed a novel method of scoring the test. This consists of a time sampling technique, which involves not only scoring the frequency of immobility, but also ‘active’ behaviours, namely swimming and climbing (Figure 1.11 (A), respectively), by recording the predominant behaviour elicited every 5 seconds of the test swim. With this scoring method, distinct active behaviours emerge in the FST, which can be related to monoaminergic mechanism of action, with 5-HT compounds increasing swimming counts, while NA compounds increase climbing behaviour (Detke et al., 1995, Page et al., 1999) (Figure 1.11 (B and C, respectively)).
Figure 1.11: The behaviours of swimming (left) and climbing (right) in the FST (A) and the effects of the serotonergic–altering compound FLX (B) and the noradrenergic–altering compound DMI (C) in the modified FST (images adapted from Cryan et al. (2002) (A) and Detke et al. (1995) (B and C).

Although the FST is the most commonly used preclinical screening assay for detecting antidepressant properties, it is not without its limitations. For example, although the subacute dosing regime is seen as a major advantage of the test due to its practicality and the ability to gain results quickly (Cryan et al., 2002), this short-term dosing regime is also thought to be a major weakness of the FST as a valid preclinical model, given that clinically, antidepressants have a therapeutic lag of several weeks (Nierenberg et al., 2000, Nierenberg et al., 2007). Thus, the test relies completely on predictive validity. Moreover, due to the complete reliance of the test on motor movements, or lack thereof, the test has been subject to scrutiny for lack of specificity of the test. For example, drugs such as amphetamines were found to reduce immobility, but rather due to a stimulant effect on general locomotor activity (Kitada et al., 1981). As such, to avoid false positive effects, assessment of general locomotor activity has been incorporated into several FST study designs to ensure that drugs are not stimulating...
general activity. However, an additional locomotor test prior to the FST can be quite stressful on the rat, which may subsequently affect the rat’s FST behaviour and cause complications for inter-laboratory comparisons of drugs. Despite this, however, the FST still remains a staple, reliable test for detection of antidepressant properties of drugs.

1.6.1.2. **Resident-intruder paradigm**

The resident-intruder paradigm is based on the fact that when an adult male rodent is given sufficient living space, it establishes a territory within that space. Therefore, when an unfamiliar male enters the cage (intruder), the resident rat will attack this intruder in defence of its established territory. The paradigm allows examination of psychotropic drug effects on both social and agonistic rodent behaviour. Three experimental designs have been developed to allow complete profiling of a drug (Mitchell, 2005): acute, chronic and intermittent study designs. For the acute and chronic study designs, each group of animals is tested once weekly on four occasions, with resident rats being singly housed for 3 days prior to each test exposure. On the test day, the resident’s cage is placed beneath a recording camera and the rat is allowed 30 minutes to habituate to its surroundings, after which an intruder rat is introduced to the resident’s home cage. The test is video recorded for 10 minutes for later analysis of the residents’ behaviour, and rats are then returned to their original home cages. Typically, acute drug effects are assessed by administration of drug 30-60 minutes prior to testing. Resident rats receive four treatments (one at every testing session – vehicle and three doses of drug), and are exposed to each of the corresponding intruder conspecifics over the four test sessions. This study design has proven robust at detecting the ability of several classes of antidepressant drugs to acutely reduce agonistic behaviour, whilst anxiolytic drugs such as DZP only induce effects at doses that are also sedative, making effects invalid (Mitchell and Redfern, 1992a) (Figure 1.12 (A, B and C)).
Figure 1.12: The effect of acute treatment with the TCA clomipramine (A), the SSRI FLX (B) and the anxiolytic DZP (C) on investigation, aggression and flight submit behaviour in drug-treated resident rats (black bars) and non-treated intruder rats (white bars) in the resident-intruder paradigm. *p<0.05, **p<0.01, ***p<0.001 vs. control (images adapted from Mitchell and Redfern (1992a)).

In chronic treatment studies, resident rats are not drug treated prior to the first test session to allow a baseline behavioural profile to be established. Chronic treatment commences after the first test session, via osmotic mini pumps and subsequent tests are carried out 7 and 14 days later, with the final encounter occurring 7 days after the drug treatment ceased. For this experimental design resident rats also encounter each of the corresponding intruder conspecifics over the four test sessions (Mitchell, 2005). Contrary to the acute study design, antidepressants have been consistently shown to increase aggressive behaviour when assessed using this chronic method (which has been suggested to represent an increased assertiveness and externalization of emotions in the rat), whilst again, no effects of anxiolytic drugs such as DZP are evident after chronic administration (Mitchell and Redfern, 1992a) (Figure 1.13 (A, B and C)).
Figure 1.13: The effect of chronic treatment with the TCA clomipramine (A), the SSRI fluoxetine (B) and the anxiolytic diazepam (C) on investigation, aggression and flight submit behaviour in drug-treated resident rats (black bars) and non-treated intruder rats (white bars) in the resident-intruder paradigm. *p<0.05, **p<0.01, ***p<0.001 vs. Day 0 (images adapted from Mitchell and Redfern (1992a)).

For the time-course study design rats are also singly housed for three days prior to testing, although they remain singly housed for the duration of the experiment. For this study design, resident rats are exposed to a total of 8 testing sessions, whereby there are two groups with 4 rats in each group with each resident group having a corresponding intruder group). Similar to the chronic design, resident rats are first tested without any treatment to establish a baseline behavioural profile, after which, osmotic mini pumps are implanted. Testing sessions are carried out daily and it is ensured that each resident rat is tested with each of the corresponding 8 intruder conspecific rats over the testing phase. This study design allows determination of the onset of drug-induced effects over the first week of drug treatment.

A series of behaviours are recorded during the scoring of the resident-intruder paradigm sessions and the scores for each of these behaviours are grouped according to their motivational category (Table 1.18). Whilst the resident-intruder paradigm possesses the characteristics of construct and predictive validity, ‘face’ validity is absent in this
model. However, overall, the resident-intruder paradigm provides an ethologically relevant animal model for detecting both acute and chronic effects of antidepressants.

<table>
<thead>
<tr>
<th>Motivational Category</th>
<th>Behavioural Element</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploration</td>
<td>Locomotion, rearing</td>
</tr>
<tr>
<td>Investigation</td>
<td>Approach, follow, stretched attention, to-fro, walk round/circle/side, nose and investigate, sniff genitalia, tail rattle</td>
</tr>
<tr>
<td>Sexual</td>
<td>Mount*, attempt mount, lick penis</td>
</tr>
<tr>
<td>Aggression</td>
<td>Aggressive groom, aggressive posture, attack, bite, offensive sideways, offensive upright, pull, threat/thrust</td>
</tr>
<tr>
<td>Flight-submit</td>
<td>Defensive sideways, defensive upright, submit</td>
</tr>
<tr>
<td>Flight-escape</td>
<td>Attend, crouch, elevated crouch, flag and evade, retreat, under food hopper</td>
</tr>
<tr>
<td>Maintenance</td>
<td>Digging, drinking, eating, licking, scratching, head/body shake, washing</td>
</tr>
</tbody>
</table>

Table 1. 18: Behaviours recorded during the resident-intruder paradigm, grouped according to motivational category (information adapted from Mitchell (2005)). *Where a female conspecific is used.

1.6.1.3. Saccharin preference test

The saccharin preference test is based on the symptom of depression referred to as anhedonia. Anhedonia is defined as diminished interest or pleasure in response to stimuli that were previously perceived as rewarding in a premorbid state (American Psychiatric Association, 2013). Along with depressed mood, anhedonia is one of two symptoms required for a diagnosis of MDD (American Psychiatric Association, 2013). In rodents, anhedonia has been described as reduced social investigation (Fishkin and Winslow, 1997), as well as a reduction in sexual behaviour and the consumption of highly palatable substances (Yirmiya, 1996). Of these, the assessment of highly palatable substances is the most commonly assessed anhedonia parameter.

Rats have a strong preference for sweet solutions such as those containing sucrose or saccharin and the consumption of these compounds is said to be due to their hedonic
properties (Willner, 2005). The saccharin preference test (SPT) is therefore highly utilised across preclinical laboratories as a test of anhedonia. Typically, rats are trained for several days to develop a steady baseline preference for a sweet saccharin substance over plain water. To do this, rats are presented with a ‘two-bottle’ choice for several hours a day – one bottle which contains saccharin and another which contains water. At the end of the two-bottle exposure, total fluid consumption is determined by adding together the amount of water and the amount of saccharin solution consumed. The saccharin preference is then calculated by expressing the amount of saccharin consumed as a percentage of the total amount of fluid consumed. Rats will generally develop a steady saccharin preference baseline within several days of training (Pastor-Ciurana et al., 2014). Although some studies incorporate water deprivation prior to the SPT to encourage fluid consumption over a short period of time (Guan et al., 2015, Seiglie et al., 2015), the SPT has also been reliably carried out without prior fluid deprivation (Pastor-Ciurana et al., 2014), whereby saccharin preference is measured over a longer period of time (e.g. overnight or 24 hours).

The SPT is typically performed in combination with animal models that induce a depressive-like phenotype, such as the CUMS model or an inflammatory challenge (discussed in the next section). In the CUMS model, animals elicit an anhedonic response (reduced saccharin preference) compared to control animals. Several classes of antidepressants have been reported to attenuate the anhedonia that is induced by CUMS (Liu et al., 2014, Wang et al., 2016, Xing et al., 2013), therefore illustrating predictive validity and increasing the overall validity of this test. When performed in combination with a model such as the CUMS, or the acute systemic LPS (described below), the SPT thus possesses the three characteristics of construct, face and predictive validity, and is therefore a valuable behavioural screen for antidepressant action.

1.6.1.4. LPS-induced depressive-like behaviour

LPS-induced depressive-like behaviour in rodents is based upon the neuroinflammatory theory of depression, which proposes that depression is associated with excessive production of inflammatory mediators. As previously mentioned, LPS is an endotoxin that stimulates the production of pro-inflammatory cytokines in rodents, such as IL-1, IL-6, TNFα and IFNγ (van Dam et al., 1992, Quan et al., 1999, Gatti and Bartfai, 1993, Laye et al., 1994, Breder et al., 1994). Indeed, when administered peripherally, LPS induces a biphasic profile of behavioural symptoms including initial sickness-like
behaviour, followed at a later point by depressive-like behaviour. The initial sickness-like symptoms are elicited for several hours following LPS and include decreased locomotor activity (Engeland et al., 2003), decreased social exploration (Castanon et al., 2001, Pitychoutis et al., 2009) as well as a reduction in body weight (Yirmiya, 1996, Castanon et al., 2001), and food consumption (Yirmiya et al., 2001). These symptoms are characteristic of what is reported in depressed patients and the role of inflammatory markers in mediating these effects has been well established in rodents (O'Connor et al., 2009, Yirmiya et al., 2001, Bluthe et al., 2000b, Bluthe et al., 2000a).

After the initial sickness-like behaviour has subsided, a depressive-like phenotype has been characterized at least 24 hours after an LPS challenge. For example, Frenois et al. (2007) illustrated that approximately 24 hours after LPS or saline challenge, animals administered LPS showed increased depressive-like behaviour in the FST, compared to saline treated animals. Similar effects of LPS on FST results have also been reported in other studies (O'Connor et al., 2009, Yang et al., 2013, Hosseini et al., 2012). Furthermore, several studies have reported an anhedonia response induced by LPS exposure, marked by a reduction in sucrose or saccharin preference (Yirmiya, 1996, Pitychoutis et al., 2009) (Figure 1.14 (A)). Similar to the sickness-like symptoms, these LPS-induced depressive-like symptoms have been reported to be mediated by several inflammatory markers (O'Connor et al., 2009, Yang et al., 2013). Further validating LPS-induced behaviours as an animal model of depression are the findings that prior chronic antidepressant treatment attenuates or normalises both the sickness-like (Yirmiya et al., 2001) and depressive-like (O'Connor et al., 2009, Yirmiya, 1996) (Figure 1.14 (B)) behaviours elicited by LPS. The combination of this acute systemic LPS with subsequent anhedonia testing such as the SPT provides a valuable model of depression, meeting the criteria for face, construct and predictive validity.
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Figure 1.14: Effects of LPS administration on sucrose consumption (A) and the effects of chronic imipramine treatment on LPS administration in the sucrose preference test (B). *p<0.05 vs. VEH (A) and *p<0.05 vs. Sal-Sal (B) (images adapted from Pitychoutis et al. (2009) (A) and Yirmiya (1996) (B)).

1.6.2. Models for screening anxiolytic efficacy

1.6.2.1. EPM

The EPM is one of the most commonly used animal tests of anxiety. The test was first proposed by Handley and Mithani (1984) and was further developed by Pellow and File (1986). The EPM consists of a plus-shaped apparatus, with two open arms (no walls surrounding the arms) and two closed arms (a wall surrounding the edge of the entire arm). The four arms meet at a central square and the apparatus is elevated off the ground (Figure 1.15). The EPM is based on the principle of thigmotaxis, whereby rodents have a natural aversion to open spaces and thus have a tendency to remain close to vertical surfaces (i.e. the walls of the closed arms). Rodents are placed on the centre square of the EPM, usually facing one of the open arms, and are left to explore the arena for 5 minutes. Typically, rodents will avoid the open arms of the maze and will thus enter and spend more time in the closed arms. For analysis, an arm entry is registered once all four of the rodent’s paws have entered an arm. Total arm entries and total arm time is calculated by adding open and closed arm entries, and open and closed arm time, respectively. Anxiety is then measured by the percentage of open arm entries (number of open arm entries/total arm entries*100) and the percentage of open arm time (time spent in the open arm/total arm time*100). When administered acutely, anxiolytic drugs robustly reduce the tendency of the rodents to spend more time in the closed arms. Thus, there is an increase in the percentage of open arm entries and time (Kapus et al., 2008, Griebel et al., 2000).
Importantly, as previously mentioned, SSRI antidepressant drugs are currently the first-line treatment for anxiety disorders, and can reduce anxiety symptoms after chronic administration. Thus, it is important that animal models of anxiety should also detect anxiolytic properties of these other drug classes in order to completely fulfil the criteria for predictive validity. However, a review that assessed the effects of SSRIs in the EPM reported that SSRI effects are inconsistent after both acute and chronic administration, inducing either anxiogenic-like effects, or no effect at all (Borsini et al., 2002). It was concluded from this comprehensive review that the ability of the EPM to pick up anxiolytic-like effects is limited to benzodiazepine or other GABA-related compounds, and thus the test does not have predictive validity for the measurement of anxiolytic effects of other compounds. However, the EPM is a valuable tool for detecting these types of compounds, and remains one of the most commonly employed behavioural tests for screening anxiolytics.

1.6.2.2. Open field
The open field test was originally described by Hall (1934), who used defecation in the open field as an index of timidity. The test was further developed by Walsh and Cummins (1976) as a means of detecting anxiolytic or anxiogenic effects of compounds. The testing procedure involves exposing a rodent to an unknown environment from which they cannot escape due to the presence of surrounding walls (Figure 1.16). Originally, the open field consisted of a circular shape (Hall et al. 1934), although square or rectangular open fields are also used. However, importantly, the size of the open field has been found to affect both activity and defecation levels in rodents (Nagy and Holm, 1970), and is therefore an important variable to consider when testing. Typically, an animal is placed either in the centre, or near the walls of the arena, and
behaviour is assessed for a period of time (usually 5 minutes). As with the EPM, the basis of this test stems from the principle of thigmotaxis. The rats will spend most of the 5 minute trial adjacent to the walls of the arena, with their exploration inhibited due to stress. Bright lighting is also commonly used in the open field to make the test more stressful for the rat and thus make it easier to detect anxiolytic effects. However, similar to the shape of the arena, the illumination of the arena can also affect results (Nagy and Holm, 1970), and is an important variable to consider before testing. The main behaviours that are assessed in the test are distance moved (for detection of sedative or stimulatory drug effects), as well as the entries into, and time spent in the inner area of the open field (to assess anxiolytic and anxiogenic drug effects). Acute administration of anxiolytic drugs such as benzodiazepines, reduce the stress-induced inhibition of exploration behaviour that is evident in control animals (Bert et al., 2001, Nazar et al., 1997). Whilst the open field possesses the characteristics of construct and predictive validity, face validity is lacking for this test. However, the open field remains one of the most commonly used behavioural tests for assessing both anxiolytic and locomotor effects of drugs.

Figure 1. 16: The open field apparatus (figure adapted from http://researchoutdoorplay.tumblr.com/).

1.6.2.3. Novelty-induced hypophagia

Novelty-induced hypophagia (NIH) testing is based on the principle of ‘hyponeophagia’, which refers to the conflict which rodents face when given a choice of either approaching and consuming a desirable food in a novel environment, or avoiding the novel environment. In hyponeophagia tests, rodents are either food-deprived and then presented with regular chow, or are maintained with ad libitum food access, and
presented with a familiar, highly palatable food. The latency to consume and/or the amount eaten in this novel environment are recorded. Hyponeophagia testing displays strong predictive validity for anxiolytic drug effects, as well as their onset of action. For example, anxiolytic compounds that are effective in humans, such as benzodiazepines, also reduce anxiety in hyponeophagia testing (Merali et al., 2003). There is also high predictive validity for the onset of action of anxiolytics, with chronic anxiolytic effects evident in both humans and in rodent hyponeophagia testing (Bodnoff et al., 1989). Importantly, hyponeophagia models also display a strong predictive validity for the onset of action of anxiolytic effects of antidepressants. For instance, when administered chronically, the antidepressants FLX, DMI and amitriptyline have been shown to reduce hyponeophagia (Dulawa et al., 2004, Merali et al., 2003, Bodnoff et al., 1989).

In order to control for potential effects of a drug on general feeding behaviour related to appetite, it is crucial to assess the same parameters (latency to consume and amount eaten) in the homecage environment also. However, not all studies carry out this critical control step. Thus, Dulawa and Hen (2005) characterised a new paradigm, the NIH test, in which they utilised the experimental parameters which they believed would produce optimal results. The experimental parameters were as follows:

- inclusion of a valid control (i.e. testing in the home cage)
- assessment of both behaviours (latency to consume and amount consumed) in both the home cage and in the novel cage test
- use of a highly palatable familiar substance as opposed to imposing food deprivation (this was chosen because fixed food deprivation periods do not consistently induce sufficient hunger levels in different, but presumably identical batches of rodents)
- use of the home cage instead of an open field
- darker lighting in the home cage and brighter lighting in the novel cage (to produce an optimal level of hyponeophagia for detecting anxiolytic and anxiogenic effects of drugs)
- assessment of latency to consume and amount consumed every 5 minutes of a 30 minute trial

Dulawa and Hen (2005) then confirmed the predictive validity of the NIH test for detecting anxiolytic effects of antidepressants, illustrating that chronic, but not
subchronic FLX treatment reduces hyponeophagia. Although this test possesses many positive characteristics, it is important to note that, similar to the FST, its validity is primarily predictive. However, the NIH test is an important tool for drug discovery, as well as for mechanistic studies trying to unveil the neurobiology of antidepressant response.

1.6.3. Duration of treatment for preclinical testing
It is clear from the range of behavioural tests described above that different tests rely on particular dosing regimes for the successful detection of both anxiolytic and antidepressant drug properties. Typically, preclinical anxiolytic properties are detected after acute administration, and the EPM and open field test are two such tests that are sensitive to this acute treatment regime. This preclinical onset of action closely resembles the clinical situation, whereby anxiolytic actions are fast acting in humans also (Altamura et al., 2013). In the most commonly used preclinical antidepressant screening test, the FST, antidepressant properties are robustly elicited after subacute administration. This differs a great deal to the clinical situation, in which chronic treatment is required before antidepressants start to exert their effects. An animal model that does respond to chronic antidepressant treatment is the LPS challenge. With this model, chronic antidepressant treatment prior to the LPS challenge normalises depressive-like behaviour that is typically elicited, such as anhedonia in the saccharin preference test. Importantly, some tests are sensitive to detecting both acute anxiolytic effects as well as chronic antidepressant tests, such as the NIH test and the resident-intruder paradigm. The ability to pick up both acute and chronic effects makes these tests imperative in the screening and development of novel compounds, in an era where treatment for depression, anxiety, and their comorbidity is severely lacking.

1.6.4. Validity of preclinical animal models
When thinking about utilising each of these tests, one must carefully consider the advantages and disadvantages of each one in terms of their validity, so that preclinical drug screening is executed proficiently. As described earlier, the assessment of the validity of animal models is based on three main dimensions: face, construct and predictive validity. Table 1.19 illustrates the various validities that each of the tests described above possess.
### Table 1.19: Validity characteristics of animal models that are used for antidepressant and anxiolytic drug screening.

<table>
<thead>
<tr>
<th>Test</th>
<th>Construct</th>
<th>Face</th>
<th>Predictive</th>
</tr>
</thead>
<tbody>
<tr>
<td>FST</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Resident-intruder</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>LPS challenge with subsequent saccharin preference</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Elevated plus maze</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Open field</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Novelty-induced hypophagia</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

If optimal use is to be made of each of these tests, then these validity characteristics are extremely important when considering the design of future studies in the search for improved or novel antidepressant and/or anxiolytic compounds.

### 1.6.5. The principles of the 3 R’s of animal experiments

With such a wide variety of preclinical testing being carried out, animal welfare has become an important factor within the scientific world. The term ‘animal welfare’ refers to an animal’s quality of life. An animal with a good quality of life should be healthy, well fed, housed in an environment that they might themselves choose and should also be relatively free from negative states. Animals should also be able to carry out behaviours and activities that they are naturally motivated to do (Fraser, 2008).

Adoption of the principles of the 3 Rs (Replacement, Reduction and Refinement) as a framework for humane animal research over 50 years ago has resulted in continuing efforts to improve the welfare of animals used in science research (Russell and Burch, 1959). The 3 Rs have subsequently become embedded in national and international legislation that regulates the use of animals in scientific procedures. The principles are based on the idea that animals should only be used in science if a non-animal alternative is either not possible, or has failed. When animals have to be used, then only the most humane procedures should be used to achieve the experimental objective, using the smallest number of animals that will provide statistically meaningful results (Fenwick et al., 2009).

The National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://www.nc3rs.org.uk/the-3rs) describes the 3 Rs as follows:
• **Replacement**: the avoidance or replacement of the use of animals that are defined as ‘protected’ under the ‘Animals (Scientific Procedures) Act 1986, amended 2012 (ASPA)’ in an experiment where they would have otherwise been used

• **Reduction**: techniques that minimise the amount of animals used per experiment. This can be achieved by either enabling researchers to acquire comparable results from fewer animals, or to acquire more information from the same number of animals, limiting additional animal use (e.g. improved experimental design and statistical analysis or the use of technologies such as imaging to enable longitudinal studies in the same animals)

• **Refinement**: methods that minimise the pain, suffering, distress or lasting harm that may be experienced by the animals (e.g. using appropriate anaesthetics and analgesics and limiting stress by training the animals to cooperate with techniques that they may find otherwise stressful) (https://www.nc3rs.org.uk/the-3rs).

### 1.6.6. Variation in preclinical test results and sources of variability

Despite standard protocols for almost all preclinical behavioural tests, a great deal of variability still exists in results. The FST is a prime example of this. As previously mentioned, the FST consists of a 15 minute preswim, followed 24 hours later by a 5 minute test swim. The main behaviours of interest are immobility, climbing and swimming, and these are typically altered by the administration of antidepressants prior to the test swim, either subacutely or chronically. However, despite its simplicity, the FST is subject to large differences in even baseline responses, making the comparison of drug-induced effects across studies and laboratories extremely difficult. Normally, when assessing the antidepressant-like effects of a novel compound in the FST, a positive control drug is also assessed (i.e. a drug that has a known response in the test) so that this positive response can be compared to the unknown response of the novel compound. The TCA DMI is one of the most commonly used positive control drugs in the FST and therefore the vast amount of studies that have utilised this drug are ideal for interrogating the large variation that occurs in both baseline and drug-induced effects in the FST. The extent of this variation is highlighted in Figures 1.17 – 1.20 below. There are several different experimental factors that may be responsible for the vast differences in baseline and drug-induced effects in the studies (Bogdanova *et al.*, 2013),
which are outlined in Tables 1.20 – 1.23 below. Some factors that have been assessed as potential influences on results include the strain (Armario et al., 1995, Lopez-Rubalcava and Lucki, 2000), gender (Drossopoulou et al., 2004, Simpson et al., 2012b), age (Sequeira-Cordero et al., 2013, Olivares-Nazario et al., 2016), handling (Cannizzaro et al., 2002), housing (Brenes et al., 2008, Simpson et al., 2012a) and bedding type (Raineiki et al., 2012) of the rat, breeding source of the rat (Honndorf et al., 2011, Pare and Kluczynski, 1997), treatment schedule (Detke et al., 1997, Cryan et al., 2005a), testing apparatus (Detke and Lucki, 1996), and time of testing (Gomes et al., 2011). The effect of these experimental factors on FST behaviours will be discussed in detail below.

1.6.6.1. Effect of strain
The strain of the rat can significantly affect FST behaviours. Armario et al. (1995) compared the behavioural responses of 5 inbred rat strains in the FST and showed that there was a significant effect of strain on struggling behaviour and immobility. Moreover, Wistar Kyoto rats have been reported to be far more immobile (Marti and Armario, 1996, Tejani-Butt et al., 2003) and spend less time struggling (Marti and Armario, 1996) and swimming (Tejani-Butt et al., 2003) in the FST when compared with other strains. In addition, Shaw et al. (2009) reported that Long Evans rats were more immobile in the FST compared to Wistar rats. In a single 5 minute session of the FST, Flinders sensitive line (FLS) were significantly more immobile and spent less time swimming than Sprague-Dawley rats (Kokras et al., 2009).

Interestingly, Lopez-Rubalcava and Lucki (2000) showed that not only did Wistar Kyoto rats spend more time immobile and less time climbing than Sprague-Dawley rats, but also that these two strains react differently to drug challenges in the FST. For example, DMI caused a dose-dependent decrease in immobility in both strains, but Wistar-Kyoto rats were more sensitive to the drug, showing a reduction in immobility at the lowest dose of 5 mg/kg; an effect that was absent in the Sprague-Dawley rats. Strain effects were also evident in drug-induced alterations on active behaviours. DMI increased climbing in both strains, with this effect evident at a lower dose in the Sprague-Dawley rats, whilst swimming was increased with DMI treatment in Wistar-Kyoto rats, but was not affected in Sprague-Dawley rats. Furthermore, Tejani-Butt et al. (2003) showed that the TCA DMI (8 mg/kg) was effective in Wistar Kyoto, but not
Wistar or Sprague-Dawley rats, possibly due to the increased sensitivity of Wistar-Kyoto rats to this drug, as reported by Lopez-Rubalcava and Lucki (2000).

1.6.6.2. Effect of gender
The effect of gender on FST behaviour has been investigated in several studies. Although some studies have reported no effect of sex on FST behaviours (Armario et al., 1995), others have reported that female rats spend more time immobile and less time climbing compared to males (Drossopoulou et al., 2004). Similarly, Kokras et al. (2012) reported that females exhibited a higher depressive-like phenotype in the FST compared to males, although they found the opposite effect of sex in the FST in a previous study which used different strains, whereby females spent less time immobile and more time climbing (Kokras et al., 2009). Simpson et al. (2012b) also reported that females spent less time immobile, but more time swimming than males. This decrease in immobility and increase in active behaviours observed by Simpson et al. (2012b) may be explained by an increased locomotor profile, whereby females appeared to be hyperactive compared to males in the open field test.

Kokras et al. (2009) also reported a differential effect of the TCA clomipramine, depending on gender. Although the drug decreased immobility in both males and females, it increased swimming and climbing in males but did not affect these active behaviours in females. Pitychoutis et al. (2011) reported that treatment with clomipramine decreased immobility in the FST in both male and females, but this effect was only seen in high novelty seeking males and low novelty seeking females. Furthermore, DMI has been reported to decrease immobility in males, but had no effect on females (Simpson et al., 2012b), most probably due to the much lower baseline immobility observed in females, which would decrease the window for detection of drug effects (approximately 230 seconds baseline immobility in males versus under 100 seconds in females).

1.6.6.3. Effect of treatment schedule
The subacute dosing regime for the FST is seen as a major advantage of the test, due to its practicality and the ability to gain results quickly (Cryan et al., 2002). However, this short-term dosing regime is also thought to be a major weakness of the FST as a valid preclinical model as, clinically, antidepressants have a therapeutic lag of several weeks (Nierenberg et al., 2000, Nierenberg et al., 2007). Chronic study designs have become
more commonly incorporated in the literature in recent years (Piras et al., 2014, Schaffer et al., 2010). Earlier comparisons of the magnitude of effect of chronic and subacute antidepressant treatment showed that with chronic administration, antidepressant drugs did not lose their efficacy in reducing immobility, and some even became more efficacious after chronic treatment (Kitada et al., 1981, Mancinelli et al., 1987, Miyauchi et al., 1981). In more recent years, several studies have assessed all three behaviours in the FST (immobility, climbing and swimming) after short-term and chronic dosing of low doses of antidepressants (Detke et al., 1997, Cryan et al., 2005a). These studies showed that chronic administration of antidepressants are effective at doses which are ineffective when administered acutely, similar to the clinical scenario, and thus, strengthened the validity of the FST as an animal model of depression.

Tables 1.20 – 1.23 and Figures 1.17 – 1.20 below represent studies which have carried out the FST and incorporated DMI as a positive control, using either Sprague-Dawley or Wistar rats. The tables have been organised in such a manner that studies using the same strain, gender, treatment schedule, and route of drug administration (either intraperitoneal (i.p.), or subcutaneous (s.c.)) have been compiled together. In all of the studies, the two-day FST protocol was carried out (15 minute preswim followed 24 hours later by a 5 minute test swim), and animals underwent a subacute dosing regime (dosed 23-24, 5 and 1 hour prior to the FST).
<table>
<thead>
<tr>
<th>Weight (g) at start of exp.</th>
<th>Breeding Source</th>
<th>Housing Type</th>
<th>Bedding</th>
<th>Apparatus</th>
<th>Scored by observers</th>
<th>Dose (mg/kg) (d/v)</th>
<th>Time before test</th>
<th>N</th>
<th>Scoring Technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>210-230 g</td>
<td>In-house bred</td>
<td>N.S.</td>
<td>N.S.</td>
<td>(a) 45 x 20</td>
<td>3 Blind Observers</td>
<td>(a) 3 or 10 (2ml/kg)</td>
<td>6-22</td>
<td>Time sampling</td>
<td>Havdweh et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>250 g</td>
<td>Outsourced</td>
<td>Singly housed</td>
<td>Sawdust</td>
<td>(a) N.S.</td>
<td>(b) 12:12/07:00</td>
<td>(b) 24, 5 and 1 h</td>
<td>Continuous</td>
<td>Simpson et al. (2012b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300-350 g</td>
<td>In-house bred</td>
<td>Pair-Housed Contact bedding</td>
<td>(a) 45 x 35</td>
<td>Blind Observers</td>
<td>(a) 10 (1ml/kg)</td>
<td>23.5, 5 and 1 h</td>
<td>Continuous</td>
<td>Morrish et al. (2009)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>175-200 g</td>
<td>Outsourced</td>
<td>Singly housed</td>
<td>Sawdust</td>
<td>(a) 34 x 25</td>
<td>Blind Observer</td>
<td>(a) 10 (2ml/kg)</td>
<td>8</td>
<td>Continuous</td>
<td>McDermott and Kelly (2008)</td>
<td></td>
</tr>
<tr>
<td>250-275 g</td>
<td>Outsourced</td>
<td>Pair-housed</td>
<td>N.S.</td>
<td>(a) N.S.</td>
<td>(b) 12:12/08:00</td>
<td>(b) 24, 5 and 1 h</td>
<td>Continuous</td>
<td>Hoshaw et al. (2008)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N.S.</td>
<td>Outsourced</td>
<td>Housed with dam in litters of 9-10</td>
<td>N.S.</td>
<td>(a) 45 x 21</td>
<td>Blind personnel</td>
<td>(a) 1, 3, 10 or 20 (N.S.)</td>
<td>6-8</td>
<td>Time sampling</td>
<td>Reed et al. (2008)</td>
<td></td>
</tr>
</tbody>
</table>

**Chapter 1: General Introduction**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sampling Time</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008 Reed</td>
<td>Continuous</td>
<td>2008 Hoshaw</td>
</tr>
<tr>
<td>2008 Kelly</td>
<td>Continuous</td>
<td>2008 Mc Dermott and Kelly</td>
</tr>
<tr>
<td>2012a Simpson and Kelly</td>
<td>Continuous</td>
<td>2012b Simpson and Kelly</td>
</tr>
<tr>
<td>2012a Simpson and Kelly</td>
<td>Continuous</td>
<td>2012a Simpson and Kelly</td>
</tr>
<tr>
<td>2012b Simpson and Kelly</td>
<td>Continuous</td>
<td>2012a Simpson and Kelly</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous</td>
<td>Simpson et al. (2012b)</td>
</tr>
<tr>
<td>Time sampling</td>
<td>Reed et al. (2008)</td>
</tr>
<tr>
<td>(a)Weight (b)Age</td>
<td>Breeding Source</td>
</tr>
<tr>
<td>-----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>250–275g 1–2 weeks before testing</td>
<td>Outsourced</td>
</tr>
<tr>
<td>325–375g at testing</td>
<td>Outsourced</td>
</tr>
<tr>
<td>300–400g</td>
<td>Outsourced</td>
</tr>
<tr>
<td>325–375g at testing</td>
<td>Outsourced</td>
</tr>
<tr>
<td>280–400g at testing</td>
<td>Outsourced</td>
</tr>
<tr>
<td>300–325g</td>
<td>Outsourced</td>
</tr>
<tr>
<td>150–175g on arrival</td>
<td>Outsourced</td>
</tr>
<tr>
<td>350–400g</td>
<td>Outsourced</td>
</tr>
<tr>
<td>160–180g</td>
<td>Outsourced</td>
</tr>
<tr>
<td>125±50g</td>
<td>Outsourced</td>
</tr>
</tbody>
</table>
Table 1.20: Experimental parameters for studies that assessed the effects of subacute i.p. DMI administration in the FST in male Sprague-Dawley rats. N.S. = Not specified.
**Figure 1.** Variance in baseline and 10mg/kg DMI (i.p.) induced responses in the FST in male Sprague-Dawley rats (data obtained from studies reported in Table 1.20). Data are expressed as Mean±SEM. *p<0.05, **p<0.01 vs. relevant control.
### Table 1.21: Experimental parameters for studies that assessed the effects of subacute s.c. DMI administration in the FST in male Sprague-Dawley rats. N.S. = Not specified, V.A. = Videotaped from above.

<table>
<thead>
<tr>
<th>(a)Weight (b)Age</th>
<th>Breeding Source</th>
<th>Housing</th>
<th>Bedding Type</th>
<th>Apparatus (a) H x W (cm) (b) Water Depth (cm) (c) Water Temp (°C)</th>
<th>Scored by</th>
<th>(a)L:D cycle(h) (b)Time of Testing</th>
<th>(a)Dose (mg/kg) (d/v) (b)Time before test</th>
<th>N</th>
<th>Scoring Technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)180–200g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>4 per cage</td>
<td>N.S.</td>
<td>(a)46 x 20 (b)30 (c)24 ±1</td>
<td>Blind Observer</td>
<td>[a]12:12/06:00 (b)07:00-15:00h</td>
<td>(a)20 (2ml/kg) (b)23.5 and 1 h</td>
<td>14</td>
<td>Time sampling</td>
<td>Drugan et al. (2010)</td>
</tr>
<tr>
<td>(a)225–250g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>2 per cage</td>
<td>N.S.</td>
<td>[a]? x 21 (b)30 (c)23-25</td>
<td>Blind Observer</td>
<td>[a]12:12/07:00 (b)N.S.</td>
<td>(a)20 (2ml/kg) (b)23, 5 and 1 h</td>
<td>8-10</td>
<td>Time sampling</td>
<td>Carr et al. (2010)</td>
</tr>
<tr>
<td>(a)250-350g at time of testing (b) N.S.</td>
<td>Outsourced</td>
<td>4 per cage</td>
<td>N.S.</td>
<td>(a)46 x 21 (b)30 (c)25</td>
<td>Blind Observer</td>
<td>[a]12:12/06:00 (b)13:00-17:00</td>
<td>(a)20 (2ml/kg) (b)23, 5, 5 and 1 h</td>
<td>9-10</td>
<td>Time sampling</td>
<td>Slattery et al. (2005)</td>
</tr>
<tr>
<td>(a)150-175g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>3 per cage</td>
<td>N.S.</td>
<td>(a)46 x 20 (b)30 (c)23-25</td>
<td>Blind Observer</td>
<td>[a]12:12/07:00 (b)12:00-18:00</td>
<td>(a)5, 10 or 20 (2ml/kg) (b)23, 5, 5 and 1 h</td>
<td>8</td>
<td>Time sampling</td>
<td>Lopez-Rubalcava and Lucki (2000)</td>
</tr>
<tr>
<td>(a)150-175g (b) N.S.</td>
<td>Outsourced</td>
<td>2 per cage</td>
<td>N.S.</td>
<td>(a)46 x 20 (b)30 (c)23-25</td>
<td>Blind Observer (V.A.)</td>
<td>[a]12:12/07:00 (b)12:00 19:00</td>
<td>(a)1, 3.2 or 10 (2ml/kg) (b)23, 5, 5 and 1 h</td>
<td>15-22</td>
<td>Time sampling</td>
<td>Reneric and Lucki (1998)</td>
</tr>
<tr>
<td>(a)150-175g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>2-4 per cage</td>
<td>N.S.</td>
<td>(a)46 x 20 (b)30 (c)23-25</td>
<td>Blind Observer (V.A.)</td>
<td>[a]12:12/07:00 (b)12:00 18:00</td>
<td>(a)5, 10 or 20 (4ml/kg) (b)23, 5, 5 and 1 h</td>
<td>10</td>
<td>Time sampling</td>
<td>Detke et al. (1995)</td>
</tr>
<tr>
<td>(a)150-175g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>4 per cage</td>
<td>N.S.</td>
<td>(a)46x20 (b)30 (c)23-27</td>
<td>N.A.</td>
<td>[a]12:12/07:00 (b)N.S.</td>
<td>(a)10 (4ml/kg) (b)24, 5 and 1 h</td>
<td>10</td>
<td>Continuous</td>
<td>Singh and Lucki (1993)</td>
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<tr>
<td>(a)Approx. 200g (b) N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>(a)46x20 (b)30 (c)23 27</td>
<td>Blind Observer</td>
<td>(a)N.S. (b)N.S.</td>
<td>(a)10 (N.S.) (b)23, 5 and 1 h</td>
<td>12</td>
<td>Time sampling</td>
<td>Detke and Lucki (1996)</td>
</tr>
<tr>
<td>(a)Approx. 200g (b) N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>(a)46x20 (b)30 (c)23-25</td>
<td>Blind Observer</td>
<td>(a)N.S. (b)N.S.</td>
<td>(a)10 (N.S.) (b)23, 5 and 1 h</td>
<td>12</td>
<td>Time sampling</td>
<td>Detke and Lucki (1996)</td>
</tr>
<tr>
<td>(a)150-175g (b) N.S.</td>
<td>Outsourced</td>
<td>5 per cage</td>
<td>N.S.</td>
<td>(a)46x20 (b)24 (c)23-25</td>
<td>Two Blind Observers</td>
<td>[a]12:12/07:00 (b)N.S.</td>
<td>(a)15, 10 or 15 (4ml/kg) (b)23, 5, 5 and 1 h</td>
<td>10</td>
<td>Continuous</td>
<td>Wieland and Lucki (1990)</td>
</tr>
</tbody>
</table>
Figure 1.18: Variance in baseline and 10mg/kg DMI (s.c.) induced responses in the FST in male Sprague-Dawley rats (data obtained from studies reported in Table 1.21). Data are expressed as Mean±SEM. **p<0.01 vs. relevant control.
<table>
<thead>
<tr>
<th>Weight (a)</th>
<th>Age (b)</th>
<th>Breeding Source</th>
<th>Housing</th>
<th>Bedding Type</th>
<th>Apparatus (a)H x W (cm) (b)Water Depth (cm) (c)Water Temp (°C)</th>
<th>Scored by (a)D cycle(h) (b)Time of Testing (c)Time before test</th>
<th>Dose (mg/kg) (d/v)</th>
<th>N</th>
<th>Scoring Technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>175-200g on arrival</td>
<td>N.S.</td>
<td>Outsourced</td>
<td>Singly housed</td>
<td>Sawdust</td>
<td>(a)34 x 25 (b)24 (c)25</td>
<td>Blind Observer</td>
<td>(a)12:12/08:00</td>
<td>(b)N.S.</td>
<td>(c)10</td>
<td>Continuous</td>
</tr>
<tr>
<td>220-230g</td>
<td>Approx. 80 days</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>(a)7 x 21 (b)30 (c)25</td>
<td>Blind Observer</td>
<td>(a)12:12/07:00</td>
<td>(b)N.S.</td>
<td>(c)17.5 or 15</td>
<td>Time</td>
</tr>
<tr>
<td>250-300g</td>
<td>N.S.</td>
<td>N.S.</td>
<td>5 per cage</td>
<td>N.S.</td>
<td>(a)54 x 34 x 60 (b)40 (c)24±1</td>
<td>Blind Observer</td>
<td>(a)12:12/07:00</td>
<td>(b)N.S.</td>
<td>(c)15, 10 or 15</td>
<td>Time</td>
</tr>
<tr>
<td>300-400g</td>
<td>(b)4 months</td>
<td>N.S.</td>
<td>Group housed</td>
<td>N.S.</td>
<td>(a)40 x 20 (b)25 (c)25</td>
<td>N.S.</td>
<td>(a)12:12/06:00</td>
<td>(b)N.S.</td>
<td>(c)10</td>
<td>Continuous</td>
</tr>
<tr>
<td>300-350g</td>
<td>N.S.</td>
<td>N.S.</td>
<td>4 per cage</td>
<td>N.S.</td>
<td>(a)50 x 20 (b)20 (c)25</td>
<td>Blind Observer</td>
<td>(a)12:12/06:00</td>
<td>(b)N.S.</td>
<td>(c)15</td>
<td>Continuous</td>
</tr>
<tr>
<td>N.S.</td>
<td>N.S.</td>
<td>In-house bred</td>
<td>Singly housed</td>
<td>Paper trays</td>
<td>(a)35 x 20 (b)17 (c)25±1</td>
<td>N.S.</td>
<td>(a)12:12/06:00</td>
<td>(b)N.S.</td>
<td>(c)10, 10 or 20</td>
<td>Continuous</td>
</tr>
<tr>
<td>N.S.</td>
<td>N.S.</td>
<td>In-house bred</td>
<td>5-6 per cage</td>
<td>Paper trays</td>
<td>(a)35 x 20 (b)17 (c)25±1</td>
<td>N.S.</td>
<td>(a)12:12/06:00</td>
<td>(b)N.S.</td>
<td>(c)10, 10 or 20</td>
<td>Continuous</td>
</tr>
<tr>
<td>120-150g</td>
<td>In-house bred</td>
<td>6 per cage</td>
<td>Sawdust</td>
<td>N.S.</td>
<td>(a)36 x 22 (b)22 (c)24±1</td>
<td>N.S.</td>
<td>(a)12:12/08:00</td>
<td>(b)N.S.</td>
<td>(c)10 or 30</td>
<td>Continuous</td>
</tr>
<tr>
<td>170-230g</td>
<td>N.S.</td>
<td>Outsourced</td>
<td>5 per cage</td>
<td>N.S.</td>
<td>(a)23 x 30 x 40 (b)28 (c)22</td>
<td>N.S.</td>
<td>(a)12:12/06:00</td>
<td>(b)14:00-17:00</td>
<td>(c)10 or 20</td>
<td>Continuous</td>
</tr>
<tr>
<td>250±30g</td>
<td>N.S.</td>
<td>Outsourced</td>
<td>3 per cage</td>
<td>N.S.</td>
<td>(a)N.S. (b)20 (c)21±1</td>
<td>N.S.</td>
<td>(a)12:12/N.S. (b)N.S.</td>
<td>(c)2.5 or 10</td>
<td>Continuous</td>
<td>(d)2ml/kg</td>
</tr>
<tr>
<td>Weight</td>
<td>Breeding Source</td>
<td>Housing</td>
<td>Bedding Type</td>
<td>Apparatus</td>
<td>Scored by</td>
<td>L:D cycle (h)</td>
<td>Dose (mg/kg) (d/v)</td>
<td>Dose before test</td>
<td>N</td>
<td>Technique</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------</td>
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<td>--------------</td>
<td>----------------</td>
<td>---------------</td>
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<td>------------------</td>
<td>----</td>
<td>-----------</td>
</tr>
<tr>
<td>(a)180-250g</td>
<td>In-house bred</td>
<td>N.S.</td>
<td>N.S.</td>
<td>(a)20 x 22 x 50 (b)30 (c)27-28</td>
<td>Blind Observer</td>
<td>(a)12:12/07:00 (b)N.S.</td>
<td>(a)20 or 30 (N.S.) (b)24, 5 and 1 h</td>
<td>7-13</td>
<td>Continuous</td>
<td>Mannisto et al. (1995)</td>
</tr>
<tr>
<td>(b)N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)300-310g</td>
<td>Outsourced</td>
<td>5 per cage</td>
<td>N.S.</td>
<td>(a)46 x 18 (b)18 (c)25</td>
<td>N.S.</td>
<td>(a)12:12/08:00 (b)N.S.</td>
<td>(a)10 or 20 (N.S.) (b)24, 5 and 1 h</td>
<td>8-12</td>
<td>Continuous</td>
<td>Pulvirenti et al. (1990)</td>
</tr>
</tbody>
</table>

Table 1.22: Experimental parameters for studies that assessed the effects of subacute i.p. DMI administration in the FST in male Wistar rats. N.S. = Not specified.
Figure 1.19: Variance in baseline and 10mg/kg DMI (i.p.) induced responses in the FST in male Wistar rats (data obtained from studies reported in Table 1.22). Data are expressed as Mean±SEM. *p<0.05, **p<0.01 vs. relevant control.
<table>
<thead>
<tr>
<th>(a)Weight (b)Age</th>
<th>Breeding Source</th>
<th>Housing</th>
<th>Bedding Type</th>
<th>Apparatus (a)H x W (cm) (b)Water Depth (cm) (c)Water Temp °C</th>
<th>Scored by (a)L:D cycle(h) (b)Time of Testing</th>
<th>(a)Dose (mg/kg) (d/v) (b)Time before test</th>
<th>N</th>
<th>Scoring Technique</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)180–200g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>4 per cage</td>
<td>N.S.</td>
<td>(a)46 x 20 (b)30 (c)24 ±1 Blind Observer</td>
<td>(a)12:12/06:00 (b)07:00-15:00h (a)20 (2ml/kg) (b)23, 5 and 1 h</td>
<td>14</td>
<td>Time sampling</td>
<td>Drugan et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>(a)225–250g on arrival (b) N.S.</td>
<td>Outsourced</td>
<td>2 per cage</td>
<td>N.S.</td>
<td>(a)? x 21 (b)30 (c)23.25 Blind Observer</td>
<td>(a)12:12/07:00 (b)N.S. (a)20 (2ml/kg) (b)23.5, 5 and 1 h</td>
<td>8:10</td>
<td>Time sampling</td>
<td>Carr et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>(a)250–300g (b)N.S.</td>
<td>Outsourced</td>
<td>Group housed</td>
<td>N.S.</td>
<td>(a)44 x 32 (b)28 (c)22 Blind Observer</td>
<td>(a)reverse 12:12/19:00 (b)N.S. (a)15 (2ml/kg) (b)23.5, 5 and 1 h</td>
<td>8</td>
<td>Continuous</td>
<td>Hedou et al. (2001)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.23:** Experimental parameters for studies that assessed the effects of subacute s.c. DMI administration in the FST in male Wistar rats. N.S. = Not specified.
Figure 1.20: Variance in baseline and 20mg/kg DMI (s.c.) induced responses in the FST in Wistar rats (data obtained from studies reported in Table 1.23). Data are expressed as Mean±SEM. * \( p<0.05 \) vs. relevant control.
As mentioned, these tables have been organised in such a manner that studies using the same strain, gender, treatment schedule, and route of drug administration have been compiled together, so that these factors are not accountable for the differences in the results presented. It is clear from the graphs however, that even with all of these factors accounted for there are vast differences in the ages of the rats used, the housing (number per cage and bedding), breeding source, testing apparatus and the time of testing. Thus, it is possible that because these factors may not all be consistent across each of the studies, that these factors may have played a role in the variation that is evident in the graphs. The potential effects of these factors on baseline and drug-induced behaviours have indeed been investigated previously, and are further discussed below.

1.6.6.4. Effect of age
Several studies have shown that the age of the subjects used can affect the baseline time spent immobile (Turner et al., 2012, Sequeira-Cordero et al., 2013). In a study that compared adult rats (12 weeks old) versus juvenile rats (4 weeks old), adult rats spent less time immobile and more time climbing and swimming (Sequeira-Cordero et al., 2013). Furthermore, adult rats (90 days old) spent more time immobile and climbing and less time swimming, compared to young males (35-37 days old) (Martinez-Mota et al., 2011). Although Pechnick et al. (2008) reported no effect of age on immobility in pups (30 days old) versus adult rats (112 days old), age seemed to affect the active behaviours in the FST, with pups spending more time climbing and less time swimming compared to adults. Moreover, when 3-4 month old female rats were compared to 18-20 month old rats, older rats spent much more time immobile than the younger rats (Turner et al., 2012).

With regards the effect of age on antidepressant response in the FST, Karanges et al. (2011) found that treatment with the SSRI paroxetine decreased immobility and increased swimming in adult rats (70 days old), but had no effect on either behaviour in adolescent rats (28 days old). However, in another study, treatment with DMI decreased immobility and increased climbing in both adult rats (112 days old) and pups (30 days old), whilst the drug decreased swimming counts in pups, but not in adults (Pechnick et al., 2008). Very few studies actually report the age of the subjects used in the study. For example, in Table 1.20, only 4 out of 22 papers reported the age of the subjects used. With the above outlined evidence, the age of the rat could therefore be a prime cause of the variability in results (Figure 1.17-1.20).
1.6.6.5. **Effect of handling**

Another potential factor that may affect behavioural results is the handling of the rats. Freitas *et al.* (2015) compared depressive-like phenotype in male rats that had either been tactile-stimulated or unhandled from postnatal days 8-14, and found that tactile stimulation had an antidepressant-like effect, with these rats showing a decrease in immobility time in the FST. Moreover, tactile stimulation potentiated the antidepressant-like effect of the SSRI sertraline, with the drug decreasing immobility in the tactile stimulated rats; an effect that was absent in the unhandled rats. Handling from postnatal day 1-21 has also been reported to decrease time spent immobile compared to non handled rats (Tejedor-Real et al., 2007), whilst conversely, Papaioannou *et al.* (2002) found that a similar handling protocol (postnatal day 1-22) had no effect on male rats, but caused an increase in immobility time in female rats.

Although this parameter is not reported in the tables of experimental parameters above, it should be noted that very few studies reported how the rats were handled. The handling of a rat from birth up to the day of testing will likely never be completely identical between two studies due to the wide range of activities such as cage changing and weighing that occur frequently. Importantly, handling is a procedure that is mandatory if a study requires administration of a drug. An exception is the osmotic mini pump dosing regime, whereby the animals are implanted with osmotic mini pumps that slowly release the drug subcutaneously, and thus do not require the rat to be handled by humans. However, most studies incorporate dosing regimes that do require handling (i.p., s.c. or oral gavage (p.o.)). With handling evidently having an effect on FST behaviour, it is likely that this too may be a contributing factor to the high variability of the graphed results, considering the inconsistency in handling and dosing techniques across studies.

1.6.6.6. **Effect of housing**

Both social and physical housing enrichment have been assessed for their effects on FST behaviour. Simpson *et al.* (2012a) reported no difference in baseline immobility in rats housed in either social or isolated conditions. Furthermore, social housing conditions did not affect baseline immobility in singly housed versus pair housed rats (Hall *et al.*, 1998, Fischer *et al.*, 2012). On the contrary, Brenes *et al.* (2008) reported an antidepressant-like effect of social housing compared to isolated housing, having reduced baseline immobility. In terms of antidepressant activity, housing conditions...
affected drug-induced effects, with the TCA DMI reducing immobility in the animals housed in isolated conditions only (Simpson et al., 2012a).

With regard to physical housing manipulations, environmental enrichment (EE) has been reported to either have no effect on baseline immobility (Simpson et al., 2012a), or an antidepressant-like effect (Brenes et al., 2009), compared to rats housed in either social or isolated conditions. Furthermore, Bjornebekk et al. (2008) reported that physical EE, such as a running wheel, increased the active behaviour of climbing in the FST. The effect of physical EE on drug-induced effects has also been investigated. While Simpson et al. (2012a) reported a blunting effect of EE on drug-induced effects in the FST compared to those singly housed, other studies have reported a potentiation of drug-induced effects in rats housed with EE compared to social conditions (Kus et al., 2010, Nowakowska et al., 2010). With an ever growing concern for the welfare of the animals used within scientific experiments, the introduction of new legislation requirements may force behavioural laboratories to employ EE for their subjects (Council of Europe, 2007/526/EC). Thus, the potential effect of EE on behavioural responses is worrying, as it may reduce comparability across studies due to altered behavioural responses. The social housing varies substantially across the FST and DMI studies reported above. Some rats are housed singly, whilst others are housed in groups of up to 5 per cage. Importantly some studies did not even report how the rats were housed, despite social housing having a considerable effect on FST behaviour. In terms of physical enrichment, no studies reported the use of enrichment, unless the focus of the study was to assess EE protocols. The lack of consistency in the housing of these rats may therefore play a major role in the extensive variation that is evident.

1.6.6.7. Effect of bedding material

As well as the quantity of bedding material used, simply using a particular type of bedding material can have profound effects on standard behavioural tests, with Sakhai et al. (2013) illustrating that animals reared on corn cob bedding were significantly less anxious in the open field and light-dark box test compared to animals reared on wood pulp. The effects of bedding materials on behavioural tests such as the FST have therefore become a subject of investigation. For example, studies have shown that limited nesting/bedding material over the postnatal period increases immobility time in the FST (Cui et al., 2006, Raineki et al., 2012). Although no studies have been carried out to assess the potential effects of bedding type on FST behaviours, the above results
on anxiety tests illustrate the likelihood that this factor may indeed be a contributor to the large differences in FST results also. The fact that simply using a particular type of bedding can affect behavioural testing is a concern, as a lot of laboratories have started to adopt different bedding types based on increasing awareness of the occupational risks in laboratory animal facilities, such as air-borne dust particles (Kaliste et al., 2004), that can be a danger to both animals and humans. Importantly, very few of the above studies reported the bedding type that was used. In fact, only 5 out of the 22 studies in Table 1.20 reported the bedding type. Thus, inconsistencies in the bedding material used could be responsible for the variation that is evident across studies.

1.6.6.8. Effect of breeding source

Another experimental parameter that may be responsible for variation in FST results is the supplier from which the test subjects are sourced. The supplier has been found to affect rodent behaviour, with Palm et al. (2011) reporting profound differences in voluntary ethanol consumption depending on the vendor. Furthermore, studies have reported that rodents obtained from different suppliers show significantly different anxiety profiles in tests such as the open field (Honndorf et al., 2011, Pare and Kluczynski, 1997) and EPM (Honndorf et al., 2011). Although Pare and Kluczynski (1997) reported no effect of vendor on depressive-like behaviours in the FST, the above results indicate that breeding source is a factor that needs to be more thoroughly investigated as a potential source of variation in FST results. Whilst some of the studies listed in Tables 1.20 – 1.23 used in-house bred rats, and others used rats that were outsourced, some studies did not even report the source of the rat. Due to the broad global distribution of these studies, the source of the rats varied immensely. The fact that the source of the rat has such an effect on results is crucial when interrogating data such as those in the graphs above, as it may make reproducibility impossible across continents, or even countries. It is therefore clear that this factor may have played a large role in accounting for the variability between studies.

1.6.6.9. Effect of testing apparatus

The dimensions of the FST have also been found to affect results. When the rat FST was originally developed (Porsolt et al., 1978), the water was filled to a depth of 15cm, and rats soon became immobile and carried out only behaviours necessary to keep their heads above the water. However, in later years, the effects of different water depths on
Chapter 1: General Introduction

FST behaviour were assessed (Abel, 1994, Detke and Lucki, 1996). Abel (1994) compared immobility behaviour in rats exposed to 20cm versus 35cm water for both the preswim and test swim and found that rats tested at 35cm showed lower immobility times. In addition, when another group compared the effects of 15cm and 30cm water depth on FST behaviours, immobility was reduced in the greater water depth. Moreover, both climbing and swimming behaviour were increased in the 30cm water versus the 15cm water (Detke and Lucki, 1996). Calil and Marcondes (2006) also illustrated that when exposed to a 50 minute swim session, rats swam in a cylinder of dimensions 20 x 20 x 50cm with 20cm water were more immobile than rats swam in cylinders with dimensions of 50 x 50 x 50cm with 38cm water, whilst DMI decreased immobility in only the smaller cylinder dimensions.

Generally, water in the FST is between 23-25 ºC. Studies have assessed the effect of water temperature on FST behaviours and it has been reported that rats tested in cooler water (20 ºC) do not acquire an immobile posture (Jefferys and Funder, 1994), while at a higher water temperature, rats are less active (Drugan et al., 2005). Furthermore, in a 15 minute swim session, rats swim at 19 ºC were less immobile and spent more time swimming than rats swim at either 25 ºC or 35 ºC. Apparatus parameters varied across the FST and DMI studies outlined in the above tables. The water temperature was the least variable, ranging from 23-27 ºC, while the water height was generally between 15 and 30 cm. The cylinder dimensions varied the most between studies with vast differences in the height and diameter of cylinders used. It is likely that these differences in apparatus parameters may have contributed to the variability in the results graphed.

1.6.6.10. Effects of time of testing

The effect of circadian time on FST behaviours has also been investigated. Whilst Gomes et al. (2011) reported no effect of light cycle on FST immobility, another study reported that rats elicited significantly less escape-oriented behaviour when tested nocturnally compared to diurnally (Kelliher et al., 2000). In addition, the antidepressant nomifensine has been reported to be more effective when tested in the light phase compared to the dark phase (Borsini et al., 1990). As well as a potential effect of the circadian clock on FST behaviours, there may also be a circannual effect on rats in the FST. For example, one study compared FST results in male rats each month for 14 months and found that a distinct pattern emerged whereby rats were most immobile in
the winter months and least immobile during the summer months (Abel, 1995). A circannual effect has also been found in female rats, with rats spending more time immobile when tested in February and May compared to August and November (Aksoy et al., 2004). Regarding antidepressant response, several antidepressants elicited maximum response in the FST in the month of March compared to all of the other months in the year, suggesting fluctuations in internal mechanisms (Borsini et al., 1990). Interestingly, 5-HT concentrations in the rat brain have been reported to increase in the winter months and decrease as the summer months approach, which may potentially affect the efficacy of antidepressants (Borsini et al., 1990). Within the FST and DMI experiments described in Tables 1.20 – 1.23, very little studies reported the time of FST testing, whilst none reported the time of the year, despite this factor seemingly playing a role in FST behaviour. The high variability observed in these studies could therefore also be as a result of the time of day, as well as the time of year, of testing.

It is clear from the above outlined results that careful consideration needs to be taken when designing an FST experiment, with several experimental parameters having a marked effect on both baseline and drug-induced effects. This evidence emphasises the need for a move toward more standardized protocols if results are to be validly compared across laboratories.

1.7. Novel drugs and mismatch between preclinical and clinical success

For the treatment of depression, the major goal is to develop novel therapies that work via a mechanism other than altering monoaminergic transmission, and thus, do not possess some of the adverse effects that current antidepressant do. Over the past few decades, the mismatch between preclinical and clinical antidepressant efficacy of novel acting compounds has become apparent. For example, several preclinical results have shown promising results for novel antidepressant compounds that work via a mechanism other than altering monoamine transmission. However, when assessed clinically, none of these drugs have been successful enough to become new lines of treatment. An example is neurokinin receptor antagonists. Despite showing preclinical efficacy in rats and gerbils in the saccharin preference test following CUMS, and in the tail suspension test (Varty et al., 2003, Papp et al., 2000), neurokinin 1 receptor antagonists failed to show consistent antidepressant effects clinically (Griebel and Holsboer, 2012), with just 4 studies showing improvement, 6 studies showing no
effects, 3 being terminated and 1 study being inconclusive. CRF1 receptor antagonist compounds were also tested clinically after showing effects in the CUMS model (Sandi et al., 2008) and the FST (Jutkiewicz et al., 2005), among other tests. However, in the 5 clinical studies that assessed these compounds, only one showed improvement, whilst the other 4 studies showed no improvement (Griebel and Holsboer, 2012). Tables 1.24 and 1.25 illustrate the extent of preclinical tests in which these targets showed antidepressant activity, despite not showing clinical efficacy. Other compounds which showed promise preclinically were sodium channel inhibitors (lamotrigine) (Lapidus et al., 2013), but again, these compounds were found to have little efficacy as clinical antidepressants (Santos et al., 2008, Barbee et al., 2011).

<table>
<thead>
<tr>
<th>Species</th>
<th>Preclinical test in which drugs were effective</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbils</td>
<td>FST and tail suspension test</td>
<td>Varty et al. (2003); Wallace-Boone et al. (2008)</td>
</tr>
<tr>
<td>Tree shrews</td>
<td>Chronic social stress</td>
<td>van der Hart et al. (2005); Czeh et al. (2005)</td>
</tr>
<tr>
<td>Rats</td>
<td>Chronic social stress</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.24:** Behavioural tests in which NK1 receptor antagonists showed antidepressant efficacy.

<table>
<thead>
<tr>
<th>Species</th>
<th>Preclinical test in which drugs were effective</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>FST</td>
<td>Jutkiewicz et al. (2005)</td>
</tr>
<tr>
<td>Mice</td>
<td>Tail suspension test</td>
<td>Nielsen et al. (2004)</td>
</tr>
<tr>
<td>Rats</td>
<td>DRL-72 (operant procedure)</td>
<td>Louis et al. (2006)</td>
</tr>
</tbody>
</table>

**Table 1.25:** Behavioural tests in which CRF1 receptor antagonists showed antidepressant efficacy.

### 1.7.1. Reasons for this mismatch and how to improve on preclinical screening

It is obvious that there is an ongoing mismatch between drugs that are revealing efficacy preclinically and the drugs that are being marketed clinically. This mismatch is clearly evident when one looks at the wide range of drugs that have shown promising effects preclinically over the past decade but have not been successful in treating depression clinically. Further emphasizing the standstill in development of novel acting drugs is the prescription statistics reported earlier in which the top 10 antidepressant drugs prescribed in 2004 were almost the exact same as those in 2013 (Garvey and Kelly,
2015). Although one drug differed, this drug had the same fundamental mechanism of action – alteration of monoamine transmission.

The lack of development of novel acting compounds may be as a result of several factors. As can be seen throughout, depression is a highly diverse disease, made complicated by several factors such as its heterogeneity, the high incidence of comorbidity, as well as the lack of understanding of the pathophysiology and aetiology, resulting in numerous theories of the disease. Thus preclinical testing is quite varied, resulting in the now extremely diverse literature in which the types of behavioural symptoms, models and theories assessed in studies is vast. Of particular interest, is that despite the numerous possible causes of depression and the wide spectrum of symptoms, individual laboratories still tend to focus on elucidating the effects of novel compounds on just one, or very few behavioural symptoms, often incorporating just one theory within study designs. This results in a jigsaw-type picture of drug efficacy, whereby the overall effects of a drug on numerous symptoms of depression is determined by piecing together results from a multitude of studies. What makes this process even more questionable is the example provided earlier in which all seemingly identical FST studies showed extreme variability in both baseline and drug-induced effects between studies, resulting in a sense of unreliability of drug effects in what is seemingly a relatively straightforward test. Hence, one can therefore imagine the potential unreliability of results of other tests across laboratories, which may be magnified when several tests are considered together as a whole in this jigsaw picture. This could result in several false positive drugs, or worse, the dismissal of drugs that may in fact possess clinical antidepressant efficacy.

1.8. Development of a battery study design

A more efficient approach to preclinical screening may be the development of more complex study designs, whereby an array of symptoms and theories can be incorporated into one experiment when investigating potential antidepressant effects of novel drugs. Thus, an experiment would consist of a more extensive examination of the drug through the incorporation of a battery of tests. A vital part of developing such a battery design is the careful selection of the tests that would be incorporated into the experiment, as well as the treatment schedule utilised for detecting drug effects. The previously described tests of the FST, resident-intruder paradigm, LPS-induced responses in the SPT, the EPM, open field, and NIH testing all measure different behaviours and hence
different symptoms, including measures of anxiety, behavioural despair, anhedonia, and social and agonistic behaviour. The assessment of both anxiolytic and antidepressant properties that these tests comprise make them ideal for incorporation into a study design that would inform us of both of these properties of a drug – an experimental aspect that is extremely important considering the high comorbidity of depression and anxiety disorders.

Furthermore, varying dosing schedules are used for each of these tests, such as acute treatment prior to the EPM, open field, resident-intruder paradigm, NIH and FST, and chronic treatment prior to tests such as the LPS-induced responses in the SPT, the resident-intruder paradigm and the NIH test, to detect both anxiolytic and antidepressant properties of drugs. This makes these tests ideal for a battery study design that would allow assessment of both acute and chronic effects of drugs in the same rat. Considering the current treatment lag for pharmacological treatment of depression, the acute phase may be particularly beneficial for detecting early onset of action, while the chronic treatment is important for detecting potential biomarkers that may reflect the chronic onset of action in the clinical situation. Moreover, a battery of these tests would provide interrogation of more than one theory of depression. For example, the FST is renowned for detecting monoamine altering drugs, whilst the LPS-induced responses would assess potential drug effects on an inflammatory driven model. Thus, both the monoamine and neuroinflammation theory of depression are incorporated into one experiment. In addition, neurochemical investigation may be utilised to uncover the potential role of another system, the neurotrophic factors, in producing antidepressant effects after chronic treatment – an assumption that is widely speculated.

It is clear that a degree of standardization in several experimental parameters is required to avoid extensive variation between laboratories. It is important when standardising a protocol that the optimal experimental parameters are used, and thus it is vital to characterise these tests before incorporating them into the battery study design. Characterisation involves comparing options for various parameters to determine which may provide a greater window for detection of drug efficacy – i.e. to achieve the optimal baseline results that will allow even subtle drug effects to be identified. Drawing on the previous example of the FST, it is evident that several experimental parameters can cause immense variability, and thus these parameters need to be interrogated to determine those which are more favourable for increasing the detection of drug effects. These parameters include the strain, gender and age of the rat, as well
as the handling, housing, bedding type, breeding source, apparatus, time of testing, and
lastly, the drug treatment schedule used.

Finally, if such a test battery design is to be developed, an important consideration is the
principle of the 3 Rs of animal research, Replacement, Reduction, and Refinement. The
incorporation of animals for the battery study means that the Replacement principle is
not utilised. However, the development of the battery study design would allow for the
‘Reduction’ of animals, as the study design would allow for the same animals to be used
for detection of both anxiolytic and antidepressant drug properties. Furthermore,
through the characterisation process, tests will be optimized so that they are as effective
as possible at detecting drug properties, and hence the maximum amount of information
will be derived from rats that will endure as little testing as possible, thus satisfying the
principle of ‘Refinement’.

Several studies have assessed the potential effects of carrying out several behavioural
tests in rodents (McIlwain et al., 2001, Blokland et al., 2012, Paylor et al., 2006). McIlwain et al. (2001) tested to see if differences exist in mice that have undergone
testing in different tasks and mice that are naïve to the test experience. They also
assessed whether the test order affects how an animal performs on subsequent tests.
The results indicated that in some tests, mice that had undergone previous testing
behaved differently to naïve mice. As well as this, certain test variables were sensitive
to test order, whilst others were not affected by this. Another study in mice illustrated
that there was no major difference in test results of rats that were tested at intervals of 1
week, and rats tested at intervals of 1-2 days (Paylor et al., 2006). Interestingly,
Blokland et al. (2012) assessed possible order effects in rats that were tested in 3 tests –
the open field, the zero maze and the FST. They reported that the behaviour in the FST
and open field were dependent on the order of the test in the test battery. Therefore,
these studies indicate that when developing a test battery design, it is important to
consider the order of the tests, as well as the time interval between each.
1.9. **Research Objectives**

The primary objective of the work herein was to improve the methods of preclinical testing for assessment of novel drugs to treat depression. The goal was to first characterise several preclinical tests that are commonly used to screen anxiolytic and antidepressant properties of drugs, to confirm their optimal testing parameters for detecting drug efficacy. Once these experimental parameters had been decided upon, an experimental battery of tests was designed using each of the tests described above. This design included an acute phase for screening anxiolytic properties, and also a chronic phase, for detecting antidepressant effects.

As evidenced by the above review of the literature, there is an extensive amount of behavioural tests for screening anxiolytic and antidepressant properties. Currently, most laboratories tend to focus on just one, or very few behavioural tests, often incorporating just one theory within study designs, and very rarely screening for dual anxiolytic and antidepressant drug properties. Considering that depression is a highly diverse disease with an array of symptoms, several developed theories, and a high comorbidity with anxiety disorders, this approach seems primitive. The studies presented herein were intended to develop an improved preclinical screening process for the detection of antidepressant and/or anxiolytic properties. The specific aims in undertaking these studies are presented below.

**Specific Aims:**

- To characterise preclinical tests to determine the optimal experimental parameters for detecting drug effects. This included assessing the effects of the following experimental parameters: breeding source of the rat, age of the rat, bedding type used, exposure to EE, the dosing route and treatment regime, and the scoring technique of the tests
- To establish and validate an improved method for determining false positives in the FST
- To establish and validate a replicable LPS-induced anhedonia model within our laboratory
- Once optimal experimental conditions had been determined, to use well established antidepressants and anxiolytics to develop and validate a novel test battery design which would address several limitations of current preclinical literature. This battery sought to incorporate:
Chapter 1: General Introduction

- Behavioural tests to assess both antidepressant and anxiolytic drug effects
- A chronic, clinically relevant dosing regime, whereby acute and chronic behavioural effects could be elucidated, as well as potential post-mortem biomarkers of antidepressant action
- As many theories of depression as possible
- The 3 Rs principles
Chapter 2 Materials and Methods

This chapter describes the general materials and methods used in the studies that comprise the thesis. More detailed information pertaining to individual studies is provided in the ‘Materials and Methods’ sections of subsequent results chapters.

2.1. Materials

2.1.1. Animal husbandry
Sprague-Dawley rats (male): in-house bred (CNS Pharmacology Laboratory, NUI, Galway) or Charles River (Margate, United Kingdom)

Rat cages (42 cm x 25.5 cm x 13 cm, plastic bottoms with metal cage lids): North Kent Plastics (Coalville, United Kingdom)

Water bottles: North Kent Plastics (Coalville, United Kingdom)

Goldflakes bedding: LBD (Serving Biotechnology) Ltd. (Surrey, United Kingdom)

Corn cob bedding: W.M. Lillico (Surrey, United Kingdom)

3 Rs Paper bedding: Fibrecycle Ltd. (North Lincolnshire, United Kingdom)

Rat chow (Harlan Teklad global diets chow): ENVIGO RMS (Bicester, United Kingdom)

Temperature/humidity monitor: Radionics Ltd. (Dublin Ireland)

Nesting material (Safe bed fluff bedding): Petworld (Galway, Ireland)

EE:

- Physical enrichment: nesting material, plastic tube (6” x 3.5”) (constructed by Mr. Ambrose O’Halloran)
- Nutritional enrichment: hazelnuts, cocopops, sunflower seeds, muesli

Saccharin sodium salt hydrate (Cat # S1002): Sigma-Aldrich (Dublin, Ireland)

Weighing scales: Mason Technology (Dublin, Ireland)
2.1.2. **Behavioural Equipment**

EPM (arms 50 x 10 cm; Length x Width, walls of the closed arms 30 cm high): constructed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)

Open field (75 cm diameter with walls 41 cm high): constructed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)

Forced swim test cylinders:

- Original cylinders (50 x 25 cm; Height x Diameter): Lennox (Dublin, Ireland)
- Current cylinders (45 x 20 cm; Height x Diameter): apparatus constructed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)

Home cage locomotor activity assessment:

- Arena (rat’s home cage; dimensions outlined above): North Kent Plastics (Coalville, United Kingdom)
- Black wood shaving bedding: dyed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway), using Chestnut Black spirit dye: Chestnut Products (Stowmarket, England)
- Black sheets for base of cage bottom (constructed by Mr. Ambrose O’Halloran)
- Cage lids painted black (when using older home cage tracking system): painted by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)

Resident-Intruder paradigm:

- Rat’s home cage (dimensions outlined above)
- Black marker for labelling rats: Tesco (Galway, Ireland)

LPS-induced effects in the SPT:

- Rat’s home cage (dimensions outlined above)
- Water bottles: North Kent Plastics (Coalville, United Kingdom)

Novelty-induced hypophagia:

- Familiar arena (rat’s home cage; dimensions outlined above)
- Novel arena (novel cage; dimensions outlined above)
- Palatable food: Cheerios cereal
Chapter 2: Materials and Methods

- Ramekin (5 x 9 cm; Height x Diameter) to place Cheerios in: Home store and more (Galway, Ireland)
- 3 24-place home cage racks with video recording cameras: constructed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)
- Black/grey sheets for base of cage bottom: constructed by Mr. Ambrose O’Halloran (Pharmacology and Therapeutics, NUI, Galway)

Video cameras (Nikon/Ganz): Radionics (Dublin Ireland)

DVD+R: Tesco (Galway, Ireland)

DVD recorders (Sony): Currys (Galway, Ireland)

DVR Recorder (8 place Inspire DVR range): Tracksys (Nottingham, United Kingdom)

2.1.3. Drugs and drug administration

Distilled H₂O

Saline (0.89% w/v NaCl): Sigma Aldrich (Dublin, Ireland)

Desipramine hydrochloride (Cat # D3900): Sigma-Aldrich (Dublin, Ireland)

Fluoxetine hydrochloride: Cat # 11PM00777PL, Pinewood (Tipperary, Ireland) or Cat # PHR1394, Sigma-Aldrich (Dublin, Ireland)

Venlafaxine hydrochloride (Cat # V0110): Tokyo Chemical Industry UK Ltd. (Oxford, United Kingdom)

Ketamine (Ketamidor ®): Chanelle (Galway, Ireland)

Diazepam (Diazemuls emulsion): Chanelle (Galway, Ireland)

Lipopolysaccharide (LPS) from Escherichia coli 0111:B4 (Cat # L2630): Sigma-Aldrich (Dublin, Ireland)

1ml syringes: BD Microlance (Oxford, UK)

Needles (25G x 5/8”): BD Microlance (Oxford, UK)
2.1.4. Computer Software
Microsoft Office: Microsoft Ireland (Dublin, Ireland)
IBM SPSS Statistics 21: SPSS Inc. (Chicago, IL, USA)
GraphPad Prism 5: GraphPad Software Inc. (La Jolla, CA, USA)
Ethovision ® XT 8.5: Noldus (Wageningen, The Netherlands)

2.2. Methods

2.2.1. Animals
All experimental procedures on animals were conducted with the approval of the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland, Galway (12/NOV/07), under licence from the Irish Department of Health and Children and in compliance with European Communities Council directive 86/609 guidelines. Male Sprague-Dawley rats were used for all of the experiments described and were either obtained from Charles River (U.K.) or bred in-house. Rats were individually housed in plastic-bottomed cages (42 x 25.5 x 13 cm; L x W x H), with wood shavings, corn cob or paper bedding, depending on the nature of the experiment. Bedding was changed weekly. Rats were maintained on a 12-h light/dark cycle (lights on at 08:00 h) in a temperature controlled room (23 ± 2 °C) with relative humidity ranging at 35-60%. Standard rat chow pellets and water were available ad libitum. In studies where anhedonia was assessed, a saccharin drinking solution was also provided to the rats. Animals were weighed at least once weekly to ensure normal growth patterns were being maintained. In all cases, animals were randomly assigned to their treatment groups.

2.2.2. Behavioural testing

2.2.2.1. Elevated plus maze
As previously mentioned, the EPM is a test for anxiety-like behaviour (Pellow and File, 1986). This test is based on the principle of thigmotaxis, whereby the rat has a natural aversion to the open arms and has a tendency to remain close to vertical surfaces, and thus, on the closed arms. The EPM in which all animals were tested consisted of a plus-shaped apparatus – two open arms and two closed arms that are raised approximately 55
cm above the floor (Figure 2.1). All arms are 50 cm long and the walls surrounding the closed arms are 30 cm high. Four 60 watt bulbs are placed above the EPM – one bulb over every arm. These bulbs are powered by dimmer switches and the light intensity was manipulated so that the end of each closed arm was approximately 55-60 lux and the end of each open arm was approximately 110-120 lux. Each rat was placed in the centre square of the EPM, facing an open arm and was allowed to freely explore the arena for 5 minutes. If the rat fell from the EPM it was discounted from analysis. When the effect of a drug was assessed in the EPM, the drug was administered via the i.p. or s.c. route 30 minutes prior to testing at a dose volume of 1-2 ml/kg (specified in particular chapters). After each rat trial, the rats were placed either in the home cage or onto the open field for further analysis, after which they were returned to their home cage. The maze was cleaned and wiped down with warm soapy water and tissue in between trials.

All trials were recorded on a DVD or DVR recorder from a camera that was placed approximately 1.3 metres above the centre of the EPM. Trials were later scored in a blind fashion, i.e. with the treatment received unknown to the observer. An arm entry was defined as all four of the rat’s paws being on the arm. A rat was therefore considered to have left an arm once all four of his paws were no longer in an arm (i.e. one or more paws in the centre square). Open and closed arm entries and time were manually scored with the Ethovision® XT 8.5 system using the ‘mutually exclusive’ setting. Total arm entries (open arm + closed arm) and total arm time (open arm + closed arm) were calculated to derive the parameters of interest – percentage open arm entries (% OAE; open arm entries/total arm entries*100) and percentage open arm time (% OAT; open arm time/total arm time*100).
Figure 2.1: The EPM apparatus illustrated from a screenshot of the video scoring image (A) and a diagrammatic depiction of the apparatus (B) (figure adapted from http://www.augusta.edu/).

2.2.2.2. Open Field

The open field test was used to measure general locomotor activity and emotionality in a novel environment (Hall et al. 1934). As mentioned earlier, the test involves exposing a rodent to an unknown environment from which they cannot escape. The apparatus consists of a white circular base (75 cm in diameter), surrounded by silver mirrored walls (41 cm high) (Figure 2.2). Four 60 watt bulbs are placed above the open field apparatus. These bulbs are powered by dimmer switches and the light intensity was manipulated so that it measured between 190-210 lux throughout the arena on the circular white base. In all cases, rats were tested in the open field immediately after EPM testing, as results from our laboratory previously showed no test order effects when these tests are carried out in this manner (unpublished data). Each rat was placed in the centre of the open field and allowed to freely explore the arena for 5 minutes. For this test, drugs were administered via i.p. or s.c. injection 35-37 minutes prior to testing (i.e. immediately after the EPM) at a dose volume of 1-2 ml/kg (specified in particular chapters). After each trial, the rat was placed back into its home cage and the apparatus was cleaned and wiped down with warm soapy water and tissue. All trials were recorded on a DVD or DVR recorder from a camera that was placed above the centre of the open field. Trials were later scored in a blind fashion. The distance that the rat moved (cm) was the parameter of interest for this test. Distance moved (cm) was
calculated automatically by calibrating the diameter of the arena and using the ‘detection determines speed’ trial setting on the Ethovision ® XT 8.5 system.

![Image A](image1.png)

![Image B](image2.png)

**Figure 2.2:** Open Field apparatus. The open field apparatus illustrated from a screenshot of the video scoring image (A) and side profile of the apparatus (B).

### 2.2.2.3. Forced Swim Test
The FST is the most widely used preclinical behavioural test for assessing antidepressant efficacy of compounds (Porsolt *et al.*, 1978). In the FST, rats are forced to swim for 15 minutes in a water-filled cylinder from which they cannot escape. 24 hours later, the animals are exposed to the same conditions, except for 5 minutes – this 5 minute swim is the test that is scored. The rats soon develop an ‘immobile’ posture which is said to reflect behavioural despair. For the current FST experiments each rat
was placed individually in a clear cylinder (50 cm x 25 cm (old apparatus) or 45 cm x 20 cm (new apparatus)) filled to 30 cm with water (23-25 °C), for a 15 minute preswim (see Figure 2.3). Then, 24 hours later, the animals were exposed to the same conditions, but for 5 minutes. When the effect of a drug was assessed in the FST, the drug was administered either subacutely (24, 5 and 1 hour prior to the 5 minute test swim, with the first dose administered 15 minutes after the preswim) or chronically (once daily for 14 days with the last dose administered 1 hour prior to the 5 minute FST or 21 days with the last dose administered 24 hours prior to the 5 minute FST) (specified in particular chapters). Drugs were always administered via s.c. injection, except for one study in Chapter 3 in which the aim was to determine the optimal route of administration. After each swim, the rats were towel dried and placed back in their home cage. Cylinders were filled with fresh water in between each rat.

All trials were recorded by a DVD or DVR recorder from a camera that was placed at a certain distance from the apparatus so that all four cylinders could be viewed together and clearly. The 5 minute trials were later scored individually on video in a blind fashion. The parameters of interest were immobility, climbing and swimming behaviour. Immobility was assigned when the rat exhibited only the minimum amount of activity required to keep its head above the water. Climbing behaviour consisted of forceful movements of the four limbs, with upward-directed movements of the forepaws, usually along the side of the cylinder. Swimming was defined as movement (usually horizontal) throughout the cylinder. For all studies, these behaviours were scored using the continuous scoring technique which involved scoring the entire amount of time the rat spent undergoing each of the three behaviours over the 5 minute test. An exception was one study in Chapter 3 in which the aim was to compare the sensitivity of the two scoring techniques at detecting drug effects, and for this study both the continuous scoring and the time sampling scoring were used. The time sampling scoring consisted of recording the predominant behaviour elicited by the rat every 5 seconds of the test (Detke et al., 1997). Both scoring techniques were carried out using the manual scoring setting on the EthoVision XT 8.5 software package.
2.2.2.4. Home cage activity monitoring prior to the FST and nocturnal

Home cage activity monitoring was carried out for two purposes: 1) to assess the effects of drugs on general locomotion prior to the FST (to detect false positives in this test) and 2) to assess nocturnal locomotor activity the final test battery (Chapter 5). The animals were housed in plastic-bottomed cages (42 x 25.5 x 13 cm; L x W x H) (Figure 2.4). When the home cage activity monitoring was carried out prior to the FST, the protocol was as follows: where wood shavings was used as bedding, the animals were placed back into their home cage with fresh black bedding (wood shavings dyed black) after the 5 hour dosing time point for the FST and a black plate was placed at the base of the cage to allow optimal video tracking of the rat’s movement. Where paper bedding was used, animals remained in the bedding that they were in, as the grey colour of the paper bedding displayed enough of a contrast for optimal video tracking of the rat. Immediately after dosing at the 1 hour FST time point, animals were transferred in their cage to a home-cage activity monitoring rack (in earlier studies in which home cage racks did not have cameras above every cage position) or placed back in their usual cage position (in later studies in which cameras were fixed above every cage position). Immediately after the hour of home cage tracking was complete, rats were placed in the 5 minute test swim for the FST, after which they were towel dried and returned to their home cage in their original cage position. When the home cage activity monitoring was carried out for the purpose of nocturnal home cage activity, animals remained in their home cage, in which a camera was fixed above to allow monitoring of the rat and 12 hour nocturnal home cage locomotor activity was assessed. For both home cage activity protocols, all trials were recorded on a DVD or DVR recorder from the camera that was placed above the cage and video trials were scored later. The
distance that the rat moved (cm) was the parameter of interest and this was calculated automatically by calibrating the diameter of the arena and using the ‘detection determines speed’ trial setting on the Ethovision® XT 8.5 system.

(A)

(B)

(C)

**Figure 2.4:** Home cage monitoring rack (A), and a screen shot view of the videos for scoring when animals were housed in paper bedding (B) and black sawdust bedding (C).
2.2.2.5. **Resident-intruder paradigm**

As discussed previously, the resident-intruder paradigm is based on the principle that when adult male rodents are given sufficient living space, they establish a territory within that space, and therefore an unfamiliar intruder placed in that cage will cause the resident to attack. Thus, the rat’s own homecage was the apparatus for this experiment. The acute protocol was adopted for use in the current experiments, with two minor adjustments – the animals were singly housed for at least 7 days prior to testing, and the test was only carried out once, as opposed to four times. On the day of testing, the resident rat was dosed, and a black mark was placed on its back to distinguish the resident from the intruder. The resident rat was placed back into its home cage which was then placed on a different cage rack in the same room with a built-in camera above the cage. The resident was allowed 30 minutes to habituate to this new cage position. The corresponding intruder was administered the same drug dose immediately after the resident rat was dosed, and was placed back in its original cage position for the 30 minutes. When the 30 minutes had passed, the intruder rat was placed inside the resident’s cage with the resident rat for a total of 10 minutes. After each trial, the intruder was placed back in its home cage and the cage of the resident rat was placed back in its original position.

All trials were recorded by a DVR recorder from a camera that was placed above each individual test cage, as in the home cage activity monitoring. Trials were later scored on video in a blind fashion. There were several parameters of interest, divided into six distinct motivational categories: exploration (locomotion, rearing, non-social exploration), investigation (approach, follow, stretched attention, to-fro, walk round/circle/side, nose and investigate, sniff genitalia, tail rattle), aggression (aggressive groom, aggressive posture, attack, bite, offensive sideways, offensive upright, pull, threat/thrust), flight-submit (defensive sideways, defensive upright, submit), flight-escape (attend, crouch, elevated crouch, flag and evade, retreat), and maintenance (digging, drinking, eating, licking, scratching, head/body shake, washing/grooming) (Figure 2.5). These behaviours were manually scored with the Ethovision ® XT 8.5 system using the ‘mutually exclusive’ setting.
Figure 2.5: Social exploration (A), aggressive (rat on top) and flight submit (rat on bottom) behaviour (B) and flight escape (left) and chase (right) behaviour (C) in the resident-intruder paradigm (images adapted from Koolhaas et al. (2013)).

2.2.2.6. Saccharin preference test and LPS-induced responses

The SPT is a valuable test in that it represents a clinically translatable symptom – anhedonia. Rats have a strong preference for sweet solutions such as saccharin, and when the SPT is combined with a challenge of the pro-inflammatory endotoxin LPS, a rat’s preference for saccharin will typically reduce, indicating a depressive-like anhedonic effect of LPS. A 0.5% saccharin solution was used for all studies. In all instances, rats were exposed to one of two protocols.

The first protocol was carried out for the first three battery studies. In this protocol, animals were trained to develop a preference for saccharin by adding an extra bottle which contained saccharin to the front of the rat’s home cage (beside the water bottle) so that they then had access to both water and saccharin (Figure 2.6 (B)). For each drug group, saccharin was placed on the left side (usual bottle position) for half of the animals and on the right side for the other half to account for any preference in bottle positioning, as the rats are familiar with the left position from the water bottle. For the training period, animals were given access to the two bottles for 3 hours per day for 2 days. On the second training day rats received a final dose of chronic drug treatment. 24 hours later, rats received an i.p. injection of LPS (200 µg/kg). Animals were placed back in their home cage and saccharin was made available to the animals in the same
manner as previous days. After 3 hours, the saccharin bottle was removed and the water bottle placed back in the left position, if not already there. To determine saccharin preference, water and saccharin bottles were weighed immediately prior to, and immediately after testing to determine the total fluid consumption over the test. Saccharin preference was determined by expressing the amount of saccharin consumed as a percentage of the overall fluid consumption (saccharin consumption/total fluid consumption*100). Calculations were carried out using Microsoft Office Excel 2007.

In protocol 2, animals were trained to develop a preference for saccharin in the same manner as above, but the training period consisted of 3 overnight exposures to a choice of both water and saccharin, in which the saccharin was made available approximately 1-2 hours prior to the dark cycle (between 18:00-19:00 hr) and removed approximately 0.5-2 hours after onset of the light cycle the next morning. On the fourth day, rats received an i.p. injection of LPS (50, 100, 150 or 200 µg/kg) in the middle of their light cycle (approximately 24 hours after the previous chronic drug administration) and rats were not administered a chronic drug treatment on this day. Animals were placed back in their home cage and after approximately 4 hours, saccharin was made available to the animals overnight in the same manner as previous days. The next day, animals returned to their chronic dosing regime and overnight saccharin preference testing continued for three nights after. Saccharin preference was calculated as described above.

**Figure 2.6:** LPS induced anhedonia testing. Injection of LPS via the i.p. route (A), followed by the SPT (B).
2.2.2.7. **Novelty-induced hypophagia test**

The NIH test is based on the principle of ‘hyponeophagia’ – the conflict which rodents face when given a choice of approaching and consuming a desirable food in a novel environment, or avoiding the novel environment. All animals were tested using the same protocol. The rats were not food deprived at any stage. They were allowed at least 2 days training (2 days was sufficient to develop a high baseline consumption) in their home cage in which they were trained to eat a highly palatable food (Figure 2.7 (A)), which in this instance was ‘Cheerios’ cereal. For this training, 10 Cheerios were placed in a ramekin, and the ramekin was placed at the back of the cage in the same position every day. Ramekins were removed from the rat’s cage after 30 minutes and the Cheerios consumption was recorded. The day after the last training session, the animals underwent a home cage test day in which the same procedure was carried out as previous days, except the ramekins were removed from the home cage after 15 minutes. Approximately 24 hours following this, rats underwent the novel arena test. The novel arena was in a different room to the holding room and consisted of a novel cage with no bedding, with black bases to enhance observation (Figure 2.7 (B)). Four of these novel cages were placed side by side. Four 60 watt bulbs were placed above the cages. These bulbs were controlled by dimmer switches and the light intensity was manipulated so that light intensity in all four cages was very high (between 200-300 lux). Ramekins were filled with 20 Cheerios on this day. Rats were placed in the novel arena and the ramekins were placed in a similar position to previously. After 15 minutes, the ramekins were removed, the rats were placed back in their home cage and the number of Cheerios eaten was recorded. The novel arenas were cleaned and wiped down with warm soapy water and tissue in between trials. In all instances of this test, the effect of a drug was assessed. A drug was administered s.c. either 30 minutes (acute treatment) or 24 hours (after chronic treatment) prior to testing at a dose volume of 2 ml/kg.

All trials were recorded on a DVR recorder from a camera that was placed above each individual test cage (home cage testing) or above all four novel cages (novel arena test day). All trials were later scored in a blind fashion. The parameters of interest were latency to consume the first Cheerio and the amount of Cheerios eaten in both the home cage test and the novel cage test. The latency to consume the first Cheerio was manually scored with the Ethovision ® XT 8.5 system, using the ‘start-stop’ function, which allowed the latency to the first consumption to be calculated once the button was pressed. The amount of Cheerios consumed was recorded immediately after testing.
Figure 2. NIH testing. Rats receiving Cheerios in ramekins in the home cage (A) and in a novel, brightly lit cage (B).
2.2.3. Drug Dosing

Table 2.1 represents the route of administration, the dose volumes, and the vehicle used to make up each drug.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Route(s)</th>
<th>Dose volume</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desipramine hydrochloride</td>
<td>2.5, 5, 10 or 20 mg/kg</td>
<td>s.c., i.p. or p.o.</td>
<td>1 ml/kg or 2 ml/kg</td>
<td>DH₂O</td>
</tr>
<tr>
<td>Fluoxetine hydrochloride</td>
<td>5, 10 or 20 mg/kg</td>
<td>s.c.</td>
<td>2 ml/kg</td>
<td>DH₂O</td>
</tr>
<tr>
<td>Diazepam</td>
<td>0.5, 1, 1.5 or 2 mg/kg</td>
<td>s.c. or i.p.</td>
<td>1 ml/kg or 2 ml/kg</td>
<td>Sterile 0.89% NaCl or DH₂O</td>
</tr>
<tr>
<td>Venlafaxine hydrochloride</td>
<td>20 mg/kg</td>
<td>s.c.</td>
<td>2 ml/kg</td>
<td>DH₂O</td>
</tr>
<tr>
<td>Ketamine</td>
<td>10 mg/kg</td>
<td>s.c.</td>
<td>2 ml/kg</td>
<td>DH₂O</td>
</tr>
<tr>
<td>LPS</td>
<td>0, 50, 100, 150 or 200 µg/kg</td>
<td>i.p.</td>
<td>1 ml/kg</td>
<td>Sterile 0.89% NaCl</td>
</tr>
<tr>
<td>Saccharin sodium salt hydrate</td>
<td>0.5% solution</td>
<td>Drinking fluid</td>
<td>Saccharin consumption</td>
<td>Drinking water</td>
</tr>
</tbody>
</table>

Table 2.1: Preparation of drug and drinking solutions for testing. The dose, route of administration, dose volume and vehicle used for all compounds.

The specific drug dose, route, volume and vehicle used for each study are provided in the ‘Experimental protocol’ section of each results chapter (Chapters 3-5). All parameters were decided based on either previous studies within our laboratory (Simpson et al., 2012a, Simpson et al., 2012b) and within the literature (Enginar et al., 2016, Olivares-Nazario et al., 2016, Engin et al., 2009, Rogoz et al., 2002, Dwyer et al., 2015, Yirmiya, 1996, Hadweh et al., 2010) or on studies that were carried out in the current thesis as part of characterisation. For subacute FST dosing studies, desipramine hydrochloride was prepared by dissolving the compound in DH₂O to the required concentration and the same batch of prepared drug was used over the two days (refrigerated overnight). For chronic dosing studies, desipramine hydrochloride, fluoxetine hydrochloride and venlafaxine hydrochloride were prepared by dissolving in the specified vehicles to the required concentration. The drugs were then divided into aliquots according to how much drug was required for each dosing day, and frozen at -20°C over the course of the study. Aliquots were thawed and brought to room temperature on the day that they were required for dosing. Diazepam and ketamine
were prepared by diluting these suspensions in the specified vehicle to the required concentration. Diazepam and ketamine were made up fresh on all dose days, as the laboratory vet advised that these suspensions should not be used after 24 hours from when they were removed from their original vials.

2.2.4. Animal sacrifice and tissue collection

2.2.4.1. Decapitation
For post-mortem investigations, animals were sacrificed by decapitation and fresh-frozen brain tissue was collected. Immediately following decapitation, an incision was made in the skin on the top of the head using a scissors. The skin was pulled back to expose the skull. The optic ridge between the eyes was then cracked using a rongeur and a shallow cut was made along the midline of the skull to peel back the parietal and frontal bones. The optic nerve was teased out and the whole brain was removed from the skull using a forceps. The brains were snap frozen on a bed of solid CO₂ pellets and stored at -80 ºC until analysis.

2.2.4.2. Brain Dissection and tissue collection
Brains were removed from the -80 ºC freezer and placed in a Styrofoam box containing solid CO₂ pellets prior to dissection to allow for ease of access. Brains were taken out from the Styrofoam box and placed on an ice cold plate. The left hippocampus was isolated and approximately 2/3 of the tissue was used for PCR analysis.

2.2.5. Analysis of gene expression using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.2.5.1. RNA isolation
Total RNA was extracted from homogenised discrete hippocampus samples using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Fisher Scientific, Ireland). 354µl of RA1 lysis buffer containing 1% β-mercaptoethanol (M6250: Sigma-Aldrich, Ireland) was added to approximately 30mg tissue prior to homogenisation with an Ultra-Turrax Polytron tissue disrupter (Fisher Scientific, Ireland). Homogenates were then transferred to a Nucleospin filter column (purple) and centrifuged at 11,000g for 1 min. 350µl of 70% molecular grade ethanol (E7023: Sigma-Aldrich, Ireland) was
added to the lysates and mixed by pipetting up and down 10 times. The samples were then transferred to another set of Nucleospin RNA II columns (blue) and centrifuged at 11,000g for 30 seconds to allow RNA bind to the column. Following centrifugation, the columns were placed in new collection tubes and 350µl of membrane desalting buffer (MDB, supplied with kit) was added prior to centrifugation at 11,000g for 1 minute. Genomic DNA was digested using a 10% v/v rDNase solution prepared in DNase reaction buffer (supplied). 95µl of the rDNase solution was pipetted directly onto the centre of each column and allowed to stand for 15 minutes at room temperature, following which 200µl RA2 buffer was added to each column prior to centrifugation at 11,000g for 30 seconds. The columns were then placed in new collection tubes and 600µl of RA3 wash buffer was added followed by centrifugation at 11,000g for 30 seconds. The eluent was discarded and 250µl of RA3 wash buffer was added followed by centrifugation at 11,000g for 2 minutes. The columns were then placed in RNase-free collection tubes and the RNA was eluted by the addition of 60µl of RNase-free water (W4503: Sigma-Aldrich, Dublin) followed by centrifugation at 11,000g for 1 minute. The eluted RNA was stored on ice until RNA quantification was complete and then stored at -80°C until reverse transcription.

2.2.5.2. RNA quantification and equalization

The quantity, purity and quality of RNA were assessed using a Mastero Nano drop spectrophotometer (Medical Supply Co. Dublin). RNA quantity was determined by measuring optical density (OD) at 260nm (1 OD unit at 260 nm corresponds to 40µg/ml RNA). RNA quality was determined by measuring the OD260/OD280 ratio where a value of approximately 1.6-2.0 was deemed indicative of pure RNA. All RNA samples with a ratio >1.6 were accepted. Prior to cDNA synthesis, all samples were equalised to the same concentration of RNA (2µg/20µl) by addition of RNase free water. Equalised samples were then stored at -80°C until reverse transcribed.

2.2.5.3. Reverse Transcription of mRNA to cDNA

A high capacity complementary DNA (cDNA) kit (Cat # 4368814: Applied Biosystems, UK) was used to reverse transcribe RNA samples. 10µl of equalised RNA was added to an equal volume of 2X master mix in a PCR mini-tube. The 2X master mix was prepared as follows: 2.0µl 10X RT buffer, 0.8µl 25X dNTP mix, 2.0µl 10X RT random primers, 1.0µl Multiscribe Reverse Transcriptase and 4.2µl RNase free water. Samples
were then placed in an ‘MJ research’ thermal cycler (Bio-Rad, Fannin, Dublin) and incubated at 25°C for 10 minutes, 37 °C for 2 hours and 85 °C for 5 minutes. The final cycle in the program maintained the samples at 4 °C. The resultant cDNA was diluted 1 in 4 with RNase free water and stored at -80°C until quantification by qRT-PCR.

2.2.5.4. Quantitative Real-time PCR (qRT-PCR) analysis of gene expression

Gene expression of target proteins were determined using commercially available TaqMan gene expression assays (Applied Biosystems, UK) containing specific forward and reverse target primers and FAM-labelled MGB probes (Table 2.2.). β-actin was used as an endogenous control to normalise gene expression between samples and was quantified using a β-actin endogenous control assay (cat #4352340E Applied Biosystems, UK) containing specific primers and a VIC-labelled MGB probe. Assay IDs for the genes examined are given in Table 2.2.

A reaction master mixture was first prepared and stored on ice for each target gene. This consisted of 0.625 µl target primers, 0.625 µl β-Actin (multiplex version) and 6.25 µl TaqMan Universal PCR Master Mix (Cat # 4324018: Applied Biosystems, UK) per sample. 5 µl of each sample was pipetted in duplicate onto a MicroAmp® optical 96 well plate (Applied Biosystems, UK). 7.5 µl of the relevant reaction mixture was then added to each well giving a total reaction volume of 12.5 µl. Non template controls (NTC) containing the master mix and RNase free water without cDNA for each target gene were also included. Plates were then covered with optical adhesive covers and spun at 1000 g for 1 minute to ensure complete mixing and elimination of bubbles. The plate was then placed in the real time PCR thermocycler (StepOnePlus™, Applied Biosystems, UK) pre-set to run the following Relative Quantification protocol: step 1: 95 °C for 10 minutes, step 2: 95 °C for 15 seconds followed by one minute at 60 °C. Step 2 was repeated 40 times and the fluorescence read during the annealing and extension phase (60 °C) for the duration of the programme.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Assay number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>Rn02532967_s1</td>
</tr>
<tr>
<td>Ntrk2</td>
<td>Rn01441749_m1</td>
</tr>
<tr>
<td>β-actin</td>
<td>4352340E</td>
</tr>
</tbody>
</table>

Table 2.2: List of Taqman gene expression assays used.
2.2.5.5. Analysis of qRT-PCR Data

Amplification plots and copy threshold (Ct) values were examined using Applied Biosystems StepOne software V2.2.2. Ct values for each sample were analysed after setting the threshold to the linear exponential phase of the amplification plots and exporting to Microsoft Excel for final analysis (Figure 2.8). The $2^{-\Delta\Delta Ct}$ method was used to determine gene expression (Livak and Schmittgen, 2001). This method is used to assess relative gene expression by comparing gene expression of experimental samples to control samples, allowing determination of the fold change in mRNA expression between experimental groups. This method involves 3 steps: (1) Normalisation to endogenous control (β-actin) where ΔCt is determined: ΔCt = Ct Target gene - Ct Endogenous control; (2) Normalisation to control sample where ΔΔCt is determined: ΔΔCt = ΔCt Sample - average ΔCt of Control group; and (3) where the fold difference is given by $2^{-\Delta\Delta Ct}$. The $2^{-\Delta\Delta Ct}$ values for each sample were then expressed as a percentage of the average of the $2^{-\Delta\Delta Ct}$ values for the control group. In this manner the percentage increase or decrease in mRNA expression between experimental groups was determined.
Figure 2. 8: Sample Amplification Plots for (a) the endogenous control β-actin, (b) BDNF and (c) Ntrk2 receptor.
2.2.6. Statistical analyses and graph construction

All statistical analyses were carried out using IBM SPSS Statistics Version 21. A $p$ value of less than 0.05 was considered statistically significant in all tests. Full details of statistical analyses are provided in the ‘Results’ section of each Results chapter (Chapters 3-5). Details on graphical and tabular representation of data are outlined in each Results chapter. Graphs were constructed in GraphPad Prism Version 5.
Chapter 3  Potential sources of variation in behaviour in the EPM and FST

3.1. Introduction
As previously mentioned, the EPM and FST are the most commonly used behavioural tests for preclinical assessment of anxiolytic and antidepressant properties of drugs, respectively. Therefore, it is imperative that these tests are reliable and their results are replicable. However, a major problem that exists within the literature is the large variations in both baseline and drug-induced responses in such tests, as could be seen with the FST and DMI examples outlined in Chapter 1. Various experimental parameters have started to be investigated for the possible cause of variation in these results. As specified earlier, these factors include the strain, gender, age, handling, housing, bedding type and breeding source of the rat, as well as the treatment schedule, testing apparatus and the time of testing. The potential effects of all of these parameters are important when bearing in mind the development of the test battery study design outlined in the aims of this thesis. Moreover, the idea that such parameters as housing conditions may potentially affect behavioural testing is disconcerting, considering that the growing concern for animal welfare has seen the development of legislation which may require laboratories to change the housing conditions of experimental subjects. For example, the EU Directive 2010/63/EU on the protection of animals used for scientific purposes now states that rodents should be socially housed and provided with environments in which sufficient enrichment is provided to promote physical exercise, foraging, as well as manipulative and cognitive activities, as appropriate for the species. In addition, the idea that the breeding source of the rat may potentially affect results is worrying, as animal rights activism has increased the likelihood of animal blockade in research, with only a few remaining airlines that can carry animals to Ireland from the UK, where the majority of our animals are sourced. Hence, if an in-house colony was developed, concerns about changes in phenotype would arise which may affect reliability and comparability of results. Therefore, we sought to investigate the effect of the above outlined parameters on behavioural testing within our own laboratory to (1) contribute important information to the literature that may prove helpful in the adaptation of laboratories to new inevitable protocols, and (2) to determine the optimal experimental parameters to use when developing the battery study design for this project.
Based on previous experience within our laboratory, it was decided from the outset that we would use male Sprague-Dawley rats, that would be handled in the usual manner carried out within our laboratory, and that would be tested during the light-phase of their light/dark cycle. Furthermore, all of our testing would be carried out using our existing apparatus’ in the same way that we have previously used them, except in the case of the FST, where several refinements were assessed and outlined later. Thus, the factors that remained to be investigated prior to development of the battery study design were the breeding source and age of the rat, the bedding and overall housing type, and the treatment schedule and route of administration that would be employed. Each of these factors had to be carefully decided upon to ensure that the battery study design would utilise the optimal parameters for sensitive and robust detection of drug effects.

As previously mentioned in Chapter 1, the breeding supplier from which the rat is sourced can cause variation in many important behavioural tests. The supplier of the rat has been found to affect varying aspects of rodent behaviour, ranging from ethanol consumption, anxiety tests, depressive-like tests, and general locomotion (Palm et al., 2011, Honndorf et al., 2011, Pare and Kluczynski, 1997). Interestingly, Rex et al. (2007) showed that even when rats are from the same source, the maintenance of these rats on two different animal holding sites produced differences in responses to tests such as the free exploration paradigm, the hole-board test and the elevated plus maze (Figure 3.1 (A) for EPM results). Moreover, studies have reported that rodents obtained from different suppliers show significantly different anxiety profiles in tests such as the open field (Honndorf et al., 2011, Pare and Kluczynski, 1997) and EPM (Honndorf et al., 2011) (Figure 3.1 (B) for open field results). Taken together, this evidence suggests that careful consideration is required in deciding which commercial vendor to obtain rodents from when planning an experiment that is sensitive to anxiety profiles.
Figure 3.1: Effect of breeding source on EPM and open field behaviour. Open arm time in the EPM differed between rats obtained from the commercial vendor Charles River (white bar) and from the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia (grey bar) (A) and number of entries into the centre zone of an open field differed between rats from Harlan-Winkelmann (white bar), Charles River (dark grey bar) and Janvier (light grey bar) (B). Data are mean±SEM and mean±SD, respectively. *p<0.05 vs relative control. Images adapted from Honndorf et al. (2011) and Rex et al. (2007).

In relation to the FST, Pare and Kluczynski (1997) reported no effect of vendor on depressive-like behaviours (Figure 3.2 (A)). However, rats obtained from four different breeding suppliers have shown differences in basal monoamine levels in several brain regions (Figure 3.2 (B)), and these rats have also responded differently to drugs such as monoamine oxidase inhibitors (Miller et al., 1968). Therefore, with monoamines playing a pivotal role in behaviours elicited in the FST (Detke et al., 1995), it is important to further investigate the effects of breeding supplier on FST behaviour within our own laboratory, before carrying out the final battery study design.
Figure 3.2: Effect of breeding source on FST and brain monoamine levels. Time spent immobile in the FST in Wistar-Kyoto rats did not differ between animals obtained from Charles River, Taconic or Harlan laboratory (A), while midbrain NE and 5-HT levels differed between breeding sources (B) Data are mean±SEM. *p<0.05 vs. the Hormone Assay Lab. Image (A) and information (B) adapted from Pare and Kluczynski (1997) and Miller et al. (1968), respectively.

Age is an experimental parameter that has been more commonly investigated with regards to its effects on EPM and FST behaviour in particular. In relation to the EPM, several studies have shown that the age of the subjects used can affect baseline behaviour. Studies that have reported the absolute OAE and OAT values have found that as the rats increase with age, they become more anxious in the EPM, displaying less entries into, as well as less time spent, in the open arms (Imhof et al., 1993, Ferguson and Gray, 2005, Turner et al., 2012). Similarly, when open arm results are expressed as a percentage of total arm behaviour, both %OAE and %OAT have been found to decrease as the age of the rat increases, signifying an increased anxiety profile in older rats (Boguszewski and Zagrodzka, 2002, Andrade et al., 2003, Bessa et al., 2005) (Figure 3.3 (A)). Of the studies that have investigated the effects of age in the EPM, Bessa et al. (2005) also assessed the potential effects of age on drug-induced behaviour. They found that older rats (12 months old) did not respond to the anxiolytic midazolam in the EPM, whilst a dose response effect was evident for the drug in 3 month old rats (Figure 3.3 (B) for data).
Chapter 3: Sources of Variation

Figure 3.3: Effect of age on EPM behaviours. %OAE in the EPM was significantly lower in old (24 months old) rats compared to young (4 months old) rats (A) whilst the anxiolytic midazolam 0.5mg/kg (grey bar) or 1mg/kg (black bar) significantly increased %OAT in 3 month old but not in 12 month old rats (B). Data are expressed as mean±SEM. *p<0.05, ***p<0.001 vs. relative control. Images adapted from Boguszewski and Zagrodzka (2002) and Bessa et al. (2005), respectively.

With regards the FST, several studies have shown that the age of the subjects used can affect the baseline time spent immobile (Turner et al., 2012, Sequeira-Cordero et al., 2013), as well as climbing and swimming in the FST (Sequeira-Cordero et al., 2013) (Figure 3.4 (A)). In addition, the age of the rat has also been shown to alter drug-induced effects in the FST after both subacute (Olivares-Nazario et al., 2016) and chronic (Karanges et al., 2011) drug treatment. For example, Olivares-Nazario et al. (2016) found that DMI and FLX reduced immobility in the FST in 3-5 month old and 12 month old rats, but that drug effects were completely lacking in 23-25 month old rats. Moreover, Karanges et al. (2011) reported that chronic paroxetine decreased immobility in adult rats; an effect that was not evident in adolescent rats (Figure 3.4 (B)). With age illustrating an immense effect on baseline and drug-induced behaviour in both anxiety and depressive-like tests, it suggests that emotional behaviour differs depending on the age of the rat, and careful consideration should be taken when deciding on the age of the subjects used in studies incorporating such tests.
Figure 3.4: Effect of age on FST behaviours. Baseline time spent immobile, swimming and climbing in the FST was significantly different in juvenile (4 weeks old) and adult (12 weeks old) rats (A), whilst baseline and paroxetine (PRX)-induced immobility significantly differed between adolescent (4 weeks old) and adult (10 weeks old) rats (B). Data are mean±SEM. *p<0.05 vs relative control. Images adapted from Sequeira-Cordero et al. (2013) and Karanges et al. (2011), respectively.

Investigations into the potential effects of bedding materials on behavioural tests have only started to emerge within the last decade. To our knowledge, there are no studies that have specifically assessed the effects of bedding material on EPM behaviour. However, one study has compared the effect of bedding type on other behavioural tests of anxiety. Within this study, which compared wood pulp and corn cob bedding, rats were much more anxious in both the open field and the light-dark box when housed in wood pulp bedding (Sakhai et al., 2013) (Figure 3.5(A) and (B)).
Figure 3.5: Effect of bedding type on open field and light dark box results. Animals reared on wood pulp bedding were much more anxious in the open field (A) and the light-dark box (B) compared to animals reared on corn cob bedding. Data are mean±SEM. ****p<0.0001, ***p<0.001 vs relative control. Images adapted from Sakhai et al. (2013).

Similar to the EPM, no studies have been carried out to assess the potential effects of bedding material on FST behaviours. However, Cui et al. (2006) and Raineki et al. (2012) have shown that limited nesting/bedding material over the postnatal period increases immobility time in the FST in adulthood and adolescence, respectively, highlighting the importance of the home cage environmental parameters in depressive-like behaviour (Figure 3.6). Taken together, these EPM and FST results illustrate the likelihood that bedding type may play an important role in determining how rats will perform in both anxiety and depressive-like tests, and thus, is an important parameter to decide upon for future testing within the laboratory.
Chapter 3: Sources of Variation

Figure 3.6: Effect of EE on FST behaviour. EE (Str-EE) normalised the increase in depressive-like behaviour in the FST that was seen in animals that were provided with limited nesting/bedding material over the postnatal period (Str). Data are mean±SEM. *p<0.05 vs control. Image adapted from Cui et al. (2006).

With regard to physical housing manipulations, the effect of EE on EPM behaviour varies across studies. Simpson et al. (2012a) reported no effect of EE on %OAE or %OAT behaviour (Figure 3.7 (A)), whilst similarly, another study reported no effect of EE on either %OAT or OAE (Brenes et al., 2009). Conversely, Galani et al. (2007) found that rats housed in enriched housing conditions showed a reduced anxiety profile, with increased %OAE and %OAT compared to rats housed in standard conditions (Figure 3.7 (B)). In agreement with these findings, other studies have reported the anxiolytic-like effect of enriched housing conditions, illustrated by an increase in OAE and %OAE (Pena et al., 2006, Pena et al., 2009). Further evidence for an anxiolytic-like effect of EE is supported in several other studies (Santucci et al., 1994, Hellemans et al., 2004). Simpson et al. (2012a) was the only one of the above studies that assessed the effect of EE on drug-induced EPM behaviours, however, the drug failed to induce an effect on EPM behaviours in any of the groups, regardless of housing conditions.
Figure 3.7: Effect of housing conditions on EPM behaviour. There was no difference in %OAE in animals housed in standard (SC), isolated (IC) or EE housing conditions (A) whilst in another study, enriched housing conditions (S-EC) produced an anxiolytic-effect in the EPM compared to standard housing conditions (S-SC) (B). Data are expressed as mean±SEM. *p<0.05 vs control. Images adapted from Simpson et al. (2012a) and Galani et al. (2007), respectively.

In the FST, EE has been reported to either have no effect on baseline immobility (Simpson et al., 2012a), or an antidepressant-like effect (Brenes et al., 2009) (Figure 3.8(A)), compared to rats housed without EE, either in social or isolated conditions. Furthermore, Bjornebekk et al. (2008) reported that physical EE, such as a running wheel, increased the active behaviour of climbing in the FST. The effect of EE on drug-induced effects has also been investigated in the FST. Although Simpson et al. (2012a) reported a blunting effect of EE on drug-induced effects compared to those singly housed (Figure 3.8 (B)), other studies have reported a potentiation of drug-induced effects in rats housed with EE compared to social conditions (Kus et al., 2010, Nowakowska et al., 2010). Importantly, EE is often used as somewhat of a therapeutic intervention, such as in animal models of stroke (Kuptsova et al., 2015, Greifzu et al., 2014), or specifically incorporated into study designs with the aim of inducing altered behaviour, and thus, can be quite elaborate. Indeed the above mentioned studies in which EE induced behavioural alterations were in fact seeking to alter behaviours, and used quite elaborate enrichment protocols.
As mentioned earlier, there is an ever growing concern for the welfare of the animals used within scientific experiments, and thus, the introduction of new legislation requirements may force behavioural laboratories to employ EE for their subjects (EU Directive 2010/63/EU). This is concerning when the evidence cited above has shown that EE affects behavioural test results, and thus may affect comparability of studies within and across laboratories. However, one factor that is clear within these studies is the vast inconsistencies in environmental protocols, such as the types of physical objects used, and the age of onset and duration of EE, among other factors, with several studies incorporating excessive EE. It is important therefore, that if a mandatory EE protocol is required within laboratories for legislative purposes, that it is modest enough to maintain standard baseline and drug-induced effects whilst also satisfying the criteria for the legislation. Conversely, whilst the above studies used quite elaborate EE, under the ‘Enrichment’ section of the EU Directive 2010/63/EU the provision of enrichment is described as providing the animals with a degree of control and choice over their environment to reduce stress-induced behaviour. Moreover, enrichment techniques should be in place to extend the range of activities available to increase the animals coping activities, such as physical exercise, foraging, and manipulative and cognitive activities, as appropriate to the species. As such, it is clear that efforts can be made to incorporate an enrichment protocol that satisfies this legislation, whilst also being much milder and user friendly, compared to the protocols used in the above studies.
However, no studies have yet assessed the potential effect of such an EE protocol, should laboratories have to adopt these legislative policies.

With the FST playing such a large role in the screening of antidepressant properties of novel compounds, several studies have sought to improve its validity and translatability. For example, although the subacute dosing regime for the FST is seen as a major advantage of the test due to its practicality and the ability to gain results quickly (Cryan et al., 2002), this short-term dosing regime is also thought to be a major weakness of the FST as a valid preclinical model as, clinically, antidepressants have a therapeutic lag of several weeks (Nierenberg et al., 2000, Nierenberg et al., 2007). Therefore, chronic study designs have become more commonly incorporated in the literature in recent years (Piras et al., 2014, Schaffer et al., 2010). Earlier comparisons of the magnitude of effect of chronic and subacute antidepressant treatment showed that with chronic administration, antidepressant drugs did not lose their efficacy in reducing immobility, and some even became more efficacious after chronic treatment (Kitada et al., 1981, Mancinelli et al., 1987, Miyauchi et al., 1981). In more recent years, several studies have assessed all three behaviours in the FST (immobility, climbing and swimming) after short-term and chronic dosing of low doses of antidepressants (Detke et al., 1997, Cryan et al., 2005a). These landmark studies showed that chronic administration of antidepressants are effective at doses which are ineffective when administered acutely, similar to the clinical scenario, and thus, strengthened the validity of the FST as an animal model of depression.

However, these studies were not without their limitations. For example, the effectiveness of the chronic treatment design compared to the more commonly used subacute dosing design could not be deciphered, as the acute methods that were used deviated from the traditional 3 dose regime over 24 hours. Furthermore, Cryan et al. (2005a) used a modified version of the FST, which consisted of just a 15 minute swim, despite the fact that the traditional two day FST protocol is still utilised by the majority of research laboratories today. Ideally, the battery study design should include a chronic treatment end point so that both behavioural and neurochemical measurements may be more translatable to clinical antidepressant activity. Thus, with the shortfalls of the above studies, it is important for us to specifically compare the traditional subacute dosing method that is standard within our laboratory, with a chronic dosing design to verify that drugs are comparably efficacious in both regimes. This would therefore
provide a reassurance that the chronic FST design could be incorporated into the test battery for the reliable detection of antidepressant properties and potential biomarker identification.

As well as the subacute dosing regime, the scoring technique for the FST has also been modified in a bid to improve the sensitivity of the test for detecting a wider range of compounds. The FST was originally assessed using a continuous scoring method in which the total amount of time the animal spends immobile in the 5 minute test is scored (Porsolt et al., 1978). However, Detke et al. (1995) developed a novel method of scoring the test. This consists of a time sampling technique, which involves scoring the frequency of immobility, as well as the active behaviours, climbing and swimming, by recording the predominant behaviour elicited every 5 seconds of the test swim. With this scoring method, distinct active behaviours emerge in the FST, which can be related to monoaminergic mechanism of action. For example, serotonergic compounds increase swimming counts, while noradrenergic compounds increase climbing counts (Detke et al., 1995, Page et al., 1999). Since its validation, this scoring method has been adopted by several laboratories (Cryan et al., 2005b, Carr et al., 2010, Morrish et al., 2009, Hadweh et al., 2010), due to its sensitivity at detecting active behaviours produced by drugs, and its ease of use compared to constant observation of the rat. However, this time sampling method has not been universally adopted, with many laboratories still using the traditional scoring method. The use of both scoring methods poses complications for inter-laboratory comparisons of drug efficacy in the FST. Although validity correlations have been carried out to compare both scoring techniques (Detke et al., 1995), the two scoring methods have not yet been comprehensively compared for sensitivity at detecting behavioural effects in the FST. With so many other experimental variables having an effect on FST results, it is important to compare these scoring techniques, to ensure that they are comparable in their ability to detect drug effects.

Despite its now universal use, in the beginning, the FST was under scrutiny for lack of specificity of the test. For example, drugs such as amphetamines were found to reduce immobility, but rather due to a stimulant effect on general locomotor activity (Kitada et al., 1981), as opposed to having an antidepressant-like effect. As such, to avoid any false positive effects, assessment of general locomotor activity has been incorporated into several study designs that use the FST, to ensure that drugs are not stimulating
general activity. For example, the open field test is often embedded into the study design and carried out prior to the FST as a measure of locomotor activity (Zhu et al., 2013, Harvey et al., 2010), to ensure that any reduction in immobility in the FST is due to an antidepressant-like mechanism alone, as opposed to a general stimulant effect (Figure 3.9).

**Figure 3.9:** Incorporation of open field test in FST study. Example of an experiment in which the open field was carried out prior to the FST to ensure that the drug being tested in the FST does not alter locomotor activity per se. Image adapted from Zhu et al. (2013).

However, carrying out an additional test prior to the FST can be quite stressful on the rat. This may subsequently affect the rat’s FST behaviour, and thus the experimenter’s interpretation of the results, causing complications for inter-laboratory comparisons of antidepressant-like drug effects. To avoid this potential interaction effect, some laboratories have assessed open field activity in separate groups of animals that have received the same treatment as animals undergoing the FST (Kawaura et al., 2010), although this requires an increase in animal numbers for the experiment, and it is then only assumptions of locomotor activity that can be concluded for the actual FST animals. An alternative to carrying out an extra behavioural test is to assess locomotor effects in the animals own home cage. This would avoid any extra stress on the rat, ensuring a valid reflection of the animals’ general locomotor activity for identifying false positive drugs, and importantly, will reduce the number of animals required for the experiment, an important aspect in laboratory research, as outlined by the 3 Rs principles.
As could be seen in Chapter 1, the route of drug administration also varies between studies, with the i.p. route being the most commonly employed for the FST and DMI studies, followed by the s.c. route. Both of these routes of administration can induce effects in the FST, but the success rate of each route at producing effects differs. Let us take the Sprague-Dawley FST and DMI (10mg/kg) studies mentioned in Chapter 1 as an example. Although the i.p. route was the most commonly used, a significant reduction in immobility was only evident in 46% of these studies. However, when we look at the studies that incorporated the s.c. route of administration, although much less studies used this route, immobility was significantly reduced in 100% of these studies. Importantly, the pharmacokinetics of a drug differ depending on the route by which the drug was administered, with the highest bioavailability of substances typically produced with routes that avoid the first pass metabolism effect, such as the s.c. route.

Considering that many studies are designed based on published experimental protocols, the current findings are important to note, as many researchers may choose the most commonly used injection route for comparability, but this may not be the best route to produce an effect. Therefore, it is vital to determine the optimal route of drug administration before investing time and resources into a study.

Behavioural baseline is a critical determinant of response to drugs, and with so many experimental factors potentially affecting behavioural results in tests, it is important that all of these parameters are assessed within our laboratory before the development of the test battery design. Due to time constraints, it was not possible to assess the effects of these parameters in all of the behavioural tests that would be carried out within the battery study design. Therefore, we chose to carry out these characterisation studies with the most popular tests used for anxiolytic and antidepressant screening, the EPM and the FST, respectively. Thus, the current studies used the benzodiazepine DZP and the antidepressant DMI in the EPM and FST, respectively, to investigate the effects of the breeding source and the age of the rat, as well as bedding material and EE on baseline and drug-induced effects. Importantly, prior to these investigations, the optimal route of drug administration for the FST was also determined, so that this route was incorporated in these FST characterisation studies. Furthermore, DMI was used to investigate whether antidepressant and distinct active behavioural effects are evident in the FST after subacute or chronic treatment. Assessment of home cage locomotor activity in the hour prior to the FST was also incorporated into this study design to help establish a more reliable and efficient method of detecting false positives in the FST.
Finally, we investigated if there were any differences between the two scoring techniques for detecting antidepressant-like and active behaviours in the FST.

3.2. Experimental protocols
A detailed description of the apparatus and test procedure for the use of the EPM and FST is provided in Chapter 2.

3.2.1. Experiment 1: Profile of EPM and FST baseline behaviours
This experiment was carried out to determine baseline responses in the EPM and FST within our laboratory, using a large number of rats. Male rats (Charles River, UK) were group housed throughout the testing period. Rats underwent the EPM protocol without any drug treatment, as outlined in Chapter 2, section 2.2.2.1. Approximately 1 week later, rats underwent the FST protocol without any drug treatment, as outlined in Chapter 2, section 2.2.2.3. The frequency distribution of each behavioural parameter for both tests was graphed to determine the distribution of the data across their particular measurement scale. The representation of the data in this way allowed us to determine the rat’s typical behavioural characteristics within these tests when carried out within our laboratory, and thus, provided a standard for us to compare future results to.

3.2.2. Experiment 2: Comparison of baseline results in the EPM and FST in commercially bred and in-house bred rats
The goal of this experiment was to compare baseline EPM and FST results in rats that were bred at different sources. Commercially bred rats arrived to the laboratory 6 days prior to the commencement of testing. On the day of arrival, both in-house bred, and commercially obtained male rats were singly housed in wood shavings. Rats (8 weeks old) underwent the EPM protocol without any drug treatment, as outlined in Chapter 2, section 2.2.2.1. Approximately two weeks later, the same rats underwent the FST protocol without any drug treatment, as outlined in Chapter 2, section 2.2.2.3. Results were scored as described in the respective sections in Chapter 2.

3.2.3. Experiment 3: Comparison of route of administration of DMI in the FST
The goal of this experiment was to compare the three main routes of administration in the FST to determine the optimal route for future studies. Male, in-house bred rats (14
weeks old at testing) were group housed in cages of four with wood shavings as bedding. Rats underwent the FST protocol with subacute DMI treatment as outlined in Chapter 2, section 2.2.2.3. DMI was administered at a dose of 20mg/kg either via the s.c, i.p. or p.o. route in a dose volume of 1ml/kg. All rats received three injections at every dosing point of the subacute regime (one injection of each route). Control animals received vehicle drug. Results were scored as described in Chapter 2, section 2.2.2.3.

3.2.4. **Experiment 4: Effect of age on baseline and drug-induced effects in the EPM and FST**
The goal of this experiment was to assess the effect of age on baseline and drug-induced effects in the EPM and FST. Male rats (Charles River, UK) were singly housed in wood shavings for 6 days prior to testing. Rats of three different ages were tested. Rats were approximately 9 weeks old, 7 months old and 16 months old when EPM testing began. All rats underwent the EPM protocol as outlined in Chapter 2, section 2.2.2.1. Approximately one week later, rats underwent the FST protocol with subacute vehicle or DMI treatment (10mg/kg s.c., 2ml/kg) as outlined in Chapter 2, section 2.2.2.3. Results were scored as described in the respective sections in Chapter 2.

3.2.5. **Experiment 5: Effect of bedding on baseline and drug-induced effects in the EPM and FST**
The goal of this experiment was to assess the effect of bedding type on baseline and drug-induced behaviours in the EPM and FST. Male rats (Charles River, UK) were singly housed for 6 days prior to testing. Half of the rats were housed in wood shavings and the rest were housed in corn cob bedding. Rats were 9 weeks old when EPM testing began and they underwent the EPM protocol as outlined in Chapter 2, section 2.2.2.1. Approximately one week later, rats underwent the FST protocol with subacute vehicle or DMI treatment (10mg/kg s.c., 2ml/kg) as outlined in Chapter 2, section 2.2.2.3. Results were scored as described in the respective sections in Chapter 2.

3.2.6. **Experiment 6: Effect of housing environment on baseline and drug-induced effects in the EPM and FST**
The goal of this experiment was to assess the effect of housing environment (bedding and/or EE) on baseline and drug-induced effects in the EPM and FST. Male, in-house bred rats were singly housed for two weeks prior to testing. At this point, rats were
housed in either wood shavings or paper bedding, with or without the incorporation of a modest EE protocol. EE consisted of the addition of nesting material and a plastic tube affixed to the inside of the cage. Additionally, EE rats received a weekly novel food supplement (hazelnuts, muesli, chocolate rice puffs) to encourage foraging behaviour. Rats were 11 weeks old when EPM testing began and they underwent the EPM protocol as outlined in Chapter 2, section 2.2.2.1. Approximately one week later, rats underwent the FST protocol with subacute vehicle or DMI treatment (10mg/kg s.c., 2ml/kg) as outlined in Chapter 2, section 2.2.2.3. Results were scored as described in the respective sections in Chapter 2.

3.2.7. Experiment 7: Comparison of drug treatment regime and scoring techniques in the FST
The goal of this experiment was to compare the effects of subacute and chronic drug treatment, as well as the scoring techniques used in the FST. Furthermore, the incorporation of a home cage monitoring protocol immediately prior to the FST was assessed for its ability to detect false positives in the FST. Male, in-house bred rats (approximately 10 weeks old at beginning of dosing) were singly housed in wood shavings for 8 days prior to dosing. Rats underwent the FST protocol with either subacute DMI or amphetamine (AMP; false positive in the FST) treatment, or chronic (14 days) treatment, as outlined in Chapter 2, section 2.2.2.3. For both treatment regimes DMI was administered s.c. at a dose of 10mg/kg. AMP was administered s.c. at a dose of 1mg/kg. Control rats received vehicle injections. All drugs were administered in a dose volume of 2ml/kg and all animals received the same amount of injections, regardless of treatment group. Home cage activity monitoring was carried out in the hour prior to the FST as described in Chapter 2, section 2.2.2.4. Results were scored as described in Chapter 2, section 2.2.2.4.

3.2.8. Statistical analysis
All of the data were tested for normality and homogeneity of variance, using Shapiro-Wilks and Levene tests, respectively. Data were analysed parametrically if they met the criteria of fulfilling two of the following three characteristics: even n numbers across groups, normality, and homogeneity. If data did not meet these criteria, they were analysed using non parametric statistical tests. The specific tests carried out are described under each particular results section. The level of statistical significance was
set at \( p = 0.05 \). Data were analysed using SPSS statistics software. Graphs were constructed using GraphPad Prism ® software. Expression of data is specified under each graph.

3.3. Results

3.3.1. Experiment 1 – Behavioural Characterisation of the EPM

3.3.1.1. OAE and % OAE frequency of distribution

This data represents the frequency of distribution of OAE and % OAE in the EPM. The graph is divided into segments which specify the percentage of rats that entered the open arms a specific number of times (Figure 3.10 (A)) or a specific percentage of times (Figure 3.10 (B)). For OAE, 37% of animals entered the open arms 7-8 times, followed by 21, 18, 15, 5, 1 and 3% of animals entering the arms 9-10, 10-12, 5-6, 13-14, 15-16 and 3-4 times, respectively. No animals entered the open arms fewer times than twice. This OAE data did not follow normal distribution (Shapiro-Wilk \( p < 0.05 \)). For % OAE, 36% of animals fell into the 40-49 and 50-59 %OAE categories, followed by 25 and 4% of animals within the 30-39 and 60-69 % OAE categories, respectively. No animals fell into the other % OAE categories. This % OAE data followed normal distribution (Shapiro-Wilk \( p > 0.05 \)).

![OAE and % OAE frequency of distribution](image)

Figure 3. 10: Frequency distribution of OAE and % OAE in the EPM. Baseline frequency distribution of OAE (A) and % OAE (B) (n=73). Mean±SD OAE: 9±3.

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3.3.1.2. **OAT and % OAT frequency of distribution**

This data represents the frequency of distribution of OAT and % OAT in the EPM. The graph is divided into segments which specify the percentage of rats that spent time in the open arms for a specific amount of time (Figure 3.11 (A)) or a specific percentage of time (Figure 3.11 (B)). For OAT, 37% of animals spent 60-89 seconds in the open arms, followed by 32, 18, 8 and 5% of animals spending 90-119 and 120-149, 30-59 and 150-179 seconds, in the open arms, respectively. No animals fell into the other time categories. This OAT data followed normal distribution (Shapiro-Wilk p>0.05). For % OAT, 40% of animals spent 30-39% of their time in the open arms, followed by 21% of animals spending 40-49 and 50-59 seconds and 12, 4 and 3% of animals spending 20-29, 60-69 and 10-19 seconds in the open arms, respectively. No animals fell into the other % OAT categories. This % OAT data followed normal distribution (Shapiro-Wilk p>0.05).

![OAT and % OAT frequency](image)

**Figure 3.11: Frequency distribution of OAT and % OAT in the EPM.** Baseline frequency distribution of OAT (A) and % OAT (B) (n=73). Mean±SD OAT (s): 97±29.

3.3.2. **Experiment 1 – Behavioural Characterisation of the FST**

3.3.2.1. **Immobility frequency of distribution in the FST**

This data represents the frequency of distribution of immobility time. The graph is divided into segments which specify the percentage of rats that displayed immobility for a specific amount of time (Figure 3.12). 38% of animals spent 150-199 seconds immobile, followed closely by 36% spending 200-249 seconds immobile. 10, 5 and 3% of animals spent 100-149, 250-299 and 50-99 seconds immobile, respectively. No
animals fell into the other time categories for immobility. This immobility data did not follow normal distribution (Shapiro-Wilk $p<0.05$).

**Figure 3. 12: Frequency distribution of time spent immobile in the FST.** Baseline frequency distribution of time spent immobile in the FST ($n=92$).

### 3.3.2.2. Climbing frequency of distribution in the FST

This data represents the frequency of distribution of climbing time. The graph is divided into segments which specify the percentage of rats that displayed climbing for a specific amount of time (Figure 3.13). 57% of animals spent 50-99 seconds climbing, followed by 22, 16 and 5% spending 0-49, 100-149 and 150-199 seconds climbing, respectively. No animals fell into the other time categories for climbing. This climbing data did not follow normal distribution (Shapiro-Wilk $p<0.05$).
3.3.2.3. **Swimming frequency of distribution in the FST**

This data represents the frequency of distribution of swimming time. The graph is divided into segments which specify the percentage of rats that displayed swimming for a specific amount of time (Figure 3.14). 85% of animals spent 0-49 seconds swimming, followed closely by 14 and 1% that spent 50-99 and 100-149 seconds swimming, respectively. No animals fell into the other time categories for swimming. This swimming data did not follow normal distribution (Shapiro-Wilk $p<0.05$).
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3.3.3. Experiment 2 – Effect of breeding source on baseline EPM behaviour

3.3.3.1. Effect of breeding source on baseline % OAE
This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if the breeding source of the rats affected % OAE. No significant effect of breeding source was found (t(22)=1.45, p=0.160) (Figure 3.15).

Figure 3. 14: Frequency distribution of time spent swimming in the FST. Baseline frequency distribution of time spent swimming in the FST (n=92).

Figure 3. 15: % OAE in in-house bred and commercially bred rats in the EPM. The breeding source of the rat did not affect % OAE. Data are expressed as mean±SD (n=12 per group). Mean±SD OAE for in-house bred rats: 11±2.
3.3.3.2. **Effect of breeding source on baseline % OAT**

This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if the breeding source of the rats affected % OAT. No significant effect of breeding source was found ($t_{(22)}=1.49$, $p=0.150$) (Figure 3.16).

![%OAT in in-house bred and commercially bred rats in the EPM](image)

Figure 3. 16: % OAT in in-house bred and commercially bred rats in the EPM.

The breeding source of the rat did not affect % OAT. Data are expressed as mean±SD (n=12 per group). Mean±SD OAT (s) for in-house bred rats: 86±21.

3.3.4. **Experiment 2 – Effect of breeding source on baseline FST behaviour**

3.3.4.1. **Effect of breeding source on immobility**

This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if the breeding source of the rats affected time spent immobile. No significant effect of breeding source was found ($t_{(22)}=1.34$, $p=0.193$) (Figure 3.17).
3.3.4.2. **Effect of breeding source on climbing**

This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if the breeding source of the rats affected time spent climbing. No significant effect of breeding source was found ($t_{(22)}=-0.90, p=0.379$) (Figure 3.18).

**Figure 3. 17: Time spent immobile in in-house bred and commercially bred rats in the FST.** The breeding source of the rat did not affect time spent immobile. Data are expressed as mean+SD (n=12 per group).

**Figure 3. 18: Time spent climbing in in-house bred and commercially bred rats in the FST.** The breeding source of the rat did not affect time spent climbing. Data are expressed as mean+SD (n=12 per group).
3.3.4.3. **Effect of breeding source on swimming**

This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if the breeding source of the rats affected time spent swimming. No significant effect of breeding source was found ($t_{(22)} = -0.62, p=0.379$) (Figure 3.19).

![Figure 3.19: Time spent swimming in in-house bred and commercially bred rats in the FST.](image)

The breeding source of the rat did not affect time spent swimming. Data are expressed as mean+SD (n=12 per group).

3.3.5. **Experiment 3 – Effect of route of administration on DMI-induced responses in the FST**

3.3.5.1. **Effect of DMI route of administration on immobility**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI-induced effects on immobility differed depending on the route of administration. A significant effect of route was found [$F_{(3,36)}=2.84, p=0.049$]. *Post-hoc* Dunnett’s test revealed that DMI decreased the time spent immobile only when administered via the s.c. route ($p<0.05$) (Figure 3.20).
Figure 3. 20: Effect of DMI on immobility in the FST when administered via different routes. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. DMI decreased the time spent immobile when administered via the s.c. route. Data are expressed as mean+SD (n=10 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

3.3.5.2. Effect of DMI route of administration on climbing
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI-induced effects on climbing differed depending on the route of administration. A significant effect of route was found \[F_{(3,36)}=3.53, p=0.024\]. Post-hoc Dunnett’s test revealed that DMI increased the time spent climbing only when administered via the s.c. route \((p<0.05)\) (Figure 3.21).
Figure 3. 21: Effect of DMI on climbing in the FST when administered via different routes. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. DMI increased the time spent climbing when administered via the s.c. route. Data are expressed as mean±SD (n=10 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

3.3.5.3. Effect of DMI route of administration on swimming
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI-induced effects on swimming differed depending on the route of administration. No significant effect of route was found $[F(3,36)=0.37, p=0.777]$ (Figure 3.22).
Figure 3.22: Effect of DMI on swimming in the FST when administered via different routes. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. DMI had no effect on swimming regardless of the route of administration. Data are expressed as mean+SD (n=10 per group).

3.3.6. Experiment 4 – Effect of age on baseline responses in the EPM

3.3.6.1. Effect of age on baseline % OAE
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if age affected % OAE. No significant effect of age was found \( [F(2,14)=2.38, p=0.129] \) (Figure 3.23).

Figure 3.23: Effect of age on % OAE in the EPM. The age of the rat did not affect % OAE. Data are expressed as mean+SD (n=5-6 per group). Mean±SD OAE for 9 week old rats: 5±2.
3.3.6.2. **Effect of age on baseline % OAT**

This data did not meet the criteria to be analysed parametrically, and thus it was subjected to cubed transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine if age affected % OAT. No significant effect of age was found \[ F(2,14)=1.00, p=0.392 \] (Figure 3.24).

![Figure 3.24: Effect of age on % OAT in the EPM.](image)

The age of the rat did not affect % OAT. Data are expressed as mean+SD (n=5-6 per group). Mean±SD OAT (s) for 9 week old rats: 46±13.

3.3.7. **Experiment 4 – Effect of age on baseline and DMI-induced responses in the FST**

3.3.7.1. **Effect of age and DMI on immobility**

This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if age and DMI affected immobility. A significant effect of age and DMI was found \[ F(2,30)=8.04, p=0.002; F(1,30)=18.68, p<0.001 \], whilst there was no significant interaction effect \[ F(2,30)=2.99, p=0.066 \] (Figure 3.25). Post-hoc Student-Newman-Keuls test revealed no difference in baseline immobility between age groups. However, age affected the ability of DMI to decrease time spent immobile, with post-hoc Student-Newman-Keuls test revealing that DMI decreased time spent immobile in the 10 week old and the 16 month old rats, but not the 7 month old rats.
Figure 3. 25: Effect of age and DMI on immobility in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Age did not affect baseline time spent immobile. DMI decreased time spent immobile in 10 week old and 16 month old rats. Data are expressed as mean+SD (n=6 per group). Student-Newman-Keuls post-hoc test: *p<0.05 vs. control.

3.3.7.2. Effect of age and DMI on climbing
This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if age and DMI affected climbing. A significant effect of age, DMI, and an interaction effect was found \[F_{(2,30)}=7.53, p=0.002; F_{(1,30)}=16.71, p<0.001; F_{(2,30)}=3.52, p=0.042,\text{ respectively}\] (Figure 3.26). Post-hoc Student-Newman-Keuls test revealed no difference in baseline climbing between age groups. However, age affected the ability of DMI to increase time spent climbing, with post-hoc Student-Newman-Keuls test revealing that DMI increased time spent climbing in the 10 week old and the 16 month old rats, but not the 7 month old rats.
Figure 3.26: Effect of age and DMI on climbing in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Age did not affect baseline time spent climbing. DMI increased time spent climbing in 10 week old and 16 month old rats. Data are expressed as mean+SD (n=6 per group). Student-Newman-Keuls post-hoc test: *p<0.05 vs. control.

3.3.7.3. Effect of age and DMI on swimming
This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if age and DMI affected swimming. There was no significant age, DMI or interaction effect on swimming \([F(2,30)=0.80, p=0.460; F(1,30)=0.59, p=0.447; F(2,30)=0.44, p=0.650\), respectively (Figure 3.27).
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Figure 3.27: Effect of age and DMI on swimming in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Age did not affect baseline time spent swimming. DMI did not alter time spent swimming in any of the age groups. Data are expressed as mean+SD (n=6 per group).

3.3.8. Experiment 5 – Effect of bedding type on baseline responses in the EPM

3.3.8.1. Effect of bedding type on baseline % OAE

This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if bedding type affected % OAE. A significant effect of bedding type was found ($t_{(10)}=2.45, p=0.034$) (Figure 3.28).
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Figure 3.28: Effect of bedding type on % OAE in the EPM. Rats housed in corn cob bedding displayed lower % OAE than rats housed in sawdust bedding. Data are expressed as mean±SD (n=6 per group). *p<0.05 vs. sawdust bedding. Mean±SD OAE for rats housed in sawdust: 5±2.

3.3.8.2. Effect of bedding type on baseline % OAT
This data met the criteria to be analysed parametrically. An independent t-test was carried out to determine if bedding type affected % OAT. No significant effect of bedding type was found ($t_{(10)}=1.65$, $p=0.130$) (Figure 3.29).

Figure 3.29: Effect of bedding type on % OAT in the EPM. The bedding type did not affect % OAT. Data are expressed as mean±SD (n=6 per group). Mean±SD OAT (s) for rats housed in sawdust: 46±13.
3.3.9. Experiment 5 – Effect of bedding type on baseline and DMI-induced responses in the FST

3.3.9.1. Effect of bedding type and DMI on immobility

This data did not meet the criteria to be analysed parametrically, and thus it was subjected to cubed transformation prior to statistical analysis. A Two-Way ANOVA was carried out to determine if bedding and DMI affected immobility. There was a significant effect of bedding on baseline time spent immobile \([F_{(1,19)}=6.15, p=0.023]\). There was a significant effect of DMI on time spent immobile \([F_{(1,19)}=41.35, p<0.001]\), whilst there was also a significant bedding and DMI interaction effect \([F_{(1,19)}=6.99, p=0.016]\) (Figure 3.30). Post-hoc Student-Newman-Keuls test revealed that baseline immobility was lower in rats housed in corn cob bedding compared to sawdust bedding, whilst DMI decreased time spent immobile in both sawdust and corn cob bedding.

![Graph showing time spent immobile in the FST for sawdust and corn cob bedding with DMI and control conditions.](graph.png)

**Figure 3.30:** Effect of bedding and DMI on immobility in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Rats housed in corn cob bedding spent less time immobile than rats housed in sawdust. DMI decreased time spent immobile in rats housed in sawdust and corn cob bedding. Data are expressed as mean±SD (n=5-6 per group). Student-Newman-Keuls post-hoc test: *p<0.05 vs. control drug, #p<0.05 vs. sawdust bedding.

3.3.9.2. Effect of bedding type and DMI on climbing

This raw data did not meet the criteria to be analysed parametrically, and thus it was subjected to log transformation prior to statistical analysis. A Two-Way ANOVA was carried out to determine if bedding and DMI affected climbing. There was no
significant effect of bedding on baseline time spent climbing \( [F_{(1,19)}=3.79, \ p=0.067] \). There was a significant effect of DMI on time spent climbing \( [F_{(1,19)}=30.87, \ p<0.001] \), whilst there was no significant bedding and DMI interaction effect \( [F_{(1,19)}=3.24, \ p=0.088] \) (Figure 3.31). Post-hoc Student-Newman-Keuls test revealed that DMI increased time spent climbing in the rats housed in sawdust and corn cob bedding.

![Bar chart showing time spent climbing with sawdust and corn cob.](image)

**Figure 3.31: Effect of bedding and DMI on climbing in the FST.** Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Time spent climbing did not differ between rats housed in sawdust and corn cob bedding. DMI increased time spent climbing in rats housed in sawdust and corn cob bedding. Data are expressed as mean±SD (n=5-6 per group). Student-Newman-Keuls post-hoc test: *p<0.05 vs. control drug.

3.3.9.3. Effect of bedding type and DMI on swimming

This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if bedding and DMI affected swimming. There was no significant bedding, DMI or interaction effect on swimming \( [F_{(1,19)}=0.00, \ p=0.996; F_{(1,19)}=0.01, \ p=0.939; F_{(1,19)}=0.11, \ p=0.742, \text{ respectively}] \) (Figure 3.32).
Figure 3.32: Effect of bedding and DMI on swimming in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Time spent swimming did not differ between rats housed in corn cob bedding and rats housed in wood shavings. DMI had no effect on time spent swimming in rats housed in sawdust or corn cob bedding. Data are expressed as mean±SD (5-6 per group).

3.3.10. Experiment 6 – Effect of housing environment on baseline responses in the EPM

3.3.10.1. Effect of housing environment on baseline % OAE

This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if bedding and EE affected % OAE. There was no significant bedding, EE or interaction effect on % OAE \([F_{(1,20)}=0.20, p=0.657; F_{(1,20)}=0.00, p=0.981; F_{(1,20)}=0.08, p=0.781\), respectively\) (Figure 3.33).
Figure 3.33: Effect of housing environment on % OAE in the EPM. The housing environment of the rat did not affect % OAE. Data are expressed as mean+SD (n=5-7 per group). Mean+SD OAE for rats housed in sawdust: 4±3.

3.3.10.2. Effect of housing environment on baseline % OAT

This data met the criteria to be analysed parametrically. A Two-Way ANOVA was carried out to determine if bedding and EE affected % OAT. There was no significant bedding, EE or interaction effect on % OAT [$F_{(1,20)}=0.56$, $p=0.462$; $F_{(1,20)}=0.05$, $p=0.824$; $F_{(1,20)}=0.01$, $p=0.936$, respectively] (Figure 3.34).
Figure 3. 34: Effect of housing environment on % OAT in the EPM. The housing environment of the rat did not affect % OAT. Data are expressed as mean+SD (n=5-7 per group). Mean+SD OAT (s) for rats housed in sawdust: 45±40.

3.3.11. Experiment 6 – Effect of housing environment on baseline and DMI-induced responses in the FST

3.3.11.1. Effect of housing environment and DMI on immobility

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in immobility between any of the groups. There was a significant effect of group ($K(7)=26.46, p<0.001$) (Figure 3.35). Post-hoc analysis using the Bonferroni correction revealed no significant difference in baseline time spent immobile between sawdust vs. sawdust EE ($p=1.000$), sawdust vs. paper ($p=1.000$) or sawdust vs. paper EE ($p=1.000$). Whilst there was no significant effect of DMI on immobility in rats housed in sawdust ($p=0.210$), sawdust EE ($p=1.000$) or paper ($p=1.000$), DMI decreased immobility in the animals housed in the paper EE environment ($p=0.042$).
Figure 3.35: Effect of housing environment and DMI on immobility in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Housing environment did not affect baseline immobility. DMI decreased time spent immobile in the paper EE housing environment. Data are expressed as median and interquartile range (n=5-6 per group). Bonferroni correction post-hoc test: *p<0.05 vs. control drug.

3.3.11.2. Effect of housing environment and DMI on climbing

This data met the criteria to be analysed parametrically. A Three-Way ANOVA was carried out to determine if bedding type, EE and DMI affected climbing. There was no significant effect of bedding \([F_{(1,39)}=0.10, p=0.751]\) or EE \([F_{(1,39)}=0.16, p=0.694]\) on baseline climbing, whilst DMI significantly affected this behaviour \([F_{(1,39)}=45.42, p<0.001]\) (Figure 3.36). Whilst there was no significant interaction effect of bedding and DMI \([F_{(1,39)}=0.00, p=0.953]\), EE and DMI \([F_{(1,39)}=0.108, p=0.745]\) or bedding and EE \([F_{(1,39)}=3.87, p=0.056]\), there was a significant bedding x EE x DMI interaction effect \([F_{(1,39)}=5.44, p=0.025]\). Post-hoc Tukey’s test revealed that DMI increased time spent climbing in only two housing environments – the rats housed in sawdust and the rats housed in paper EE \((p<0.01)\).
Figure 3.36: Effect of housing environment and DMI on climbing in the FST. Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Housing environment did not affect baseline climbing. DMI increased time spent climbing in rats housed in sawdust bedding and paper EE environments. Data are expressed as mean+SD (n=5-6 per group). Tukey’s post hoc test: **p<0.01 vs. control drug.

3.3.11.3. Effect of housing environment and DMI on swimming

This data met the criteria to be analysed parametrically. A Three-Way ANOVA was carried out to determine if bedding type, EE and DMI affected swimming. There was no significant effect of bedding $[F_{(1,39)}=0.57, p=0.454]$, EE $[F_{(1,39)}=0.07, p=0.792]$ or DMI on swimming behaviour $[F_{(1,39)}=0.43, p=0.517]$ (Figure 3.37). There was also no significant interaction effect of bedding and DMI $[F_{(1,39)}=0.44, p=0.509]$, EE and DMI $[F_{(1,39)}=0.00, p=0.976]$, bedding and EE $[F_{(1,39)}=2.92, p=0.096]$, or bedding x EE x DMI $[F_{(1,39)}=1.00, p=0.323]$. 
Rats were injected with vehicle or DMI 24, 5 and 1 hour prior to the FST. Housing environment did not affect baseline swimming. DMI did not affect swimming behaviour in any housing environment. Data are expressed as mean+SD (n=5-6 per group).

3.3.12. Experiment 7 – Comparison of drug treatment regime and scoring techniques in the FST

3.3.12.1. Effect of drug treatment on immobility using two scoring methods

The ‘Time spent immobile’ data did not meet the criteria to be analysed parametrically, and thus it was subjected to square root transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine the effect of drug treatment on time spent immobile. A significant effect of drug treatment was found \(F_{(3,35)}=45.93, p<0.001\) (Figure 3.38 (A)). Post-hoc Dunnett’s test revealed that chronic DMI \(p<0.01\) and subacute AMP \(p<0.001\) treatment decreased immobility time. A One-Way ANOVA was carried out to determine the effect of drug treatment on immobile counts. A significant effect of drug treatment was found \(F_{(3,35)}=23.85, p<0.001\) (Figure 3.38 (B)). Post-hoc Dunnett’s test revealed that chronic DMI \(p<0.001\), subacute DMI \(p<0.05\) and subacute AMP \(p<0.001\) treatment decreased immobile counts.
3.3.12.2. **Effect of drug treatment on climbing using two scoring methods**

The data for continuous and time sampling scoring met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine the effect of drug treatment on time spent climbing. A significant effect of drug treatment was found \( F(3,35)=8.77, \ p<0.001 \) (Figure 3.39 (A)). *Post-hoc* Dunnett’s test revealed that chronic DMI \( (p<0.01) \) and subacute AMP \( (p<0.001) \) treatment increased climbing time. A One-Way ANOVA was carried out to determine the effect of drug treatment on climbing counts. A significant effect of drug treatment was found \( F(3,35)=10.39, \ p<0.001 \) (Figure 3.39 (B)). *Post-hoc* Dunnett’s test revealed that chronic DMI \( (p<0.01) \), subacute DMI \( (p<0.05) \) and subacute AMP \( (p<0.001) \) treatment increased climbing counts.
Figure 3.39: Effect of DMI and AMP on climbing in the FST using two scoring methods. With the continuous scoring method chronic DMI and subacute AMP increased climbing, whilst with the time sampling scoring, chronic DMI, subacute DMI and subacute AMP increased climbing. Data are expressed as mean±SD (n=7-13 per group). Dunnett’s post-hoc test: *p<0.05, **p<0.01, ***p<0.001 vs. control.

3.3.12.3. Effect of drug treatment on swimming using two scoring methods

The data for continuous scoring and time sampling scoring did not meet the criteria to be analysed parametrically, and thus both data sets were subjected to log transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine the effect of drug treatment on time spent swimming. A significant effect of drug treatment was found [$F_{(3,35)}=4.12, p=0.013$] (Figure 3.40 (A)). However, post-hoc Dunnett’s test revealed no significant effect of any of the drug treatments on time spent swimming. A One-Way ANOVA was carried out to determine the effect of drug treatment on swimming counts. A significant effect of drug treatment was found [$F_{(3,35)}=9.44, p<0.001$] (Figure 3.40 (B)). Post-hoc Dunnett’s test revealed that subacute AMP (p<0.001) treatment increased swimming counts.
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Figure 3. 40: Effect of DMI and AMP on swimming in the FST using two scoring methods. With the continuous scoring technique none of the treatments affected swimming, whilst with the time sampling scoring, subacute AMP increased swimming. Data are expressed as mean+SD range (n=7-13 per group). Dunnett’s post-hoc test: ***p<0.001 vs. control.

3.3.12.4. Effect of drug treatment on distance moved in the home cage
This data did not meet the criteria to be analysed parametrically, and thus it was subjected to log transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine the effect of drug treatment on distance moved in the home cage. A significant effect of drug treatment was found \(F_{(3,35)}=81.51, p<0.001\) (Figure 3.41). Post-hoc Dunnett’s test revealed that chronic \((p<0.001)\) and subacute \((p<0.01)\) DMI decreased distance moved, whilst subacute AMP increased distance moved \((p<0.001)\).

Figure 3. 41: Effect of DMI and AMP on distance moved in the home cage. Chronic and subacute DMI decreased distance moved, and subacute AMP increased distance moved. Data are expressed as mean+SD (n=7-13 per group). Dunnett’s post-hoc test: **p<0.01, ***p<0.001 vs. control.
3.4. Discussion

The aim of the research covered in this chapter was to assess the effects of the breeding source and the age of the rat, as well as the effect of bedding material and EE on baseline and drug-induced effects, in the EPM and FST. Furthermore, we sought to determine the optimal route of drug administration in the FST before carrying out these characterisation studies. The final goal of the research within this chapter was to further refine the FST for incorporation into the test battery study design. This included comparing subacute and chronic dosing effects in the FST, as well as the two scoring techniques used. In addition, a home cage locomotor activity element was incorporated into the study design of the FST for more efficient detection of false positives in the test. Each study will be considered separately, with the effects on the EPM and the FST discussed.

In the first study, we sought to characterise the typical baseline behavioural responses of male Sprague-Dawley rats in the EPM and FST. This allowed us to depict frequency distribution curves for the parameters of each test. The high n numbers meant that the data should be quite reliable, and thus provided us with an initial standard with which to compare all of our future studies to, to verify typical baseline behaviour. Under normal circumstances, group sizes within studies would typically range from about 6 to 12 animals per group and assumptions are often made that if a higher group size could have been included, then normality may improve. For the EPM, % OAE and % OAT followed normal distribution within this baseline profiling (Table 3.1), a characteristic of the data that was evident throughout the rest of these characterisation studies, except one, suggesting that these parameters robustly display normal distribution within our laboratory. With regards the FST, the parameters of immobility, climbing and swimming in the control groups throughout this chapter varied in normality across studies, with the maximum number of animals in a control group being 10. However, even with an n number of 92, these behavioural parameters did not display normal distribution in the baseline characterisation study (Table 3.1), suggesting that they cannot be robustly detected as normal, regardless of the number of animals employed. These results therefore provided important information on the characteristics of several common behavioural parameters of the EPM and FST.
Table 3.1: Summary of baseline results for EPM and FST for Study 1.

In study 2, the breeding source of the rat did not affect baseline EPM behaviour, with no difference between in-house bred and commercially bred rats in the parameters of % OAE and % OAT. This is consistent with previous findings for the EPM, in which entries into, and time spent in the open arms did not differ between rats obtained from three different commercial breeders (Honndorf et al., 2011). It is important to note however, that female rats were the focus of that study, and thus no evidence has previously been reported for an effect of breeding source on EPM behaviours in male Sprague-Dawley rats. Furthermore, in the current study, the element of transport differed between the sources, in that the in-house bred rats did not have to be transported. This allowed for the potential effects of transport to also be encapsulated within the comparison, in contrast to the study carried out by Honndorf et al. (2011), in which rats from the three different sources were all transported to the laboratory. Similar to the EPM, the FST behaviours of immobility, climbing and swimming did not differ between the in-house bred and commercially bred rats. Consistent with these results, previous findings have also shown no effect of vendor on immobility in the FST (Pare and Kluczynski, 1997). However, Pare and Kluczynski (1997) simply reported ‘struggling’ for the time in which rats were not immobile, and did not distinguish between climbing and swimming behaviour. The current study therefore verifies that when these active behaviours are considered separately, the breeding source does not alter their patterns. Therefore, like the EPM studies, the current findings provide novel evidence which informs us more specifically about in-house versus commercially bred profiles, as opposed to comparing rats from different sources that all had to be transported to the laboratory. An important limitation of this study is the lack of assessment of drug effects. However, knowing that the control data is a crucial factor in the induction of chemical effects (Moser et al., 1997), we would hope that with similar baseline results, drug-induced effects would not differ drastically between these two groups of animals. These findings provide a reassurance that results, at least at baseline level, should not differ whether we use in-house bred or commercially bred rats, and thus in-house bred rats could be reliably used in the future battery study experiments.
In study 3, which compared three different routes of administration of DMI in the FST, s.c. administration was the only route to significantly decrease immobility and increase climbing behaviour. As previously mentioned, probably the most common route of administration, at least in terms of DMI administration in the FST, is the i.p. route. The s.c. route is also used quite frequently whilst the p.o. route is very rare in these experiments. When we compared DMI drug effects in studies that used i.p. and s.c. route in the FST and DMI studies mentioned in Chapter 1, it was clear that similar to the current findings, the s.c. route seemed to be the most reliable at inducing drug effects, with 100% of studies reducing immobility at a dose of 10 mg/kg dose, whilst only 46% of i.p. studies were effective at the same dose. It is well known that the route of drug administration affects the pharmacokinetics of a drug. The two main types of drug administration include enteral (through the digestive tract) and parenteral (outside the digestive tract) administration. The oral gavage route therefore falls under the enteral category, whilst the s.c. and i.p. route fall under the parenteral category. The highest bioavailability of substances is typically seen with parenteral administration because these methods avoid the first pass effect of hepatic metabolism, which can occur with orally administered compounds (Turner et al., 2011). This first pass effect may therefore explain the lack of effect of DMI in the p.o. group. Whilst no studies have compared the bioavailability of DMI after different routes of administration, clinical studies have shown that when imipramine, the parent drug of DMI, is administered orally or parenterally, imipramine predominates in the plasma after parenteral administration, whilst DMI predominates after oral administration, illustrating the increased metabolism, and hence reduced overall bioavailability of the orally administered drug compared to parenteral administration (Rigal et al., 1989, Nagy and Johansson, 1975), an outcome that likely occurs with DMI administration also. Although the s.c. and i.p. routes both fall under the parenteral category of drug administration, the pharmacokinetics of substances administered i.p. is more similar to those seen after oral administration because substances administered i.p. can also undergo hepatic metabolism before reaching systemic circulation (Turner et al., 2011). Therefore, the s.c. route would provide higher bioavailability of the drug, which may explain why this route induced an effect and the i.p. route did not. Bearing future studies in mind, the s.c. route seems to be the most reliable at inducing drug effects, and should be employed over the i.p. and p.o. routes where possible.
With regards the effect of age on EPM behaviour, there was no significant difference in % OAE or % OAT between any of the age groups. Most age studies that have been carried out have reported that as a rat gets older, its anxiety increases in the EPM, as shown by a decrease in open arm parameters (Imhof et al., 1993, Ferguson and Gray, 2005, Andrade et al., 2003, Bessa et al., 2005, Turner et al., 2012, Boguszewski and Zagrodzka, 2002), albeit that most of these studies have focused on Wistar rats. Of the two studies that did incorporate Sprague-Dawley rats, the results showed that in this strain also, older rats were more anxious. However, these studies used either female rats alone or in combination with males (as a pooled result). Thus, to our knowledge, this is the first study that has reported the effects of age on male Sprague-Dawley rats alone in the EPM. Hence, these results suggest that male Sprague-Dawley rats are not as susceptible to aging-induced anxiogenic effects as other strains when tested in the EPM. It is important to note that the potential effect of age on DZP-induced responses in the EPM was also assessed within this study (data not shown), although DZP did not induce an anxiolytic-like response in any of the groups. This is surprising, considering the substantial literature which shows potent DZP effects at this dose in the EPM using male Sprague-Dawley rats (Engin et al., 2009, Griebel et al., 1999, Pietraszek et al., 2005). A possible reason for the lack of effect of DZP is the containers in which the drug was prepared and stored in thereafter. Plastic drug containers were used, and it was only later that we became aware that the storage of DZP in plastic containers decreases drug availability (https://www.glowm.com/resources/glowm/cd/pages/drugs/d028.html). DZP was prepared identically for all of the other characterisation studies, and hence, was not active in any of these further studies. Thus, only baseline results were reported for the EPM for all characterisation studies.

In the FST, there was no significant effect of age on baseline immobility, climbing or swimming behaviour. This lack of effect of age in the FST contrasts to other research findings. For example, in a study which compared 4 week old and 12 week old Sprague-Dawley rats, the older rats were found to be less immobile and displayed more climbing and swimming behaviour than the younger rats (Sequeira-Cordero et al., 2013). Furthermore, differential effects of age were reported in a study which compared rats of 92 days old and 32 days old, whereby immobility and climbing were increased in older rats, and swimming was decreased (Martinez-Mota et al., 2011). It should be noted though, that Wistar rats were used in that particular study, and therefore
the strain may account for discrepancy of effects. In agreement with the latter study, Turner et al. (2012) reported that rats aged 18-20 months were more immobile than animals aged 3-4 months, although it was female rats that were assessed within this study. Despite no effect of age on baseline results in the current study, age did affect DMI-induced responses, with DMI decreasing immobility and increasing climbing in the 10 week old and 16 month old rats, but not the 7 month old rats. It is important to note however, that for this study, home cage locomotor activity was not monitored in the hour preceding the FST. Therefore, it may be possible that the DMI effect evident in the 16 month old animals may be a false positive effect. For instance, the time spent immobile in the 16 month old rats is very high. In general, these animals appeared quite inactive in their home cages, suggesting that the high FST immobility score might reflect a general decrease in activity as opposed to a specific behavioural despair response. Regardless, this high immobility provided a greater chance of a DMI effect emerging, but whether it was a true antidepressant-effect remains unanswered due to the lack of locomotor activity measurements prior to the FST. An effect of age on subacute drug-induced FST responses has also been reported in the literature. For example, Pechnick et al. (2008) reported that although DMI had similar effects on immobility and climbing in peripubertal and adult rats, the effect of DMI on swimming behaviour differed between the two age groups. Moreover, in a study that looked at a wider age range, it was reported that subacute DMI was only effective at reducing immobility in 3-5 month and 12-15 month old rats, and had no effect in 23-25 month old rats (Olivares-Nazario et al., 2016). In addition, the age of the rat has also been shown to alter drug-induced effects after chronic treatment, with Karanges et al. (2011) illustrating that the SSRI paroxetine decreased immobility and increased swimming in adult rats; effects that were absent in adolescent rats. There is a lot of variation in these studies in terms of the ages of rats that are compared. However, one can conclude from the results of the current study as well as the above mentioned literature that age plays an important role in both baseline and drug-induced effects in the FST, and should be carefully considered when planning a study. Thus, it was concluded that for future studies, we would use animals between the ages of 7-10 weeks, ages at which we have previously been able to reliably reproduce effects within our laboratory.

The fifth and sixth studies of this chapter dealt with comparing EPM and FST results in animals housed in different bedding types. The fifth study reported a significant effect of bedding on EPM behaviour, with animals housed on corn cob eliciting increased
anxiety-like behaviour compared to those housed in traditional sawdust bedding. Conversely, in the next study, EPM behaviour did not differ between rats housed in sawdust bedding and paper bedding. To our knowledge, there are no other studies that have assessed the effects of bedding type on EPM behaviour specifically. However, some studies have compared the effects of bedding type in other behavioural tests of anxiety such as the light dark box and the open field (Sakhai et al., 2013). Similar to the first bedding study, Sakhai et al. (2013) compared corn cob bedding to a wood based bedding. However, unlike the finding of the current study, corn cob bedding seemed to elicit a potent anxiolytic-like effect in both the light-dark box and the open field test. It should be noted however that these effects were only evident in animals that were housed in corn cob bedding from birth. Contrasting to the current results, when animals were placed on the corn cob bedding at adulthood, an effect on anxiety was absent (Sakhai et al., 2013). Whilst the current study assessed effects in Sprague-Dawley rats, Sakhai and colleagues used Long-Evans rats, and it is well known that different strains react very differently in behavioural tests of anxiety (McDermott and Kelly, 2008, Schmitt and Hiemke, 1998, Mechan et al., 2002, Ramos et al., 1997, Kulikov et al., 1997). The current findings therefore provide novel evidence that in the case of male Sprague-Dawley rats, the introduction of paper bedding at adulthood has no effect on EPM behaviour, whilst corn cob bedding has an anxiogenic-like effect.

Baseline FST behaviour was also significantly altered in rats housed in corn cob bedding compared to sawdust. Rats housed in corn cob bedding spent less time immobile than those housed in sawdust. The second bedding study reported no difference in baseline immobility, climbing or swimming behaviour between the bedding groups (sawdust vs. paper). The finding that corn cob bedding had antidepressant-like properties in the FST has never before been reported. However, it is now known that corn cob bedding has powerful estrogenic properties (Villalon Landeros et al., 2012) and interestingly, estrogens have powerful and rapid effects on rodent behaviour, including alterations in aggression, sexual behaviour, communication and learning and memory (Laredo et al., 2014). The effect of estrogens on depressive-like behaviour has also been reported. For example, studies which have assessed the effects of selective estrogen receptor modulators (SERMS) administration have found that SERMS have potent antidepressant-like effects in the FST, with a reduction in immobility and an increase in climbing and swimming time that were similar to the effects of DMI (Walf et al., 2004, Walf and Frye, 2007). Thus, the antidepressant-like
effects of corn cob bedding seen in the present study may be explained by the powerful estrogenic effects of this bedding. DMI reduced immobility in rats housed in sawdust and corn cob bedding (Experiment 5), but not those housed in paper bedding (Experiment 6), whilst a DMI-induced increase in climbing was also only observed in the rats housed in sawdust and corn cob bedding. Whilst no other studies have compared drug-induced effects in the FST in different bedding types, the current results would therefore suggest that corn cob and paper bedding affect baseline and drug-induced effects, respectively, and thus are not suitable substitutions for the traditional sawdust bedding used within our laboratory.

Within study 6 of this chapter the housing element of EE was also assessed. The results showed that the EE protocol that we employed did not alter baseline % OAE or % OAT in the EPM. Although these results are consistent with the results of some other EE studies (Simpson et al., 2012a, Brenes et al., 2009), the majority of EE based research has illustrated a potent anxiolytic-like effect of EE in the EPM (Galani et al., 2007, Pena et al., 2006, Santucci et al., 1994, Hellemans et al., 2004). Importantly, the EE protocol that we employed consisted of mild EE, whilst in contrast, the studies mentioned above have all employed an extensive EE protocol, with animals housed in large groups, and in cages with different levels and several toys. However, the aim in those studies was to assess EE as a possible intervention for alleviation of anxiety-like symptoms, and thus, their goal was to try to induce an effect with EE. In contrast, we wanted to develop a protocol that would maintain previous baseline effects within our laboratory to allow continuous comparability of past, present and future studies, whilst also fulfilling the requirements of the animal welfare legislation. Thus, this objective was successfully achieved.

In the FST, EE did not affect baseline immobility, climbing or swimming. The results of other studies that have assessed the effects of EE in this test are variable. For example, although Simpson et al. (2012a) reported no effect of EE on FST behaviour, several other studies have found that EE can significantly reduce immobility time (Brenes et al., 2009) and increase climbing time (Bjornebekk et al., 2008). The Paper EE housing environment was the only housing environment that revealed a similar DMI pattern in the FST to results previously reported within our laboratory for rats housed in the traditional sawdust bedding. Thus, this housing condition met the requirements of maintaining similar baseline and drug-induced FST responses to our previous
experiments, whilst also fulfilling the criteria in the legislation for the housing of the rat and is therefore ideal to use in further studies.

The seventh and final study of this chapter compared the effects of subacute and chronic drug treatment, as well as scoring techniques used in the FST. Furthermore, the incorporation of a home cage monitoring protocol immediately prior to the FST was assessed for its ability to detect false positives in the FST. The finding that subacute DMI at a dose of 10 mg/kg did not decrease immobility using the continuous scoring method differs to the results of our previous experiments (Chapter 3; Experiment 3, Experiment 4 and Experiment 5) and to results reported in the literature (Yamada et al., 2013, Drugan et al., 2013). However, similar to results reported in the literature (Morrish et al., 2009, Hadweh et al., 2010), subacute DMI decreased immobility using the time sampling scoring method. The decrease in immobility induced by chronic DMI at a dose of 10 mg/kg in the current study has also been reported in the literature, using the continuous (Popik et al., 2008) and time sampling (Hadweh et al., 2010) scoring method. There are no studies however, that have systematically compared the two dosing regimes within the same study design. In earlier years, studies assessed both subacute and chronic DMI treatment in the FST (Kitada et al., 1981, Miyauchi et al., 1981, Mancinelli et al., 1987), although experimental flaws were evident in each of these studies. For example, Kitada et al. (1981) assessed subacute and chronic treatment regimes as two independent studies as opposed to one systematic experiment, whilst Miyauchi et al. (1981) administered different doses for acute and chronic treatment. Furthermore, Mancinelli et al. (1987) did not include valid controls that received the same amount of injections regardless of treatment type.

The validity of the FST as a model for clinical depression was strengthened when a series of studies illustrated that chronic antidepressant treatment produced antidepressant-like effects at doses that did not show effects after short-term treatment (Detke et al., 1997, Cryan et al., 2005a). However, the short-term treatment that was used in both studies deviated from the traditional subacute method. Furthermore, one of these studies used a modified FST (Cryan et al., 2005a), which consisted of just a 15 minute swim, rather than the traditional two day FST that is still utilised by the majority of research laboratories today. The current study therefore addressed the experimental question that was not directly assessed within the previous studies, and confirmed that indeed, the chronic antidepressant treatment regime that was employed has similar
antidepressant-like effects to the traditional subacute treatment in the FST, except that the effects are more pronounced after chronic treatment.

Despite the past finding that distinct active behaviours emerge in the FST depending on the mechanism of action of the drug (Detke et al., 1995), not all studies assess the active behaviours in the FST. However, the current finding that DMI treatment increased climbing behaviour for both dosing regimes is consistent with what has been previously reported in studies assessing subacute (Drugan et al., 2013, Piras et al., 2014) and chronic (Jeannotte et al., 2009) DMI treatment. Furthermore, in studies that have assessed subacute or short-term versus chronic treatment (Detke et al., 1997, Cryan et al., 2005a), similar to this study, the decrease in immobility after chronic DMI shows a concomitant increase in climbing behaviour. Also, consistent with the current study, these studies found no effect of DMI on swimming behaviour, a finding that was anticipated, since DMI alters NA neurotransmission, which we know increases climbing behaviour (Detke et al., 1995).

Both scoring techniques revealed similar patterns for all three behaviours, except in the case of subacute DMI, whereby the time sampling method appeared to be more sensitive than the continuous scoring method at detecting drug-induced alterations in immobile and climbing behaviour. For the chronic dosing regime both scoring techniques revealed similar patterns for all three behaviours, supporting the idea that both techniques are equally valid for detecting chronic antidepressant-like and active behaviours of DMI in the FST. Although the time sampling technique had been validated to detect patterns of active behaviour depending on drug type (Detke et al., 1995), a comparison of the sensitivity of both scoring techniques for detecting chronic antidepressant-like and active behaviours in the FST had not yet been thoroughly assessed in the literature. Detke et al. (1995) carried out validity correlations comparing the time sampling to the continuous scoring technique, although only the results of the time sampling scoring were reported. As a result, it is not possible to discern from this previous study whether there are any subtle discrepancies in their sensitivity of detecting behavioural effects, which could be an important factor when screening novel drugs for potential antidepressant effects. Therefore, the current findings provide a reassurance that both scoring techniques are equally reliable for detecting chronic DMI effects in the FST.
The false positive AMP was employed within this study to confirm the validity of the study design to detect false positives in the FST. Indeed, AMP significantly decreased immobility and increased both climbing and swimming behaviour in the FST, suggesting it has antidepressant-like effects. However, as expected, AMP increased the distance moved in the home cage immediately prior to the FST. This illustrates its general stimulation effects, and invalidates it as having antidepressant-like properties, thus confirming its status as a false positive for the FST. With the home cage analysis illustrating valid sensitivity to detect false positives, it can be confirmed that the chronic effects elicited by DMI in the FST are purely antidepressant-like, as DMI did not induce a locomotor stimulation effect, but rather an inhibitory effect, for both dosing regimes.

Although the FST relies completely on motor movements, or lack thereof, none of the mentioned short term versus chronic treatment studies reported general locomotor activity immediately prior to the FST. This is surprising, as all antidepressants work by altering monoamines, several of which are important modulators of psychomotor activity (Brown and Gershon, 1993, Fishman et al., 1983, Geyer, 1996, Ressler and Nemeroff, 1999, Stone et al., 2003). Some researchers do, however, employ tests such as the open field to assess locomotor activity prior to the FST (Zhu et al., 2013, Harvey et al., 2010), although some of these studies use an experimental group independent from the FST animals to represent the locomotor effects of the drug (Kawaura et al., 2010). Even when the same experimental group is used for both tests, there remains the concern that the animal is exposed to a somewhat invasive novel environment before the FST, making the results unreliable and harder to compare to other studies that have not employed this additional element in their study design. Thus, the home cage locomotor assessment employed within the current study would be an optimal alternative for assessing locomotor effects for several reasons. Firstly, it allows the same experimental group to be used for locomotor and FST assessment without the added stress of an additional locomotor test prior to the FST and thus, decreases the amount of animals required for the experiment. Secondly, the results are more reliable as they represent the animals’ movement in its home environment, in which replicate variation has proven quite minimal.

Little is known about the effects of subacute versus chronic antidepressant treatment on locomotor activity in rodents. Acute administration of DMI at doses of 30 and 60 mg/kg have been shown to decrease distance moved in rats 3 hours after treatment
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(Artaz et al., 2005), but this regime does not represent effects after subacute dosing, and these doses used would not typically be employed in the FST. Mitchell et al. (2006) compared acute and chronic effects of antidepressants on locomotor behaviour in mice, however, the acute and chronic treatments were administered via different routes. As an addition to assessing antidepressant effects in the FST, Reneric and colleagues also assessed the effects of subacute (Reneric and Lucki, 1998) and subacute versus chronic (Reneric et al., 2002) antidepressant administration on locomotor activity. They showed that antidepressants decreased distance moved, confirming that drug effects were not secondary to an increase in locomotor activity. However, the locomotor activity was assessed in groups of animals that were independent from those that underwent the FST. It is clear therefore that the current study provides novel findings that the antidepressant-like effects of subacute or chronic DMI treatment are not associated with an increase in locomotor activity in FST tested animals, as assessed non-invasively in the homecage, verifying that both treatment regimes are working through an antidepressant-like mechanism.

In conclusion, moving on from here and thinking about the future battery study designs, these results taken together suggest that rats from either source can be reliably used and compared in the behavioural tests within our laboratory. Thus, in-house bred rats would be used for the succeeding battery studies for this project. The optimal route of administration for the FST is the s.c. route and thus this route should be incorporated in future studies. Care needs to be taken when considering the age of the rats we will use, whilst the housing environment of paper bedding with the mild EE protocol should be incorporated for the battery studies in the next phase of the project. Finally, regardless of scoring technique, a chronic dosing regime can be reliably incorporated prior to the FST, which suggests that this dosing design may be reliably utilised within a battery study, with an improved method of screening for false positives.
Chapter 4  Development and assessment of a test battery study design using standard drugs

4.1. Introduction
As previously mentioned in Chapter 1, there are numerous limitations of current pharmacological treatment of depression, including several adverse side effects (Bet et al., 2013, Anderson, 2000) as well as a treatment lag in the onset of antidepressant action (Katz et al., 2004, Stassen et al., 2007). The reason that all currently marketed antidepressants have these limitations is probably due to all of them working by the same fundamental mechanism of action – by altering central monoamine transmission. Indeed the inadequacy of drug treatments is not just a problem for depression, but for all psychiatric disorders, whereby issues of treatment resistance, sub-optimal response and unwanted side effects prevail (Dean et al., 2014). Despite this, there is a severe lack of development of novel acting psychiatric drugs. For example, in the year 2012, the FDA’s Centre for Drug Evaluation and Research approved 39 new molecular entities, which was the highest number approved in over a decade. However, most of the approvals were for rare diseases or for cancer, with no psychiatric drugs approved. In particular, the standstill in novel antidepressant development is clearly evident when we draw on the previously reported statistics in which global prescription patterns for the treatment of depression highlight the leading role of monoamine-altering compounds (Ilyas and Moncrieff, 2012, Chee et al., 2015). Therefore, novel drugs that work via an alternative mechanism of action, and thus, that may not possess these limitations are highly sought after. Indeed, efforts are being continuously made to uncover antidepressants with other mechanisms of action. For example, since about the early 2000’s, compounds that work via an alternative mechanism have shown promising antidepressant effects in several preclinical models (Papp et al., 2000, Sandi et al., 2008, Lapidus et al., 2013). However, a mismatch between preclinical and clinical antidepressant efficacy of these compounds became apparent when these drugs failed to show antidepressant efficacy clinically (Griebel and Holsboer, 2012, Santos et al., 2008, Barbee et al., 2011). Hence, some of the most recent ‘novel’ antidepressants still work largely by altering monoaminergic transmission. For example, the mechanism of action of the ‘new’ drug vortioxetine which was approved by the FDA in September 2013, includes 5-HT₃ and 5-HT₇ receptor antagonism, 5-HT₁B receptor partial agonism, 5-HT₁₄ receptor agonism and SERT inhibition.

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Importantly, the ‘placebo’ effect is becoming a topic of discussion as a potential cause for the lack of novel drug developments. For example, Mora et al. (2011) have described the ‘publication year effect’, whereby the placebo effect in antidepressant trials has increased over the last decades (Figure 4.1). The reasons for this rise in placebo effect may be due to several factors. Firstly, the belief in the effectiveness of antidepressant medication has probably increased over the decades, due to the millions of depressed patients that have been treated with these drugs since they were first developed. Indeed, for study investigators, positive expectations and confidence in the drug have increased (Rief et al., 2009). Other potential factors responsible for the increase in placebo effects may be differences in clinical study design and implementation. Regardless of the reasoning, this rise in the placebo effect has reduced the margin of benefit for the active drugs, and thus has made it more difficult for novel drugs to elicit a significant effect greater than placebo to render them effective.

Figure 4.1: Correlations of placebo effect sizes with publication year in clinical antidepressant studies. Image adapted from (Mora et al., 2011)

In addition to this rising placebo effect, another potential cause for the lack of novel antidepressants may be due the extreme diversity of the disease, its heterogeneity, the high incidence of comorbidity, and the severe lack of understanding of the pathophysiology and aetiology of the disease, which has led to several distinct theories of depression. As a result of these factors, preclinical testing has become extremely diverse in the types of behavioural symptoms, models and theories that are assessed. Surprisingly, despite the numerous possible causes of the disease and the wide spectrum of symptoms, individual laboratories still tend to focus on elucidating the effects of
novel compounds on just one, or very few behavioural symptoms, often incorporating just one theory within a study design. Therefore, a broader understanding of the effects of a drug on several different symptoms of depression can only be achieved by piecing together results from a multitude of studies. Considering the large variations in results illustrated in the FST and DMI examples in Chapter 1, as well as the characterisation studies in Chapter 3, one can imagine the unreliability of this ‘piecing together’ approach. Such unreliability may well be the cause of the several false positive drugs that have been ineffective clinically over the past decade.

A more efficient approach might be the development of more complex study designs, whereby an array of symptoms and theories can be incorporated into one experimental study to determine potential antidepressant effects of novel drugs. Thus, a study design that would include a battery of tests would allow for a more extensive examination of the drug, allowing several behavioural symptoms and theories of depression to be incorporated. Due to the high comorbidity of depression and anxiety, behavioural tests that would assess both anxiolytic and antidepressant properties of drugs would be ideal in such a study design. Furthermore, the assessment of different theories of depression within this study design would be advantageous. The behavioural tests that would be used within such a study design are important factors to consider in order to fulfil these criteria. After careful consideration, we concluded that a combination of the previously described tests of the EPM, the open field, the resident-intruder paradigm, the FST, and LPS-induced anhedonia would successfully fulfil the above criteria for a more advanced antidepressant screening process. Inclusion of all of these tests would allow the assessment of both acute (EPM, open field, resident-intruder test) and chronic (FST and LPS-induced responses in the SPT) properties of a drug. Furthermore, they would provide examination of a wide spectrum of symptoms, including measures of anxiety, social and agonistic behaviour, behavioural despair and anhedonia, as well more than one theory of depression, elements that have been lacking in preclinical studies to date.

Once the tests were decided on, the next important step was to decide the specific design of the study and the order of the testing to provide the most informative and efficient screening process. As mentioned, one of the major limitations of antidepressants is the treatment lag. Bearing this in mind, as well as the acute efficacy of anxiolytic drugs, we decided to incorporate both an acute phase and a chronic phase into the study design, speculating that the acute phase may be particularly beneficial for detecting early onset of action, whilst the chronic phase would be important for
detecting chronic onset of action that may be more clinically translatable. Thus, this
might allow a somewhat time course of effects to be depicted. Therefore, the varying
dosing schedules that are used for each of these tests (acute treatment prior to EPM,
open field and resident intruder paradigm, and chronic treatment prior to FST and LPS-
induced responses) also make them ideal for the battery study design that would allow
the assessment of both acute anxiolytic and chronic antidepressant effects of drugs.
Hence, the acute phase would involve testing in either the resident-intruder paradigm or
the EPM followed by the open field, whilst the same rats would go on to be assessed in
either LPS-induced responses, or the FST, respectively. Importantly, the amalgamation
of these particular behavioural tests would allow the interrogation of more than one
theory of depression within one study design, which is an important aspect considering
the evolving theories of an integrated model of depression, like that outlined in Chapter
1 (Maletic et al., 2007). For example the FST is well established for its ability to detect
monoamine altering drugs (monoamine theory), whilst the acute systemic LPS model
would assess drug effects on an inflammatory driven model of depressive symptoms
(neuroinflammation theory). In addition, neurochemical analysis may be carried out to
uncover the potential role of another system, the neurotrophic factors (neurotrophin
theory), in producing antidepressant effects after chronic treatment – an assumption that
is widely speculated.

An important aspect of developing this test battery design was to determine the ideal
parameters to use to allow an optimal window for detection of drug efficacy – i.e. to
achieve optimal baseline results that would allow even subtle drug effects to be
identified. The importance of baseline results was highlighted in Chapter 3, Experiment
5, in which DMI typically increased the time spent climbing in the FST in animals
housed in sawdust, whilst, although there was a trend for the same effect in animals
housed in corn cob bedding, this did not reach statistical significance. The results for
climbing in both DMI groups were quite similar, however, the slight rise in baseline
climbing in the animals housed on corn cob meant that the drug effect did not come
through. This is a prime example of how even an experimental parameter such as
bedding can affect interpretation of results of a well-established positive control drug in
the FST. Thus, Chapter 3 was extremely important for progress into the current chapter,
so that we could complete the battery studies in confidence that we were using the
experimental parameters that would ensure optimal drug screening. The results of
Chapter 3 therefore led us to conclude the following in relation to experimental parameters for the battery studies:

- The s.c. route of administration was the most effective at inducing drug effects in the FST, and hence this route would be used.
- In-house bred rats did not differ from commercially obtained rats, and therefore we would use in-house bred rats.
- The age of the rats used is important for detecting drug effects, and thus we would use animals between the ages of 7-10 weeks, ages at which we have previously been able to reproduce effects within our laboratory.
- Paper bedding with a mild EE protocol produced the most similar drug-detection ability to our conventional sawdust bedding, and also improves health concerns associated with sawdust bedding, as well as fulfilling the legislation criteria for improved housing conditions of laboratory rats. Therefore, paper bedding + EE would be used when sawdust bedding was no longer available within our laboratory.
- A chronic (14 day) drug treatment regime can detect effects of the antidepressant DMI in the FST, and therefore this chronic regime would be employed. Furthermore, home cage activity monitoring can be successfully integrated into the FST experimental design for more robust detection of false positive drugs in this test. Finally, the two scoring techniques are both effective at detecting effects in the FST, and thus, the more commonly employed continuous scoring method would be used.

Another important consideration in the development of this battery study design is the principle of the 3 Rs of animal research, Replacement, Reduction, Refinement. The incorporation of animals for the battery study means that the Replacement principle is not utilised. However, the development of the battery study design allows for the ‘Reduction’ of animals, as the study design would allow for the same animals to be used for detection of both anxiolytic and antidepressant drug properties. Furthermore, through the characterisation process, the behavioural tests have been optimized so that they are as effective as possible at detecting drug properties, and hence the maximum amount of information will be derived from rats with as little testing as possible, thus satisfying the principle of ‘Refinement’. The incorporation of the principle of ‘Reduction’ means that the design that we ultimately decided on involved rats undergoing more than one behavioural test, and hence animals that undergo the FST.
and LPS-induced anhedonia test will have been exposed to prior testing. One needs to ensure that behavioural results are not affected due to prior test exposure. Indeed, several studies have assessed the potential effects of carrying out several behavioural tests in rodents. These studies indicate that in some tests, mice that have undergone previous testing behave differently to naïve mice (McIlwain et al., 2001), whilst another study that compared the interval times between testing found that there were no major differences in test results in mice tested at intervals of 1-2 days and mice tested at intervals of 1 week (Paylor et al., 2006). Interestingly, in rats, Blokland et al. (2012) reported that behaviour in FST and open field were dependent on the order in which they were tested in a test battery. Hence, in order to validate the behavioural test battery, already well established drugs must be assessed in the study design to ensure that when each of the tests is carried out as part of the behavioural test battery, that they will still be as efficient at detecting drug effects. The three drugs chosen to validate the test battery design were DMI, DZP and FLX. Therefore, it was important to know what effects these drugs typically produce in the tests that we are using.

As previously mentioned, DMI is a TCA. This drug therefore inhibits the reuptake of the neurotransmitter NA, and to a lesser extent, 5-HT by binding to their reuptake inhibitors, and thus increasing the levels of these neurotransmitters in the synapse. Despite the acute neurochemical effects of DMI, as previously mentioned, it is thought that the drug may elicit its effects through an alternative, adaptive mechanism, as depressive symptoms are only alleviated after weeks of treatment. DMI is commonly used as a positive control when screening novel compounds for antidepressant efficacy. As mentioned in Chapter 1, in particular, DMI is regularly used as a positive control in the FST. For example, when the search terms ‘forced swim test and desipramine and rat’ are entered into the online PubMed.gov search engine, 212 items are returned in the search results. When the search terms ‘forced swim test and rat’ are entered into this search engine, 2,666 items are returned, meaning that almost one tenth of these studies possibly incorporate DMI as a positive control drug in the FST. The refinement of the FST in the mid 1990’s saw the distinction of different active behaviours emerging depending on the antidepressant’s mechanism of action, with noradrenergic altering compounds increasing climbing, whilst serotonergic altering compounds increase swimming behaviour. Therefore, the typical effects of DMI in the FST include a decrease in immobility (Yamada et al., 2013, Morrish et al., 2009, Hadweh et al., 2010) and an increase in climbing behaviour (Piras et al., 2014, Drugan et al., 2005), with no
effect on swimming behaviour (Drugan et al., 2005, Wasik et al., 2014). DMI is much less commonly employed in LPS based studies, however it has been reported that other antidepressants attenuate LPS-induced responses (Yirmiya et al., 2001). DMI is not typically known for having anxiolytic properties, and therefore its effects in anxiety tests such as the EPM and resident-intruder paradigm are rarely assessed. However, studies which have assessed DMI effects in the EPM have shown that the drug is neither anxiolytic nor anxiogenic in this test (Bondi et al., 2008, Drapier et al., 2007, Durand et al., 2000). Similarly, DMI has not been commonly assessed in the resident-intruder paradigm. However, acute administration of several classes of antidepressants have been shown to decrease aggressive-like behaviour in this test, including SSRIs, SNRIs, atypical antidepressants and TCAs (Mitchell, 2005).

The benzodiazepine DZP works by binding to a specific site on the GABA<sub>A</sub> receptor, resulting in an enhanced effect of the inhibitory neurotransmitter GABA. The property that DZP is most commonly known for is its anxiolytic effects, and it is commonly used as a positive control when screening novel compounds for anxiolytic efficacy. In particular, DZP is regularly used as a positive control in the EPM. For example, when the search terms ‘elevated plus maze and diazepam and rat’ are entered into the online PubMed.gov search engine, 376 items are returned in the search results. When the search terms ‘elevated plus maze and rat’ are entered into this search engine, 3,859 items are returned, meaning that again, almost one tenth of these studies possibly incorporate DZP as a positive control drug in the EPM. The typical effects of DZP in the EPM include an increase in both % OAE and % OAT (Cosquer et al., 2005a, Engin et al., 2009, Griebel et al., 1999). DZP is much less commonly employed in the resident-intruder paradigm, but in a study carried out by Mitchell and Redfern (1992a), DZP was reported to only decrease aggressive-like behaviour at doses that were also sedative. This drug does not possess any antidepressant-like properties, and therefore its effects in antidepressant screening tests such as the FST and LPS-induced responses are rarely assessed. However, studies which have assessed DZP effects in the FST have reported no effect of the drug on any of the behaviours (Marti and Armario, 1993, Detke et al., 1995). In relation to LPS-induced responses, again, DZP has rarely been assessed, but in a study carried out by Vinkers et al. (2009), DZP did not attenuate LPS-induced effects.

The SSRI FLX selectively blocks the reuptake of 5-HT into synaptic neurons. The neuronal transporter for 5-HT is inhibited, thus resulting in an increase in synaptic 5-
Similar to DMI, FLX is also commonly used as a positive control when screening novel compounds for antidepressant efficacy, in particular, in the FST. For example, when the search terms ‘forced swim test and fluoxetine and rat’ are entered into the online PubMed.gov search engine, 312 items are returned in the search results, meaning that over 10% of the studies utilising the FST in rats (2, 666) incorporated FLX into the study. It was discovered in earlier years that the FST did not reliably detect SSRI effects such as FLX, with some studies reporting that this drug was inactive (Paul et al., 1990, Maj et al., 1992), some reporting it was active, but only at high doses (Porsolt et al., 1979), whilst others reported that it increased or decreased immobility (Gorka et al., 1979). However, the refinement of the FST by Detke et al. (1995) which involved a new scoring method, resulted in more sensitive detection of SSRIs in the FST. Moreover, with a decrease in immobility, SSRIs such as FLX cause a concomitant increase in the active behaviour of swimming as opposed to climbing (Fernandez-Guasti et al., 2016, Sumaya et al., 2016), due to the serotonergic mechanism of action. However, it is still controversial as to whether SSRIs such as FLX induce reliable effects in the FST, with a lot of discrepancy evident between studies. The effects of FLX on LPS-induced responses are not as commonly assessed in the literature, however studies that have investigated FLX effects in this model in rodents have found that FLX attenuates LPS-induced depressive-like behaviour (Ohgi et al., 2013, Yirmiya et al., 2001). Importantly, although SSRIs such as FLX are the first-line treatment for anxiety disorders, a review that assessed the effects of SSRIs in the EPM reported that SSRI effects are inconsistent after both acute and chronic administration, inducing either anxiogenic effects, or no effect at all (Drapier et al., 2007, Silva and Brandao, 2000, Robert et al., 2011, Borsini et al., 2002). On the other hand, this drug has not been commonly assessed in the resident-intruder paradigm. However, acute administration of FLX has been reported to decrease aggressive-like behaviour in this test (Mitchell and Redfern, 1992a).

To summarise, it is clear that there is a great need for new antidepressants that work via a novel mechanism of action and thus, that may not incur the many disadvantages of current monoaminergic-altering compounds. The mismatch between preclinical and clinical antidepressant efficacy is a major cause of the lack of development of such drugs. This mismatch is possibly due to the primitive way in which drugs are currently screened for such a complex disorder. For example, despite its wide spectrum of symptoms, high comorbidity and lack of clear understanding of the disease, preclinical
antidepressant drug screening remains quite basic, with laboratories often only investigating the effects of drugs on very few symptoms, or incorporating just one theory of depression. There is a need for a more elaborate screening process of anxiolytic and antidepressant drugs, and thus, we propose the development of a battery study design that would advance on several of the limitations of current preclinical assessment. For example, instead of measuring just one symptom within a study design, numerous tests would be included, for the measurement of several different symptoms of anxiety and depression, such as innate anxiety, locomotor activity, sociability, behavioural despair and anhedonia, several of which make up the criteria for clinical anxiety or depression. Moreover, numerous theories of depression would be incorporated within one study design, via the implementation of the several previously mentioned behavioural tests and models. In addition, a time-course aspect is included in the proposed battery study whereby acute anxiolytic and chronic antidepressant effects can be elucidated in the same study design and in the same animals, whilst also allowing assessment of chronic tolerability of these drugs by measuring daily body weight as well as food and water consumption. Therefore, the aim of the studies within this chapter was to validate this novel test battery design for both anxiolytic and antidepressant screening of novel compounds, using the TCA DMI, the benzodiazepine DZP and the SSRI FLX.

4.2. Experimental protocols

A detailed description of the apparatus and test procedures for the use of the EPM, open field, resident-intruder paradigm, FST, home cage activity monitoring and LPS-induced responses in the SPT is outlined in Chapter 2, and thus, the respective sections will be referred to in these methods for a more detailed protocol description.

Three different studies were carried out to determine if the test battery would successfully detect acute and chronic effects:

**Experiment 1:** Assessment of the acute and chronic effects of the antidepressant DMI (2.5, 5 or 10 mg/kg)

**Experiment 2:** Assessment of the acute and chronic effects of the benzodiazepine DZP (0.5, 1 or 1.5 mg/kg)

**Experiment 3:** Assessment of the acute and chronic effects of the antidepressant FLX (5, 10 or 20 mg/kg)
Male rats (in-house bred) were reared on sawdust from birth and singly housed either in sawdust (Study 1) or in paper bedding with EE (Study 2 and Study 3; as described in Chapter 3, Experiment 6), for at least 7 days prior to testing. Rats were approximately 7 weeks old when dosing began. Body weight, food and water consumption were recorded each day. For each experiment drugs were made up in distilled DH$_2$O and administered s.c. at a dose volume of 2ml/kg. Control rats received the same amount of vehicle (DH$_2$O) injections as drug treated rats.

The test battery design comprised of two subsets, with the following sequence of testing:

**Subset 1:** The first dose of drug was administered and 30 minutes later acute anxiolytic effects were assessed in the EPM, followed immediately by the open field test (Day 1), as described in Chapter 2, section 2.2.2.1 and 2.2.2.2, respectively. Drug treatment was continued for a further 13 days (one dose daily) at which point the chronic antidepressant effects were assessed in the FST. Rats received their 13\textsuperscript{th} dose 15 minutes after the preswim (Day 13) and their 14\textsuperscript{th} dose 1 hour prior to the test swim (Day 14). The FST was carried out according to the protocol described in Chapter 2, section 2.2.2.3 (14 day treatment). Home cage activity monitoring was assessed in the hour preceding the FST for detection of false positive drugs in the FST, as described in Chapter 2, section 2.2.2.4. Results of each test were scored as described in the respective sections in Chapter 2.

**Subset 2:** The first dose of drug was administered and 30 minutes later acute anxiolytic effects were assessed in the resident-intruder paradigm, as described in Chapter 2, section 2.2.2.5. Drug treatment was continued for a further 13 days (one dose daily), after which the chronic antidepressant effects were assessed in the SPT after acute systemic LPS administration. Rats received their 14\textsuperscript{th} injection (Day 14) at the usual daily dosing time, and approximately 24 hours later all rats were injected with LPS (200µg/kg, i.p.) (Day 15). Immediately after LPS injection, rats underwent the SPT as described for these test battery studies in Chapter 2, section 2.2.2.6. Results of each test were scored as described in the respective sections in Chapter 2.
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4.3. Statistical analysis

All of the data were tested for normality and homogeneity of variance, using Shapiro-Wilks and Levene tests, respectively. Data were analysed parametrically if they met the criteria of fulfilling two of the following three characteristics: even n numbers across groups, normality, and homogeneity. If data did not meet these criteria, they were analysed using non-parametric statistical tests. The specific tests carried out are described under each particular results section. The level of statistical significance was set at $p = 0.05$. Data were analysed using SPSS statistics software. Graphs were constructed using GraphPad Prism ® software. Expression of data is specified under each graph.

4.3. Results

4.3.1. Experiment 1 – Effect of DMI in the test battery study design

4.3.1.1. Effect of DMI on body weight

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if body weight differed between any of the groups during the entire drug treatment period. A significant effect of time was found [$F(3.34,197.32) = 2901.54$, $p < 0.001$], with all groups gaining weight throughout the treatment period. No significant treatment effect was found [$F(3,59) = 0.97$, $p = 0.412$], although there was a significant time x treatment interaction effect [$F(10.03,197.32) = 16.24$, $p < 0.001$] (Figure 4.3 (A)). However, a One-Way ANOVA for each day revealed no significant difference between groups on any of the days [$F(3,60) = 0.34$, $p = 0.798$;...
$F_{(3,60)}=0.28, p=0.840; F_{(3,60)}=0.35, p=0.798; F_{(3,60)}=0.43, p=0.730; F_{(3,60)}=0.67, p=0.575; F_{(3,60)}=0.70, p=0.557; F_{(3,60)}=0.88, p=0.458; F_{(3,60)}=1.26, p=0.297; F_{(3,60)}=1.68, p=0.182; F_{(3,60)}=1.95, p=0.131; F_{(3,60)}=1.84, p=0.150; F_{(3,60)}=2.37, p=0.080; F_{(3,60)}=2.37, p=0.079$ and $F_{(3,60)}=2.63, p=0.059$ for days 1-14, respectively. A significant effect of treatment was found for total body weight gain from pre-dosing to final dose [F(3,60)=39.34, $p<0.001$] (Figure 4.3 (B)). Post-hoc Dunnett’s test revealed that the DMI 5 mg/kg and 10 mg/kg groups gained less weight over the entire treatment period ($p<0.001$).
Figure 4. 3: Effect of DMI on body weight. DMI had no effect on body weight on each day throughout the dosing period (A), whilst total weight gain over the entire treatment period was reduced in DMI 5 mg/kg and 10 mg/kg groups (B). Data are expressed as mean±SD (n=16 per group). Dunnett’s post-hoc test: ***p<0.001 vs. control.

4.3.1.2. Effect of DMI on food consumption
This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if food consumption differed between any of the groups during the entire drug treatment period. A significant effect of time
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[F(6,63,357.83)=7.28, \ p<0.001] and treatment [F(3,54)=16.99, \ p<0.001] was found, whilst there was no significant time x treatment interaction effect [F(19.88,357.83)=0.76, \ p=0.760] (Figure 4.4 (A)). A One-Way ANOVA for each day revealed a significant difference between groups on all days [F(3,60)=10.51, \ p<0.001; F(3,60)=8.83, \ p<0.001; F(3,60)=11.84, \ p<0.001; F(3,60)=13.55, \ p<0.001; F(3,60)=8.13, \ p<0.001; F(3,60)=7.01, \ p<0.001; F(3,60)=13.65, \ p<0.001; F(3,60)=9.87, \ p<0.001; F(3,60)=12.87, \ p<0.001; F(3,60)=4.11, \ p=0.010; F(3,60)=12.31, \ p<0.001; F(3,60)=11.45, \ p<0.001 and F(3,60)=2.87, \ p=0.045 for days 1-13, respectively]. Post-hoc Dunnett’s test revealed that all DMI groups consumed less food on days 1, 3, 4, 6, 9 and 11. Less food was also consumed in the DMI 2.5 mg/kg group on days 2 and 7, the DMI 5 mg/kg group on days 8 and 12, and in the DMI 10 mg/kg group on days 2, 5, 7, 8, 10, 12 and 13.

Figure 4.4: Effect of DMI on food consumption. Less food was consumed in all DMI treatment groups on days 1, 3, 4, 6, 9 and 11. Less food was also consumed in the DMI 2.5 mg/kg group on days 2 and 7, the DMI 5 mg/kg group on days 8 and 12 and in the DMI 10 mg/kg group on days 2, 5, 7, 8, 10, 12 and 13. Data are expressed as mean±SD (n=16 per group).

4.3.1.3. Effect of DMI on water consumption

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if water consumption differed between any of the groups during the entire drug treatment period. A significant effect of time
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[F(5.45, 288.85)=14.51, \( p<0.001 \)] and treatment \([F(3.53)=3.93, \ p=0.013] \) was found, whilst there was no significant time \times \) treatment interaction effect \([F(16, 35.288.85)=0.88, \ p=0.594]\) (Figure 4.5 (A)). A One-Way ANOVA for each day revealed no significant difference between groups on days 1, 2, 6, 8, 10, 12 and 13 \([F(3, 58)=0.30, \ p=0.827; \ F(3, 58)=2.64, \ p=0.058; \ F(3, 58)=2.75, \ p=0.051; \ F(3, 58)=0.62, \ p=0.607; \ F(3, 58)=1.88, \ p=0.143; \ F(3, 58)=1.01, \ p=0.396\) and \( F(3, 58)=1.83, \ p=0.152\), respectively\], whilst there was a significant difference between groups on days 3, 4, 5, 7, 9 and 11 \([F(3, 58)=3.93, \ p=0.013; \ F(3, 58)=4.07, \ p=0.011; \ F(3, 58)=5.13, \ p=0.003; \ F(3, 58)=5.09, \ p=0.003.; \ F(3, 58)=5.74, \ p=0.002; \ F(3, 58)=5.32, \ p=0.003\), respectively\]. Post-hoc Dunnett’s test revealed that less water was consumed in the DMI 10 mg/kg group on days 3, 4, 5, 6, 7, 9 and 11.

![Water consumption graph](image)

**Figure 4.5: Effect of DMI on water consumption.** Less water was consumed in the DMI 10 mg/kg group on days 3, 4, 5, 6, 7, 9 and 11. Data are expressed as mean±SD (n=16 per group).

### 4.3.1.4. Effect of DMI on % OAE in the EPM

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected % OAE. No significant effect of group was found \([F(3, 27)=0.96, \ p=0.425]\) (Figure 4.6).


Figure 4.6: Effects of DMI on % OAE in the EPM. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. DMI had no effect on % OAE at any of the doses. Data are expressed as mean±SD (n=7-8 per group). Mean±SD OAE for control group: 3±3.

4.3.1.5. Effect of DMI on % OAT in the EPM
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected % OAT. No significant effect of group was found [F(3,27)=1.17, p=0.341] (Figure 4.7).

Figure 4.7: Effects of DMI on % OAT in the EPM. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. DMI had no effect on % OAT at any of the doses. Data are expressed as mean±SD (n=7-8 per group). Mean±SD OAT (s) for control group: 28±30.
4.3.1.6. Effect of DMI on distance moved in the Open Field

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected distance moved. No significant effect of group was found \( F_{(3,28)}=2.11, p=0.121 \) (Figure 4.8).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4.8}
\caption{Effects of DMI on distance moved in the open field. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the open field was assessed approximately 35 minutes later. DMI had no effect on distance moved at any of the doses. Data are expressed as mean±SD (n=7-8 per group).}
\end{figure}

4.3.1.7. Effect of DMI on social investigation behaviour in the Resident-Intruder Paradigm

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in social investigation time and social investigation counts between any of the groups. Whilst there was no significant effect of treatment group on social investigation counts \( K_{(3)}=3.52, p=0.318 \) (Figure 4.9 (B)), there was a significant effect of group on social investigation time \( K_{(3)}=9.64, p=0.022 \) (Figure 4.9 (A)). However, further post-hoc analysis using the Bonferroni correction revealed no effect of any of the treatment groups on social investigation time.
4.3.1.8. Effect of DMI on aggressive behaviour in the Resident-Intruder Paradigm

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in aggression time and aggression counts between any of the groups. Whilst there was no significant effect of treatment group on aggression counts ($K(3)=7.71, p=0.052$) (Figure 4.10 (B)), there was a significant effect of group on aggression time ($K(3)=7.94, p=0.047$) (Figure 4.10 (A)). However, further post-hoc analysis using the Bonferroni correction revealed no effect of any of the treatment groups on aggression time.

Figure 4. 9: Effects of DMI on social investigation behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on social investigation time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).

Figure 4. 10: Effects of DMI on aggression behaviour in the resident intruder-paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on aggression time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).
4.3.1.9. Effect of DMI on flight submit behaviour in the Resident-Intruder Paradigm

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in flight submit time and flight submit counts between any of the groups. No significant effect of group was found for flight submit time or counts ($K_{(3)}=6.80, p=0.079$; $K_{(3)}=67.81, p=0.050$, respectively) (Figure 4.11 (A + B)).

Figure 4.11: Effects of DMI on flight submit behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on flight submit time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).

4.3.1.10. Effect of DMI on grooming behaviour in the Resident-Intruder Paradigm

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in grooming time and grooming counts between any of the groups. No significant effect of group was found for grooming time or counts ($K_{(3)}=1.60, p=0.659$; $K_{(3)}=2.80, p=0.423$, respectively) (Figure 4.12 (A + B)).
Figure 4. 12: Effects of DMI on grooming behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on grooming time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).

4.3.1.11. Effect of DMI on rearing behaviour in the Resident-Intruder Paradigm

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in rearing time and rearing counts between any of the groups. No significant effect of group was found for rearing time or counts ($K_3 = 2.47, p = 0.481; K_3 = 3.83, p = 0.280$, respectively) (Figure 4.13 (A + B)).

Figure 4. 13: Effects of DMI on rearing behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on rearing time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).
4.3.1.12. **Effect of DMI on walking behaviour in the Resident-Intruder Paradigm**

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in walking time and walking counts between any of the groups. No significant effect of group was found for walking time or counts ($K_{(3)}=4.85$, $p=0.183$; $K_{(3)}=7.22$, $p=0.065$, respectively) (Figure 4.14 (A + B)).

![Figure 4.14: Effects of DMI on walking behaviour in the resident-intruder paradigm.](image)

Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on walking time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).

4.3.1.13. **Effect of DMI on non-social exploration behaviour in the Resident-Intruder Paradigm**

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in non-social exploration time and non-social exploration counts between any of the groups. No significant effect of group was found for non-social exploration time or counts ($K_{(3)}=6.25$, $p=0.100$; $K_{(3)}=5.67$, $p=0.129$) (Figure 4.15 (A + B)).
Figure 4. 15: Effects of DMI on non-social exploration behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DMI had no effect on non-social exploration time (A) or counts (B) at any of the doses. Data are expressed as median and interquartile range (n=3-4 per group).

4.3.1.14.  Effect of DMI on immobility in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected time spent immobile. A significant effect of group was found \([F_{(3,25)}=7.49, p=0.001]\) (Figure 4.16). Post-hoc Dunnett’s test revealed that DMI 5 mg/kg and 10 mg/kg decreased the time spent immobile \((p<0.01)\).

Figure 4. 16: Effects of DMI on time spent immobile in the FST. Rats received chronic daily injections of vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DMI 5 mg/kg and 10 mg/kg decreased time spent immobile. Data are expressed as mean+SD (n=6-8 per group). Dunnett’s post-hoc test: **\(p<0.01\) vs. control.
4.3.1.15. **Effect of DMI on climbing in the FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected time spent climbing. A significant effect of group was found \([F_{(3,25)}=5.04, p=0.007]\) (Figure 4.17). Post-hoc Dunnett’s test revealed that DMI 5 mg/kg \((p<0.05)\) and 10 mg/kg \((p<0.01)\) increased the time spent climbing.

![Figure 4.17: Effects of DMI on time spent climbing in the FST.](image)

Rats received chronic daily injections of vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DMI 5 mg/kg and 10 mg/kg increased time spent climbing. Data are expressed as mean+SD (n=6-8 per group). Dunnett’s post-hoc test: *\(p<0.05\), **\(p<0.01\) vs. control.

4.3.1.16. **Effect of DMI on swimming in the FST**

This data did not meet the criteria to be analysed parametrically, and thus it was subjected to log transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine the effect of drug treatment on time spent swimming. No significant effect of drug treatment was found \([F_{(3,25)}=0.74, p=0.538]\) (Figure 4.18).

![Diagram of swimming test](image)
Figure 4. 18: Effects of DMI on time spent swimming in the FST. Rats received chronic daily injections of vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DMI had no effect on time spent swimming at any of the doses. Data are expressed as mean+SD (n=6-8 per group).

4.3.1.17. Effect of DMI on home cage activity in the hour preceding the FST

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI affected home cage locomotor activity in the hour preceding the FST. No significant effect of group was found \([F(3,21)=2.95, p=0.056]\) (Figure 4.19).

Figure 4. 19: Effects of DMI on home cage locomotor activity. Rats received chronic daily injections of vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) for 14 days and home cage locomotor activity was assessed immediately after the last injection (in the hour preceding the FST). DMI had no effect on home cage locomotor activity at any of the doses. Data are expressed as mean+SD (n=5-7 per group).
4.3.1.18. Effect of DMI on saccharin preference after systemic LPS challenge
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DMI altered % saccharin preference after systemic LPS. An anhedonic effect was not evident after LPS administration, and no significant effect of group was found $[F(3,20)=0.17, p=0.913]$ (Figure 4.20).

![Bar chart](image)

Figure 4.20: Effects of DMI on % saccharin preference after systemic LPS challenge. Rats received chronic daily injections of vehicle or DMI (2.5, 5 or 10 mg/kg s.c.) for 14 days and % saccharin preference was assessed approximately 24 hours after the final dose. DMI had no effect on % saccharin preference at any of the doses. Data are expressed as mean±SD (n=6 per group). Mean±SD saccharin consumption (ml): 14±3.

4.3.2. Experiment 2 – Effect of DZP in the test battery study design

4.3.2.1. Effect of DZP on body weight
This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if body weight differed between any of the groups during the entire drug treatment period. A significant effect of time was found $[F(1.98,119.04)=2882.86, p<0.001]$, with all groups gaining weight throughout the treatment period. No significant treatment effect was found $[F(3,60)=0.46, p=0.709]$, although there was a significant time x treatment interaction effect $[F(5.95,119.04)=3.69, p=0.002]$ (Figure 4.21 (A)). However, a One-Way ANOVA for each day revealed no significant difference between groups on any of the days $[F(3,60)=0.02, p=0.995; F(3,60)=0.08, p=0.972; F(3,60)=0.13, p=0.945; F(3,60)=0.18, p=0.909; F(3,60)=0.27, p=0.847; F(3,60)=0.36, p=0.784; F(3,60)=0.43, p=0.730; F(3,60)=0.63, p=0.600; F(3,60)=0.62, p=0.607]$.
$F(3,60)=0.67, p=0.577; F(3,60)=0.98, p=0.407; F(3,60)=1.01, p=0.394; F(3,60)=0.93, p=0.431; F(3,60)=1.05, p=0.378$, for days 1-14 respectively. A significant effect of treatment was found for total body weight gain from pre-dosing to final dose [$F(3,60)=4.13, p=0.010$] (Figure 4.21 (B)). *Post-hoc* Dunnett’s test revealed that the DZP 1.5 mg/kg group gained less weight over the entire treatment period ($p<0.01$).

**Figure 4.21: Effect of DZP on body weight.** DZP had no effect on body weight on each day throughout the dosing period (A), whilst total weight gain over the entire treatment period was reduced in the DZP 1.5 mg/kg group (B). Data are expressed as mean±SD ($n=16$ per group). Dunnett’s *post-hoc* test: *$p<0.01$ vs. control.
4.3.2.2. Effect of DZP on food consumption

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if food consumption differed between any of the groups during the entire drug treatment period. A significant effect of time was found \( F(7.08,361.28) = 3.66, p=0.001 \), whilst no significant group or group x time interaction effect was found \( F(3,51) = 1.30, p=0.286; F(21.25,361.28) = 0.67, p=0.864 \), respectively (Figure 4.22).

![Figure 4.22: Effect of DZP on food consumption.](image)

DZP had no effect on food consumption throughout the dosing period. Data are expressed as mean±SD (n=16 per group).

4.3.2.3. Effect of DZP on water consumption

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if water consumption differed between any of the groups during the entire drug treatment period. A significant effect of time was found \( F(6.32,373.10) = 20.20, p<0.001 \), whilst there was no significant group or group x time interaction effect \( F(3,59) = 0.37, p=0.772; F(18.97,373.10) = 0.74, p=0.776 \), respectively (Figure 4.23).
Figure 4. 23: Effect of DZP on water consumption. DZP had no effect on water consumption throughout the dosing period. Data are expressed as mean±SD (n=16 per group).

4.3.2.4. Effect of DZP on % OAE in the EPM
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected % OAE. A significant effect of group was found [$F_{(3,25)}=3.65$, $p=0.026$] (Figure 4.24). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg increased % OAE ($p<0.05$).
Figure 4.24: Effects of DZP on % OAE in the EPM. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. DZP 1 mg/kg and 1.5 mg/kg increased % OAE. Data are expressed as mean±SD (n=6-8 per group). Dunnett’s post-hoc test: *p<0.05 vs. control. Mean±SD OAE for control group: 3±2.

4.3.2.5. Effect of DZP on % OAT in the EPM

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected % OAT. A significant effect of group was found [F(3,25)=4.27, p=0.015] (Figure 4.25). Post-hoc Dunnett’s test revealed that DZP 1.5 mg/kg increased % OAT (p<0.01).

Figure 4.25: Effects of DZP on % OAT in the EPM. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. DZP 1.5 mg/kg increased % OAT. Data are expressed as mean±SD (n=6-8 per group). Dunnett’s post-hoc test: **p<0.01 vs. control. Mean±SD OAT (s) for control group: 37±23.
4.3.2.6. **Effect of DZP on distance moved in the Open Field**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected distance moved. A significant effect of group was found \([F(3,27)=9.52, p<0.001]\) (Figure 4.26). *Post-hoc* Dunnett’s test revealed that DZP 1.5 mg/kg decreased distance moved \((p<0.01)\).

![Figure 4.26: Effects of DZP on distance moved in the open field.](image)

Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the open field was assessed approximately 35 minutes later. DZP 1.5 mg/kg decreased distance moved. Data are expressed as mean±SD \((n=7-8 \text{ per group})\). Dunnett’s *post-hoc* test: **\(p<0.01\) vs. control.

4.3.2.7. **Effect of DZP on social investigation behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected social investigation time and social investigation counts. No significant effect of group was found for social investigation time \([F(3,12)=0.69, p=0.575]\) (Figure 4.27 (A)), whilst a significant effect of group was found for social investigation counts \([F(3,12)=4.93, p=0.019]\) (Figure 4.27 (B)). *Post-hoc* Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg decreased social investigation counts \((p<0.05)\).
Figure 4. 27: Effects of DZP on social investigation behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on social investigation time (A) at any of the doses. DZP 1 mg/kg and 1.5 mg/kg decreased social investigation counts (B). Data are expressed as mean+SD (n=4 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

4.3.2.8. Effect of DZP on aggressive behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected aggression time and aggression counts. A significant effect of group was found for aggression time and counts \( [F(3,12)=4.55, p=0.024; F(3,12)=5.60, p=0.012, \text{respectively}] \) (Figure 4.28 (A + B)). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg decreased aggression time and counts (p<0.05).

Figure 4. 28: Effects of DZP on aggression behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP 1 mg/kg and 1.5 mg/kg decreased aggression time (A) and counts (B). Data are expressed as mean+SD (n=4 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.
4.3.2.9. **Effect of DZP on flight submit behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected flight submit time and flight submit counts. A significant effect of group was found for flight submit time and counts \([F_{(3,12)}=7.26, p=0.005; F_{(3,12)}=6.94, p=0.006]\) (Figure 4.29 (A + B)). However, further analysis with a *post-hoc* Dunnett’s test revealed that DZP had no effect on flight submit time or counts at any of the doses.

![Figure 4.29: Effects of DZP on flight submit behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on flight submit time (A) or counts (B) at any of the doses. Data are expressed as mean+SD (n=4 per group).](image)

4.3.2.10. **Effect of DZP on grooming behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected grooming time and grooming counts. No significant effect of group was found for grooming time or counts \([F_{(3,12)}=1.23, p=0.341; F_{(3,12)}=1.02, p=0.420, \text{ respectively}\) (Figure 4.30 (A + B)).
Figure 4. 30: Effects of DZP on grooming behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on grooming time (A) or counts (B) at any of the doses. Data are expressed as mean±SEM (n=4 per group).

4.3.2.11. Effect of DZP on rearing behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected rearing time and rearing counts. No significant effect of group was found for rearing time \( [F_{(3,12)}=2.02, p=0.164] \) (Figure 4.31 (A)), whilst a significant effect of group was found for rearing counts \( [F_{(3,12)}=7.20, p=0.005] \) (Figure 4.31 (B)). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg decreased rearing counts \( (p<0.01) \).

Figure 4. 31: Effects of DZP on rearing behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on rearing time at any of the doses (A). DZP 1 mg/kg and 1.5 mg/kg decreased rearing counts (B). Data are expressed as mean±SD (n=4 per group). Dunnett’s post-hoc test: **\( p<0.01 \) vs. control.
4.3.2.12. **Effect of DZP on walking behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected walking time and walking counts. No significant effect of group was found for walking time \( [F(3,12)=3.16, \, p=0.064] \) (Figure 4.32 (A)), whilst a significant effect of group was found for walking counts \( [F(3,12)=5.19, \, p=0.016] \) (Figure 4.32 (B)). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg decreased walking counts \( (p<0.05) \).

![Graph showing effects of DZP on walking behaviour](image)

**Figure 4.32:** Effects of DZP on walking behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on walking time (A) at any of the doses. DZP 1 mg/kg and 1.5 mg/kg decreased walking counts (B). Data are expressed as mean±SD (n=4 per group). Dunnett’s post-hoc test: *\( p<0.05 \) vs. control.

4.3.2.13. **Effect of DZP on non-social exploration behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected non-social exploration time and non-social exploration counts. No significant effect of group was found for non-social exploration time \( [F(3,12)=1.66, \, p=0.227] \) (Figure 4.33 (A)), whilst a significant effect of group was found for non-social exploration counts \( [F(3,12)=5.34, \, p=0.014] \) (Figure 4.33 (B)). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg decreased non-social exploration counts \( (p<0.05) \).
Figure 4. 33: Effects of DZP on non-social exploration behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. DZP had no effect on non-social exploration time at any dose (A). DZP 0.5 mg/kg decreased non-social exploration counts (B). Data are expressed as mean±SEM (n=4 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

4.3.2.14. Effect of DZP on immobility in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected time spent immobile. No significant effect of group was found \([F(3,27)=0.20, p=0.895]\) (Figure 4.34).

Figure 4. 34: Effects of DZP on time spent immobile in the FST. Rats received chronic daily injections of vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DZP had no effect on time spent immobile at any of the doses. Data are expressed as mean±SD (n=7-8 per group).
4.3.2.15. **Effect of DZP on climbing in the FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected time spent climbing. No significant effect of group was found \([F(3,27) = 1.36, p = 0.276]\) (Figure 4.35).

![Graph showing time spent climbing in the FST](image)

**Figure 4.35**: Effects of DZP on time spent climbing in the FST. Rats received chronic daily injections of vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DZP had no effect on time spent climbing at any of the doses. Data are expressed as mean+SD (n=7-8 per group).

4.3.2.16. **Effect of DZP on swimming in the FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected time spent swimming. No significant effect of group was found \([F(3,27) = 0.32, p = 0.814]\) (Figure 4.36).
Figure 4.36: Effects of DZP on time spent swimming in the FST. Rats received chronic daily injections of vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. DZP had no effect on time spent swimming at any of the doses. Data are expressed as mean+SD (n=7-8 per group).

4.3.2.17. Effect of DZP on home cage activity in the hour preceding the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP affected home cage locomotor activity in the hour preceding the FST. A significant effect of group was found \([F(3,28)=3.56, p=0.027]\) (Figure 4.37). Post-hoc Dunnett’s test revealed that DZP 1 mg/kg and 1.5 mg/kg decreased home cage locomotor activity \((p<0.05)\).
Figure 4.37: Effects of DZP on home cage locomotor activity. Rats received chronic daily injections of vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) for 14 days and home cage locomotor activity was assessed immediately after the last injection (in the hour preceding the FST). DZP 1 mg/kg and 1.5 mg/kg decreased home cage locomotor activity. Data are expressed as mean+SD (n=8 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

4.3.2.18. Effect of DZP on saccharin preference after systemic LPS challenge

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if DZP altered % saccharin preference after systemic LPS. An anhedonic effect was not evident after LPS administration, and no significant effect of group was found [F(3,28)=0.36, p=0.786] (Figure 4.38).
Figure 4.38: Effects of DZP on % saccharin preference after systemic LPS challenge. Rats received chronic daily injections of vehicle or DZP (0.5, 1 or 1.5 mg/kg s.c.) for 14 days and % saccharin preference was assessed approximately 24 hours after the final dose. DZP had no effect on % saccharin preference at any of the doses. Data are expressed as mean±SD (n=8 per group). Mean±SD saccharin consumption (ml): 13±11.

4.3.3. Effect of FLX in the test battery study design

4.3.3.1. Effect of FLX on body weight

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if body weight differed between any of the groups during the entire drug treatment period. A significant effect of time was found [$F(1.52,88.37)=1353.76$, $p<0.001$], with all groups gaining weight throughout the treatment period. No significant treatment effect was found [$F(3,58)=0.97$, $p=0.411$], although there was a significant time x treatment interaction effect [$F(4.57,88.37)=3.69$, $p<0.01$] (Figure 4.39 (A)). A One-Way ANOVA for each day revealed no significant difference between groups on days 1-12 [$F(3,59)=0.13$, $p=0.944$; $F(3,59)=0.09$, $p=0.964$; $F(3,59)=0.33$, $p=0.805$; $F(3,59)=0.62$, $p=0.606$; $F(3,59)=0.81$, $p=0.495$; $F(3,59)=1.01$, $p=0.394$; $F(3,59)=1.35$, $p=0.268$; $F(3,59)=1.67$, $p=0.183$; $F(3,59)=1.76$, $p=0.164$; $F(3,59)=2.26$, $p=0.091$; $F(3,59)=2.42$, $p=0.075$; $F(3,59)=2.48$, $p=0.070$, respectively], whilst there was a significant effect of treatment on days 13 and 14 of dosing [$F(3,59)=3.03$, $p=0.036$; $F(3,59)=3.26$, $p=0.028$, respectively]. However, further analysis with a post-hoc Dunnett’s test revealed that none of the drug treatment groups differed in body weight compared to control animals on days 13 and 14. A significant effect of treatment was found for total
body weight gain from pre-dosing to final dose [$F(3,59)=22.51$, $p<0.001$] (Figure 4.39 (B)). Post-hoc Dunnett’s test revealed that the FLX 20 mg/kg group gained less weight over the entire treatment period ($p<0.001$).

![Graph (A)](#)

**Figure 4.39: Effect of FLX on body weight.** FLX had no effect on body weight on each day throughout the dosing period (A), whilst total weight gain over the entire treatment period was reduced in the FLX 20 mg/kg group (B). Data are expressed as mean±SD ($n=16$ per group). Dunnett’s post-hoc test: **p<0.001 vs. control.**
4.3.3.2. Effect of FLX on food consumption

This data did not meet the criteria to be analysed parametrically. A Friedman’s ANOVA was carried out to determine if food consumption differed between any of the groups during the entire drug treatment period. A significant overall effect was found ($\chi^2(12)=108.16, p<0.001$) (Figure 4.40). No effect of drug treatment was found on days 7 or 8 ($K(3)=7.14, p=0.068$ and $K(3)=7.40, p=0.060$, respectively), whilst a significant effect was found on days 1, 2, 3, 4, 5, 6, 9, 10, 11, 12 and 13 ($K(3)=20.52, p<0.001$; $K(3)=17.52, p=0.001$; $K(3)=29.17, p<0.001$; $K(3)=32.75, p<0.001$; $K(3)=25.60, p<0.001$; $K(3)=10.59, p=0.014$; $K(3)=10.47, p=0.015$; $K(3)=8.91, p=0.030$; $K(3)=9.97, p=0.019$; $K(3)=12.14, p=0.007$ and $K(3)=12.50, p=0.006$, respectively). Post-hoc analysis using the Bonferroni correction revealed that the FLX 20 mg/kg group consumed less food than the control group on days 1 ($p<0.01$), 2 ($p<0.05$), 3 ($p<0.001$), 4 ($p<0.001$) and 12 ($p<0.05$).

Figure 4.40: Effect of FLX on food consumption. Less food was consumed in the FLX 20 mg/kg group on days 1, 2, 3, 4, 5 and 12. Data are expressed as median and interquartile range (n=15-16 per group).

4.3.3.3. Effect of FLX on water consumption

This data did not meet the criteria to be analysed parametrically. A Friedman’s ANOVA was carried out to determine if water consumption differed between any of the groups during the entire drug treatment period. A significant overall effect was found
No effect of drug treatment was found on days 1, 3, 5, 8 and 13 ($K(3) = 3.70, p = 0.296$; $K(3) = 5.91, p = 0.116$; $K(3) = 5.06, p = 0.168$; $K(3) = 5.13, p = 0.163$ and $K(3) = 4.37, p = 0.224$, respectively), whilst a significant effect was found on days 2, 4, 6, 7, 9, 10, 11 and 12 ($K(3) = 8.69, p = 0.034$; $K(3) = 10.06, p = 0.018$; $K(3) = 8.47, p = 0.037$; $K(3) = 10.07, p = 0.018$; $K(3) = 11.70, p = 0.008$; $K(3) = 13.79, p = 0.003$; $K(3) = 12.89, p = 0.005$ and $K(3) = 10.63, p = 0.014$, respectively). Post-hoc analysis using the Bonferroni correction revealed that the FLX 20 mg/kg group consumed less water than the control group on days 10 and 11 ($p < 0.05$).

Figure 4.41: Effect of FLX on water consumption. Less water was consumed in the FLX 20 mg/kg group on days 10 and 11. Data are expressed as median and interquartile range (n=15-16 per group).

4.3.3.4. Effect of FLX on % OAE in the EPM
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected % OAE. No significant effect of group was found [$F(3,27) = 0.89, p = 0.458$] (Figure 4.42).
Figure 4.42: Effects of FLX on % OAE in the EPM. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. FLX had no effect on % OAE at any of the doses. Data are expressed as mean±SD (n=7-8 per group). Mean±SD OAE for control group: 2±1.

4.3.3.5. Effect of FLX on % OAT in the EPM
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected % OAT. No significant effect of group was found [$F(3,27)=0.82, p=0.493$] (Figure 4.43).

Figure 4.43: Effects of FLX on % OAT in the EPM. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the EPM was assessed 30 minutes later. FLX had no effect on % OAT at any of the doses. Data are expressed as mean±SD (n=7-8 per group). Mean±SD OAT (s) for control group: 24±21.
4.3.3.6. **Effect of FLX on distance moved in the Open Field**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected distance moved. No significant effect of group was found \[F(3,28)=0.15, p=0.927\] (Figure 4.44).

![Distance Moved (cm) graph](image)

**Figure 4.44: Effects of FLX on distance moved in the open field.** Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the open field was assessed approximately 35 minutes later. FLX had no effect on distance moved at any of the doses. Data are expressed as mean+SD (n=8 per group).

4.3.3.7. **Effect of FLX on social investigation behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected social investigation time and social investigation counts. No significant effect of group was found for social investigation time or counts \[F(3,12)=0.12, p=0.380; F(3,12)=1.95, p=0.175\, respectively\] (Figure 4.45 (A+B)).
4.3.3.8. **Effect of FLX on aggression behaviour in the Resident-Intruder Paradigm**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected aggression time and aggression counts. A significant effect of group was found for aggression time and counts \( F_{(3,12)}=10.70, \ p=0.001; \ F_{(3,12)}=10.96, \ p=0.001, \) respectively] (Figure 4.46 (A + B)). *Post-hoc* Dunnett’s test revealed that FLX 10 mg/kg \( (p<0.05) \) and 20 mg/kg \( (p<0.01) \) decreased duration of aggression, whilst aggression counts were also reduced in the FLX 20 mg/kg group \( (p<0.01) \).
4.3.3.9. Effect of FLX on flight submit behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected flight submit time and flight submit counts. No significant effect of group was found for flight submit time \( [F_{(3,12)}=2.08, p=0.156] \) (Figure 4.47 (A)), whilst a significant effect of group was found for flight submit counts \( [F_{(3,12)}=5.62, p=0.012] \) (Figure 4.47 (B)). However, post-hoc Dunnett’s test revealed that FLX had no effect on flight submit counts at any of the doses.

![Figure 4.47](image)

**Figure 4.47:** Effects of FLX on flight submit behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. FLX had no effect on flight submit time (A) or counts (B) at any of the doses. Data are expressed as mean+SEM \( (n=4 \text{ per group}) \).

4.3.3.10. Effect of FLX on grooming behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected grooming time and grooming counts. A significant effect of group was found for grooming time \( [F_{(3,12)}=4.69, p=0.022] \) (Figure 4.48 (A)), whilst no significant effect of group was found for grooming counts \( [F_{(3,12)}=2.03, p=0.163] \) (Figure 4.48 (B)). Post-hoc Dunnett’s test revealed that FLX 20 mg/kg increased grooming time \( (p<0.05) \).
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Figure 4. 48: Effects of FLX on grooming behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. FLX 20 mg/kg increased grooming time (A). FLX had no effect on grooming counts at any of the doses (B). Data are expressed as mean+SEM (n=4 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

4.3.3.11. Effect of FLX on rearing behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected rearing time and rearing counts. A significant effect of group was found for rearing time \([F_{(3,12)}=5.23, p=0.015]\) (Figure 4.49 (A)), whilst no significant effect of group was found for rearing counts \([F_{(3,12)}=2.60, p=0.100]\) (Figure 4.49 (B)). Post-hoc Dunnett’s test revealed that FLX 10 mg/kg increased rearing time (\(p<0.05\)).

Figure 4. 49: Effects of FLX on rearing behaviour in the resident-intruder paradigm. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. FLX 10 mg/kg increased rearing time (A). FLX had no effect on rearing counts at any of the doses (B). Data are expressed as mean+SD (n=4 per group). Dunnett’s post-hoc test: *\(p<0.05\) vs. control.
4.3.3.12. Effect of FLX on walking behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected walking time and walking counts. A significant effect of group was found for walking time \( F(3,12)=3.64, p=0.045 \) (Figure 4.50 (A)), whilst no significant effect of group was found for walking counts \( F(3,12)=1.29, p=0.321 \) (Figure 4.50 (B)). Post-hoc Dunnett’s test failed to show any difference between the groups for walking time.

![Figure 4.50: Effects of FLX on walking behaviour in the resident-intruder paradigm.](image)

Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. FLX had no effect on walking time (A) or counts (B) at any of the doses. Data are expressed as mean+SD (n=4 per group).

4.3.3.13. Effect of FLX on non-social exploration behaviour in the Resident-Intruder Paradigm

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected non-social exploration time and non-social exploration counts. A significant effect of group was found for non-social exploration time \( F(3,12)=6.51, p=0.007 \) (Figure 4.51 (A)), whilst no significant effect of group was found for non-social exploration counts \( F(3,12)=2.99, p=0.073 \) (Figure 4.51 (B)). Post-hoc Dunnett’s test revealed that FLX 20 mg/kg increased non-social exploration time (p<0.01).
Figure 4. 51: Effects of FLX on non-social exploration behaviour in the resident intruder paradigm. Rats were acutely injected with vehicle or FLX (5, 10 or 20 mg/kg s.c.) and behaviour in the resident-intruder paradigm was assessed 30 minutes later. FLX 20 mg/kg increased non-social exploration time (A). FLX had no effect on non-social exploration counts at any of the doses (B). Data are expressed as mean±SD (n=4 per group). Dunnett’s post-hoc test: **p<0.01 vs. control.

4.3.3.14. Effect of FLX on immobility in the FST

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected time spent immobile. No significant effect of group was found \[F(3,25)=1.42, p=0.259\] (Figure 4.52).

Figure 4. 52: Effects of FLX on time spent immobile in the FST. Rats received chronic daily injections of vehicle or FLX (5, 10 or 20 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. FLX had no effect on time spent immobile at any of the doses. Data are expressed as mean±SD (n=7-8 per group).
4.3.3.15. **Effect of FLX on climbing in the FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected time spent climbing. No significant effect of group was found \(F_{(3,25)}=1.80, p=0.174\) (Figure 4.53).

![Graph showing time spent climbing in the FST](#)

**Figure 4.53: Effects of FLX on time spent climbing in the FST.** Rats received chronic daily injections of vehicle or FLX (5, 10 or 20 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. FLX had no effect on time spent climbing at any of the doses. Data are expressed as mean+SD (n=7-8 per group).

4.3.3.16. **Effect of FLX on swimming in the FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected time spent swimming. No significant effect of group was found \(F_{(3,25)}=0.55, p=0.656\) (Figure 4.54).
Figure 4. 54: Effects of FLX on time spent swimming in the FST. Rats received chronic daily injections of vehicle or FLX (5, 10 or 20 mg/kg s.c.) for 14 days and behaviour in the FST was assessed 60 minutes after the final dose. FLX had no effect on time spent swimming at any of the doses. Data are expressed as mean±SD (n=7-8 per group).

4.3.3.17. Effect of FLX on home cage activity in the hour preceding the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX affected home cage locomotor activity in the hour preceding the FST. A significant effect of group was found \([F_{(3,27)}=3.61, p=0.026]\) (Figure 4.55). Post-hoc Dunnett’s test revealed that FLX 20 mg/kg significantly decreased home cage locomotor activity \((p<0.05)\).
Effect of FLX on home cage locomotor activity.

Rats received chronic daily injections of vehicle or FLX (5, 10 or 20 mg/kg s.c.) for 14 days and home cage locomotor activity was assessed immediately after the last injection (in the hour preceding the FST). FLX 20 mg/kg decreased home cage locomotor activity. Data are expressed as mean±SD (n=7-8 per group). Dunnett’s post-hoc test: *p<0.05 vs. control.

Effect of FLX on saccharin preference after systemic LPS challenge

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if FLX altered % saccharin preference after systemic LPS. An anhedonic effect was not evident after LPS administration and significant effect of group was found \( F_{(3,24)}=1.66, p=0.202 \) (Figure 4.56).
Figure 4.56: Effects of FLX on % saccharin preference after systemic LPS challenge. Rats received chronic daily injections of vehicle or FLX (5, 10 or 20 mg/kg s.c.) for 14 days and % saccharin preference was assessed approximately 24 hours after the final dose. FLX had no effect on % saccharin preference at any of the doses. Data are expressed as mean±SD (n=6-8 per group). Mean±SD saccharin consumption (ml): 9±8.
4.4. Discussion

The aim of the research covered in this chapter was to validate a novel battery study design for the efficient screening of anxiolytic and antidepressant properties of novel compounds. Validation was achieved by assessing both antidepressant and anxiolytic drug effects in the test battery design using already well established drugs. Two different classes of antidepressants were utilised for this, including the TCA DMI and the SSRI FLX, whilst the effects of the anxiolytic DZP was also assessed. Thus, three experiments were carried out – one experiment for each drug, over the course of 10 months. The effects that these drugs elicited in the battery study design would confirm whether this novel experimental design is as sufficient at detecting effects as when these behavioural tests are carried out in isolation. If so, then it may provide a more efficient and advanced screening process of novel compounds.

Firstly, in terms of drug tolerability, none of the drugs reduced body weight on any of the dose days. However, all three drugs (DMI; 5 and 10 mg/kg, DZP; 1.5 mg/kg and FLX; 20 mg/kg) significantly reduced body weight gain over the entire dosing period. This is consistent with previous findings in which animals chronically treated with DMI (Shirayama et al., 1996, D’Aquila et al., 2000), FLX (Thompson et al., 2004, McGuirk et al., 1992) and DZP (Grimm and Jancourt, 1983), have gained less weight over the dosing period, compared to control rats. Whilst similar to previous findings (Grimm and Jancourt, 1983), chronic DZP had no effect on food and water intake, DMI and FLX decreased food and water consumption on several days throughout the dosing period, effects that have also been previously reported for these drugs (Nobrega and Coscina, 1987, McGuirk et al., 1992). Thus, effects of these drugs on body, food and water weight are in accordance with what has previously been reported in the literature. Table 4.1 provides a summary of the effect of each drug in all behavioural tests. Each experiment will be discussed individually, with the acute phase of the study being addressed first, followed by the chronic phase.
### Table 4.1: Effect of DMI, DZP and FLX on each behavioural test in the test battery.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Acute anxiolytic phase</th>
<th>Chronic antidepressant phase</th>
</tr>
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<tr>
<td></td>
<td>EPM</td>
<td>Open Field</td>
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<tr>
<td>DMI 2.5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DMI 5</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DMI 10</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DZP 0.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DZP 1</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>DZP 1.5</td>
<td>+</td>
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<tr>
<td>FLX 5</td>
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<td>FLX 10</td>
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<td>FLX 20</td>
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</table>

0=no effect, +=anxiolytic/antidepressant-like effect (depending on the test), and - =anxiogenic/depressant-like/sedative effect.

In the first study, which assessed the effects of DMI in the battery study design, the drug had no effect on % OAE or % OAT in the EPM at any of the doses employed. These findings are in agreement with the limited supply of studies that have looked at the acute effects of DMI in the EPM in rats. Similar to the current study, Drapier et al. (2007) reported that acute DMI at the doses of 2.5, 5 and 10 mg/kg had no effect on % OAT. Furthermore, another study found that DMI had no effect on % OAE or % OAT at a dose of 10 mg/kg (Durand et al., 2000). Although these studies found a similar effect of acute DMI, it should be noted that they employed different strains of rats than those used within the current study. DMI has indeed been reported to be neither anxiolytic nor anxiogenic in the EPM in the Sprague-Dawley rats, although this was after chronic administration (Bondi et al., 2008). In the open field, DMI did not alter locomotor activity at any dose. This contrasts to DMI locomotor effects reported in other studies, whereby DMI induced a sedative effect at doses of 10 and 20 mg/kg (Reneric and Lucki, 1998, Rex et al., 2004). However, importantly, DMI was administered subacutely in these studies, as opposed to the acute administration in the current study, which may be responsible for the discrepancy in results. Overall, our DMI effects in the EPM are in-keeping with what is generally reported within the literature. In terms of acute drug effects in the other subset of animals, DMI had no effect on any of the behaviours in the resident-intruder paradigm. In contrast, other TCAs such as clomipramine have been reported to induce a reduction in aggression, at doses which did not display a sedative effect (Mitchell and Redfern, 1992a). A possible reason for the lack of effect of DMI on behaviours in this test may be the small group
sizes, with only 3 animals present in some groups. An increased group size may have increased the power of the study and the drug effects, and from looking at the graphs, it is evident that an effect may have come through if we had increased power in our experiment. Interestingly, Mitchell and Redfern (1992a) have shown consistently that when an antidepressant reduces aggression acutely in this test, chronic treatment increases aggression, which is suggested to represent an externalization of emotions that is associated with remission of depression. Therefore, these acute effects are an important aspect to the battery study design, as they are crucial for promptly identifying promising compounds.

The acute resident-intruder protocol that was used within these battery studies was slightly different to what is commonly carried out, in that the animals were singly housed for over 7 days, and were only exposed to an intruder on one occasion. Due to the lack of power within this DMI experiment, we were unable to determine whether our resident-intruder protocol was sufficient at detecting similar effects to what has previously been reported in the literature. However, with our DZP and FLX results (discussed later) replicating what has previously been reported in the literature, we are satisfied that these minor alterations in the test procedure did not change the typically observed results with these drug classes, and that this version of the test can still reliably detect acute antidepressant effects on aggressive behaviour.

In the chronic phase of the study DMI dose dependently decreased immobility and increased climbing, with no effect on swimming behaviour. This is consistent with our findings in Chapter 3, Experiment 7 (validation of this treatment protocol), as well as what is reported in the literature after a similar treatment regime in Sprague-Dawley rats (Schaffer et al., 2010, Hadweh et al., 2010), whereby two weeks administration of DMI 10 mg/kg decreased immobility (Schaffer et al., 2010, Hadweh et al., 2010) and increased climbing behaviour (Hadweh et al., 2010). However, an important limitation of our current studies is that the last drug dose occurred one hour prior to the FST, and thus, one cannot be sure if the effects seen in the FST truly are of chronic nature. In the study by Hadweh et al. (2010), the FST was carried out approximately 24 hours after the last drug injection, thus ensuring that any effects seen in the FST truly were induced by chronic treatment. Importantly, home cage activity in the hour prior to the FST was not increased, confirming that DMI effects in the FST were purely antidepressant-like. In relation to the LPS model, in the animals that had received chronic vehicle injections, saccharin preference did not seem obviously low, with an average of 84% saccharin preference, differing from what is reported in the literature (Yirmiya, 1996, Pitychoulis
et al., 2009). Thus, DMI did not alter the rats’ preference for saccharin, probably due to a ceiling effect. However, there were several methodological limitations to this particular testing protocol that may have accounted for the lack of effect observed. When planning the methods, it was our intention to pick a time point that may display both molecular and behavioural antidepressant effects. It is reported in the literature that 3 hours post LPS injection is the time point at which pro-inflammatory cytokines peak and which can be reduced by prior antidepressant treatment (Castanon et al., 2004, Molteni et al., 2013). Thus, we chose this time point for sacrifice of our animals, in the hopes that even a slight anhedonic effect might be evident within this 3 hour period. Our effect was most likely lacking due to the typical sickness-like behaviour that is induced by acute systemic LPS, which would have resulted in the rats drinking very little and thus no evidence of an anhedonia effect could be revealed. Another limitation is the fact that all of the animals received LPS, and thus no valid comparison could be made to verify that LPS was in fact inducing an anhedonia-like effect.

The second study involved the assessment of DZP in the battery study design. In the EPM, acute DZP had a dose-dependent anxiolytic effect, with the doses of 1 mg/kg and 1.5 mg/kg increasing % OAE and 1.5 mg/kg increasing % OAT. The anxiolytic effect of DZP at these doses mirrors what is consistently reported in the literature. For example, several studies that have administered DZP to male Sprague-Dawley rats at a dose of 1 mg/kg have reported a significant increase in % OAE and % OAT (Cosquer et al., 2005a, Griebel et al., 1999), whilst similar effects have also been reported at a dose of 1.5 mg/kg (Zanoli et al., 2002). However, with the most common route of administration being i.p. for detection of acute anxiolytic effects, very few studies have utilised the s.c. route of administration when assessing DZP effects. However, when this route has been employed, consistent with the current findings, the doses of 1 and 1.5 mg/kg have been reported to increase % OAE and % OAT (Braun et al., 2011, Silvestre et al., 1996). Thus, this confirms the reliability of our EPM protocol for detecting drugs with anxiolytic properties. Upon the discovery of the interaction of DZP with plastics (explained in Chapter 3, Discussion), care was taken within the current study to ensure that the drug was made up in glass vials as opposed to plastic containers. Moreover, as could be seen in Chapter 3, Experiment 3, the s.c. route is more efficacious at inducing effects, probably due to the reduced first pass effect, which may also have played a role in the improved drug effects within this study. In the open field, the highest dose of DZP, 1.5 mg/kg, induced a sedative effective, illustrated by
reduced distance moved. A sedative effect of DZP at this dose is consistent with what has been reported in the literature, with doses from 1 mg/kg having been found to decrease distance moved (Dunne et al., 2007). In terms of acute drug effects in the other subset of animals, DZP, at the two highest doses, decreased social investigation counts, as well as aggression time and counts. However, it is important to note that DZP also induced a sedative-like effect on rats, having decreased both walking and rearing counts at 1 mg/kg and 1.5 mg/kg, whilst a decrease in non-social exploration counts was also observed in the 0.5 mg/kg. The finding that the highest DZP dose reduced general activity is consistent with the open field results observed in the other subset of animals, whereby, distance moved was also reduced after acute administration of DZP 1.5 mg/kg. These resident-intruder results are in agreement with what has previously been reported in the literature, whereby DZP has only decreased aggression behaviour at doses that were also sedative (Mitchell and Redfern, 1992a), confirming the ability of our resident-intruder protocol to detect false positive drugs such as DZP.

In the latter phase of the study, DZP had no effect on immobility, climbing or swimming behaviour in the FST. This is consistent with the findings of the few other studies that have reported the effects of DZP in the FST (Detke et al., 1995, Marti and Armario, 1993). Again, the limitations mentioned earlier in relation to the timing of the last injection before the FST also applies here, however, DZP has not been reported to induce an effect on the FST regardless of treatment regime, and thus, we suspect that regardless of the timing of the last injection, DZP still would not have induced effects in the FST. In the hour prior to the FST, DZP 1 mg/kg and 1.5 mg/kg decreased locomotor activity in the home cage, similar to prior home cage locomotor observations within our laboratory (Dunne et al., 2007). Results in the LPS model were similar to those mentioned in the DMI battery study, whereby a “control” group was not present to reliably detect an LPS-induced anhedonia effect, and a sufficient amount of time did not elapse for any preference to be detected due to the initial sickness behaviour that is typically associated with LPS administration. Therefore, no difference could be detected between any of the groups. However, we would not have expected that DZP would have altered LPS-induced responses, due to its lack of antidepressant properties as well as other published findings that have reported that DZP does not alleviate LPS-induced effects (Vinkers et al., 2009).

In the third and final study, the effects of FLX were assessed. There was no significant effect of FLX on % OAE or % OAT in the EPM. Whilst several studies have reported
an anxiogenic effect of FLX at doses ranging from 5-20 mg/kg (Drapier et al., 2007, Robert et al., 2011, Silva and Brandao, 2000, Caille et al., 1996), it should be noted that most of these studies utilised male Wistar rats as opposed to Sprague-Dawley rats. In a study which did use Sprague-Dawley rats (Caille et al., 1996), importantly, whilst FLX decreased % OAE and % OAT, the open field test was carried out prior to the EPM. As mentioned earlier, previous work has shown that prior testing can affect subsequent results in other tests (McIlwain et al., 2001, Blokland et al., 2012), and thus, one cannot reliably interpret FLX as being anxiogenic in this instance. Furthermore, the i.p. route was used for this study as opposed to the s.c. route that was used within the current study. Interestingly, the only other study which assessed the effects of FLX in male Sprague-Dawley rats via the s.c. route reported no effect of FLX at doses up to 10 mg/kg (Griebel et al., 1997). Therefore, we can verify that our EPM protocol is reliable at replicating results within the literature in which the same strain and route were used. In the open field test FLX had no effect on distance moved at any dose, similar to what has been reported in the literature (Wang et al., 2012, Zienowicz et al., 2006). For acute testing in the resident-intruder test, FLX 10 mg/kg and 20 mg/kg dose-dependently decreased aggression time. Moreover, FLX 20 mg/kg decreased aggression counts and increased non-social exploration and grooming time, whilst the 10 mg/kg dose increased rearing time. Similarly, Mitchell and Redfern (1992a) reported that FLX dose-dependently decreased aggression behaviour at doses that were not sedative, verifying the reliability of our resident-intruder protocol.

After chronic treatment, FLX had no significant effects on immobility, climbing or swimming behaviour in the FST at any of the doses employed. As mentioned earlier, SSRIs are not consistently effective in the FST. FLX has been reported to reduce immobility and increase swimming behaviour (Cryan et al., 2005a) or have no effect at all (Thompson et al., 2004, Vazquez-Palacios et al., 2004). Whilst the latter two studies used Wistar rats, Cryan and colleagues used Sprague-Dawley rats. However, it is important to note that a ‘modified’ forced swim test was used by Cryan et al. (2005a), which deviated from the typical two-day swim which was incorporated within the current study, and this may be responsible for the disparity between drug effects. In relation to home cage activity in the hour prior to the FST, as the dose of FLX increased, locomotor activity decreased. Previous assessment of the effect of FLX on locomotor activity has reported similar effects, with a trend for a decrease in locomotor activity (Reneric et al., 2002). In the LPS model, results were similar to the previous
two studies, with no anhedonia effect, and hence no antidepressant effect could be observed. Had the LPS induced anhedonia protocol worked however, we would have expected FLX to normalise saccharin preference, as other studies have found that prior chronic FLX treatment alleviates LPS-induced depressive-like behaviour (Ohgi et al., 2013, Yirmiya et al., 2001).

An important factor of a battery study like that outlined in the current Chapter is the reproducibility of both baseline and drug-induced effects. Table 4.2 illustrates the baseline behavioural responses in the control groups across all three battery studies. Although there was slight variance within most of the parameters across all three studies, importantly, the only behaviour which was significantly different between studies was ‘swimming’ in the FST. The FST is a highly subjective test, and thus, the fact that scores varied between studies even with the same person scoring, suggests that extra care needs to be taken when scoring this test. However, the finding that no other parameters differed between studies suggests that these tests are robust and reproducible, which is an encouraging finding for the development of a test battery that would include these behavioural tests.

<table>
<thead>
<tr>
<th>Behavioural parameter</th>
<th>DMI Study (May 2014)</th>
<th>FLX Study (Nov 2014)</th>
<th>DZP Study (Jan 2015)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% OAE</td>
<td>19±17</td>
<td>12±9</td>
<td>21±11</td>
</tr>
<tr>
<td>% OAT</td>
<td>16±16</td>
<td>14±13</td>
<td>20±12</td>
</tr>
<tr>
<td>Immobility (s)</td>
<td>231±37</td>
<td>174±51</td>
<td>192±80</td>
</tr>
<tr>
<td>Climbing (s)</td>
<td>59±39</td>
<td>73±48</td>
<td>54±20</td>
</tr>
<tr>
<td>Swimming (s)</td>
<td>10±4</td>
<td>52±37*</td>
<td>54±73*</td>
</tr>
<tr>
<td>Distance Moved (cm) (HCA)</td>
<td>4794±1217</td>
<td>4462±1192</td>
<td>5997±1326</td>
</tr>
<tr>
<td>Social investigation (s)</td>
<td>175±59</td>
<td>233±35</td>
<td>250±61</td>
</tr>
<tr>
<td>Aggression (s)</td>
<td>145±29</td>
<td>159±40</td>
<td>74±46</td>
</tr>
<tr>
<td>Saccharin preference (%)</td>
<td>84±20</td>
<td>85±11</td>
<td>83±15</td>
</tr>
</tbody>
</table>

Table 4.2: Baseline behavioural responses in the control groups across all three battery studies. Data are expressed as Mean±SD. *p<0.05 vs. the first (DMI) study.

In conclusion, the three drugs DMI, DZP and FLX provided valuable information about the validity of this novel battery study for detection of anxiolytic and antidepressant drug effects. In the acute phase, all three drugs produced the expected results within the EPM and the resident-intruder test, with only DZP showing anxiolytic effects in the
EPM, whilst only the antidepressants induced reliable effects in the resident-intruder paradigm. In the chronic phase, both DMI and DZP produced the expected effects in the FST. With FLX being so unreliable at inducing effects in the FST, it was uncertain what exactly to expect, however, the finding that FLX had no effect is consistent with many other studies within the literature. Finally, the protocol used for the LPS model within this study was unsuccessful at inducing anhedonia. This may be due to several limitations. Firstly, the behavioural time point was not appropriate for the detection of anhedonia effects, with a sickness-like behaviour evident in the rats. Moreover, there was an insufficient ‘control’ group, as all animals received LPS, and thus there was no way of verifying if LPS did indeed alter anhedonia compared to animals who did not receive an inflammatory challenge. Other than the LPS model, the testing protocols used for all of the tests appeared adequate at detecting the acute and chronic effects that are typically reported in these drugs in the literature. Therefore, although the test battery did not successfully incorporate several theories of depression, it fulfilled its objective of successfully screening both anxiolytic and antidepressant properties of drugs through assessment of an array of symptoms in a design that promotes the ‘Reduction’ and ‘Refinement’ aspects of the 3 Rs. The test battery provides a more clinically translatable study design for investigating the neurochemical mechanisms behind chronic antidepressant action. In addition, these studies allowed the determination of the optimal drug doses to incorporate in future studies for chronic dosing, in terms of efficacy and tolerability. Future studies may focus on characterisation of the LPS model for successful induction of anhedonia within this laboratory. In addition, other battery study designs may be considered that would include other behavioural tests and study designs, in order to provide information on symptoms other than those investigated within the current study, as well as an accurate time-course effect of both anxiolytic and antidepressant properties.
Chapter 5  Further development and assessment of a test battery study design using standard drugs

5.1. Introduction
The previous chapter described the validation of a novel test battery design for anxiolytic and antidepressant screening of drugs, incorporating the EPM, open field, resident-intruder paradigm, FST and LPS-induced responses in the SPT. All of these tests accurately detected anxiolytic or antidepressant properties as predicted, except for the LPS model, in which peripheral administration of LPS failed to induce an anhedonic effect in the SPT. Thus, with an absence of a depressive-like phenotype, no consequent attenuation of these effects by the antidepressants could be detected. It is clear that there were several methodological limitations to our LPS testing protocol that may have accounted for the lack of effect observed in the SPT. As previously mentioned, typically, when LPS is administered peripherally it induces a biphasic profile of behavioural symptoms, including an initial sickness-like behaviour, followed later by a depressive-like phenotype. As well as these behavioural symptoms, LPS also produces several distinct neurochemical changes in the brain, including an increase in many pro-inflammatory cytokines that have been suggested to mediate the behavioural symptoms induced by LPS (O'Connor et al., 2009, Yirmiya et al., 2001, Bluthe et al., 2000a, Yang et al., 2013). This rise in pro-inflammatory cytokines has been reported to peak at 3 hours post LPS injection – an effect that can be normalised by prior chronic antidepressant treatment (Castanon et al., 2001). Thus, with the intention of also assessing molecular markers in the battery studies, we chose to sacrifice the animals 3 hours post LPS, in the hope that this 3 hour window would also be sufficient to detect differences in saccharin preference that would signify an anhedonic effect. However, it is clear that the protocol we used was not satisfactory for this purpose, and thus, we needed to further characterise this model within our laboratory to ensure that an anhedonic effect was produced after LPS exposure.

The characterisation of this model involved a re-examination of the literature to determine the optimal protocol, including the training period, the dose of LPS to use, and the testing period after LPS exposure. As previously mentioned, a sickness-like behaviour is first elicited by the animals in the hours following LPS exposure. The behavioural sickness-like symptoms include a decrease in several parameters including
locomotor activity (Engeland et al., 2003), social exploration (Pitychoutis et al., 2009), body weight (Castanon et al., 2001) and food consumption (Yirmiya et al., 2001). With both a decrease in locomotor activity and food consumption evident after LPS, saccharin preference testing may not be suitable within the first couple of hours after LPS exposure, as it is evident that the rats are not as motivated to move in the home cage or consume substances. After this initial sickness-like behaviour subsides, a depressive-like phenotype has been characterized. For example in a study carried out by Frenois et al. (2007), approximately 24 hours after LPS administration (a time at which motor activity had returned to normal and no obvious sickness-like behaviour was evident), mice showed increased depressive-like behaviour in the FST compared to saline-treated animals. Furthermore, in the same study, animals showed a decreased preference for a sweet sucrose drinking solution, at a time point when water and food consumption had returned to normal.

Bearing the above findings in mind, we concluded that in order for true depressive-like behaviour to be signified with an anhedonic response in the SPT, testing would need to occur after the initial ‘sickness’ phase and continue for a period of time such that sufficient fluid would be consumed to adequately detect an anhedonic response. In order for an anhedonic response to be detected, the rats must have first developed a steady saccharin preference baseline before testing, and thus, a training period whereby rats are allowed access to a saccharin solution is required to induce this steady baseline. Rats consume most of their food and water during the dark phase of their daily cycle, and thus, this was an important factor to consider for the training period in this characterisation study. Another important aspect of characterisation studies is a dose response component, and we therefore included several doses of LPS within the experiment, with 200 µg/kg being the highest dose, as for the SPT in particular, this dose has proven effective at inducing an anhedonic effect (Yirmiya, 1996). The emergence of an anhedonic response at approximately 24 hours after LPS administration, and its lasting effect for up to 2 days after (Frenois et al., 2007) suggests that assessment of anhedonic responses should be employed for several days post LPS to characterise the time course effects, and thus, this was another important factor to consider in the development of our LPS model.

As well as the lack of effect of the LPS model in Chapter 4, another limitation was the inability to detect a time course of both anxiolytic and antidepressant drug effects. Although the previous battery studies provided valuable information about the acute
anxiolytic and chronic antidepressant effects of drugs, different tests were incorporated in the acute and the chronic phase, which meant that the time course of drug effects on distinct symptoms could not be detected. In order to resolve this, and provide important time course information, the effect of drugs on particular symptoms may be assessed both acutely and chronically. One of the many downfalls of current antidepressant screening tests is their restriction to a particular dosing regime in order to determine drug effects, making time course effects harder to uncover. However, one particular test that unveils both acute and chronic drug efficacy is NIH testing. As mentioned in Chapter 1, the NIH test is based on ‘hyponeophagia’ – the conflict which rodents face when given a choice of either approaching and consuming a desirable food in a novel environment, or avoiding the novel environment. NIH testing exhibits strong predictive validity for both acute and chronic anxiolytic effects of drugs (Merali et al., 2003, Bodnoff et al., 1989), as well as the onset of anxiolytic action of several antidepressants (Merali et al., 2003, Dulawa et al., 2004, Bodnoff et al., 1989). Dulawa and Hen (2005) further confirmed the predictive validity of the NIH test for detecting anxiolytic effects of antidepressants, showing that chronic, but not subchronic FLX treatment reduces hyponeophagia. Thus, this test may provide crucial information for unveiling the neurobiology of antidepressant response and therefore would be a valuable test to incorporate into the battery study design for the time course of anxiolytic efficacy. In terms of detecting antidepressant efficacy, we decided to incorporate the FST both subacutely and chronically for a time course element in antidepressant effects.

As previously mentioned, the chronic treatment within the battery study design is a crucial element, as it may more accurately reflect what molecular markers are altered clinically, where chronic treatment is required for antidepressant efficacy. With the existence of several different theories of depression, there is a wide spectrum of molecules that are assessed as potential biomarkers of antidepressant activity. In particular, the neurotrophin theory of depression has been commonly assessed in recent years for its potential role in mediating antidepressant effects. More specifically, alterations in BDNF and that of its receptor tyrosine receptor kinase B (TrkB) levels have been correlated to depressive-like behaviour and antidepressant activity, with the hippocampus proving one of the main brain structures in which these changes occur. For example, several models of depression have been associated with decreased levels of BDNF in the hippocampus, including CUMS (Liu et al., 2016, Wang et al., 2015), LPS inflammation models (Nowacka et al., 2014, Lin and Wang, 2014) and social
isolation models (Sun et al., 2013), among others. In addition, TrkB has also been implicated in models of depression, with CUMS resulting in depressive-like behaviour that correlates to decreased hippocampal levels of TrkB (Sun et al., 2016). Further validating the role of BDNF-TrkB signalling in depression is their alterations with antidepressant treatment. Studies have shown that different classes of antidepressants normalise or increase BDNF levels in the hippocampus with a concomitant decrease in depressive-like behaviour, including the TCA DMI (Liu et al., 2014), the SSRI FLX (Wang et al., 2016, Xie et al., 2015) and the SNRI VLX (Huang et al., 2014, Feng et al., 2012). Moreover, antidepressant drugs have also been found to alleviate depressive-like behaviour, with a concomitant increase in hippocampal TrkB levels (Sun et al., 2016).

Interestingly, not only is BDNF-TrkB signalling associated with antidepressant action of conventional monoamine acting antidepressants, but a role for this signalling pathway has also been indicated in the action of novel antidepressant compounds that work via an alternative mechanism of action. The NMDA receptor antagonist ketamine (KET) is a prime example of this. Over the past decade KET has been shown to exert antidepressant properties in both animals (Yang et al., 2012, Sun et al., 2016) and humans (Katalinic et al., 2013) and several preclinical studies have focused on assessing the potential role of the BDNF-TrkB signalling pathway in its therapeutic effects. Similar to the above mentioned studies, KET has been shown to normalise or increase BDNF and/or TrkB levels in the hippocampus in a manner which correlates to a reduction in depressive-like behaviour. These effects have been observed in different tests and models of depression including the FST (Reus et al., 2014, Zhou et al., 2014, Yang et al., 2012) and CUMS (Liu et al., 2016, Sun et al., 2016).

Bearing the above information in mind, another test battery was designed to include the NIH test, the FST, and our newly characterised LPS-induced anhedonia model. As previously mentioned, we decided to incorporate the NIH test and the FST both acutely and chronically, by incorporating a two-subset design similar to the previous battery studies that would allow a time course element to be included for the symptoms assessed in these tests. Importantly, in the chronic phase of the current study, we ensured that behavioural testing was carried out at least 24 hours after the final drug treatment, to ensure that any effects observed were truly chronic effects, a limitation that was evident within Chapter 4. The LPS model was incorporated into one of these subsets, and was carried out after chronic drug administration. Furthermore, a post-
mortem element which assessed the involvement of neurotrophin factors in antidepressant effects was included within this test battery design, whereby hippocampal BDNF and TrkB levels were assessed as potential biomarkers of antidepressant response. Similar to the previous test batteries, validation of this battery design was required using several well established drugs. The three drugs used in Chapter 4, DMI, FLX and VLX were used, with doses chosen based on results from Chapter 4, while the SNRI VLX and the novel acting antidepressant drug KET were also assessed, with doses chosen based on those effective within the literature. As with the prior battery studies, it was important to know what effects these drugs typically induce in the tests to be employed.

In relation to NIH testing, the standard anxiolytic DZP has been found to reliably induce an anxiolytic effect after acute (Bodnoff et al., 1989, Rex et al., 1998, Shephard and Broadhurst, 1982a), subchronic (Shephard and Broadhurst, 1982a, Shephard and Broadhurst, 1982b) and chronic (Bodnoff et al., 1988, Bodnoff et al., 1989) treatment. Interestingly, chronic, but not acute DMI induces an anxiolytic effect (Bodnoff et al., 1988, Bodnoff et al., 1989), whilst similar effects are evident with FLX (Bodnoff et al., 1989). To our knowledge, the effects of VLX on NIH have not been studied, whilst acute KET has been shown to induce an anxiolytic effect (Burgdorf et al., 2013). For the FST, the effects of DMI, FLX and DZP have been reported in Chapter 4, whilst antidepressant-like effects of VLX (Feng et al., 2012, Silva et al., 2012) and KET (Reus et al., 2014, Zhou et al., 2014) have also been reported in the FST.

To summarize, it is clear that further characterisation of LPS-induced anhedonia within our laboratory was required for the successful incorporation of this test in a battery study design. Furthermore, although the previous battery studies provided important information about the acute and chronic effects of anxiolytics and antidepressants, the assessment of individual tests both acutely and chronically would provide an important time course element for the effects of drugs on individual symptoms. Therefore, the experiments within this final chapter were designed for the following purposes:

**Study 1**: To further characterise the LPS-induced model of anhedonia within our laboratory

**Study 2**: To assess whether a time course aspect could be successfully incorporated into a battery study design to assess acute and chronic drug effects. In addition, the potential
involvement of BDNF-TrkB signalling in mediating antidepressant efficacy was also assessed.

5.2. Experimental protocols

A detailed description of the apparatus and test procedure for the use of the LPS model, the NIH test and the FST is provided in Chapter 2.

Two studies were carried out. Experiment 1 involved characterisation of the LPS-induced anhedonia model within our laboratory, whilst Experiment 2 involved a final battery study which would incorporate this characterised LPS model as well as the NIH test and the FST.

5.2.1. Experiment 1: Characterisation of the LPS model for induction of anhedonia

The goal of this experiment was to characterise an LPS-induced anhedonia model using the SPT within our laboratory. Male rats (in-house bred) were singly housed in paper bedding with mild EE for at least 3 weeks prior to LPS exposure. From approximately 7 weeks old (one week after single housing) animals were handled exactly as they would be in a chronic antidepressant dosing study – body weight, food and water consumption were recorded each day, and the animals skin was lightly squeezed between the fingers and the thumb at the scruff of the neck to mimic the hold for subcutaneous dosing that would occur in chronic antidepressant studies. Moreover, in the three days prior to LPS injection, animals were scruffed into a position that mimicked i.p. dosing, to allow habituation to this hold and thus avoid any extra stress on the day of LPS administration. At approximately 9 weeks old, animals underwent the LPS-induced saccharin preference protocol 2 as outlined in Chapter 2, section 2.2.2.6, but without administration of chronic drug treatment. Results were scored as described in Chapter 2, section 2.2.2.6.

5.2.2. Experiment 2: Assessment of the acute/subacute and chronic effects of different drug classes in a different test battery design

The goal of this experiment was to determine if this test battery design would successfully detect acute and chronic effects using different classes of drugs, including the TCA DMI, the SSRI FLX, the SNRI VLX, the NMDA antagonist KET and the benzodiazepine DZP.
Male rats (in-house bred) were singly housed in paper bedding with mild EE for 8 days prior to commencement of dosing. Rats were approximately 7 weeks old when dosing began. Body weight, food and water consumption were recorded each day. Drugs were made up in distilled DH$_2$O and administered s.c. at a dose volume of 2mg/kg. The doses received were DMI (10 mg/kg), FLX (10 mg/kg), VLX (20 mg/kg), KET (10 mg/kg) and DZP (1.5 mg/kg). Control rats received the same amount of vehicle (DH$_2$O) injections as drug treated rats. The test battery design comprised of two subsets, with the following sequence of testing:

**Subset 1:** On day 1 and 2 animals were exposed to the subacute FST protocol as described in Chapter 2, section 2.2.2.3. All drugs were administered in the subacute manner except for KET, which was only administered 15 minutes after the preswim (i.e. 24 hours prior to the test swim). Drug treatment was continued for a further 12 days (one dose daily), and 24 hours after drug treatment rats were administered an i.p. injection of LPS (100 µg/kg) (Day 15). The saccharin preference protocol 2, as described in Chapter 2, section 2.2.2.6 was carried out in conjunction with LPS administration. On day 16, chronic dosing commenced for a further 6 days. Rats underwent NIH testing as described in Chapter 2, section 2.2.2.7., with the novel cage test occurring approximately 24 hours after the final drug dose (Day 22). The following day rats were sacrificed via decapitation (at least 24 hours after testing) and brains were removed, stored and dissected as described in Chapter 2. Levels of BDNF and TrkB mRNA were measured in the hippocampus using qRT-PCR as described in Chapter 2.

**Subset 2:** Rats underwent NIH testing as described in Chapter 2, section 2.2.2.7., with the novel cage test occurring 30 minutes after the first drug injection (Day 1). On day 2 animals were dosed in a similar manner to the FST animals within subset 1, receiving two injections, 4 hours apart. Drug treatment was continued for a further 12 days (one dose daily), and 24 hours after drug treatment rats were administered an i.p. injection of saline (Day 15) and a saccharin preference protocol was carried out identical to that in subset 1. On day 16, chronic dosing commenced for a further 6 days. Rats underwent chronic FST testing as described in Chapter 2, section 2.2.2.3 (21 day treatment), with the FST test swim occurring 24 hours after the final drug dose (Day 22). On the day of the test swim rats received vehicle injections 5 and 1 hour prior to the test swim to ensure valid comparability to the subacute FST. The following day rats were sacrificed via decapitation (at least 24 hours after testing), and brains were removed, stored and
dissected as described in Chapter 2. Levels of BDNF and TrkB mRNA were measured in the hippocampus using qRT-PCR as described in Chapter 2.

For all animals, nocturnal home cage activity monitoring was carried out as described in Chapter 2, section 2.2.2.4. Results were scored as described in Chapter 2, section 2.2.2.4.

Figure 5.1: Experimental study design of battery study II. Days highlighted in red signify days that rats did not receive a daily dose of drug.

5.2.3. Statistical analysis

All of the data were tested for normality and homogeneity of variance, using Shapiro-Wilks and Levene tests, respectively. Data were analysed parametrically if they met the criteria of fulfilling two of the following three characteristics: even n numbers across groups, normality, and homogeneity. If data did not meet these criteria, they were analysed using non parametric statistical tests. The specific tests carried out are described under each particular results section. The level of statistical significance was set at \( p < 0.05 \). For the SPT, all groups from both subsets were analysed together, since subset 2 consisted of the saline control groups for each equivalent LPS-administered drug group. However, this data is presented on two separate graphs for clarity. Data were analysed using SPSS statistics software. Graphs were constructed using GraphPad Prism © software. Expression of data is specified under each graph.
5.3. Results

5.3.1. Characterisation of LPS-induced anhedonia

5.3.1.1. Saccharin preference

This data did not meet the criteria to be analysed parametrically. A Friedman’s ANOVA was carried out to determine if % saccharin preference differed between any of the drug groups during the saccharin and LPS exposure period. A significant overall effect was found ($\chi^2_{(9)}=73.86, p<0.001$) (Figure 5.2). A Kruskal-Wallis test was then carried out for all days to determine if there was a difference in % saccharin preference between any of the groups. A significant effect of group was found on the test night (Day 0) and the night after (Day 1 Post LPS) ($K_{(4)}=17.24, p=0.002; K_{(4)}=17.62, p=0.001$, respectively), but not on any other nights. Post-hoc analysis using the Bonferroni correction revealed that rats which received 50 µg/kg, 100 µg/kg and 150 µg/kg elicited a significant reduction in % saccharin preference when compared to controls on the test night (Night 0) ($p<0.05$), whilst rats in the 50 µg/kg ($p<0.01$), 100 µg/kg ($p<0.05$) and 200 µg/kg ($p<0.05$) demonstrated reduced % saccharin preference on the following night (Night 1).
Figure 5.2: Effect of LPS on % saccharin preference. Rats in the 50 µg/kg, 100 µg/kg and 150 µg/kg LPS groups showed decreased % saccharin preference on the test night (Night 0), whilst rats in the 50 µg/kg, 100 µg/kg and 200 µg/kg showed decreased % saccharin preference the following night (Night 1). Data are expressed as median and interquartile range (n=8 per group).

5.3.1.2. Body Weight Gain
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected body weight gain from immediately prior to LPS administration to 24 hours post LPS. A significant effect of treatment was found \([F_{(4,35)}=3.76, p=0.012]\) (Figure 5.3). Post-hoc Dunnett’s test revealed that LPS 100 µg/kg significantly decreased weight gain in the 24 hours post LPS administration \((p<0.01)\).
Figure 5.3: Effect of LPS on body weight gain. LPS 100 µg/kg reduced total weight gain in the 24 hours post LPS administration. Data are expressed as mean±SD (n=8 per group). Dunnett’s post-hoc test: **p<0.01 vs. control.

5.3.1.3. Food Consumption
This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if food consumption differed between any of the groups during the saccharin and LPS exposure period. A significant effect of time was found \( F(2.99,104.72)=29.14, p<0.001 \). There was no significant effect of group \( F(4,35)=0.78, p=0.543 \), although a significant time x group interaction effect was found \( F(11.97,104.72)=2.52, p=0.006 \) (Figure 5.4). A One-Way ANOVA for each night revealed that food consumption only differed between groups on the test night (0) \( F(4,35)=3.83, p=0.011 \). Post-hoc Dunnett’s test revealed that on the test night (Night 0), the LPS 100 µg/kg group consumed less food than the control group (p<0.01).
Figure 5.4: Effect of LPS on food consumption. Food consumption was significantly lower in the LPS 100 µg/kg group on the test night (Night 0) compared to control. Data are expressed as mean±SD (n=8 per group).

5.3.1.4. Water Consumption
This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if water consumption differed between any of the groups during the saccharin and LPS exposure period. A significant effect of time was found \( F_{(3.62,126.82)}=13.74, p<0.001 \), whilst no significant group or group x time interaction effect was found \( F_{(4,35)}=2.01, p=0.115 \) and \( F_{(14.49,126.82)}=1.72, p=0.058 \), respectively (Figure 5.5).
Figure 5.5: Effect of LPS on water consumption. LPS had no effect on water consumption at any of the doses throughout the saccharin exposure period. Data are expressed as mean±SD (n=8 per group).

5.3.1.5. Total Fluid Consumption
This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if total fluid consumption differed between any of the groups during the saccharin and LPS exposure period. A significant effect of time was found \([F(3.71,129.87)=195.44, p<0.001]\), whilst no significant group or group x time interaction effect was found \([F(4,35)=1.06, p=0.389\) and \(F(14.84,129.87)=1.46, p=0.131\), respectively] (Figure 5.6).
Figure 5. 6: Effect of LPS on total fluid consumption. LPS had no effect on total fluid consumption at any of the doses throughout the saccharin exposure period. Data are expressed as mean±SD (n=8 per group).
5.3.2. Development and assessment of a second test battery design using standard and novel drugs

5.3.2.1. Effect of drug treatment on body weight gain

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if body weight differed between any of the weeks over the three weeks of treatment. A significant time \( F_{(1.64,142.24)}=80.36, p<0.001 \), treatment \( F_{(5,87)}=13.36, p<0.001 \) and time x treatment interaction effect \( F_{(8.17,142.24)}=3.10, p<0.01 \) was found (Figure 5.7). A One-Way ANOVA for each week revealed a significant difference between groups during Week 1, 2 and 3 \( F_{(5,87)}=12.27, p<0.001; F_{(5,87)}=7.96, p<0.001; F_{(5,87)}=4.73, p=0.001 \), respectively. Post-hoc Dunnett’s test revealed that in Week 1, animals in the DMI (\( p<0.001 \)), FLX (\( p<0.01 \)), KET (\( p<0.001 \)) and DZP (\( p<0.01 \)) groups gained less weight, in Week 2, animals in the DMI (\( p<0.001 \)), FLX (\( p<0.01 \)), VLX (\( p<0.001 \)) and KET (\( p<0.01 \)) groups gained less weight, whilst in Week 3, animals in the DMI (\( p<0.001 \)) and VLX (\( p<0.05 \)) groups gained less weight.

![Figure 5.7: Effect of drug treatment on body weight gain.](image.png)

Body weight gain was reduced in the DMI, FLX, KET and DZP groups in Week 1, in the DMI, FLX, VLX and KET groups in Week 2 and in the DMI and VLX treatment groups in Week 3. Data are expressed as mean+SD (n=15-16 per group). Dunnett’s post-hoc test: *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \) vs. control.
5.3.2.2. **Effect of drug treatment on food consumption**

The data did not meet the criteria to be analysed parametrically. A Friedman’s ANOVA was carried out to determine if food consumption differed between any of the groups during the dosing period. A significant overall effect was found ($\chi^2(2) = 19.69, p<0.001$) (Figure 5.8). A Kruskal-Wallis test was then carried out for each week to determine if there was a difference in food consumption between any of the groups. A significant effect of group was found for Week 1, 2 and 3 ($K(5) = 35.09, p<0.001; K(5) = 31.54, p<0.001; K(5) = 23.44, p<0.001$, respectively). *Post-hoc* analysis using the Bonferroni correction revealed that less food was consumed in the DMI group in Week 1 ($p<0.001$), Week 2 ($p<0.001$) and Week 3 ($p<0.01$), and in the KET group in Week 1 ($p<0.01$).

![Diagram showing food consumption across weeks and drug groups](image)

**Figure 5.8: Effect of drug treatment on food consumption.** Less food was consumed in the DMI and KET groups in Week 1, and in the DMI group in Week 2 and Week 3. Data are expressed as median and interquartile range (n=15-16 per group). Bonferroni correction *post-hoc* test: **p<0.01, ***p<0.001 vs. control.

5.3.2.3. **Effect of drug treatment on water consumption**

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if water consumption differed between any of the weeks over the three weeks of treatment. A significant effect of time was found [$F(1.43,112.74) = 160.24, p<0.001$], whilst a significant treatment [$F(5,79) = 4.37, p<0.01$] and time x treatment interaction effect [$F(7.14,112.74) = 5.23, p<0.001$] was also found (Figure 5.9). A One-Way ANOVA was carried out for each week to determine if water consumption differed between any of the groups. A significant effect of drug
treatment was found in Week 1 \([F(5,85)=11.85, \ p<0.001]\) and Week 2 \([F(5,83)=3.07, \ p=0.014]\), but not in Week 3 \([F(5,86)=1.52, \ p=0.193]\). Post-hoc Dunnett’s test revealed that rats in the DMI \((p<0.001)\) and FLX \((p<0.05)\) groups consumed less water in Week 1 compared to control.

![Total Water Consumption](image)

**Figure 5.9:** Effect of drug treatment on water consumption. Less water was consumed in the DMI and FLX groups in Week 1. Data are expressed as mean+SD \((n=15-16 \ \text{per group})\). Dunnett’s *post-hoc* test: \(*p<0.05, \ ***p<0.001\) vs. control.

### 5.3.2.4. Effect of acute/subacute drug treatment on immobility in the FST

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected time spent immobile. A significant effect of drug treatment was found \([F(5,40)=7.43, \ p<0.001]\) (Figure 5.10). *Post-hoc* Dunnett’s test revealed that DMI decreased the time spent immobile \((p<0.001)\).
Figure 5.10: Effects of acute/subacute drug treatment on time spent immobile in the FST. Rats received acute (KET) or subacute (DMI, FLX, VLX and DZP) drug treatment 24 or 24, 5 and 1 hour, respectively, prior to FST. DMI decreased time spent immobile. Data are expressed as mean+SD (n=7-8 per group). Dunnett’s post-hoc test: ***p<0.001 vs. control.

5.3.2.5. Effect of acute/subacute drug administration on climbing in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected time spent climbing. A significant effect of drug treatment was found \[F_{(5,40)}=8.07, \ p<0.001\] (Figure 5.11). Post-hoc Dunnett’s test revealed that DMI increased the time spent climbing (p<0.001).
Figure 5.11: Effects of acute/subacute drug treatment on time spent climbing in the FST. Rats received acute (KET) or subacute (DMI, FLX, VLX and DZP) drug treatment 24 or 24, 5 and 1 hour, respectively, prior to FST. DMI increased time spent climbing. Data are expressed as mean+SD (n=7-8 per group). Dunnett’s post-hoc test: ***p<0.001 vs. control.

5.3.2.6. Effect of acute/subacute drug treatment on swimming in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected time spent swimming. No significant effect of drug treatment was found [F(5,40)=0.19, p=0.965] (Figure 5.12).
Figure 5.12: Effects of acute/subacute drug treatment on time spent swimming in the FST. Rats received acute (KET) or subacute (DMI, FLX, VLX and DZP) drug treatment 24 or 24, 5 and 1 hour, respectively, prior to FST. There was no effect of any drug treatment on time spent swimming. Data are expressed as mean+SD (n=7-8 per group).

5.3.2.7. Effect of acute/subacute drug treatment on distance moved in the home cage prior to FST

This data did not meet the criteria to be analysed parametrically, and thus it was subjected to log transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine the effect of drug treatment on distance moved in the home cage. A significant effect of drug treatment was found \[F(5,27)=5.03, p=0.002\] (Figure 5.13). Post-hoc Dunnett’s test revealed that DZP decreased distance moved in the home cage \(p<0.01\).
Figure 5.13: Effects of acute/subacute drug treatment on distance moved in the home cage. Rats received acute (KET) or subacute (DMI, FLX, VLX and DZP) drug treatment and locomotor activity was assessed in the hour prior to the FST. DZP reduced locomotor activity in the home cage. Data are expressed as mean+SD (n=5-6 per group). Dunnett’s post-hoc test: **p<0.01 vs. control.

5.3.2.8. Effect of acute drug treatment on latency to consume in the home cage versus a novel cage in the NIH test

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if the latency to consume differed between the home cage test day and the novel cage test day. A significant effect of time [$F_{(1.00,31.00)}=47.39, p<0.001$] was found, whilst no significant treatment [$F_{(5,31)}=1.59, p=0.193$], or time x treatment interaction effect [$F_{(5.00,31.00)}=1.88, p=0.127$] was found (Figure 5.14). Post-hoc Student-Newman Keuls test revealed that latency to consume was increased in the DMI and VLX groups on the novel cage test day compared to the home cage test day.
Figure 5.14: Effects of acute drug treatment on latency to consume in the home cage and a novel cage for NIH testing. Rats were acutely injected with vehicle or drug and behaviour in the NIH test was assessed 30 minutes later. Latency to consume was increased in the novel cage compared to the home cage in the DMI and VLX groups. Data are expressed as mean+SEM (n=5-7 per group). Student-Newman Keuls post-hoc test: *p<0.05 vs. home cage test day.

5.3.2.9. Effect of acute drug treatment on amount consumed in the NIH test

This data did not meet the criteria to be analysed parametrically. A Kruskal-Wallis test was carried out to determine if there was a difference in the number of Cheerios eaten between any of the groups. There was no significant difference between the groups ($K(5)=8.53, p=0.129$) (Figure 5.15).
Figure 5.15: Effects of acute drug treatment on number of Cheerios eaten in the NIH test. Rats were acutely injected with vehicle or drug and behaviour in the NIH test was assessed 30 minutes later. None of the drug treatments affected the number of Cheerios eaten. Data are expressed as median and interquartile range (n=4-6 per group).

5.3.2.10. Effect of chronic drug treatment on immobility in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected time spent immobile. No significant effect of drug treatment was found \[F(5,41)=1.61, p=0.180\] (Figure 5.16).
Figure 5. 16: Effect of chronic drug treatment on time spent immobile in the FST.
Rats were chronically dosed with vehicle or drug and behaviour was assessed in the FST 24 hours after the last dose. There was no effect of any drug treatment on time spent immobile. Data are expressed as mean+SD (n=7-8 per group).

5.3.2.11. Effect of chronic drug treatment on climbing in the FST
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected time spent climbing. No significant effect of drug treatment was found \([F(5,41)=2.03, p=0.094]\) (Figure 5.17).

Figure 5. 17: Effect of chronic drug treatment on time spent climbing in the FST.
Rats were chronically dosed with vehicle or drug and behaviour was assessed in the FST 24 hours after the last dose. There was no effect of any drug treatment on time spent climbing. Data are expressed as mean+SD (n=7-8 per group).
5.3.2.12. **Effect of chronic drug treatment on swimming in the FST**

This data did not meet the criteria to be analysed parametrically, and thus it was subjected to log transformation prior to statistical analysis. A One-Way ANOVA was carried out to determine if drug treatment affected time spent swimming. No significant effect of drug treatment was found \( F(5,41)=0.90, p=0.491 \) (Figure 5.18).

![Figure 5.18: Effect of chronic drug treatment on time spent swimming in the FST.](image)

Rats were chronically dosed with vehicle or drug and behaviour was assessed in the FST 24 hours after the last dose. There was no effect of any drug treatment on time spent swimming. Data are expressed as mean+SD range (n=7-8 per group).

5.3.2.13. **Effect of chronic drug treatment on distance moved in the home cage prior to FST**

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected distance moved in the home cage prior to the FST. No significant effect of drug treatment was found \( F(5,27)=0.57, p=0.725 \) (Figure 5.19).
6.4.2.14. **Effect of chronic drug treatment on latency to consume in the home cage versus a novel cage in the NIH test**

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if the latency to consume differed between the home cage test day and the novel cage test day. A significant effect of time \([F_{(1.00,29.00)}=96.66, p<0.001]\) was found, whilst no significant treatment \([F_{(5,29)}=1.51, p=0.217]\), or time x treatment interaction effect \([F_{(5,00.29,00)}=2.32, p=0.068]\) was found (Figure 5.20). *Post-hoc* Student-Newman Keuls test revealed that latency to consume was increased in the FLX, KET and DZP groups on the novel cage test day compared to the home cage test day.

**Figure 5.19: Effect of chronic drug treatment on distance moved in the home cage.**

Rats were chronically dosed with vehicle or drug and home cage locomotor activity was assessed in the hour prior to the FST. There was no effect of any drug treatment on distance moved in the home cage. Data are expressed as mean+SD (n=4-6 per group).
Figure 5. 20: Effect of chronic drug treatment on latency to consume in the home cage and a novel cage for NIH testing. Rats were chronically dosed with vehicle or drug and behaviour was assessed in the NIH test 24 hours after the last dose. Latency to consume was increased in the novel cage compared to the home cage in the FLX, KET and DZP groups. Data are expressed as mean+SEM (n=5-6 per group). Student-Newman Keuls post-hoc test: *p<0.05 vs. home cage test day.

5.3.2.15. Effect chronic drug treatment on amount consumed in the NIH test

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected the number of Cheerios eaten in the NIH test. A significant effect of drug treatment was found [$F(5,29)=3.10$, $p=0.023$] (Figure 5.21). Post-hoc Dunnett’s test revealed that DMI ($p<0.01$) and KET ($p<0.05$) decreased the number of Cheerios eaten in the NIH test.
Figure 5.21: Effects of chronic drug treatment on number of Cheerios eaten in the NIH test. Rats were chronically dosed with vehicle or drug and behaviour was assessed in the NIH test 24 hours after the last dose. DMI decreased the number of Cheerios eaten. Data are expressed as mean±SD (n=5-6 per group). Dunnett’s post-hoc test: *p<0.05, **p<0.01 vs. control.

5.3.2.16. Effect of chronic drug treatment on LPS-induced responses in the SPT

This data did not meet the criteria to be analysed parametrically. A Friedman’s ANOVA was carried out to determine if % saccharin preference differed between any of the groups during the saccharin and LPS exposure period. A significant overall effect was found ($\chi^2_{(6)}=56.95$, $p<0.001$) (Figure 5.22). A Kruskal-Wallis test was then carried out for all days to determine if there was a difference in % saccharin preference between any of the groups. There was no significant difference between the groups on baseline night 1, 2 or 3, the test night, or Post LPS nights 1, 2 or 3 ($K_{(11)}=11.25$, $p=0.423$; $K_{(11)}=17.07$, $p=0.106$; $K_{(11)}=12.37$, $p=0.336$; $K_{(11)}=4.76$, $p=0.942$; $K_{(11)}=6.74$, $p=0.819$; $K_{(11)}=8.01$, $p=0.713$; $K_{(11)}=6.98$, $p=0.800$) (Figure 5.21 A + B). There was also no difference in absolute saccharin consumption between the groups on any of the nights (data not shown).
Figure 5. 22: Effect of chronic drug treatment on LPS-induced responses in the SPT. % Saccharin preference in saline-treated animals (A) and LPS treated animals (B). Rats were chronically dosed with vehicle or drug for 14 days and 24 hours later received an acute i.p. LPS challenge (Day 0), and % saccharin preference was measured for 3 nights after. LPS did not alter saccharin preference compared to saline treated animals (A + B). Chronic drug treatment did not alter % saccharin preference in saline treated animals (A) or LPS treated animals (B). Data are expressed as median and interquartile range (n=4-7 (A) and 5-8 per group (B)).

5.3.2.17. Effect of drug treatment on nocturnal home cage activity

This data met the criteria to be analysed parametrically. A One-Way ANOVA with Repeated Measures was carried out to determine if nocturnal home cage activity differed between any of the groups at baseline, night 7, night 14 and night 21 after drug treatment. A significant time $[F(3,189)=14.18, \ p<0.001]$, treatment $[F(1,5)=2100.15, \ p<0.001]$ and group x time interaction $[F(15,189)=4.22, \ p<0.001]$ effect was found (Figure 5.23). A One-Way ANOVA for each night revealed a significant difference between groups on night 7 $[F(5,65)=4.43, \ p=0.002]$ and night 14 $[F(5,64)=8.76, \ p<0.001]$, with no difference at baseline $[F(5,66)=1.08, \ p=0.378]$ or night 21 $[F(5,66)=1.76, \ p=0.133]$. Post-hoc Dunnett’s test revealed that locomotor activity was reduced in the DMI group on night 7 ($p<0.01$), and the DMI ($p<0.001$) and VLX ($p<0.01$) groups on night 14.
Figure 5.23: Effect of chronic drug treatment on nocturnal home cage activity. Rats were chronically dosed with vehicle or drug and nocturnal home cage activity was assessed. Distance moved (cm) was decreased in the DMI and VLX groups on night 7 and night 14 of chronic dosing. Data are expressed as mean+SD (n=8-13 per group). Dunnett’s *post-hoc* test: **p<0.01, ***p<0.001 vs. control.

5.3.2.18. Effect of chronic drug treatment on BDNF mRNA levels

This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected BDNF mRNA levels. No significant effect of drug treatment was found [$F_{(5,41)}=0.99, p=0.435$] (Figure 5.24).
Figure 5.24: Effect of chronic drug treatment on BDNF mRNA levels in the hippocampus. Rats were chronically dosed with vehicle or drug and sacrificed approximately 48 hours after the final dose. Chronic drug treatment had no effect on BDNF mRNA levels. Data are mean±SD (n=7-8 per group).

5.3.2.19. Effect of chronic drug treatment on TrkB mRNA levels
This data met the criteria to be analysed parametrically. A One-Way ANOVA was carried out to determine if drug treatment affected TrkB mRNA levels. No significant effect of drug treatment was found \([F_{(5,42)}]=1.24, p=0.308\) (Figure 5.25).

Figure 5.25: Effect of chronic drug treatment on TrkB mRNA levels in the hippocampus. Rats were chronically dosed with vehicle or drug and sacrificed approximately 48 hours after the final dose. Chronic drug treatment had no effect on TrkB mRNA levels. Data are mean±SD (n=7-8 per group).
Chapter 5: Battery Study II

5.4. Discussion

The aim of the research covered in this chapter was to characterise the LPS-induced model of anhedonia within our laboratory and to assess whether a time course aspect could be reliably incorporated into a battery study design to assess acute and chronic drug effects. In addition, the potential involvement of neurotrophin signalling in mediating antidepressant efficacy was also assessed. The LPS model was characterised by incorporating a longer training period, by carefully selecting the time frame in which to assess anhedonic effects and by including a dose response element. Validation of whether a time course aspect for distinct symptoms could be assessed in a test battery manner was achieved by assessing both acute and chronic effects in the same test (NIH and FST), using standard antidepressants and anxiolytics, whilst hippocampal BDNF and TrkB mRNA levels were measured to determine their potential involvement in mediating antidepressant response. Each experiment will be discussed individually, with the LPS characterisation study addressed first, followed by the test battery, whereby the results for the FST (acute and chronic) will be discussed initially, followed by the NIH results (acute and chronic) and lastly the LPS-induced responses.

In the LPS characterisation study, LPS 50 µg/kg, 100 µg/kg and 150 µg/kg decreased % saccharin preference on the test night (Night 0), whilst % saccharin preference was decreased in the LPS 50 µg/kg, 100 µg/kg and 200 µg/kg groups the night after (Night 1), suggesting an anhedonic effect. These results are in line with previous work that has reported that LPS induces an anhedonic effect in the saccharin or sucrose preference test in rodents (Yirmiya, 1996, Frenois et al., 2007). In particular, Frenois et al. (2007) illustrated that anhedonic effects occurred 24 hours later, at a time point when water and food consumption had returned to normal, verifying that this reduction in saccharin preference truly was a depressive-like as opposed to a sickness-like effect. To verify that the reductions in saccharin preference in the current study also represented a depressive-like phenotype, body weight, as well as water, total fluid and food consumption were also measured. None of the LPS doses affected water consumption or total fluid consumption at any of the assessed time points. LPS 100 µg/kg caused a significant decrease in body weight gain from immediately prior to LPS, to 24 hours post LPS. Moreover, this dose also caused a significant decrease in food consumption on the test night (Night 0), suggesting a sickness-like effect. However, % saccharin preference remained decreased the following night, and food consumption had returned to normal, suggesting that the night after (Night 1) represented a true anhedonic
response. It is clear therefore, that this LPS and saccharin preference protocol is satisfactory to induce an anhedonic effect. As the 100 µg/kg dose was the only dose that significantly showed the biphasic sickness- and depressive-like timeline, we chose to use this dose for the next battery study design, as it would allow an insight into the time course of sickness- and depressive-like behaviour, and perhaps an insight into the effects of prior chronic antidepressant treatment on both sickness and depressive-like behaviour.

In the test battery study a reduction in body weight gain was evident in all treatment groups throughout the dosing period. As mentioned in Chapter 4, reduced body weight gain has also been reported in the literature after chronic DMI, FLX and DZP treatment (D'Aquila et al., 2000, Thompson et al., 2004, Grimm and Jancourt, 1983). With regards VLX and KET, similar to the current findings, previous work has also demonstrated that chronic administration of these drugs is associated with a decrease in body weight gain (de Oliveira et al., 2004, Venancio et al., 2013). Consistent with other work, DZP had no effect on food and water consumption throughout the dosing period (Grimm and Jancourt, 1983). Food and water consumption was not altered in the VLX group, which differs to previous work that has been carried out, whereby a decrease in food consumption was observed after chronic treatment with this drug (Jackson et al., 1997). However, it should be noted that Jackson et al. (1997) assessed food intake after only acute administration of VLX, and to our knowledge there are no other studies that have comprehensively assessed this parameter in rats after a chronic regime similar to that used in the current study. DMI reduced food and water consumption throughout the dosing period, whilst FLX also reduced water consumption, effects that have also been observed in other studies (Nobrega and Coscina, 1987, McGuirk et al., 1992). Whilst water consumption was not affected in the KET group, these rats consumed less food throughout the dosing period. To our knowledge, no other studies have reported the effect of chronic KET administration on food and water intake. Taken together, for the most part, all of the drugs produced the expected effects on body weight, as well as food and water consumption.

Subacute treatment with DMI decreased immobility, increased climbing and had no effect on swimming behaviour, whilst none of the other drugs altered any of these behaviours. The effects observed for DMI are in agreement with other studies that have assessed the subacute effects of this drug in the FST (Yamada et al., 2013, Piras et al., 2014). The lack of effect of subacute FLX in the FST is consistent with what was
reported in Chapter 4. The inconsistency of SSRIs to induce effects in the FST has been previously discussed, and therefore with FLX having showed mixed effects on FST behaviour in previous studies (Ozerov et al., 2016, Enginar et al., 2016), this finding was not unexpected. The SNRI VLX also failed to have an effect on any of the FST behaviours. Similar to FLX, reports on VLX effects in the FST have been quite mixed, with some studies reporting an antidepressant-like effect (Silva et al., 2012, Ozerov et al., 2016, Bukhari and Dar, 2013) whilst others have found no effect of the drug (Lapmanee et al., 2013, Feng et al., 2012, Martisova et al., 2013). However, within the mentioned studies that have found an effect of VLX, only one of these used the typical subacute dosing regime (Silva et al., 2012). That study used Wistar rats as opposed to Sprague-Dawley rats, and having seen the vast effect that rat strain can have on baseline and drug-induced FST behaviours, this may explain the difference in effects observed.

No effect of acute KET (24 hours prior to the FST) was observed. These findings contradict published studies in which KET administration 24 hours prior to the FST has reduced immobility (Lepack et al., 2015, Gigliucci et al., 2013, Dwyer et al., 2015). It is important to note, however, that Gigliucci et al. (2013) used a much higher dose of KET (25 mg/kg), whilst, although Lepack et al. (2015) and Dwyer et al. (2015) used the same dose as the current study, their FST protocol was slightly different to the current study, whereby the preswim occurred two days prior to the test swim. Furthermore, rats in these studies either underwent surgery (Dwyer et al., 2015) or were tested for 10 minutes in the FST (Lepack et al., 2015). These different experimental aspects therefore, may be a cause for the discrepancy in results. The lack of effect of DZP is consistent with previous work within the literature, in which DZP has had no effect on any FST behaviours (Detke et al., 1995, Marti and Armario, 1993). Therefore, for the subacute FST aspect of the battery study, we can conclude that overall, our results are in-keeping with what is generally reported within the literature, and this protocol can be reliably incorporated into the test battery design for detecting antidepressant effects.

In subset 2 there was no effect of chronic treatment of any of the drugs on immobility, climbing or swimming behaviour. The finding that chronic DMI had no effects differs to what has previously been reported within our laboratory, whereby antidepressant-like effects are evident in this test after a shorter dosing period of 14 days (Chapter 3 and 4). Importantly, however, the last dose of DMI in these previous studies was administered just one hour prior to the FST, in contrast to the current study whereby the last dose was administered 24 hours prior to the FST to ensure that any effects observed were truly
chronic. Other studies have previously reported decreases in immobility and increases in climbing behaviour when the same dose of DMI has been administered chronically (Popik et al., 2008, Hadweh et al., 2010, Jeannotte et al., 2009), although importantly, both Popik et al. (2008) and Jeannotte et al. (2009) administered the last dose of DMI within an hour of the FST, whilst Hadweh et al. (2010) administered the last dose approximately 48 hours prior to the 5 minute test swim. Similar to the subacute regime, chronic FLX failed to induce an effect in the FST, which is in line with what was reported in Chapter 4. Although there are mixed reports on the effect of VLX in the FST, the finding that chronic VLX treatment had no effects in the FST is also consistent with other chronic studies (Martisova et al., 2012, Lapmanee et al., 2013). Chronic KET had no effect on FST behaviour, contradicting what is reported in the literature, whereby chronic administration decreased immobility (Reus et al., 2013, Garcia et al., 2008) and increased swimming and climbing (Garcia et al., 2008). It is important to note however, that these studies used Wistar rats and a higher dose of 15 mg/kg, which may account for the difference in effects. The finding that chronic DZP had no effects in the FST is consistent with what has been previously reported within our laboratory (Chapter 4) and in the literature (Marti and Armario, 1993). Unlike the subacute FST protocol, it is clear that the chronic treatment regime employed prior to the FST in the current study displayed slightly different results to what is generally reported within the literature. Whilst other studies have found an effect of 14 day treatment with a well established antidepressant such as DMI (10 mg/kg) (Hadweh et al., 2010), 21 day treatment using the same dose of drug within the current study had no effect on FST behaviour, although there was a strong trend for an antidepressant effect. The only major difference between these two studies is the time point at which dosing occurred, with the current study assessing FST behaviour 24 hours after the last drug dose, whilst Hadweh et al. (2010) tested the animals approximately 48 hours after the final dose, thus suggesting that the time that elapses between the final chronic dose and testing is an important experimental factor.

In relation to NIH testing, rats in the control group did not display novelty-induced hypophagia after acute dosing, as there was no increase in latency to consume a palatable food in a novel cage compared to the home cage. Therefore, our protocol was not sufficient at inducing hypophagia. The only instance in which novelty induced hypophagia was evident was in rats that had received an acute injection of DMI or VLX immediately prior to the novel cage test. Acute administration of the anxiolytic DZP
failed to induce an anxiolytic effect by reducing the latency to consume the Cheerios in a novel cage. The lack of effect of acute DZP was unexpected, as several studies have shown an acute anxiolytic effect of this drug in the NIH test (Bodnoff et al., 1989, Shephard and Broadhurst, 1982a, Rex et al., 1998). However, unlike the current study, all of these studies employed food deprivation prior to testing. Dietze et al. (2016) recently reported that food deprivation can alter motivation to eat in a novel environment, and with baseline responses being extremely important for drug response (as seen in Chapter 3), this may account for the contrasting effects of the current study. The antidepressant drugs DMI, FLX, VLX and KET also failed to induce an acute anxiolytic effect in this test. Similarly, other studies have also reported a lack of effect of acute DMI and FLX on latency to consume food in this test (Bodnoff et al., 1988, Bodnoff et al., 1989), whilst to our knowledge, no other studies have assessed the acute effects of VLX in the NIH test. The current results therefore provide novel findings that this SNRI drug acts similarly to TCAs and SSRIs in the NIH test. Contrasting to the current results, Burgdorf et al. (2013), reported that the same dose of KET, administered 1 hour prior to testing, reduced hyponeophagia. This is the only other study that has assessed acute KET effects in the NIH test. The difference in results may be due to the inability of our protocol to induce a reliable novelty-induced hypophagia effect in even the control rats. Importantly, the amount of food consumed was not affected by acute administration with any of the drugs, suggesting that general feeding behaviour related to appetite was not affected. This finding ensures that any effects observed on the latency to consume were not due to a secondary effect on appetite.

Similar to the acute protocol, the control group in the chronic protocol did not elicit a hypophagia response in a novel cage compared to the home cage, and therefore our protocol was not sufficient at inducing hypophagia. The only instance in which novelty induced hypophagia was evident was in rats that had received chronic injections of FLX, KET or DZP prior to the novel cage test. Chronic administration of DZP failed to induce an anxiolytic effect in the novel cage. This lack of effect of chronic DZP was unexpected, as other studies have shown a chronic anxiolytic effect of this drug in the NIH test (Bodnoff et al., 1988, Bodnoff et al., 1989, Shephard and Broadhurst, 1982a, Rex et al., 1998). However, as mentioned earlier, these DZP and NIH studies employed food deprivation prior to testing, which may affect baseline and drug-induced effects. Chronic treatment with DMI, FLX, VLX and KET also failed to induce an anxiolytic effect. The lack of effect of chronic DMI differs to what has previously been reported
in the literature, in which an anxiolytic effect of chronic DMI was evident (Bodnoff et al., 1989, Bodnoff et al., 1988). Whilst no other studies have assessed the chronic effects of VLX or KET in the NIH test, Bodnoff et al. (1989) found that chronic FLX actually had an anxiolytic effect in the test. However, again, the food deprivation that was incorporated within that study may be the cause of these inconsistent FLX effects. In relation to the amount of food consumed, chronic DMI and KET affected this parameter, with rats in this group consuming less food than the control group. Importantly, neither DMI nor KET affected the amount of food consumed when the home cage testing was carried out (data not shown), verifying that these drugs were not altering the general consumption of this palatable food, and suggesting that this effect on food consumption may have been due to an anxiogenic effect of the drugs. This finding illustrates the importance of incorporating a home cage test day, as proposed by Dulawa and Hen (2005), whereby secondary effects of drugs on appetite can be eliminated as a potential cause of altered consumption in the test.

The LPS protocol that was described earlier in this chapter was incorporated into this battery study design due to its ability to induce an anhedonic effect. However, although an almost identical protocol was used, LPS failed to show anhedonic effects in the SPT at any time point in the battery study. This was disappointing, as the potential antidepressant effects of the drugs could therefore not be detected on the symptom of anhedonia within this test battery. One of the main reasons for a discrepancy of LPS-induced anhedonia effects in the literature is the existence of different LPS serotypes, whereby different serotypes have varying physiological effects on rats (Nedrebo and Reed, 2002). However, the same serotype of LPS was used for both the characterisation study and the battery study. Despite this, it is important to note that although the same serotype was used for both studies, different batches of drug were used for each study, with LPS being made up independently for each particular study. Thus, the independent LPS batches, as well as the fact that LPS has been shown to be prone to non-responders (Wurfel et al., 2005), may potentially be a contributing factor to the discrepancy in results. Moreover, a major experimental factor that may have caused the discrepancy in results is the chronic injections that occurred in the battery study that did not occur in the LPS characterisation study. Despite efforts to control for the potential effect of dosing by ‘scruffing’ the animals in a hold similar to that of injections, one cannot rule out the potential behavioural effect of actual chronic injections on animal behaviour, as
rats do experience some pain and discomfort whilst receiving an injection (Cloutier et al., 2015), which could ultimately affect behavioural testing.

The assessment of nocturnal home cage activity illustrated that there was a reduction in locomotor activity in rats treated with DMI on night 7, and DMI and VLX on night 14 of dosing, whilst none of the other drugs altered this parameter on any of the nights. The finding that DMI and VLX decreased locomotor activity is consistent with previous studies that have assessed effects of DMI (Artaiz et al., 2005, Reneric and Lucki, 1998) and VLX (Artaiz et al., 2005, Reneric and Lucki, 1998) on locomotor activity. However, it should be noted that these drug effects were elicited after acute or subacute drug treatment, and importantly, the assessment of locomotor activity was undertaken in novel environments as opposed to the home cage, which would not represent a true reflection of the animal’s typical locomotor behaviour. Whilst Wollnik (1992) assessed the effects of chronic DMI on circadian wheel-running rhythms of laboratory rats, to our knowledge, no other studies have assessed the effects of chronic DMI or VLX on general nocturnal locomotor activity in rats. FLX, KET and DZP had no effect on locomotor activity on any of the nights assessed. Whilst FLX (Reneric et al., 2002, Reneric and Lucki, 1998), DZP (Dunne et al., 2007) and KET (Becker et al., 2003) have been previously reported to reduce locomotor activity, it should be noted that all of these effects were observed after acute or short-term dosing periods, and with the exception of the study by Dunne et al. (2007), all behaviours were assessed in a novel environment. Although Wollnik (1992) also assessed the circadian behavioural effects of chronic FLX, the parameter of interest was wheel-running rhythms and not general locomotor activity. Thus, the current findings provide novel evidence of the effects of chronic administration of these drugs on general nocturnal locomotor activity in the home cage.

Hippocampal BDNF mRNA levels were not altered by prior chronic treatment with any of the drugs. Importantly, evidence suggests that BDNF levels in this region are increased in a manner that is associated with antidepressant drug efficacy. For example, chronic administration of the traditional antidepressants DMI (Liu et al., 2014, Nibuya et al., 1995), FLX (Wang et al., 2016, Xie et al., 2015) and VLX (Zhang et al., 2010, Larsen et al., 2010) have decreased depressive-like behaviour, in a manner that is associated with an increase in hippocampal BDNF. It is no surprise therefore, that with a lack of antidepressant efficacy after chronic administration, BDNF levels were not altered in any of the groups. In relation to the novel antidepressant KET, studies are
quite inconsistent in determining whether the antidepressant-like effects elicited by this drug are mediated by BDNF levels in the hippocampus, with some studies reporting decreases in BDNF levels where KET was antidepressant-like (Fraga et al., 2013), whilst others have failed to show this association (Akinfiresoye and Tizabi, 2013, Garcia et al., 2008). Thus, the KET effects within this study are not particularly contradictory to results within the literature. The effects of DZP on BDNF levels have not been commonly assessed, and thus, the DZP results are also not contradictory to previous studies.

Similar to BDNF, hippocampal TrkB mRNA levels were not altered by prior chronic treatment with any of the drugs. Investigation into the role of TrkB in mediating antidepressant response has not been as common as BDNF investigations. Studies assessing chronic effects of DMI (Nibuya et al., 1995) and FLX (Nibuya et al., 1995, Wang et al., 2015) on TrkB levels have indeed shown alterations in the hippocampus, however, the association of these alterations with a decrease in depressive-like behaviour has not been demonstrated. Whilst the effects of chronic VLX and DZP on TrkB levels have not been commonly assessed within the literature, KET has been shown to decrease depressive-like behaviour whilst showing an increase in hippocampal TrkB levels (Yang et al., 2012), although these effects were observed after short-term as opposed to long-term antidepressant activity. Thus, we can confirm that the TrkB measurements employed within this study are not contradictory to what is generally reported in the literature. Overall, we can conclude that the protocols we used for BDNF and TrkB mRNA measurement within this test battery are reliable for detecting possible alterations of these molecules in antidepressant response.

In conclusion, the LPS model developed within the characterisation study showed promising results, although a lack of reliability was observed when this protocol failed to show effects in the test battery study. Thus, further investigation and characterisation of this model is required before its successful and reliable incorporation into a test battery design. In terms of the battery study, time course effects were not adequately detected. For the FST, the subacute protocol was quite reliable in producing the typical effects observed with the drugs used. However, in the chronic phase, it is clear that the protocol used was not sufficient at detecting chronic antidepressant effects. In comparison, the 14 day dosing regime incorporated within Chapter 4 reliably induced the typical effects expected for each drug, and thus, this may be the optimal protocol for using in future battery study designs. In the NIH test the effects observed were quite
inconsistent to what is reported in the literature, suggesting that this test may also need further refinement before incorporation into a battery study design. In particular, the element of food deprivation could be assessed in further characterisation of this test. The qRT-PCR protocol used within this study proved successful at detecting mRNA levels of BDNF and TrkB in the hippocampus. Moreover, the results observed were in keeping with reports from the literature suggesting that this molecular protocol can be reliably utilised as an end point of a battery study design to determine whether these molecules are responsible for mediating the behavioural effects observed.
Chapter 6  General Discussion

The work presented herein aimed to develop an improved preclinical screening process for the detection of antidepressant and/or anxiolytic drug properties. Due to the high variability that exists in both baseline and drug-induced results between laboratories, the first studies involved characterisation of several preclinical antidepressant and anxiolytic screening tests to determine optimal parameters for detecting drug efficacy. Thereafter, several well established antidepressant and anxiolytic drugs were used to validate two novel test battery designs which would address several limitations of current preclinical testing by incorporating several behavioural symptoms, multiple theories of depression, a time course element and a more clinically relevant drug treatment regime that may provide translatable evidence for potential biomarkers of antidepressant activity. In addition, these test battery designs sought to maximise output by using refined behavioural tests in a reduced number of animals.

As could be seen in Chapter 1, there is extreme variability of results in even seemingly straightforward behavioural tests such as the FST. If such variability is evident in baseline results and effects induced by even gold standard antidepressant drugs, one can imagine how hard it might be to produce reliable and reproducible effects of a novel compound under scrutiny. Thus, this variability in behavioural test results is a major factor that needs to be addressed in order to improve preclinical drug screening. Something that is obvious across all behavioural studies is the lack of standardization of not only the behavioural apparatus’ and protocols themselves, but also in the subjects used, the conditions in which they are housed, as well as the treatment regime of the compound that is being tested. It is therefore not unreasonable to suggest that some of these experimental parameters may be responsible for the variability of results that exists between studies, and indeed several of these parameters have started to become investigated as potential sources of variation. Thus, the first results chapter (Chapter 3) describes the initial characterisation studies of this thesis, in which several experimental parameters were assessed as potential sources of variation in results in an effort to determine the optimal parameters for sensitive detection of drug efficacy.

With the EPM and the FST being the most widely used behavioural tests for screening anxiolytic and antidepressant drugs, respectively, these tests were used to investigate the effects of several experimental parameters on both baseline and drug-induced behaviour. The results of these characterisation studies illustrated the immense
importance of several experimental parameters for the optimal detection of drug effects. Whilst only the bedding type in which the animals were housed elicited an effect on EPM behaviours, the FST was sensitive to manipulation of almost all of the experimental parameters investigated. In fact, the breeding source of the rat was the only parameter that did not affect FST behaviour. The route of administration was found to be an extremely important factor, with the s.c. method being the only drug route in which typical DMI-induced behaviours were observed. Moreover, the age and housing environment (bedding type/inclusion or exclusion of EE) significantly affected baseline and/or drug-induced behaviours in the FST. The final study of this first results chapter validated a 14 day drug treatment regime prior to the FST, with both subacute and chronic DMI treatment inducing the typical behavioural profile in the FST. Although the FST is the most commonly used behavioural test for screening antidepressant efficacy, its major caveat is that drug effects are elicited after subacute drug treatment. Studies that had previously compared the two dosing regimes were experimentally flawed (Kitada et al., 1981, Mancinelli et al., 1987, Miyauchi et al., 1981, Detke et al., 1997, Cryan et al., 2005a), and thus, the current findings provided novel evidence that when systematically compared, subacute and chronic dosing induced similar effects in the FST, and therefore increased the clinical translatability of this test. Within this characterisation study, the chronic drug effects were similar when scored using both scoring techniques, confirming that both are equally valid for detecting FST behavioural effects. These findings provide reassurance that the scoring technique used in a study design does not alter chronic drug effects, and thus is not a contributing factor to the variation in drug effects reported. A more reliable and efficient screening method for false positives in the FST was also validated within this study, with successful incorporation of home cage locomotor assessment in the hour prior to the FST. This method of screening for false positives is especially important, as it avoids the incorporation of another test into the study design to assess locomotor activity. With there already being such variation in behavioural results, an additional locomotor test may only add to the variation and make reproducibility more difficult, as it has been shown that drugs induce different effects in the open field and home cage (Dunne et al., 2007). Thus, the novel method we have incorporated would be extremely beneficial in improving reproducibility and replicability of results, if a similar technique were to be adopted by other laboratories.
Taken together, this series of studies highlighted the impact that several seemingly modest experimental parameters can have on behavioural results, and may explain the extreme variation of results, and lack of replicability that exists across laboratories. Alterations in several experimental factors affected baseline results, which in turn affected the sensitivity of the tests for detecting drug efficacy, emphasising the importance of the control data in the sensitive detection of drug effects. Indeed, the importance of the control data in the outcome of a study has been highlighted previously by Moser and colleagues. Moser et al. (1997) carried out a study to determine intra- and inter-laboratory reliability of a functional observational battery and an automated assessment of motor activity in eight laboratories worldwide. Notably, they found that the control data were fundamental to the outcome of the studies in terms of sensitivity and reliability of the test measures, which ultimately impacted on the between-laboratory comparisons of chemical effects. Thus, with several of the above examined experimental parameters altering baseline results, it is clear that careful consideration is required when designing a study, or indeed when repeating a study, to ensure reliable and replicable results.

The factors of reliability and replicability are even more valuable at a time where attrition rates are high and there is a lack of translatability or predictability of drug effects clinically. For example, in 2004, the US FDA assessed the disconnect that exists between the increased expenditure on Research and Development and the high attrition rate in drug discovery (US FDA, 2004). Within this report, animal models were criticised and the need for improved models to better predict the outcome of drugs clinically was highlighted. However, despite this, the attrition rate has not improved, and in fact it is estimated that only 11% of medical compounds are approved for use after years of research and development (Glasziou et al., 2014). Green (2015) postulated that one of the main reasons for this high attrition and lack of predictability is ‘bias from irreproducibility and research misconduct’. In defence of this suggestion, Green referenced a 2012 study (Begley and Ellis, 2012) in which the full impact of irreproducibility was evident, whereby 47 out of 53 cancer studies published in top tier journals could not be replicated, even though they were published as ‘landmark’ studies (Green, 2015). It is clear therefore, that reliability and replicability are extremely important elements in drug discovery, and thus need to be considered a priority in the preclinical behavioural testing for the much sought after novel acting compounds to treat depression and/or anxiety. The best possible way to ensure reliability and
replicability of preclinical assessment of these compounds is a move toward more standardised preclinical behavioural testing. Collectively, the results of the characterisation studies within this thesis provide a valuable contribution to the literature about the optimal parameters for increasing the sensitivity of detecting drug effects in these commonly used behavioural tests and therefore, these findings will provide important insight when laboratories move toward more standardised protocols for behavioural testing. In addition, these results also provided crucial information for designing the behavioural battery studies, to ensure that the optimal parameters were used to increase the efficiency and reliability of the battery study.

When we look at the results of the second results chapter (Chapter 4), it is evident that the effect of the chronic dosing regime employed in Chapter 3 is reproducible, as the same dosing regime induced a similar behavioural pattern with DMI in this series of studies also. The overall goal of the battery studies in this chapter was to assess whether multiple tests could be incorporated into the same study design, and in the same animals, to improve the efficiency of preclinical drug screening, whereby both acute and chronic drug effects could be detected, using the minimal amount of animals. Overall, the acute assessment of compounds in the EPM and open field (arm 1) or the resident-intruder paradigm (arm 2), were reliably detected. Importantly, prior testing in the EPM and open field test did not alter the chronic effects elicited in the FST in this arm of the battery study, verifying that these tests can successfully be incorporated in the same animals without affecting the reproducibility of effects in the FST. In the second arm of this study, the LPS protocol that was utilised chronically did not induce an anhedonic response due to several methodological limitations that have been outlined previously. Thus, the effects of antidepressant drugs on anhedonia could not be assessed, nor could the potential effect of acute testing prior to LPS-induced anhedonia. Within these battery studies, a dose response element was also employed, which allowed us to determine the optimal drug doses for chronic administration with regards efficacy and tolerability, and thus provided valuable information on the drug doses to use for future battery study development. Whilst these studies provided important verification that EPM and open field testing could reliably be incorporated prior to assessment of chronic drug effects in the FST, and therefore validated a more efficacious screening of compounds, several limitations of these studies needed to be addressed within the next chapter (Chapter 5) to improve on the development and design of the test battery.
Firstly, Chapter 5 aimed to further characterise the LPS-induced anhedonia model within our laboratory, in order to establish a protocol that would successfully induce anhedonia in the SPT, and which could be incorporated into the battery study design to assess the effects of antidepressants. Indeed, an LPS-induced anhedonia protocol was successfully validated, in which saccharin preference was decreased both on the night of the injection and the following night (over 24 hours later). Whilst similar results had been observed in mice previously (Frenois et al., 2007), these findings provide novel evidence that anhedonia can be observed in rats in the SPT as a definitive delayed depressive-like behaviour, whereby a decrease in saccharin preference was not accompanied by decreases in body weight or food consumption on the second night after administration. Thus, this protocol was incorporated into the final test battery that was also carried out in Chapter 5. The final battery study sought to address several experimental limitations of the prior studies. Firstly, when the rats were dosed chronically in Chapter 3 and 4, the final dose of drug was administered only 1 hour prior to the FST, and thus, the fact that an acute effect may be induced with this dosing regime could not be ruled out. Therefore, within the final battery study, drugs were administered for 21 days, and the last drug dose occurred 24 hours prior to FST testing, to validate that any effects observed in the FST were indeed chronic effects, and not acute. An additional aim of Chapter 5 was to develop and validate an advanced battery study design to that in Chapter 4, in which the time course of drug effects could be detected for individual symptoms, using the NIH test and the FST. Finally, BDNF and TrkB hippocampal signalling were investigated as potential biomarkers of antidepressant efficacy after chronic treatment. To validate the refined test battery design, the five drugs used were DMI, FLX, VLX, KET and DZP.

Whilst the subacute dosing regime in the FST elicited the typical effects expected, for the chronic dosing regime, significant drug effects were completely absent, in contrast to the effects of the 14 day treatment regime observed in Chapter 3 and 4. Thus, the findings from this battery study would suggest that the FST effects observed within Chapter 3 and 4 were not definitively chronic, and that currently, a chronic dosing regime has not been successfully incorporated prior to the FST within our laboratory. The incorporation of a chronic dosing regime in preclinical tests of antidepressant efficacy is particularly important, as it may provide more insight into the molecular adaptations that occur clinically, whereby antidepressant efficacy is only evident after weeks of treatment, despite the fact that antidepressants work almost immediately at
their site of action. Due to this mismatch in acute antidepressant action and the delay in efficacy, the conventional ‘proximal’ explanations of how antidepressants elicit their effects are being replaced with explanations which suggest that downstream effects are responsible for the action of delayed antidepressant efficacy, such as the neuroplasticity theory of depression. Thus, if chronic administration is incorporated in the assessment of antidepressant efficacy, post-mortem analysis thereafter could provide important insights on the molecular changes that occur chronically and could uncover potential biomarkers of antidepressant efficacy to help in the discovery of novel, clinically effective compounds. For NIH testing within this study, a hypophagia effect was not evident in the control groups after acute or chronic dosing, suggesting that our protocol was not sufficient at inducing hypophagia. Therefore, the ability of our protocol to detect acute and chronic drug effects was questionable. Although some drugs induced effects acutely and/or chronically in this test, the reliability of these effects are questioned due to the finding that the standard anxiolytic DZP did not reduce anxiety in the test after either acute or chronic administration, contrasting to what is reported in the literature. Importantly, there was a crucial experimental difference that may have been responsible for the discrepancy of anxiolytic effects in the current study and previous studies. Whilst animals in the current study received food ad libitum, rats within most other comparable experiments were food deprived. Since it has been reported that food deprivation can potentially alter behavioural responses in such a test (Dietze et al., 2016), this may be a contributing factor to the variation in results that was observed between the current study and previous studies. Again, this emphasises the importance for standardisation amongst behavioural tests for reproducible and replicable results in the search for novel acting compounds.

Despite the finding that LPS induced an anhedonia response in the characterisation study, these effects could not be replicated when a similar protocol was incorporated into the battery study design. Thus, the potential antidepressant effects of the drugs could not be detected for the symptom of anhedonia. Possible reasons for the different effects between studies may be an increased number of non-responders (Wurfel et al., 2005) in the battery study compared to the characterisation study. Furthermore, one cannot rule out that prior testing in the FST, an element that was not incorporated in the characterisation study, may have affected the results, as previous work has shown that the results of some behavioural tests are sensitive to prior test exposure (Blokland et al., 2012). Moreover, whilst every effort was made in the characterisation study to mimic
Chapter 6: General Discussion

chronic injection, it also cannot be ruled out that the stress of a daily injection may have contributed to the difference in results observed between the two studies. These differences suggest that the ability of LPS to induce anhedonia may be sensitive to prior handling/injecting and behavioural testing experience, and is an important element to consider when planning future studies in which LPS-induced anhedonia is a core element. Due to the lack of sensitivity of the chronic FST design, and both the acute and chronic NIH testing, as well as the lack of effect of LPS, the time course element that we sought to incorporate was not successfully implemented. It is clear that much further characterisation of these tests and dosing regimes is required before they can be successfully incorporated into one study design. In relation to post-mortem investigations of potential biomarkers of antidepressant activity, it was hypothesised that antidepressant effects in behavioural tests would be associated with increased BDNF and TrkB levels in the hippocampus. Thus, with the lack of antidepressant effects with our chronic dosing regime, the unaltered levels of hippocampal BDNF and TrkB were anticipated.

Conclusions

The work described within this thesis contributes greatly to the preclinical field of antidepressant and anxiolytic screening. The initial characterisation studies emphasize the extreme variability that can occur in preclinical behavioural results and uncovered many seemingly insignificant experimental variables as possible causes of the inconsistency within the literature. At a time when there is such high demand for discovery of novel antidepressants, this inconsistency can be enormously detrimental, leading to results that are not reproducible and replicable, and possibly resulting in the disregard of several compounds that may otherwise have been discovered as novel acting antidepressants. The results highlight the need for conscientious experimental planning and the importance of moving toward more standardised experimental protocols and study designs in order for the preclinical field to advance in its reliability. These characterisation studies illustrate novel findings that contribute to the existing body of knowledge assessing the effects of experimental variables, and thus, provide information on the optimal experimental variables to use to maximise the sensitivity of behavioural tests such as the EPM and FST for detecting drug effects.

In terms of the two battery study designs that were employed, one of the most valuable conclusions that was drawn was that the EPM, open field and FST can be successfully
Chapter 6: General Discussion

incorporated into the same study design. Despite the possibility that effects observed within the EPM and FST are both acute, this design still provides a more efficient means of screening compounds for anxiolytic effects and antidepressant effects and will at least provide the predictive efficacy of the compound within the FST. The results from both Chapter 4 and Chapter 5 provide a great insight into the complications that can arise when trying to incorporate several diverse behavioural tests into one study design, without subsequently affecting replicability. The importance of standardisation was again emphasised, with variation observed not only between our own studies and the literature, but also between our own studies within Chapter 4 and 5. It is evident that some models may provide better stability than others for the incorporation into a battery study design, and this needs to be carefully considered. Collectively, it is clear from the results from Chapter 5 that these particular behavioural tests, using the outlined protocols, cannot be successfully incorporated into one study design to detect time course effects, as well as potential biomarkers of antidepressant efficacy. Whilst the results of these battery studies provide a base for which to further develop such a study design, thus far, the development of a test battery that would fulfil the criteria specified remains distant, with a great deal of further development and characterisation warranted.

Limitations and Future Work

Upon careful consideration of the studies incorporated within this thesis, several limitations can be identified. Firstly, rats are naturally a sociable species, and where possible should be group housed. However, for all of the battery studies rats were singly housed, and therefore this may affect their general behaviours from the start. The single housing, however, was an experimental element that could not be changed for several reasons. For example, single housing allowed for food and water consumption to be accurately detected in order to provide tolerability information about chronic administration of the drugs used. Moreover, in order for the home cage tracking data to be obtained, the tracking software which we used (Ethovision) requires that the animals are singly housed, as no more than one animal can be tracked on the same screen. However, a recent development with this software now allows the tracking of more than one animal in one cage. Studies are currently being planned within our laboratory to validate this new feature for possible incorporation into future studies so that single housing can be avoided when home cage tracking is required.
In terms of the LPS-induced anhedonia protocols, a useful addition would be the assessment of body temperature after LPS exposure. Typically when LPS is administered to rats, a biphasic body temperature response is elicited (initial hypothermia followed by hyperthermia). With LPS non-responders having been reported in other studies, body temperature assessment may have provided important information as to whether the LPS was active in the rats by signifying this alteration in body temperature. Thus, non-responders could be identified early and experimental design could be improved. Unfortunately, our ethics approval application did not allow for the procedure of body temperature assessment, although had it been carried out, we could have determined whether the lack of reproducibility of the LPS protocol was due to an unusually high amount of non-responders, or because of other experimental reasons. Nevertheless, there is still uncertainty about this protocol and future studies need to be undertaken to further refine the model.

Other future work may include further investigation of a chronic dosing regime prior to the FST, with the aim of developing a dosing regime that would be similar to the clinical scenario, and in which the effects observed could validly be interpreted as chronic, and not acute. Moreover, a comprehensive assessment of several experimental parameters could be investigated for the LPS and NIH testing. In relation to the LPS testing, experimental parameters such as prior testing, chronic injections and handling may be further assessed to help identify and eliminate parameters that may be responsible for the irreproducibility of LPS-induced anhedonia effects. For NIH testing, food deprivation is an experimental parameter that varied between the current study and most studies within the literature. With food deprivation having been found to alter motivation to eat in a novel environment, this is therefore an important factor to investigate for the possible cause of inconsistent results. In addition, upon careful interrogation and comparison of the NIH procedures within other studies, it was evident that the lux or light intensity used for novel cage testing varies immensely, and thus further investigation on the effects of this parameter may help to increase the likelihood of producing a novelty-induced hypophagia effect, as well as increasing the sensitivity of the test. Each of these experiments may therefore help in identifying the optimal experimental parameters to use in a move toward more standardised protocols that may increase reproducibility and replicability of these behavioural models.

A more refined battery study needs to be designed that may incorporate the most robust elements of Chapter 4 and 5, such as the incorporation of the EPM or resident-intruder
paradigm within the acute phase of the study. The resident-intruder paradigm would also be a useful tool for future battery study designs, as a time course element has also been already validated for this test, whereby a timeline of the emergence of drug effects can be seen. Whilst the models used within the battery studies in this thesis are relatively straightforward and labour friendly, another element that would be interesting to explore in such a battery study design would be the incorporation of models that provide more mechanistic observations, such as the olfactory bulbectomy model or the CUMS model. However, these models are more labour intensive, and would require an increased number of groups due to the controls/shams required in the study design, and would need to be validated and robust before incorporation into such a study design.
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