Author:
Kamenish, Katie A

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Exploring emotional behavior in rodents using models of affective bias and self-awareness

Exploring emotional behavior in rodents using models of affective bias and self-awareness

Katie A. Kamenish

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Abstract

The neuropsychological hypothesis of depression suggests that negative affective biases are linked to the development and maintenance of MDD symptoms as well as to the mechanisms underlying the effects of antidepressant drugs. It has been previously shown that conventional and rapid-acting antidepressants differ in their ability to modify affective biases related to learning and memory in the rodent affective bias test (ABT). This thesis investigates the effects of both amitriptyline, a tricyclic antidepressant, and (2R, 6R) hydroxynorketamine (HNK), a ketamine metabolite, on the formation of affective biases toward new reward-paired associations as well as on the modulation of previously established negative affective biases using the ABT. In addition, the effects of both drugs on reward-induced biases were tested using a rodent reward learning assay (RLA).

The ABT studies revealed that acute treatment with amitriptyline was able to positively bias learning of new reward-paired associations while HNK failed to demonstrate an effect on new learning. However, both amitriptyline and HNK were able to attenuate the retrieval of previously learnt negative affective biases in the ABT following acute administration. When tested 24 hours post-treatment, animals treated with HNK exhibited a positive bias toward the stimulus that had been previously paired with the negative affective state manipulation. Neither amitriptyline nor HNK had an effect on the formation of reward-induced biases in the RLA, suggesting that their effects are specific to biases related to affective state.

To further explore the emotional complexity of rodents, this thesis also investigated the extent to which rats demonstrate self-directed behavior in an olfactory self-recognition task. Research has shown that species known to fail vision-based measures of self-awareness may demonstrate self-directed behavior if the task caters to the primary sensory modality of that species. The experiment discussed here found that rats exhibited greater exploration of both their own scent and that of a conspecific when the conspecific's scent held a greater degree of novelty, suggesting that rats may possess social awareness rather than an introspective "self-awareness."

In summary, these results provide further evidence of the neuropsychological mechanisms associated with affective biases as well as those underlying the effects of both conventional and rapid-acting antidepressants. In addition, the results of the olfactory recognition study allow for further understanding of the cognitive capabilities of laboratory rodents which may inform both laboratory animal welfare practices as well as the preclinical study of depression and other mood disorders.

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Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University of Bristol's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, other is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

ABT	affective bias test
AD	antidepressant drug
AMPAR	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF	brain-derived neurotrophic factor
CUMS	chronic unpredictable mild stress
DA	dopamine
DSM	Diagnostic and Statistical Manual of Mental Disorders
ELA	early life stress
FST	forced swim test
HNK	hydroxynorketamine
HPA	hypothalamic-pituitary-adrenal axis
H1	histaminergic H1 receptor
JBT	judgement bias test
LE	Long Evans rat
LH	Lister Hooded rat
MAOI	monoamine oxidase inhibitor
MDD	Major Depressive Disorder
MSR	mirror self-recognition test
M1	muscarinic M1 receptor
NA	noradrenaline
NARI	noradrenaline reuptake inhibitor
NAT	noradrenaline transporter
NMDA	N-methyl-D-aspartate
NSFT	novelty suppressed feeding test
PRB	progressive ratio breakpoint
RLA	reward learning assay
RM-ANOVA	repeated measures Analysis of Variance
SERT	serotonin transporter
SNRI	serotonin-noradrenaline reuptake inhibitor
SPT	sucrose preference test
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TRD	treatment-resistant depression
TST	tail suspension test
VFX	venlafaxine
5-HT	serotonin

Chapter 1: General Introduction

1. Depression Overview

1.1 Prevalence

Major depressive disorder (MDD) (hereafter referred to as 'depression') is an affective disorder that, in recent decades, has become one of the most widespread psychiatric conditions in the world. Depression affects the lives of 3.8 percent of the global population, meaning that over 280 million people currently struggle with this disorder worldwide.[1] Depression is particularly prevalent among women, with a diagnosis rate that is almost twice as high compared to men.[2] This illness negatively impacts social and occupational functioning in a way that significantly decreases one's quality of life, especially for those who experience chronic or recurring depressive episodes. Depression can also lead to a number of negative physiological and psychological health consequences, the worst of which is death by suicide. Suicide results in the loss of nearly 750,000 lives each year, and it is currently the fourth leading cause of death in the 15 to 29-year-old population.[3] In addition, the prevalence of MDD has created a major global economic burden, with billions of dollars spent each year on patient care, antidepressant drugs, and outpatient clinics. Depression is currently the second-leading cause of disability around the world, and it is projected to become the leading cause of disease within the next ten years.[3]

1.2 Diagnostic Tools and Criteria

Depression is an extremely heterogeneous disorder, with patients presenting any number of a wide range of physical, psychological, and behavioral symptoms. Since its initial publication in 1952, the Diagnostic and Statistical Manual of Mental Disorders (DSM) has been used to diagnose depression using a set list of symptoms and predetermined diagnostic criteria. The most recent edition of the DSM[4] characterizes MDD as a mood disorder with a variety of possible symptoms including depressed mood, weight gain/loss, fatigue, recurrent suicidal ideation, feelings of guilt or worthlessness, and more (see **Table 1.1**). In order to be diagnosed with MDD, the patient must display at least five or more symptoms for a period of at least two weeks and must demonstrate either negative mood or loss of pleasure. However, making an accurate diagnosis and effective treatment plan for depression is difficult due to the wide array of symptoms and reliance on self-reporting. An additional diagnostic obstacle is the large

overlap in symptoms between MDD and other psychiatric disorders such as Generalized Anxiety Disorder[5], Post-Traumatic Stress Disorder[6], and certain personality disorders[7-8].

Major Depressive Disorder: Diagnostic Criteria	
Diagnosis requires patient to demonstrate at least 5 of the 9 listed symptoms within the same 2-week period. Symptoms must represent changes in social/occupational functioning and cannot be directly attributed to another existing medical condition. **Presentation of depressed mood (1) and/or diminish pleasure (2) is required for diagnosis.**	
Symptom	**Additional Criteria**
1. Depressed mood (can be irritable in children/adolescents)	Indicated by self-report or observations by others
2. Diminished interest/pleasure in activities	Indicated by self-report or observations by others
3. Insomnia/hypersomnia	
4. Significant weight loss/gain or significant increase/decrease in appetite	Change of > 5% body weight in one month; failure to meet expected weight gain (for children); weight loss not attributable to dieting
5. Daily fatigue/loss of energy	
6. Psychomotor retardation/agitation	Indicated by observations by others
7. Feelings of worthlessness and/or excessive guilt	Must occur nearly every day and extend beyond normal feelings of guilt
8. Decreased concentration and/or decisiveness	Indicated by self-report or observations by others
9. Recurrent thoughts of death and/or suicidal ideations; previous suicide attempt	Must extend beyond normal fear of dying; specific suicide plan not required

Table 1.1: Diagnostic criteria for Major Depressive Disorder as outlined in the DSM-5[4].

2. Depression Treatment

While there are a variety of antidepressant drugs (ADs) and psychological interventions currently available, they have only demonstrated effective symptom relief in little over half of patients[9-10]. Those who do not respond to two or more conventional antidepressants are considered to have treatment-resistant depression (TRD). Even if they do eventually respond to treatment, patients with TRD have a greater likelihood of relapse[11]. As a result, patients with TRD sometimes resort to alternative, more invasive treatments such as electroconvulsive therapy or deep brain stimulation.

The development of antidepressant drugs began in the 1950s following reports that iproniazid, an antitubercular drug, and imipramine, an antihistamine, both demonstrated antidepressant

effects in their respective patients[12-13]. The first generation of ADs were the monoamine oxidase inhibitors (MAOIs) and the tricyclic antidepressants (TCAs). MAOIs inhibit the degradation of noradrenaline (NA), serotonin (5-HT), and dopamine (DA), while TCAs inhibit NA and 5-HT transporters to prevent the reuptake of these neurotransmitters back into the presynaptic cell[14-15]. While both classes of drugs showed efficacy in treating depressed patients, they came with a host of adverse side effects including dry mouth, nausea, and paresthesia[16-17]. These drugs also were known to have potentially harmful food and drug interactions and low therapeutic index. Concerns over the side effects of these first generation ADs led to the development of new classes of antidepressants that were more selective and had less non-specific effects.

Second generation antidepressants include selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NARIs), and selective serotonin and noradrenaline reuptake inhibitors (SNRIs). This new generation of drugs showed similar efficacy to the first generation ADs but were considered to be safer and have less adverse side effects[18.] As with the MAOIs and TCAs, these drugs increase monoamine levels in the synaptic cleft, which leads to downregulation of postsynaptic monoamine receptors and an increase in overall postsynaptic transmission[19-20]. It was originally believed that such effects on monoaminergic transmission resulted in clinical improvement, however, alternative hypotheses explore the role of brain-derived neurotrophic factor (BDNF) and changes in neuroplasticity as possible mechanisms underlying the antidepressant effects of these drugs[21-22]. A 2018 meta-analysis of the therapeutic efficacy of 21 different conventional antidepressants (including two tricyclics) reported that each of the drugs evaluated demonstrated greater efficacy than placebo[23].

Despite their known clinical efficacy, there are still quite a few adverse effects associated with the second generation ADs. Some commonly reported side effects include dry mouth, cardiovascular issues, and increased suicidality[24]. As a result, over 40 percent of patients taking conventional antidepressants drop out of treatment due to adverse effects[25]. The increase in reported side effects has also led to growing concerns that these drugs may not actually be as beneficial relative to the first generation ADs as previously thought[26-28]. Another critical issue regarding the use of conventional antidepressants is their delayed onset of action. Although the inhibition of transporters is detectable shortly after administration, it typically takes weeks before there is any noticeable improvement in symptoms[29-30]. This poses a serious concern for

clinicians, as such a long delay in efficacy may discourage patients from continuing treatment as well as negatively impact those struggling with severe symptoms at the start of treatment.

The discovery of rapid-acting antidepressants has helped address the concerns regarding the delayed therapeutic efficacy of conventional antidepressants. Ketamine, a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist, has recently gained significant attention as a potential treatment due to the fast-acting nature of its effects[31-33]. Patients undergoing ketamine treatment have shown significant improvement in symptoms in as little as four hours following a single infusion[34-35]. This improvement was sustained for an average of seven days post-administration[36], and repeated treatment has been shown to maintain reduction in symptoms for even longer[37]. These findings are further supported in studies using animal models[38-39].

3. Depression Causes and Treatment – Current Theories

There are several hypotheses surrounding the various causes of MDD as well as the mechanisms underlying antidepressant drug action. The majority of theories are based on reports of effective treatments or from studies using pre-clinical animal models. However, this approach is limited due to the complex nature of the disorder and the heterogeneity of symptoms. Below is a brief explanation of three leading theories of depression etiology. Although there are other hypotheses that offer valuable insights into the pathophysiology of MDD, they are beyond the scope of this thesis and will not be discussed.

3.1 The Monoamine Hypothesis

The monoamine hypothesis argues that depression results from deficient levels of monoaminergic neurotransmitters (NA, DA, and 5-HT) in the brain[40]. Evidence in support of this theory stems from findings that conventional antidepressants increase monoamine levels by inhibiting the reuptake and/or degradation of NA and 5-HT[30, 41-43]. The key argument against this hypothesis, however, calls into question why long-term use of antidepressants is required to achieve significant symptom improvement despite the drugs' acute effects on monoaminergic transmission[44-45]. In addition, there are multiple studies reporting that other drugs known to increase monoamine activity, such as amphetamine and cocaine, do not demonstrate antidepressant effects[46-47]. To address these concerns, an updated version of the original theory suggests that acute increases in transmission produce long-term adaptations via

the downregulation of NA and 5-HT autoreceptors[48]. Despite this development, there has been a continued emphasis on moving away from monoamine-related theories of depression, as evidenced by a recent meta-analysis which reviewed clinical studies examining the role 5-HT levels, function of 5-HT receptors, and the effects of tryptophan depletion[49]. Results of this review found insufficient evidence of an association between depression and low activity and/or concentrations of 5-HT as well as minimal evidence that tryptophan depletion induces a reduction in mood in depressed or healthy participants.

3.2 The Neurotrophic Hypothesis

Another theory that aims to elucidate the etiology of MDD investigates the role of neurotrophic signaling in the development and treatment of the disorder. There is an abundance of research suggesting that exposure to stressful life events is a major risk factor for MDD, with many studies supporting the link between stress and subsequent development of depressive symptoms[50-52]. The neurotrophic hypothesis of depression suggests that elevation of glucocorticoid levels following stress exposure leads to atrophy of mature neurons and a decrease in the number of newborn neurons in the hippocampus, resulting in structural changes to this region[53-55]. This is evidenced by multiple reports of depressed patients displaying lower hippocampal volume compared to healthy controls[56-57]. The theory argues that decreased neurogenesis in the hippocampus prevents the region from providing the inhibitory feedback required for regulation of the hypothalamic-pituitary-adrenal (HPA) axis[58-59], contributing to the increased HPA activity often observed in patients with depression[60-61] (outlined in **Figure 1.1**). It is thought that brain-derived neurotrophic factor (BDNF) plays a key role in this process due to its involvement in promoting synaptic plasticity and neural growth[62-63], as depressed patients have been known to demonstrate decreased BDNF levels[64-65]. In addition, rodents subjected to chronic stress show decreased mRNA expression of BDNF in the hippocampus, and this is reversed by treatment with various antidepressants[66-69]. The ability to increase neurogenesis is believed to be a potential mechanism driving the behavioral effects of antidepressant drugs, evidenced by reports that chronic treatment with SSRIs and tricyclics increase BDNF expression in the hippocampus[70-73]. The neurotrophic hypothesis of depression may offer an explanation into the mechanisms underlying the delayed efficacy of conventional antidepressants as well as to the effects of rapid-acting antidepressants.

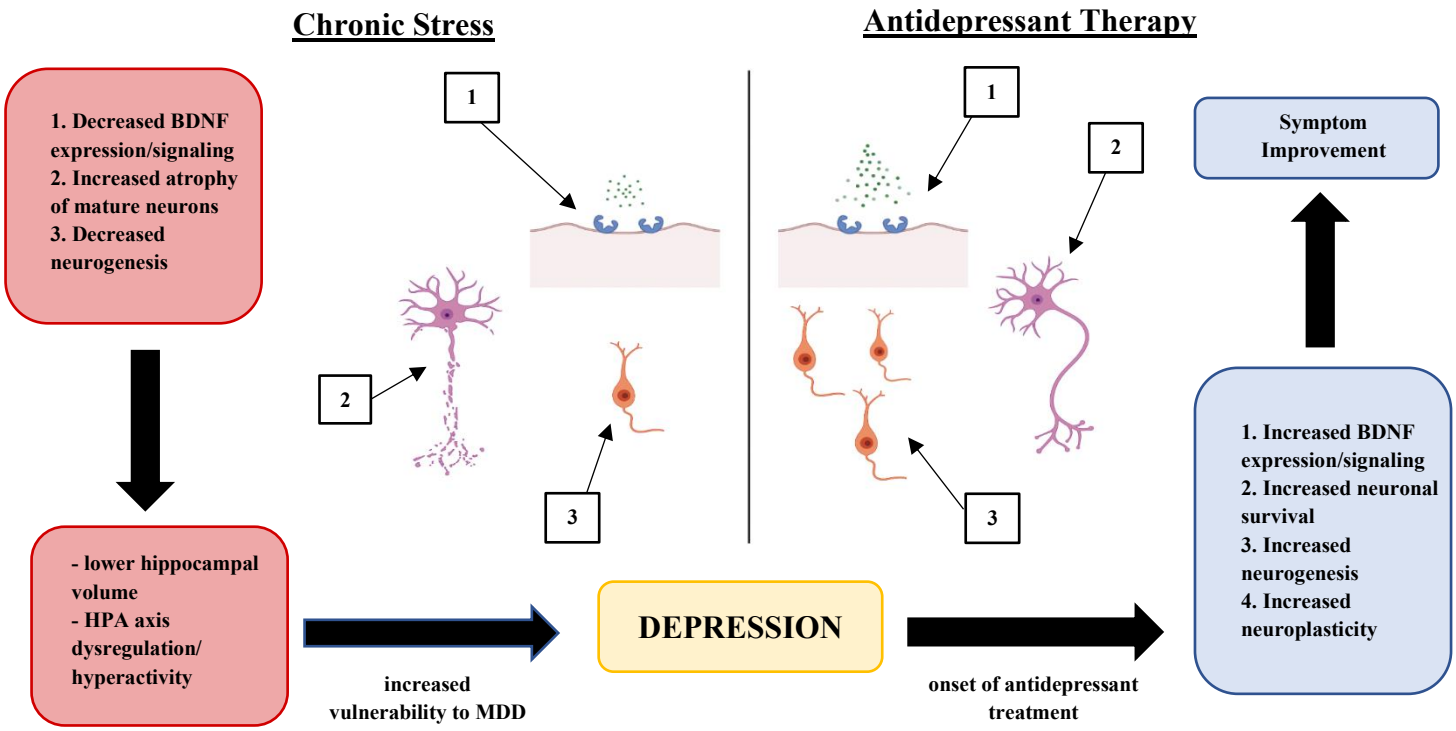

Figure 1.1: Overview of the opposing effects of stress and antidepressant therapy on BDNF expression and neurogenesis. Chronic stress has been shown to decrease BDNF signaling resulting in decreased neurogenesis and increased atrophy of mature hippocampal neurons, and this often leads to the lower hippocampal volume and HPA axis hyperactivity seen in depressed patients. Treatment with antidepressants has been theorized to improve symptoms by increasing BDNF expression and promoting both neurogenesis and plasticity.

3.3 The Cognitive Neuropsychological Hypothesis

3.3.1 Overview

In 1970, Aaron Beck proposed a theory advocating for the role of cognition in the development, maintenance, and treatment of MDD. He argued that depressed patients engage in a "negative cognitive triad" in which they hold distorted thoughts about themselves, their external environment, and the future[74-75]. This cognitive theory of depression suggests that such dysfunctional thought patterns arise from the formation of negative schemata following stressful life events[76-77]. Once formed, these patterns are activated by subsequent stressors, and the pervasiveness of these maladaptive cognitive distortions eventually lead to the onset of depression in vulnerable individuals[78-79] (see **Figure 1.2**). This biased way of thinking negatively influences several areas of information processing including memory, attention, and perception of emotionally-valenced stimuli[77, 80].

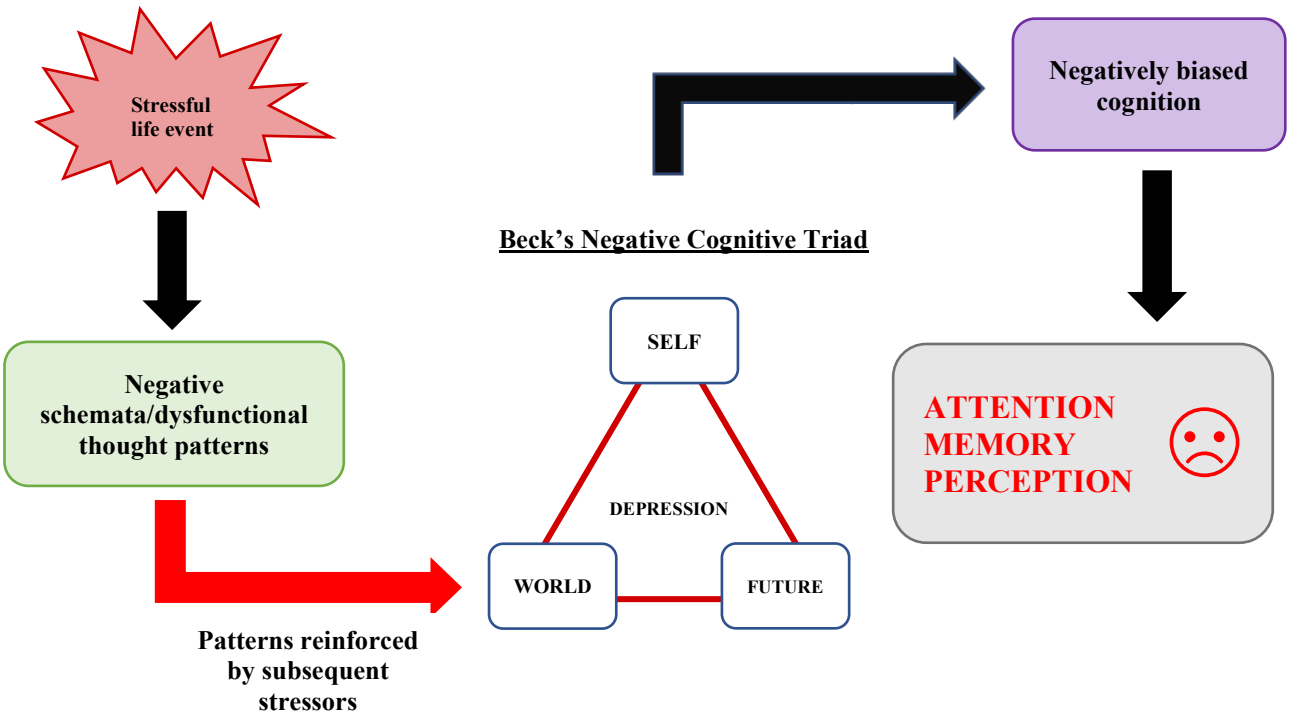

Figure 1.2: Overview of Beck's cognitive theory of depression. Beck's theory argues that stressful life events can lead to the development of dysfunctional and negative thought patterns, and such cognitive distortions contribute to the triad of negative beliefs linked to the onset of depression in vulnerable individuals. These beliefs continue to negatively bias cognitive functions in a way that perpetuates depressive symptoms.

3.3.2 Affective Biases in Depression

Research surrounding Beck's theory has continued to expand on the relationship between negative affective processing and depression. It has been argued that one's emotional state can influence a multitude of cognitive processes such as attention, learning, and memory, resulting in an "affective bias" that can skew these processes in either a positive or negative direction[81-84]. This updated version of Beck's original theory, deemed the cognitive neuropsychological model, suggests that negative affective biases are critical in the development and maintenance of depressive symptoms and play a key role in the mechanisms underlying antidepressant drug action[82, 85-86]. The model states that negative biases in affective processing lead to the formation of the negative thought patterns outlined in Beck's cognitive triad[82, 84, 87]. Depressed patients demonstrate negative biases across several domains of affective processing. For example, people with MDD are more likely to recall negative information concerning themselves[80], are more likely to interpret ambiguous stimuli as negative[88], and demonstrate an attentional bias toward negatively-valenced emotional information[89]. Negative affective biases are associated with a higher risk of relapse[90] and can persist even in recovered patients[91]. In addition, an

increase in negative affective processing has been observed in healthy volunteers deemed to be at high risk of developing MDD[92-94].

3.3.3 Antidepressant Drug Action

The cognitive neuropsychological model also proposes that affective biases are key to understanding the mechanisms of antidepressant drug action. It is thought that improvement of MDD symptoms following treatment with ADs is a result of a reversal of negatively-biased affective processing[82, 87]. More specifically, antidepressants shift biases toward emotionally-valenced stimuli from negative to positive, and this is likely due to neural modulation in areas of the limbic system and prefrontal cortex[82, 95-96]. This model also offers an explanation regarding the delayed efficacy of conventional ADs. Although the subconscious, neuropharmacological changes in affective processing occur soon after drug administration, time is needed before the patient experiences subjectively noticeable changes in mood[85, 97]. The reason for this delay is that, while ADs themselves do not directly improve mood, they allow the patient to process emotional stimuli more positively. Over time, the patient is able to relearn and reconsolidate previous negative emotional associations, and this results in gradual yet beneficial changes in behavior[82, 85] (see **Figure 1.3**). This theory is supported by findings demonstrating that acute treatment with antidepressants i.e., SSRIs and SNRIs reverses negative biases in tasks measuring various aspects of affective processing[95, 98-101].

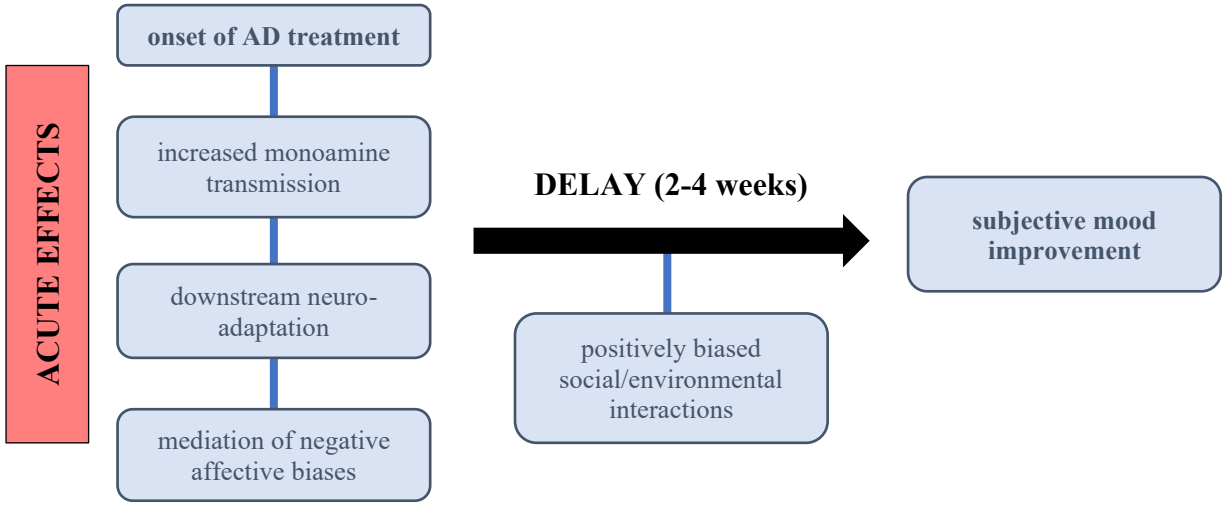

Figure 1.3: Cognitive neuropsychological theory of mechanisms underlying the delayed onset of action in conventional antidepressants. Adapted from Harmer *et al*. (2017)[85].

These findings, among many others, provide strong evidence for affective biases as a potential biomarker for depression and as a means of developing novel treatment strategies. However, rather than one hypothesis capturing MDD in its entirety, it is likely that the disorder's true etiology involves an overlapping of multiple mechanisms. Each of the theories outlined above, as well as those not discussed here, provide valuable insights into the pathophysiology of depression. Integration of these hypotheses is critical in furthering our understanding of MDD and in aiding those who do not respond to current treatments.

4. Preclinical Research in Depression

Preclinical research of depression involves the use of animal models and behavioral assays designed to elucidate the etiology of depression and investigate the mechanisms of potential antidepressants. Animal models are invaluable in furthering our understanding of depression as they allow us to study the neurobiology of MDD without being constrained by the experimental and ethical considerations associated with human studies. Unfortunately, the heterogeneous nature of MDD symptoms make it nearly impossible to develop a single animal model that captures the full complexity of the disorder. Instead, models aim to emulate one or a few depression-like behaviors e.g., anhedonia or behavioral despair using objective measures. Animal models of depression are typically based on observed responses to various stressors, such as chronic mild stress or early life adversity, or on the pharmacological action of known antidepressants. Below is a discussion of the importance of valid animal models in depression

research as well as an overview of a few key behavioral models. Alternative methods, including genetic and inflammatory models[102-03], are not discussed here.

4.1 Validity in Animal Models of MDD

An ideal animal model is one that appropriately models symptoms of MDD and is able to generate sound, testable hypotheses surrounding the disorder. To do so, the model must meet several criteria in terms of its validity. A review by Willner (1984) argues that animal models must fulfill three main types of validity: face (how well the model recapitulates symptoms observed in humans), construct (whether the behavior or symptom being modelled matches the disorder's theoretical background), and predictive validity (how accurate the model is in predicting the effects of antidepressant treatments in humans)[104]. There has also been a recent push to include assessments such as mechanistic[105] and homological validity[106-07] when considering the use of animal disease models. An overview of various forms of validity and associated criteria can be found below in **Table 1.2**.

Criteria of Validity for Animal Disease Models		
Type of Validity	**Validity Subtypes**	**Aim of Validation**
face validity	ethological validity	mimics behavioral symptoms of disease
	biomarker validity	similar biomarkers to human disease
construct validity	homological validity	validates species/strain used in animal model
	ontopathogenic validity	similar early environmental factors increase vulnerability to disease (theory of diathesis)
	triggering validity	similar factors induce pathology in vulnerable organisms during adulthood
predictive validity	induction validity	effects of etiological factors on animal disease resemble effects observed in humans
	remission validity	action of treatment in model resembles action in human disease
mechanistic validity		similarity between cognitive/neurobiological mechanisms underlying animal disease and presumed mechanisms of human disease

Table 1.2: Criteria of validity for animal models. Adapted from Willner (1984)[104] and Belzung & Lemoine (2011)[105].

4.2 Animals Models of MDD

One of the most widely known animal models of depression is the learned helplessness model, in which animals are repeatedly exposed to unpredictable mild stressors such as inescapable foot shocks. Exposure to these aversive, uncontrollable events induces a state of "helplessness," meaning that, when opportunity for escape is provided, the animal takes longer to escape or fails to escape entirely[108]. This form of "default passivity" has been linked to similar behaviors observed in depressed patients[109-11]. Animals subjected to learned helplessness demonstrate several depression-like behaviors such as anhedonia[112], increased corticosterone levels[113], and changes in REM activity[114]. These animals have also been known to show decreased motor activity[115] as well as increased aggression[116]. Helpless behaviors can be reversed following chronic administration of antidepressants including TCAs[117-18] and SSRIs[119]. In addition, treatment with anxiolytic and neuroleptic drugs do not have similar effects, suggesting the specificity of the model to depressive behavior[119]. Overall, learned helplessness is considered to be a well-established, highly valid means of modelling depression in laboratory animals[120]. However, the model does have several limitations including variable success in inducing helplessness[121] and in maintenance of depression-like behavior following cessation of the stressor[122]. Additional criticisms of this model point to differences in helpless behavior between strains[113] and general concerns regarding animal welfare[123].

Adverse events which occur during critical periods of development can lead to changes in cognitive function and contribute to later development of MDD[124-25]. There are several ways early life stress is modelled in animals including maternal separation, prenatal stress, and exposure to infection[126]. Rodents subjected to early life adversity (ELA) show altered neurotransmission[127-28], hyperactivity of the HPA axis[129], deficits in social behavior[130-31], weight loss[132], and changes in synaptic plasticity[133]. This model has even been shown to induce sex-related differences in symptoms similar to those observed in depressed humans[134]. Treatment with various antidepressants can reverse some of these behavioral and neurobiological impairments[135-37]. The ELA model is widely considered to have both good construct and predictive validity as well as high translational value[129,136, 138].

The chronic unpredictable mild stress (CUMS) model is considered to be one of the most valid and translatable means of modelling depression in animals[139]. Similar to the learned helplessness model, the CUMS model involves exposing animals to mild yet unpredictable

stressors in order to induce depression-like behaviors but differs in that exposure can last anywhere from one to eight weeks[139-40]. Examples of stressors used in the CUMS model include restricted access to food/water, temperature changes, and psychosocial manipulations[141-42]. Animals who undergo CUMS demonstrate signs of anhedonia[143], altered sleep patterns[144], decreased motor activity[145], and other behavioral changes relevant to MDD[146], and these deficits persist following cessation of the stressor[141,147]. Such changes can be reversed following chronic treatment with conventional antidepressants[104,148] as well as with acute treatment of certain rapid-acting antidepressants[149]. This reversal does not occur when treated with anxiolytic or antipsychotic drugs, indicating a specificity of the model to depression-like behaviors[145]. The CUMS model is seen as having high validity[150] but falls short due to its labor-intensive nature and poor inter-laboratory reliability[141].

Other psychosocial models include the chronic social defeat stress (CSDS) model in which animals experience "social subordination" following repeated encounters with an unfamiliar, dominant aggressor of the same species[151]. This model is thought to mimic a psychopathology similar to that induced by social conflict in humans[152], as animals subjected to CSDS often demonstrate social avoidance, decreased grooming, and increased sensitivity to subsequent stressors[153-56]. Chronic treatment with SSRIs and tricyclics can reverse behavioral impairments in CSDS animals[157-59], and acute administration of ketamine has also shown potent antidepressant effects in studies using this model[160]. However, the deficits in social behavior thought to be modelled by CSDS are associated with a multitude of other disorders including GAD, PTSD, and social phobias[158]. In addition, there has been very limited research into how CSDS influences behaviors more closely associated with depression such as learned helplessness and reduced reward sensitivity[154]. Animal studies using CSDS have also reported both sex and age differences in response to social defeat exposure[161-62].

4.3 Behavioral Assays to Investigate Depression-Like Behaviors and for Measurement of Antidepressant Drug Efficacy

Developed in the 1970s by Porsolt and colleagues, the forced swim test (FST) provides a measure of behavioral despair and was developed as a screen for antidepressant drugs in rodents[163]. The test involves placing animals in a water tank and recording how long they engage in escape behaviors before becoming immobile, with increased immobility time representing greater behavioral despair[164]. This is evidenced by findings that animals subjected

to chronic stress show increased immobility compared to controls[165]. Pre-treatment with conventional antidepressants reduces time spent immobile in the FST[166-68], and this effect is also observed following acute administration of ketamine[169]. Similar to the FST, the tail suspension test (TST) is another assay which uses immobility as a measure of behavioral despair[170]. Animals treated with both conventional[171] and rapid-acting antidepressants[172] demonstrate reductions in immobility in the TST. Historically, both the FST and TST have been praised for their high predictive validity as well as for their high reproducibility between laboratories[173]. However, these tests are known to deliver false negatives[174], and results often show inconsistent effect sizes as well as considerable variability between strains[175-76]. A recent review found that reduced immobility in the FST accurately predicted clinical outcomes in less than 25 percent of antidepressants evaluated[177], suggesting that the predictive validity of such assays may be lower than previously thought. It has also been called into question whether the FST and TST demonstrate adequate face and construct validity[177-78]. Such criticisms arise from concerns that, rather than indicating behavioral despair, immobility instead measures the animal's adaptive reaction to an acute stressor[179-81]. In addition, the acute efficacy of conventional antidepressants in both tests are inconsistent with clinical findings in which chronic treatment is required to achieve therapeutic effects in depressed patients[181-83]. Arguments against the use of these behavioral tests in preclinical research are further supplemented by concerns regarding animal welfare due to the acutely stressful nature of both assays[184].

Anhedonia, defined as loss of interest in pleasurable or rewarding activities, is a core symptom of depression, and there are several behavioral assays which attempt to recapitulate this deficit in rodents[185.] The most widely known assay measuring anhedonia in animals is the sucrose preference test (SPT). Animals subjected to depression models such as learned helplessness or CUMS demonstrate reduced preference for sucrose solution compared to plain drinking water[143,186]. This deficit is reversed following treatment chronic treatment with conventional ADs[143,187-88] and acute treatment with ketamine[189]. Reduced sucrose preference is also reversed following certain psychosocial interventions such as environmental enrichment[190]. A major criticism of the SPT is that depressed patients often fail to demonstrate deficits in similar tasks of reward sensitivity[191-92]. Furthermore, some argue that the reduced sucrose consumption observed in the SPT may be influenced by metabolic changes caused by the food/water restriction required in the protocols of certain stress models[193]. Another preclinical measure of anhedonia involves the use of a progressive ratio breakpoint (PRB) procedure. In this assay,

animals press levers to obtain a reward, but the number of presses required to obtain the reward increases between trials. The "breakpoint" occurs when the value of the reward fails to outweigh the effort required for lever pressing[194-95]. There is evidence that stress exposure reduces the breakpoint in this task[196], and this is consistent with findings of similar deficits in depressed patients[197-98]. Treatment with both conventional and rapid-acting antidepressants has been shown to increase the breakpoint in rodents[196, 199-200]. However, the validity of the PRB procedure is limited by findings showing that stress models often fail to reduce breakpoint in the task[201-02]. There is also an argument suggesting that changes in breakpoint can be influenced by factors other than motivation such as satiety and motor processes.

Anxiety and depression are highly comorbid and share great overlap in their symptomology[23-04]. As a result, many preclinical tests of anxiety-related behavior are also used in depression research to both assess anxiety-related behaviors in rodents and measure the effects of pharmacological interventions. Assays such as the open field test, elevated plus maze, and novelty suppressed feeding test (NSFT) use exploration and reward seeking as behavioral readouts of anxiety-like symptoms. This framework is based on the natural tendency of rodents to avoid open, unprotected spaces, a behavior which can be amplified by prior stress exposure[205] and attentuated by treatment with anxiolytic drugs such as benzodiazepines[206]. Chronic stress exposure decreases exploration time in both the open field test[141,207] and elevated plus maze[208-09], and similar deficits are seen using alternative depression models[210-11]. In the NSFT, animals demonstrate an increased latency to feed following CUMS and other stress models[212-13]. Chronic treatment with conventional antidepressants reverses behavioral impairments in all three assays[214-16] However, treatment with ketamine decreases latency to feed in the NSFT[217] but increases anxiety-related behaviors in the open field and elevated plus maze[218-19]. These findings suggest that while these tasks are helpful in elucidating the extent to which antidepressants also exert anxiolytic effects, they may be limited when investigating a drug's uniquely antidepressant properties. An additional limitation of these assays is the variability of results between laboratories as well as an inability to replicate findings from studies using assays such as the forced swim test [220-21].

Many studies use changes in an animal's natural behavior as indicators of a depression-like condition. For example, rodents often groom themselves as a means of self-soothing, and this behavior is observed in both normal and stressed conditions[222-23]. Rodents tend to demonstrate an increased duration of self-grooming in various experimental models of both depression and

anxiety[224-25], and these stress-induced changes in grooming behavior are reversed following administration of antidepressants[226-27]. Urination and defecation are also used to measure an animal's response to stress and subsequent antidepressant treatment. Defecation has been shown to increase under stressful conditions and decline after AD treatment[141,224,228]. Other behaviors used as indices of depressive symptoms include general locomotion, sleep patterns, and aggressive behavior[147, 224, 229]. Using natural, ethologically relevant behaviors holds many advantages for preclinical research as such readouts are non-invasive, easily quantified, and show greater sensitivity to stressors. In addition, these tests address some of the confounds associated with assays involving more "artificial" behaviors[230-31]. However, such measures are still subject to many of the same critiques as other assays including poor reliability and lack of consideration for behavioral contexts[224, 232-33].

4.4 Behavioral Assays to Investigate Affective Biases

Despite their invaluable contributions to preclinical mental health research, each of the models and tests discussed above are limited in their ability to capture a depressive phenotype in animals. As a result, there has been a recent emphasis on developing translational models which aim to mimic the neuropsychological symptoms of MDD. One approach has been to look at the way in which depression influences affective processing and develop tasks designed to measure similar deficits in animals. While there have been attempts to quantify emotional processing in animals[234], these measures often produce results that are difficult to interpret. It has been argued that previous measures of affective state in animals are more sensitive to behavioral and physiological arousal rather than emotional valence, meaning that stimuli which induce different affective states may result in similar responses[235]. Therefore, it is important to consider the cognitive components of emotional processing when developing tests of affective state in animals.

It has been well-established that cognition plays a key role in one's emotional state, and emotion greatly influences several cognitive functions such as learning, memory, and attention. Negatively skewed affective processing is a phenomenon commonly reported in conjunction with MDD, as depressed patients often demonstrate negative affective biases in a number of cognitive domains. For example, depressed patients are more likely to interpret facial expressions as negative in emotional recognition tasks[84,90], and this pessimistic interpretation extends to other forms of ambiguous stimuli[236-37]. In addition, depressed patients demonstrate

faster response times to negative words in an emotional Stroop test[238-39], are more likely to recall negative self-descriptors[82], are slower to disengage from negative emotional information[89], and tend to perceive rewarding experiences as less valuable than healthy controls[240-41]. Imaging studies have found that people with MDD show increased activity of the amygdala and other areas of the limbic system in response to negative stimuli[242-43], with activity in these areas decreasing when presented with positive stimuli[100,244].

In order to study affective processing in a preclinical setting, it is important to develop tests using emotional stimuli that is appropriate for animals. Based on the findings discussed above, two tasks have been developed: the judgment bias task (JBT)[245] and the affective bias task (ABT)[246].

4.4.1 Judgment Bias Task

The judgment bias task aims to test biases in decision-making and interpretation relating to ambiguous stimuli. Developed in 2004, the first version of the rodent JBT[245] involved training rats to discriminate between two tones of differing frequencies. One tone was associated with a food reward acquired via a lever press, and the other was associated with a white noise punishment that could be avoided by not pressing the lever. When presented with a tone of an ambiguous, intermediate frequency, the animal's subsequent decision to press the lever or withhold a response is hypothesized to reflect either a positive or negative judgement bias[245,247]. Animals subjected to mild stress (i.e., unpredictable housing) were less likely to interpret the ambiguous tone as the reward-associated cue, indicating a negative judgment bias. This result is supported by findings of similar response patterns in human versions of the task[248-49]. Later versions of the rodent JBT have adapted the original protocol to address potential confounds and explore different types of cues[250-51].

In terms of pharmacology, treating animals with anxiogenic drugs as well as subjecting animals to various stress models induces a negative affective state which leads to an increase in responses to negative cues[252-54]. It has been shown that acute treatment with conventional antidepressants typically fails to have an effect or reduces the number of reward-associated responses[250,252,255], with the exception of one study reporting a reduced negative bias following chronic treatment with fluoxetine[256]. Interestingly, acute ketamine treatment was able to induce a positive bias in the JBT[257]. These inconsistent findings, as well as those reporting failure of

prodepressant drugs to induce a negative bias[258], suggest that the validity of the JBT as a tool for screening antidepressant compounds requires improvement.

4.4.2 Affective Bias Test and Reward Learning Assay

The affective bias test was developed in 2013 by the Robinson group and is designed to measure affective biases related to learning and memory. It has been reported clinically that depressed patients tend to attribute less value to rewarding experiences compared to healthy controls[241,259]. These findings suggest that one's affective state during the learning period can bias the subsequent recall of that experience, and this hypothesis provides the theoretical framework for the ABT.

The ABT is a bowl-digging task in which animals are trained to associate digging substrates with a food pellet reward. One substrate-reward pairing is made following treatment/manipulation, while the other is made under control/vehicle conditions. Affective biases are measured by presenting animals with both previously rewarded substrates and recording the number of choices made for each substrate throughout the preference testing session (details of ABT methodology discussed in Chapter 2, Section 2.3).

Acute treatment with a variety of conventional antidepressants and psychosocial manipulations such as environmental enrichment has been shown to induce a positive bias toward the treatment/manipulation-paired substrate[246]. In contrast, treatments/manipulations which induce a negative bias in this task include psychosocial stress, treatment with prodepressant compounds, and administration of corticosterone[246,260-62]. The ABT can be used to examine biases associated with learning (when treatment is given prior to substrate-reward pairing sessions) as well as how certain treatments are able to modify previously developed biases (when administered prior to preference testing). For example, treatment with the SNRI venlafaxine positively biases learning but fails to mediate previously acquired negative biases, and the opposite is true following treatment with rapid-acting antidepressants such as ketamine[263]. Interestingly, drugs of abuse such as cocaine and amphetamine fail to produce significant effects in the ABT[246], suggesting a relationship between the mechanisms underlying the formation of affective biases and those underlying antidepressant drug action (see **Table 2.1** in Chapter 2, Section 2.2). It is also important to note that a study using both male and female rats found no significant sex differences in the task[262].

The ABT protocol has also been modified into an assay which measures reward learning, referred to as the reward learning assay (RLA). Following a similar protocol to the ABT, the RLA involves pairing one substrate with a high-value reward (two food pellets) and another with a low-value reward (one food pellet). Under control conditions, animals demonstrate a preference for the substrate associated with the higher value reward[246,261]. Chronic treatment with prodepressant compounds has been shown to induce a negative bias in the RLA[259,261]. Although originally developed to control for acute effects on memory for retrieval experiments in rats, a version of the RLA has recently been validated for use in mice[264].

Together, these findings provide cogent evidence of the ABT and RLA as translational tools for preclinical mental health research that not only demonstrate high predictive and construct validity[265], but also offer more refined, less stressful methods for studying affective biases and reward learning in animals.

5. Self-Awareness in Laboratory Rodents

5. 1 Overview

Part of what makes developing quality animal models of mental health disorders difficult is determining exactly where humans and animals overlap in terms of their cognitive capabilities. A critical question to be answered in this endeavor is the extent to which animals exhibit self-awareness. The subjective experience of animals is a subject of great debate as few agree on what it means to possess self-awareness. Early research into animal awareness has argued that self-awareness requires the ability of an organism to reflect inward and evaluate the relationship between itself and the external environment[266-68], and this differs from simply being aware of surroundings or responding to external stimuli[269-70]. More recently, it has been proposed that there are distinct levels of awareness, and the types of awareness an organism is capable of is determined by its physiological and behavioral responses to the environment. While the exact categorization of different forms of awareness is still being debated, there is a general consensus that self-awareness requires an organism to be able to engage in some form of introspection and recognize itself as an environmental entity[267,271-73]. There have been many attempts to quantify the concept of self-awareness in a wide range of species, and such studies

have offered valuable insights into both the mental experience of animals and to the importance of awareness in preclinical animal research.

5.2 Measures of Self-Awareness in Animals

5.2.1 Gallup's Mirror Self-Recognition Test

The first attempt at quantifying self-awareness in animals was reported by Gallup (1970) using a novel mirror self-recognition (MSR) test. The test involved dyeing a red mark onto the face of chimpanzees and then placing the animals in front of a mirror. Upon looking in the mirror, the chimpanzees proceeded to touch the marks on their own faces. This behavior suggested that the chimpanzees recognized that the animal in the mirror was a reflection of themselves, offering the earliest evidence of self-awareness in non-human primates[274]. This finding has since been replicated in other primate species including orangutans[275], bonobos[276], and gorillas[277]. In addition to non-human primates, animals that have been reported to demonstrate self-directed behavior in the MSR test include elephants[278], dolphins[279], and certain species of birds[280-82] (see **Table 1.3**). A 2019 study found that cleaner wrasse who were given colored throat markings exhibited self-scraping behavior when given access to a mirror, offering some of the first evidence of a fish species passing the MSR test[283]. However, whether the increased scraping behavior could be interpreted as "passing" the test remains controversial[284-85].

Gallup's MSR test has been the subject of extensive criticism since its initial publication. A primary criticism is that several reports of certain species passing the test have yet to be replicated. In addition, many species who exhibit self-directed behavior in the MSR test fail to do so upon initial testing and only do so after extensive training and reinforcement[286-88]. Others argue that the behaviors observed during the test are more likely to be a reflection of either social behavior between conspecifics[289] or of kinesthetic-visual matching to what the animal believes is another organism[290]. It has been theorized that there is a difference between social awareness and the introspective awareness required for a sense of self[273], and whether the MSR test distinguishes between the two is unclear. Furthermore, it has been shown that children from rural, non-Westernized cultures who are able to verbally express self-awareness often fail to recognize themselves in a mirror, suggesting that factors other than cognitive capability can influence behavior in the MSR test[291]. Despite these criticisms, the MSR test is still widely considered to be the gold standard of assessing self-awareness in non-human species.

Species Demonstrating Self-Directed Behavior in Gallup's MSR Test		
Classification	**Species**	**References**
primates	chimpanzee	Gallup (1970)[274]; Povinelli *et al*., (1997)[292]; Suarez & Gallup (1981)[275]
	orangutan	Suarez & Gallup (1981)[275]
	bonobo	Walraven (1997)[276]; Inoe-Nakamura (1997)[293]
	gorilla	Parker (1994)[294]; Swartz & Evans (1994)[295]; Posada & Colell (2007)[296]
	capuchin monkey	Roma *et al*., (2007)[287]
	rhesus monkey	Chang *et al*., (2015)[288]
mammals (non-primates)	Asian elephant	Plotnik *et al*., (2006)[278]
	bottlenose dolphin	Reiss & Marino (2001)[297]; Morrison & Reiss (2018)[279]
	orca whale	Delfour & Marten (2001)[298]
	domestic horse	Baragli *et al*., (2017)[299]
birds	Eurasian magpie	Prior *et al*., (2008)[280]
	Indian house crow	Buniyaadi *et al*., (2020)[281]
	scrub jay	Clary *et al*., (2020)[282]
fish	giant manta ray	Ari & D'Agostino (2016)[300]
	cleaner wrasse	Kohda *et al*., (2019)[283]
insects	myrmicine ants	Cammaerts & Caemmaerts (2015)[301]

Table 1.3: Overview of species reported as demonstrating self-directed behavior in Gallup's mirror self-recognition test.

5.2.2. Olfactory Self-Recognition

Another common criticism of the MSR test is the lack of consideration for species who do not use vision as their primary sense[302-03]. For example, species who use olfaction as their primary sensory modality, such as canines, typically fail the MSR test despite displaying similar levels of social cognition as species who do pass[304-05]. A key aspect of self-awareness is the ability to distinguish oneself from conspecifics[306], therefore a valid measure of self-awareness should incorporate apparatuses and stimuli most ecologically relevant to the species being tested. Olfactory self-recognition has been assessed in several species using various species-specific

protocols. Animals such as cichlids[307], storm petrels[308], and domesticated dogs[309-10] have all been shown to demonstrate a preference for their own scent over that of a conspecific.

The primary criticism of such studies argues that an animal's preference for its own scent may simply indicate preference for familiarity over novelty rather than reflecting true self-recognition[311]. A second argument against reports of olfactory awareness in animals posits that animals who appear to recognize themselves in one sensory modality should do so in others, as has been demonstrated in humans[312]. However, little research has been conducted examining multi-modal self-recognition in animals. Ultimately, the ability of a species to "pass" one test and "fail" another evidences the importance of accounting for an animal's perceptual capabilities when designing tests of cognition in non-human species.

5.3 Measuring Self-Awareness in Rodents

Despite their widespread use in preclinical research, little is known about the extent to which rodents exhibit self-awareness. There are several studies suggesting that rodents demonstrate awareness across a number of domains. Studies have shown that mice who have had their tails stroked while simultaneously watching a rubber tail being stroked will exhibit a defense withdrawal response when the rubber tail is grabbed alone[313-14]. These results mimic the rubber hand illusion observed in humans[315] and provide evidence of bodily ownership in rodents. In an adapted version of Gallup's MSR test, mice who had tape applied to their heads spent more time in front of a mirror than non-taped controls, and these mice also spent more time examining photographs of themselves compared to photos of familiar and unfamiliar conspecifics[316]. Rodents have also been known to exhibit "awareness" in more complex behavioral assays. An example is a duration-discrimination task in which rats could choose to either accept or decline to participate in a trial. Declining the trial would result in a guaranteed, low-value reward, whereas accepting the trial gave the animal an opportunity to receive a higher-value reward if the trial was performed accurately (inaccurate performance resulted in no reward). The study found that rats were more likely to decline trials categorized as higher in difficulty, and these results were interpreted as the rats demonstrating awareness of their knowledge regarding the task[317]. There are additional studies that have used discrimination tasks as measures of self-awareness, and these results are discussed further in Chapter 3, Section 3.2. It has also been theorized that foraging behavior and performance in maze-related tasks may be evidence of subjective awareness in rodents, as knowing where to forage or which

direction to go in a maze requires an animal to call upon memories of relevant experiences and determine the differences and similarities between the past memory and present situation[318]. There has yet to be a study investigating olfactory self-awareness in rodents, most likely due to the limited research in the area of rodent self-awareness as a whole. As with reports of self-directed behavior in other species, it is unclear whether the results of the aforementioned studies can be interpreted as definitive evidence of self-awareness. Regardless, these findings offer insight into the cognitive capabilities of laboratory rodents, and such information may have important implications for the future of animal research and welfare.

6. *Thesis Aims and Objectives*

The aim of this thesis is to investigate the mechanisms underlying the effects of antidepressants on affective biases in rodents and determine the extent to which rodents exhibit olfactory self-recognition. The hypotheses to be tested are as follows:

- Based on evidence that conventional, delayed-onset antidepressants positively bias learning in the ABT, a similar effect is predicted following treatment with amitriptyline due to the drug's effects on monoaminergic transmission.
- Treatment with both ketamine and scopolamine has been shown to modulate previously established negative affective biases in the ABT. HNK is a ketamine metabolite, and amitriptyline and scopolamine both demonstrate strong binding affinity for the muscarinic M1 receptor. Therefore, both HNK and amitriptyline are predicted to attenuate a FG7142-induced negative bias in the ABT with effects sustained 24 hours post-treatment.
- In an olfaction-based measure of recognition, rats will demonstrate a preference for their own scent over that of a conspecific as a result of self-recognition. Furthermore, preference magnitude will differ if the conspecific is of a different strain than the test subject due to the enhanced novelty of the foreign sample.

Chapter 2: The effects of pharmacological manipulations on affective biases in male Lister Hooded rats

2.1 Chapter Aims and Objectives

The aim of this chapter is to examine the effects of two pharmacological manipulations of affective state in rodents using the affective bias test and reward learning assay. Specific chapter objectives are as follows:

- To investigate the abilities of amitriptyline and hydroxynorketamine to bias learning of new reward associations in rodents using the ABT.

- To investigate both the acute and sustained effects of amitriptyline and hydroxynorketamine on previously established negative affective biases in rodents using the ABT.

- To investigate any non-specific effects of amitriptyline and hydroxynorketamine on memory using the control RLA.

2.2 Introduction

Previous experimental work using the affective bias test has shown that both pharmacological and non-pharmacological manipulations of affective state induce biases in the task that mimic effects reported in human studies[87,251]. Acute treatment with stress hormones and drugs known to increase risk of depression in humans result in a negative bias toward the treatment-paired substrate in the ABT[261]. In contrast, acute administration of both SSRIs and SNRIs positively biases learning of new substrate-reward associations, and similar effects are seen following treatment with atypical antidepressants that act via non-monoaminergic mechanisms[246,319] (see **Figure 2.1**). The ABT has also been used to demonstrate that conventional and rapid-acting antidepressants differ in their ability to modulate affective biases. Systemic administration of ketamine, a drug with rapid-onset antidepressant effects, was shown to attenuate previously acquired negative biases, an effect not replicated with the SNRI venlafaxine[263]. It is important to note that non-antidepressant anxiolytics and various drugs of abuse fail to induce biases in the ABT, indicating the task's sensitivity to the specific effects of antidepressants[246].

Psychosocial and environmental manipulations of affective state have also been reported to induce biases in the ABT[320-21](see **Table 2.1** for a detailed list of previous experimental findings). Together, these findings suggest that the ABT is a task with high predictive, translational, and mechanistic validity. Additional data has shown no significant effects of sex or strain on induction of positive and negative affective biases[321], indicating that the task also demonstrates strong homological validity.

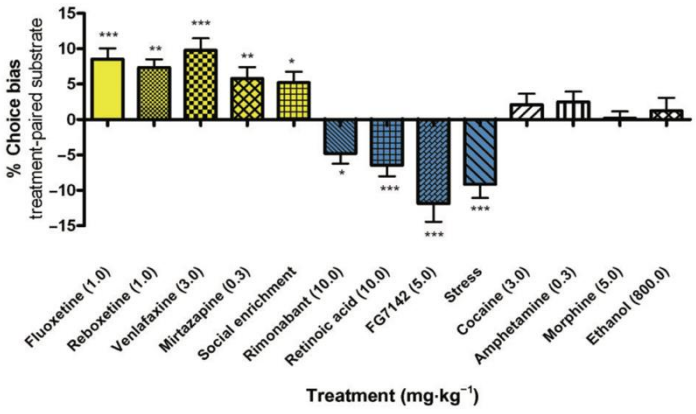

Figure 2.1: Summary of validation data for the ABT. Acute pharmacological and psychosocial manipulations of affective state induce biases in the rodent ABT which align with the effects observed in human studies. Yellow bars represent manipulations inducing a positive bias, and blue bars are those shown to induce a negative bias. White bars indicate drugs that failed to induce a significant bias in the task. Adapted from Hales *et al*. (2014)[251].

Acute Manipulations in the ABT			
Treatment	**Dose (effect)**	**Treatment**	**Dose (effect)**
Antidepressants		**Exogenous Hormones**	
agomelatine[a]	0.1 mg/kg (-) 0.3 mg/kg (-) 1.0 mg/kg (+)	**corticosterone**[c,d,e]	0.1 mg/kg (0) 1.0 mg/kg (0) 10.0 mg/kg (-) 30.0 mg/kg (-)
citalopram[a]	0.1 mg/kg (0) 0.3 mg/kg (0) 1.0 mg/kg (+) 3.0 mg/kg (0)	**oestradiol**[f]	1.0 µg/kg (+) 10.0 µg/kg (+)
fluoxetine[a]	0.3 mg/kg (+) 1.0 mg/kg (+) 3.0 mg/kg (+)	**progesterone**[f]	1.0 µg/kg (-) 10.0 µg/kg (0)
ketamine[b]	1.0 mg/kg (0) 3.0 mg/kg (0)	**Substances of Abuse**	
reboxetine[a]	0.1 mg/kg (+) 0.3 mg/kg (0) 1.0 mg/kg (-)	**amphetamine**[a]	0.3 mg/kg (0)
sertraline[c]	1.0 mg/kg (+) 3.0 mg/kg (0) 10.0 mg/kg (-)	**cocaine**[a]	3.0 mg/kg (0)
venlafaxine[a,e]	1.0 mg/kg (+) 3.0 mg/kg (+) 10.0 mg/kg (+)	**ethanol**[a]	800.0 mg/kg (0)
vortioxetine[c]	1.0 mg/kg (0) 3.0 mg/kg (+) 10.0 mg/kg (+)	**morphine**[a]	5.0 mg/kg (-)
Anxiolytic		**nicotine**[a]	0.06 mg/kg (+)
diazepam[a]	0.3 mg/kg (0) 1.0 mg/kg (0) 3.0 mg/kg (0)	**Psychosocial**	**Effect**
Pro-depressants		**playpen**[h]	+
FG7142[a,b,e]	1.0 mg/kg (0) 3.0 mg/kg (-) 5.0 mg/kg (-) 6.0 mg/kg (-)	**restraint stress and social isolation**[a]	-
retinoic acid[a]	1.0 mg/kg (-) 3.0 mg/kg (0) 10.0 mg/kg (+)	**social play**[a]	+
tetrabenazine[d]	1.0 mg/kg (0) 3.0 mg/kg (0) 10.0 mg/kg (-)	**tickling**[g]	+

Table 2.1: Summary of acute pharmacological and psychosocial manipulations in the ABT in rats. Adapted from Hinchliffe (2019)[321]. Effect: positive bias (+), negative bias (-), no effect (0). References: (a) Stuart *et al.* (2013)[246], (b) Stuart *et al.* (2015)[263], (c) Refsgaard *et al*. (2016)[319], (d) Stuart *et al*. (2017)[261], (e) Hinchcliffe *et al*. (2017)[262], (f) Hinchcliffe *et al*. (2020a)[322], Hinchcliffe *et al*. (2020b)[323], (h) Hinchcliffe & Jackson (2022)[320].

The reward learning assay is another bowl-digging task designed to measure cognitive biases in rodents. Proof of concept data for the RLA was obtained by testing whether pairing one digging substrate with a higher value reward would positively bias an animal to that substate during a preference test. An initial study found that, under control conditions, rats demonstrate

an increased preference for the higher reward-paired substrate[246]. Additional studies using the RLA have shown that drugs associated with pro-depressant risk in humans impair formation of a reward-induced positive bias in the rodent RLA[261]. Animals subjected to early life stress via maternal separation show similar reward learning deficits, suggesting the task holds strong construct validity[202]. The RLA was also recently validated in mice[264]. When testing effects of pharmacological affective state manipulations, the RLA is often used alongside the ABT as a means of controlling for any non-specific effects a drug may have on memory. The task requires that animals are kept in a "normal" affective state throughout the substrate-reward pairing sessions, meaning that the bias toward the high reward-paired substrate is due to the change in reward value rather than a dynamic change in affective state. Therefore, a drug which induces or attenuates biases in the ABT but fails to show an effect in the RLA can be assumed to have specific effects on affective processing unrelated to general memory impairment.

The aim of the experiments discussed in this chapter is to examine the effects of two pharmacological manipulations – amitriptyline and hydroxynorketamine. Amitriptyline belongs to the tricyclic class of antidepressants and for many years was one of the most widely used pharmacological treatments for MDD. Amitriptyline is known to increase transmission of NA and 5-HT by inhibiting NA transporters (NAT) and 5-HT transporters (SERT) at presynaptic terminals, leading to long-term changes in neurotransmission via desensitization of presynaptic autoreceptors[324-25]. The drug is thought have greater effects on noradrenergic transmission as a result of having nortriptyline as its main active metabolite[336]. In addition, amitriptyline acts as a competitive antagonist at both histaminergic (H1) and alpha-adrenergic receptors and has a particularly strong binding affinity for muscarinic (M1) receptors[327-28]. Amitriptyline demonstrates efficacy as an antidepressant in clinical studies[329-330] and has shown effectiveness in several physiological and behavioral animal models of depression[141,331-33]. However, it is no longer considered a first-line treatment for MDD due to its poor tolerability and prominent side effects, especially for those in outpatient treatment[334-36].

Hydroxynorketamine (HNK) is a minor metabolite of ketamine, formed as a result of the hydroxylation of norketamine[337]. Following reports of the rapid-acting antidepressant effects of ketamine[338-39], it has hypothesized that HNK may play a critical role in this mechanism[340]. Unlike ketamine, HNK has low affinity for NMDA receptors and has actually been found to act at the a_7-nicotinic acetylcholinergic receptor[341]. Research into the antidepressant effects of HNK has produced varied results, with the majority of studies having been conducted using

animal models. HNK has been reported to decrease immobility in the FST[342-43], reduce latency to feed in the NSFT[344], and reverse escape deficits in the learned helplessness model[345]. However, there are a seemingly equal number of studies using these assays in which HNK fails to produce antidepressant effects[346-48]. Although research into HNK as a novel antidepressant is still in its infancy, the compound is already proving to be an important part of understanding the underlying mechanisms of ketamine and other rapid-onset antidepressants.

2.3 Methods

2.3.1 Animals and Housing

The animals used in the following studies were a cohort of 16 male Lister Hooded rats weighing approximately 310 grams at the start of experimental manipulations (Envigo, UK). Animals were 9 weeks old at the start of experimental manipulations. The same rats were used in all experiments discussed in this chapter, and animals were given a minimum of 24 hours between treatments to potential carry-over effects. Animal weights were recorded daily, and growth was monitored against a standard growth curve for male Lister Hooded rats. Only males were used as previous studies comparing males and females in the ABT reported no sex differences[321]. Animals were housed in pairs in enriched home cages (55 x 35 x 21 cm) containing sawdust bedding, cardboard tubes, wood block chews, cotton rope, and red Perspex platforms (30 x 17 x 10 cm), under temperature-controlled conditions (21±1°C) and a 12-hour reverse light-dark cycle (lights off at 0815h). All behavioral testing was conducted during the dark cycle between 0900 and 1700h. Animals were maintained at approximately 90% of their free-feeding weight via restriction of access to laboratory chow (Purina, UK) to approximately 18g daily per rat. Water was provided *ad libitum* in the home cage but was not provided during behavioral procedures/testing. All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 as well as with University of Bristol guidelines and were approved by the University's Animal Welfare and Ethical Review Board. All experiments used *N* numbers based on previous studies[262-63] and power estimate $\alpha = 0.05$, $\beta >$ 80%.

2.3.2 Affective Bias Test (ABT)

Apparatus

All experiments using the ABT and RLA were conducted in a clear Perspex arena (40 x 40 cm) with digging substrates contained in two ceramic bowls (∅ = 10 cm) placed against the back wall of the arena. Each testing week of the experiment introduced the animal to three new digging substrates – two reward-paired substrates ('CS+A' or 'CS+B) and one unrewarded substrate ('CS-') that were matched for type of material as well as digging effort. The substrates were presented in two bowls in a pseudo-random order in the left or right position in the arena (see **Figure 2.2**).

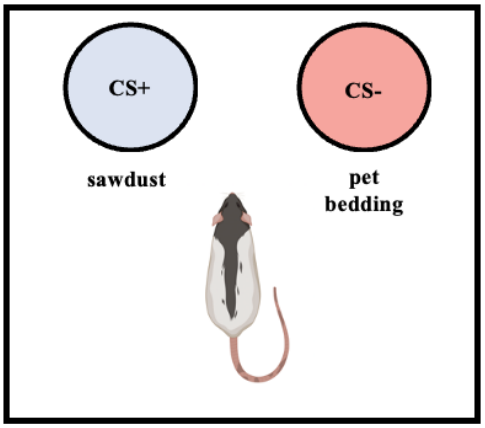

Figure 2.2: Experimental apparatus for the ABT and RLA (overhead view).

ABT/RLA Substrate Pairing			
	Substrate 'A'	**Substrate 'B'**	**Substrate 'Blank'**
test 1	coloured felt	shredded dishcloth	exfoliating gloves
test 2	fur	polyester	pompoms
test 3	cellulose sponge	corrugated paper	perlite
test 4	chubby wool	shoelaces	velcro
test 5	newspaper	tissue paper balls	paper pet bedding
test 6	gift ribbon	tights	umbrella
test 7	organza	silk	twine
test 8	timothy hay	coconut fibre	hessian sack
test 9	denim	rucksack straps	foam shapes
test 10	crepe paper	scarf yarn	sparkling fibre

Table 2.2: Examples of substrates used in the ABT and RLA.

Digging Training

The ABT and RLA training protocol requires the animals to complete five stages of digging training (one stage per day over a five-day period). During the first stage, each animal was

placed in an empty arena for 10 minutes for habituation. The next three stages of training involved training the animals to dig in a bowl filled with sawdust to obtain a single food reward pellet (45mg purified rodent tablets, containing sucrose, maltodextrin, corn starch, casein, corn oil, cellulose, minerals, silicon dioxide, vitamins, DL-methionine, magnesium stearate; Test Diet, Sandown Scientific, UK, catalogue number #1811155). On the day following habituation, each animal was placed in the arena and allowed to approach and explore two bowls containing no substrate and one reward pellet. The trial was considered complete once the rat had consumed the pellet and was removed from the arena, and the animal had to complete 12 trials in order to end the training session. During the next training stage, one of the two bowls was filled with 1cm of sawdust with a single reward pellet buried underneath. Each animal was given a cut-off time of 30s to begin exploring the bowls and start digging in the substrate-filled bowl. Once the animal had consumed the reward pellet, the bowl that did not contain sawdust was removed from the arena and replaced prior to the start of each new trial. As with the previous training stage, each rat was required to complete 12 trials before ending the training session. The next stage of digging training required the animal to complete 12 trials of digging through a bowl containing 2cm of sawdust and a reward pellet. On the final day of training, the animals underwent a discrimination task during which they were presented with bowls containing two novel digging substrates – a reward-paired substrate CS+ (mouse bedding), paired with a single reward pellet, and an unrewarded substrate CS- (shredded dishcloth). During each trial, the animal was placed in front of both bowls and allowed to choose one in which to dig for the pellet. Once the rat chose a substrate and began digging, the experimenter immediately removed the other bowl from the arena to prevent foraging. Trials were marked as either ‘correct’ or ‘incorrect’ depending on whether the animal chose the reward-paired substrate. A trial was marked as an ‘omission’ if the animal failed to approach the bowls within 30s of being placed in the arena. Prior to the start of the session, a reward pellet was crushed and sprinkled into each bowl to prevent the animals from making choices based on odor. The session ended once the rat achieved 6 correct trials in a row by choosing the reward-paired substrate.

ABT/RLA Training Stages				
Stages	**# of Bowls**	**Digging Substrates**	**# of Food Pellets**	**Criteria**
Habituation	0	None	0	10 min. of arena exploration
Digging Training 1	2	None	1	Completion of 12 trials
Digging Training 2	2	1 cm sawdust	1	Completion of 12 trials
Digging Training 3	2	2 cm sawdust	1	Completion of 12 trials
Discrimination	2	Mouse bedding and dishcloth squares	1	Completion of 6 correct consecutive trials (max. 20 trials)

Table 2.3: Summary of ABT and RLA training stages.

Pairing Sessions and Preference Testing

Each experiment using the ABT was based on four substrate-reward pairing sessions over four consecutive days followed by a preference test on the fifth day. Each week, the animal was presented with two different substrate-reward pairings, with each pairing presented during two different pairing sessions (one pairing was presented during sessions 1 and 3 and the other during sessions 2 and 4). During these pairing sessions, each animal learnt to associate two different substrates (CS+ A/B) with a food reward pellet while under either control/vehicle conditions or the manipulation condition. Each trial involved presentation of two digging substrates – one reward-paired substrate (CS+ A/B) and one unrewarded or 'blank' substrate (CS-). The CS- substrate was kept the same for all four pairing sessions in order to keep the context of both substrate-reward pairings as similar as possible. The reward was the same value for both conditions (a single reward pellet), and all additional factors (i.e., treatment, substrates, bowl location, pellet location) were fully counterbalanced. As with the discrimination session, a reward pellet was crushed and sprinkled into each substrate prior to the start of each session to avoid any odor-based decision making.

Presentation of one of the reward-paired substrates (the manipulation-paired substrate) was learnt following a treatment (e.g., drug administration) while the other was learnt under control conditions (e.g., vehicle administration). For each trial, the rat was placed in front of the two substrate-filled bowls and allowed to choose one in which to dig for a reward pellet. Once the animal made a choice, the other bowl was immediately removed from the arena by the

experimenter. A trial was marked as 'correct' if the animal chose the reward-paired substrate and 'incorrect' if they chose the unrewarded substrate. If the animal refused to approach and explore the bowls after 30s, the trial was marked as an 'omission.' If the animal approached the bowls but refused to make a choice or dug through the correct substrate but did not consume the pellet, the trial was marked as a 'fail.' Each pairing session continued until the animal had achieved 6 consecutive correct trials. A latency to dig was recorded for each trial. Following completion of four pairing sessions, a preference test was conducted in order to assess the animal's affective bias toward the reward-paired substrates. During each trial of the preference test, the rat was presented with both reward-paired substrates at the same time. Both substrates were reinforced with a single reward pellet under a random reinforcement schedule with a reward probability of one in three over 30 trials. Random reinforcement ensured the animals remained motivated to continue the task while preventing them from gaining new information about the substrate-reward association. The animals' choices, latency to dig, number of omissions, and number of pellets consumed were recorded by the experimenter. Choice bias was determined by comparing the number of choices made for the manipulation-paired substrate against the total number of trials. A similar calculation was also used to determine if there were any biases toward a particular substrate (A vs. B) or bowl location (left vs. right) across the cohort.

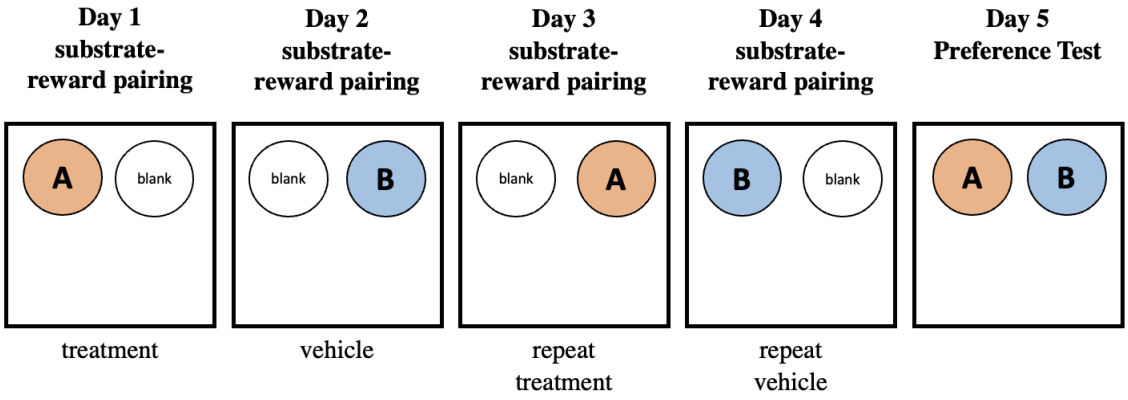

Figure 2.3: ABT methodology overview.

Pharmacological manipulation of affective state – acute retrieval

Each experiment followed the same protocol for the pairing sessions and preference testing as described above. For each week's pairing sessions, animals learnt to associate one of the substrate-reward pairings following administration of FG7142 (details below) and the other following administration of vehicle (see **Figure 2.4**). Subcutaneous injections utilized a procedure requiring minimal restraint of the animal and injection to their left or right flank (alternated daily) to reduce any stress associated with the injection process. On the fifth day of each week, the animals were administered the treatment prior to the preference testing session (pre-treatment times varied according to treatment/route of administration). For each experiment, the experimenter was blind to treatment and utilized the randomized, fully counterbalanced within-subject treatment design as outlined in **Table 2.4.**

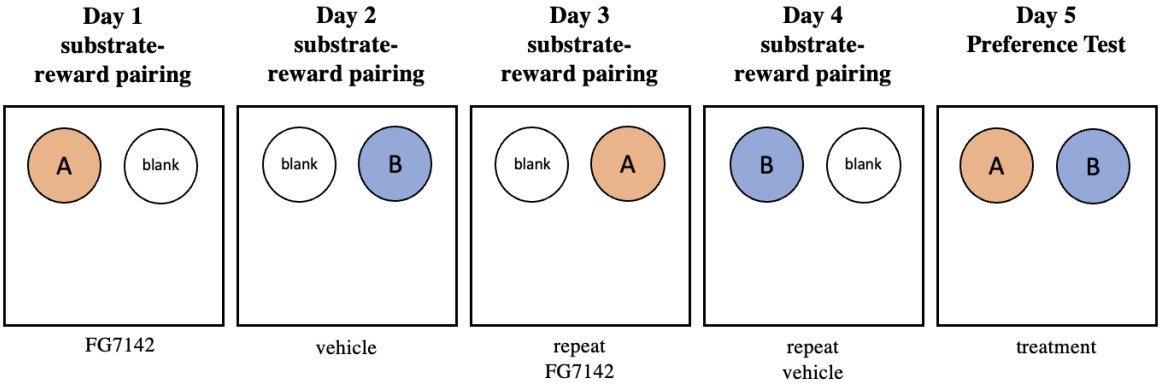

Figure 2.4: Methodological overview for testing acute effects of pharmacological affective state manipulations. Animals are treated with FG7142 to induce a negative bias toward one of the substrate-reward associations. On Day 5, animals are administered treatment prior to the preference test to determine the effect of treatment on substrate preference.

Treatment dose (mg/kg)				
Rat ID	**Week 1**	**Week 2**	**Week 3**	**Week 4**
Group 1	Dose 1	Dose 2	Vehicle	Dose 3
Group 2	Dose 2	Dose 3	Dose 1	Vehicle
Group 3	Dose 3	Vehicle	Dose 2	Dose 1
Group 4	Vehicle	Dose 1	Dose 3	Dose 2

Table 2.4: Example of randomized treatment over four weeks. All doses are fully counterbalanced using a Latin square design.

Pharmacological manipulation of affective state – sustained effects on retrieval

The pairing session protocol for these experiments was the same as for acute retrieval, with FG7142 being used to manipulate affective state. On the fifth day of each week, the animals were administered the treatment, and a preference test was conducted 24hrs after treatment to examine the effects of treatment on sustained retrieval (see **Figure 2.5**).

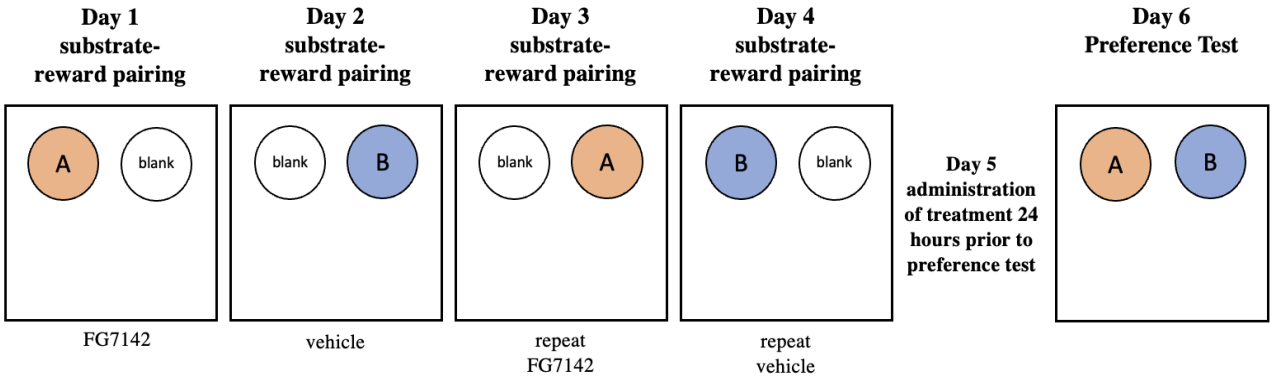

Figure 2.5: Methodological overview for testing sustained effects of pharmacological affective state manipulations. Animals are treated with FG7142 to induce a negative bias toward one of the substrate-reward associations. On Day 5, animals are administered treatment in their home cage, and a preference test is carried out 24 hours later to determine the effect of treatment on substrate preference.

Pharmacological manipulation of affective state during new learning

The ABT for these experiments followed the protocol outlined in the above section titled, *Pairing Sessions and Preference Testing*. During the pairing sessions, rats learnt to associate one of the substrate-reward pairings following administration of treatment and the other following administration of vehicle. On the fifth day of each week, a preference test was conducted to determine the effects of treatment on learning new substrate-reward associations (see **Figure 2.6**).

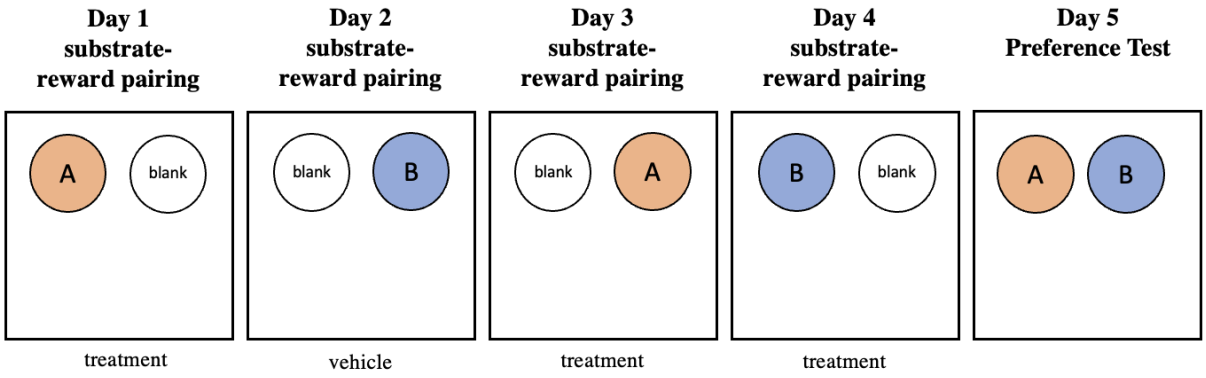

Figure 2.6: Methodological overview for testing the effects of pharmacological affective state manipulations on new learning. Animals are given treatment during pairing for one of the substrate-reward associations to determine if the treatment induces a bias toward the substrate during preference testing.

2.3.3 Reward Learning Assay (RLA)

The RLA employs the same digging training and discrimination task as outlined above. This assay involves inducing a reward-induced positive bias by training the animals to associate a digging substrate (CS+A) with two reward pellets and another substrate (CS+B) with one reward pellet. Each association was presented to the animal over four alternating pairing sessions (two per substrate). On the fifth day, the animals underwent a preference test during which they were presented with both reward-paired substrates and allowed to choose one of them. During this preference test, both substrates were equally rewarded with a single reward pellet using a random reinforcement design over 30 trials. A rat was considered to demonstrate a positive bias if they made a greater percentage of choices toward the two pellet-paired substrate.

2.3.4 The effects of amitriptyline (Experiment 1) and (2R, 6R) hydroxynorketamine (Experiment 2) on affective biases in male Lister Hooded rats

Experiments 1 and 2 followed the same protocols as described above for acute and sustained retrieval and new learning in the ABT as well for the RLA. Dose-response experiments were conducted over several weeks with animals receiving one treatment per week (fully counterbalanced), with animals receiving all drug treatments by the conclusion of each experiment.

The drugs used were FG7142 (benzodiazepine receptor inverse agonist, Sigma-Aldrich, UK; 3.0 mg/kg, administered subcutaneously with a 30 min. pre-treatment time), venlafaxine (serotonin-noradrenaline selective reuptake inhibitor, LKT Laboratories, UK; 3.0 mg/kg, administered orally with a 2hr pre-treatment time [Experiment 1] and intraperitoneally with a 1hr pre-treatment time [Experiment 2]), amitriptyline (Sigma-Aldrich, UK; 0.0, 0.3, 1.0, and 3.0 mg/kg, administered orally with a 2hr pre-treatment time, 24hr for sustained retrieval), and (2R, 6R) hydroxynorketamine (ketamine metabolite, Sigma-Aldrich, UK; 0.0, 0.3, 1.0, or 3.0 mg/kg administered intraperitoneally with a 1hr pre-treatment time, 24hr for sustained retrieval) (HNK). All drugs were dissolved in vehicle solutions: for FG7142 it was 0.9% sterile saline with Tween 80 (2 drops per 10ml saline) for Experiments 1 and 2.

For Experiment 1, both venlafaxine (VFX) and amitriptyline were prepared in a solution of condensed milk (Golden Acre, UK) and 0.9% sterile saline (2ml condensed milk per 10ml of solution). Prior to the start of the experiment, all animals were trained to consume a solution of sterile saline and condensed milk from a 1ml syringe to maintain oral drug administration. For acute and sustained retrieval and for reward-induced positive bias, animals were administered either 0.0, 0.3, or 1.0 mg/kg of amitriptyline. For new learning, animals received either 0.0, 0.3, 1.0, or 3.0 mg/kg of amitriptyline or 3.0 mg/kg of VFX.

For Experiment 2, both VFX and HNK were dissolved in a 0.9% sterile saline solution. For acute retrieval, animals were administered either 0.0, 0.3, 1.0, or 3.0 mg/kg of HNK. For sustained retrieval and reward-induced positive bias, animals were administered 0.0 or 3.0 mg/kg of HNK. For new learning, animals were administered either 0.0, 1.0, or 3.0 mg/kg of HNK or 3.0 mg/kg of VFX.

For all experiments, drugs were prepared on the day of treatment and administered in a dose volume of 1ml/kg. The doses for FG7142 and VFX were based on previous studies using these drugs in the ABT[246,261]. For amitriptyline, doses were chosen based on those reported as not impairing task performance in previous pilot studies. For HNK, doses were chosen based on those from previous studies using ketamine in the ABT[263].

2.3.5 Data Analysis

All data were analyzed using GraphPad Prism 9.0 (GraphPad Software, USA). The percentage of choice bias was calculated as the number of choices made for the treatment-paired substrate divided by the total number of preference test trials multiplied by 100 to give a percentage value. A value of 50 was then subtracted from this percentage to give a percentage choice bias score in which a positive bias toward the treatment-paired substrate gave a positive value, and a bias toward the vehicle-paired substrate gave a negative value.

The Shapiro-Wilk test was used to test data normality. For normally distributed data, the % choice bias was analyzed using a repeated-measures Analysis of Variance (RM-ANOVA) with TREATMENT as the within-subject factor for all dose-response studies. Post hoc analysis for each drug dose used Dunnett's test of multiple comparisons as well as a one-sample t test against a null hypothesized mean of 0% choice bias. Preference test data was also analyzed to detect the presence of any cohort bias toward a particular substrate or toward a particular bowl location. Analysis of the latency to respond as well as number of omissions was made using RM-ANOVA to determine any non-specific effects of treatment such sedation or changes in locomotor activity. For the pairing sessions, parameters including trials to criterion and latency to respond were analyzed using a paired t-test to compare vehicle vs. treatment data for each animal to assess any non-specific effects. Sphericity was checked for all data analyzed using ANOVA, and Geisser-Greenhouse corrected values were reported if data failed to meet sphericity assumptions. Significance was defined as $p < 0.05$, and exact p-values were reported except those where $p < 0.0001$.

2.4 Results

2.4.1 Experiment 1: The effects of amitriptyline on affective biases in male Lister Hooded rats

Acute administration of amitriptyline attenuated the FG7142-induced negative bias (one sample t-test, $t_{15} = 8.720$, $p < 0.0001$) toward the treatment-paired substrate (0.3-1.0 mg/kg, RM ANOVA, $F_{(1.827, 27.40)} = 23.43$, $p < 0.0001$, **Figure 2.7, panel A**). The effects were observed for both 0.3 mg/kg (post hoc Dunnet's test, $p < 0.0001$) and 1.0 mg/kg (post hoc Dunnet's test, $p < 0.0001$) doses. Neither the 0.3 mg/kg (one sample t-test, $t_{(15)} = 1.464$, $p = 0.1639$) nor 1.0 mg/kg dose (one-sample t-test, $t_{(15)} = 0.2997$, $p = 0.7685$) induced a significant bias when

compared to a hypothetical mean of zero percent choice bias. Treatment with amitriptyline resulted in no change in either omissions (0.3-1.0 mg/kg, RM ANOVA, $F_{(1.676, 25.14)} = 0.9268$, $p = 0.3935$) or latency to respond ($F_{(1.879, 28.18)} = 0.8873$, $p = 0.4171$) during the preference testing session. Treatment with FG7142 resulted in no change in response latency or trials to criterion during the pairing sessions (see **Table 2.5**).

When administered 24 hours prior to preference testing, amitriptyline attenuated the FG7142-induced negative bias (one sample t-test, $t_{(15)} = 4.333$, $p = 0.0006$) toward the treatment-paired substrate (0.3-1.0 mg/kg, RM ANOVA, $F_{(1.773,26.59)} = 7.081$, $p = 0.0045$, **Figure 2.7, panel B**). Effects were observed for the 1.0 mg/kg (post hoc Dunnet's test, $p = 0.0008$) dose in which there was no significantly positive or negative bias (one sample t-test, $t_{(15)} = 1.649$, $p = 0.1200$) toward the FG7142-paired substrate. During preference testing, administration of amitriptyline resulted in no change in omissions (0.3-1.0 mg/kg, RM ANOVA, $F_{(1.616, 24.23)} = 0.0617$, $p = 0.9078$) or latency to respond ($F_{(1.576, 23.64)} = 2.305$, $p = 0.1309$). Treatment with FG7142 resulted in no change in response latency or trials to criterion during the pairing sessions (see **Table 2.6**).

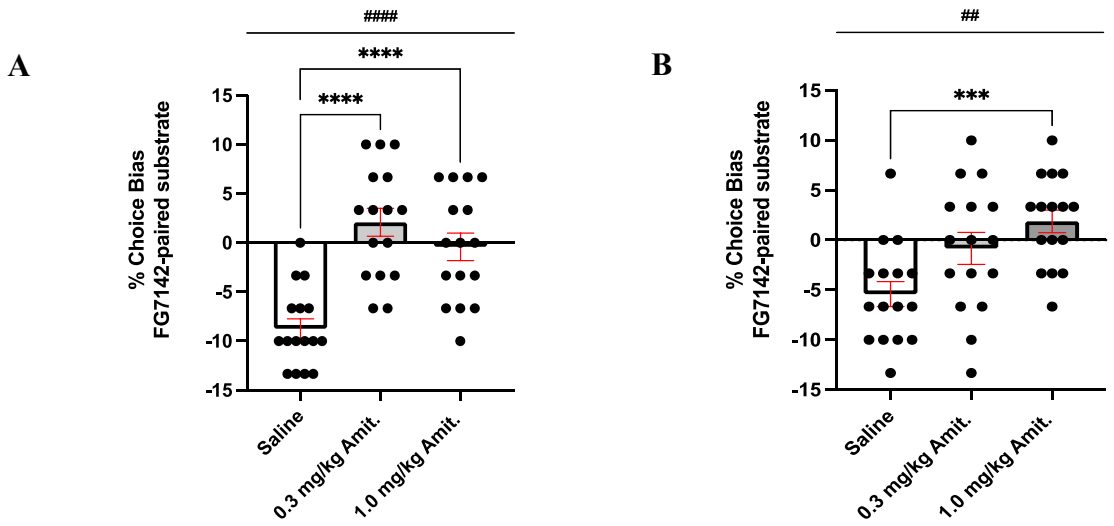

Figure 2.7: Acute administration of amitriptyline attenuates FG7142-induced negative biases with effects sustained 24 hours following treatment. Doses presented as mg/kg. Acute treatment with certain antidepressants can attenuate and/or reverse previously established negative biases induced via treatment with FG7142. The results shown in panel A demonstrate that administration of the tricyclic antidepressant amitriptyline attenuated this negative bias when tested 2 hours following treatment (RM ANOVA, $F_{(1.827, 27.40)} = 23.43$, $p < 0.0001$), while panel B illustrates amitriptyline's sustained effect when tested 24 hours following treatment (RM ANOVA, $F_{(1.773,26.59)} = 7.081$, $p = 0.0045$). Data shown as mean (n = 16 animals per group) % choice bias ± SEM, RM ANOVA with TREATMENT as factor ($^{\#\#\#\#}p < 0.0001$, $^{\#\#}p < 0.01$), post hoc Dunnet's test ($^{****}p < 0.0001$, $^{***}p < 0.005$).

Treatment	Response Latency (s)	Trials to Criterion
Vehicle	1.7 ± 0.1	6.9 ± 0.2
FG7142 (3.0 mg/kg)	1.6 ± 0.1	7.1 ± 0.3

Table 2.5: Pairing session data following treatment with FG7142 (acute retrieval). Treatment with anxiogenic compound FG7142 resulted in no change in response latency or trials to criterion during the substrate-reward pairing sessions. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

Treatment	Response Latency (s)	Trials to Criterion
Vehicle	1.5 ± 0.1	7.2 ± 0.2
FG7142 (3.0 mg/kg)	1.7 ± 0.1	7.3 ± 0.2

Table 2.6: Pairing session data following treatment with FG7142 (sustained effects on retrieval). Treatment with FG7142 resulted in no change in response latency or trials to criterion during the substrate-reward pairing sessions. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

Acute administration of amitriptyline positively biased new learning toward the treatment-paired substrate (0.3-3.0 mg/kg, RM ANOVA, $F_{(2.712, 40.68)} = 5.414$, $p = 0.004$, **Figure 2.8**) compared to the vehicle group. The effects were observed for the 0.3 mg/kg (post hoc Dunnet's

test, $p = 0.0181$) and 1.0 mg/kg (post hoc Dunnet's test, $p = 0.0047$) doses. Venlafaxine, serving as a secondary control, also induced a positive bias toward the treatment-paired substrate (one sample t-test, $t_{(15)} = 13.19$, $p < 0.0001$). During the pairing sessions, response latency (0.3-3.0 mg/kg amitriptyline and 3.0 mg/kg VFX, RM ANOVA, $F_{(2.627, 39.41)} = 1.049$, $p = 0.3749$) and trials to criterion (RM ANOVA, $F_{(3.087, 46.31)} = 1.571$, $p = 0.2082$) did not change following treatment. (see **Table 2.7**).

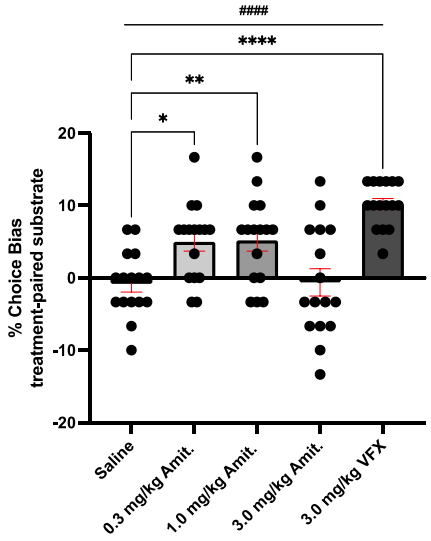

Figure 2.8: Amitriptyline induces a positive affective bias following acute treatment. Doses presented as mg/kg. Acute treatment with both typical and atypical antidepressants positively biases learning of substrate-reward associations in the ABT. When administered prior to substrate-reward pairing sessions, amitriptyline induced a positive bias toward the treatment-paired substrate (RM ANOVA, $F_{(2.712, 40.68)} = 5.414$, $p = 0.004$). Data shown as mean (n = 16 animals per group) % choice bias ± SEM, RM ANOVA with TREATMENT as factor ($^{\#\#\#\#}p < 0.001$), post hoc Dunnet's test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$).

Treatment	Dose (mg/kg)	Response Latency (s)	Trials to Criterion
Vehicle		2.0 ± 0.1	6.8 ± 0.2
VFX	3.0	1.9 ± 0.1	7.0 ± 0.2
Amitriptyline	0.3	1.9 ± 0.1	7.1 ± 0.3
	1.0	1.7 ± 0.1	7.1 ± 0.3
	3.0	1.9 ± 0.1	7.6 ± 0.2

Table 2.7: Pairing session data following treatment with amitriptyline and VFX. During pairing sessions, there was no change in response latency or trials to criterion during the substrate-reward pairing sessions following treatment with either amitriptyline or VFX. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

In the reward learning assay, animals under control conditions demonstrated a reward-induced positive bias toward the two pellet-paired substrate (one sample t-test, $t_{(12)} = 3.395$, $p = 0.0053$). Acute administration of amitriptyline prior to preference testing did not disrupt the formation of this bias (RM ANOVA, $F_{(1.974, 23.69)} = 0.0782$, $p = 0.9231$), as evidenced by the positive reward bias shown in the 0.3 mg/kg (one sample t-test, $t_{(12)} = 2.843$, $p = 0.0148$) and 1.0 mg/kg ($t_{(12)} = 3.692$, $p = 0.0031$) amitriptyline doses (see **Figure 2.9**).

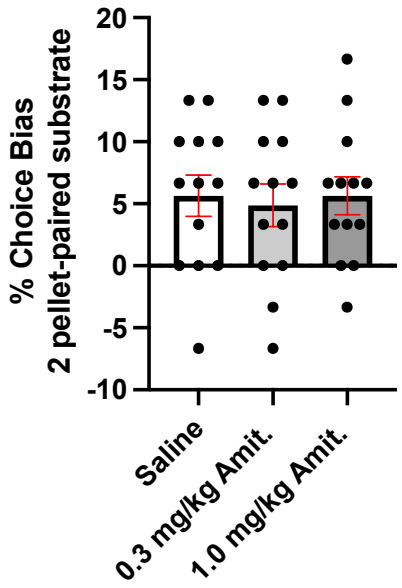

Figure 2.9: Acute treatment with amitriptyline had no effect on reward-induced positive bias. Doses presented as mg/kg. In the RLA, animals under control conditions demonstrate a positive bias toward the substrate associated with the higher-value reward (two vs. one reward pellet). Acute treatment with amitriptyline did not disrupt the formation of this reward-induced positive bias (RM ANOVA, $F_{(1.974, 23.69)} = 0.0782$, $p = 0.9231$), suggesting that the effects of amitriptyline are specific to affective state-induced biases. Data shown as mean (n = 13 animals per group) % choice bias ± SEM.

2.4.2 Experiment 2: The effects of (2R, 6R) hydroxynorketamine on affective biases in male Lister Hooded rats

Administration of FG7142 induced a negative affective bias toward the treatment-paired substrate (one sample t-test, $t_{(11)} = 8.613$, $p < 0.0001$), and this bias was attenuated via acute administration of HNK (0.3-3.0 mg/kg, RM ANOVA, $F_{(2.291,25.20)} = 4.861$, $p = 0.0133$)(see **Figure 2.10, panel A**). Effects were observed for the 1.0 mg/kg (post hoc Dunnet's test, $p = 0.0094$) and 3.0 mg/kg ($p = 0.0027$) doses, but not for the 0.3 mg/kg ($p = 0.1221$) dose. Treatment with HNK resulted in no change in number of omissions (0.3-3.0 mg/kg, RM

ANOVA, $F_{(1.1710, 18.81)} = 0.6471$, $p = 0.5115$) or latency to respond ($F_{(1.148, 12.62)} = 0.9534$, $p = 0.3607$) during the preference testing session.

When administered 24 hours prior to preference testing, HNK attenuated the FG7142-induced negative bias (one sample t-test, $t_{(11)} = 4.195$, $p = 0.0015$) toward the treatment-paired substrate (1.0-3.0 mg/kg, RM ANOVA $F_{(1.843,20.27)} = 9.733$, $p = 0.0013$) and induced a positive bias at the 3.0 mg/kg dose (one sample t-test, $t_{(11)} = 2.916$, $p = 0.0140$)(see **Figure 2.10, panel B**). Administration of HNK resulted in no change in omissions (1.0-3.0 mg/kg, RM ANOVA $F_{(1.773,19.51)} = 1.809$, $p = 0.1924$) or latency to respond (1.0-3.0 mg/kg, RM ANOVA $F_{(1.802,19.83)} = 0.2253$, $p = 0.7780$) during preference testing. During pairing sessions, there was no change in latency to respond or trials to criterion following treatment with FG7142 for both acute and sustained retrieval (see **Tables 2.8** and **2.9**).

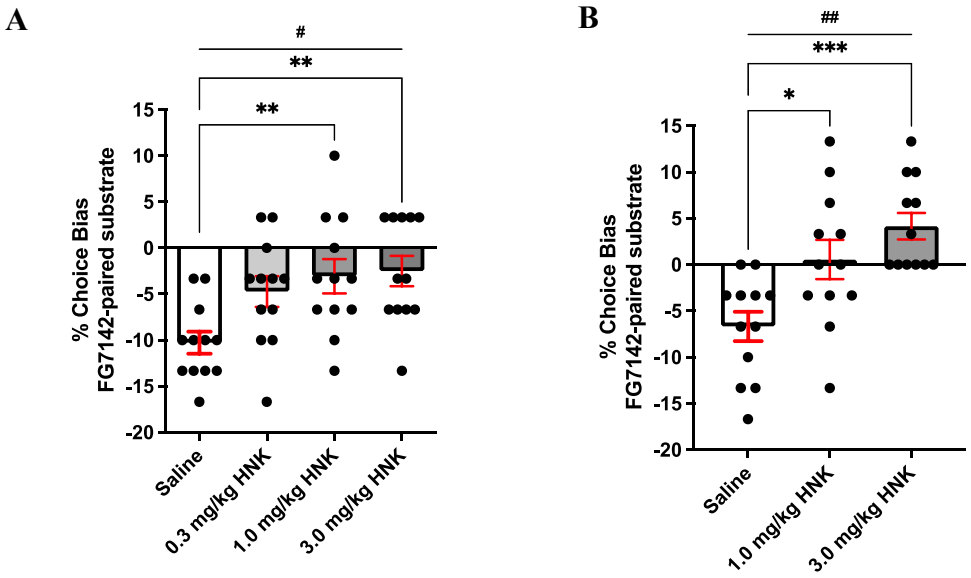

Figure 2.10: Acute administration of (2R, 6R) hydroxynorketamine attenuates FG7142-induced negative biases with effects sustained 24 hours following treatment. Doses presented as mg/kg. Panel A demonstrate that administration of hydroxynorketamine attenuated the FG7142-induced negative bias when tested 1 hour following treatment (RM ANOVA, $F_{(2.291,25.20)} = 4.861$, $p = 0.0133$), and these effects were sustained when tested 24 hours following treatment (RM ANOVA $F_{(1.843,20.27)} = 9.733$, $p = 0.0013$)(panel B). Data shown as mean (n = 16 animals per group) % choice bias ± SEM, RM ANOVA with TREATMENT as factor ($^{\#\#}p < 0.01$, $^{\#}p < 0.05$), post hoc Dunnet's test ($^{***}p < 0.005$, $^{**}p < 0.01$, $^{*}p < 0.05$). *Data illustrated in panel A collected by Julia Bartlett, University of Bristol.*

Treatment	Response Latency (s)	Trials to Criterion
Vehicle	3.2 ± 0.2	6.4 ± 0.1
FG7142 (3.0 mg/kg)	3.1 ± 0.2	6.5 ± 0.1

Table 2.8: Pairing session data following treatment with FG7142 (acute retrieval). Treatment with anxiogenic compound FG7142 resulted in no change in response latency or trials to criterion during the substrate-reward pairing sessions. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

Treatment	Response Latency (s)	Trials to Criterion
Vehicle	3.0 ± 0.2	6.8 ± 0.2
FG7142 (3.0 mg/kg)	2.9 ± 0.2	6.8 ± 0.2

Table 2.9: Pairing session data following treatment with FG7142 (sustained effects on retrieval). Treatment with FG7142 resulted in no change in response latency or trials to criterion during the substrate-reward pairing sessions. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

While there was an effect of treatment on % choice bias toward the treatment-paired substrate (RM ANOVA, $F_{2.428, 36.42)} = 5.723$, $p = 0.0044$), acute administration of HNK did not bias new learning for either the 1.0 mg/kg (post hoc Dunnet's test, $p = 0.9897$) or 3.0 mg/kg ($p = 0.9228$) doses. The effect was only observed for the 3.0 mg/kg venlafaxine group (post hoc Dunnet's test, $p = 0.0037$)(see **Figure 2.11**), in which administration of venlafaxine induced a positive affective bias compared to the vehicle group as well as against a hypothetical mean of 0% choice bias (one sample t-test, $t_{(15)} = 7.255$, $p < 0.0001$). During pairing sessions, treatment with HNK resulted in no change in trials to criterion (RM ANOVA, $F_{(1.669, 25.03)} = 3.099$, $p = 0.0708$) or latency to respond ($F_{(2.362, 35.43)} = 0.4932$, $p = 0.6455$)(see **Table 2.10**).

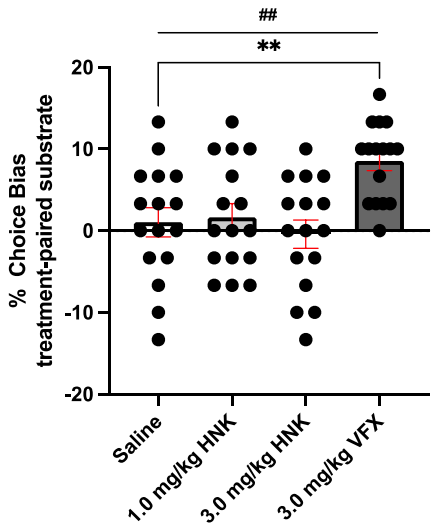

Figure 2.11: (2R, 6R) Hydroxynorketamine does not positively bias learning of substrate-reward associations. Doses presented as mg/kg. Acute treatment with both typical and atypical antidepressants positively biases learning of substrate-reward associations in the ABT, but this effect is not seen in all drugs with antidepressant effects. When administered prior to substrate-reward pairing sessions, HNK did not induce a bias toward the treatment-paired substrate (post hoc Dunnet's test, 1.0 mg/kg: $p = 0.9897$, 3.0 mg/kg: $p = 0.9228$). Data shown as mean (n = 16 animals per group) % choice bias ± SEM, RM ANOVA with TREATMENT as factor ($^{\#\#}p < 0.01$), post hoc Dunnet's test ($^{**}p < 0.01$)

Treatment	Dose (mg/kg)	Response Latency (s)	Trials to Criterion
Vehicle		1.9 ± 0.1	7.8 ± 0.4
VFX	3.0	2.0 ± 0.1	8.0 ± 0.3
HNK	1.0	2.2 ± 0.2	7.4 ± 0.2
	3.0	2.2 ± 0.2	7.7 ± 0.3

Table 2.10: Pairing session data following treatment with (2R, 6R) hydroxynorketamine and VFX. During pairing sessions, there was no change in response latency or trials to criterion during the substrate-reward pairing sessions following treatment with either HNK or VFX. Data shown as mean (n = 16 animals per group) ± SEM averaged from the pairing sessions for each treatment.

In the reward learning assay, animals under control conditions showed a positive bias toward the two pellet-paired substrate (one sample t-test, $t_{(11)} = 5.613$, $p = 0.0002$)(see **Figure 2.12**), and acute administration of 3.0 mg/kg HNK had no effect on the formation of this bias (paired t-test, $t_{(11)} = 0.4614$, $p = 0.6514$). Following treatment with HNK, animals still demonstrated a positive bias toward the two pellet-paired substrate (one sample t-test, $t_{(11)} = 5.702$, $p = 0.0001$). Treatment with HNK prior to preference testing resulted in no change in

number of omissions (paired t-test, $t_{(11)} = 1.393$, $p = 0.1911$) or latency to respond ($t_{(11)} = 1.107$, $p = 0.2920$).

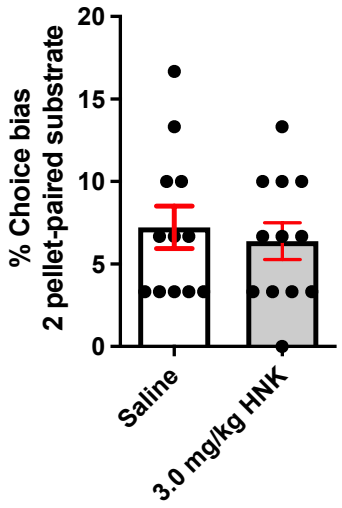

Figure 2.12: Acute treatment with (2R, 6R) hydroxynorketamine had no effect on reward-induced positive bias. Doses presented as mg/kg. Acute treatment with HNK did not disrupt the formation of the reward-induced positive bias demonstrated in the RLA under control conditions (paired t-test, $t_{(11)} = 0.4614$, $p = 0.6514$), suggesting that the effects of HNK are specific to affective state-induced biases. Data shown as mean (n = 12 animals per group) % choice bias ± SEM. *Data collected by Julia Bartlett, University of Bristol.*

2.5 Discussion

The results of the above experiment show that acute treatment with both amitriptyline and HNK attenuate FG7142-induced negative biases in the ABT with effects seen both immediately after treatment and 24 hours post-treatment. However, amitriptyline was able to positively bias learning of new substrate-reward associations while HNK failed to have an effect. In the RLA, neither amitriptyline nor HNK influenced the formation of a reward-induced positive bias when administered acutely, suggesting a specific modulation of affective state-induced biases.

These data offer further evidence that the affective bias test is a valid, highly translational assay for examining affective biases related to learning and memory. The induction of a negative bias following treatment with FG7142 replicates results of previous experiments using the ABT, as does the induction of a positive bias following treatment with the SNRI venlafaxine[246,321]. In addition, the task demonstrates mechanistic validity[105] as indicated by its ability to differentiate the effects of delayed versus rapid-acting antidepressants (discussed below). The RLA findings

also confirm these affective bias modifications are specific and not a result of any general impairments in learning and memory.

The ability of amitriptyline to positively bias new learning (**Figure 2.8**) is consistent with data from previous studies showing that acute treatment with conventional antidepressants induces a positive bias toward the treatment-paired substrate in the ABT[246,319]. The results of this experiment also concur with findings from human studies in which participants treated with antidepressants demonstrate positively biased affective processing[99-101]. These findings support the neuropsychological theory of antidepressant drug action which states that although subjective symptom improvement is delayed, treatment with antidepressants produces acute changes in emotional processing[82-87]. Acute treatment with HNK had no effect on new learning (**Figure 2.11**), and this replicates the lack of effect seen following ketamine treatment[263,321]. This suggests that affective biases in the context of new learning may be influenced by acute modulation of monoaminergic transmission following treatment with drugs that act on these neurotransmitters. Interestingly, an acute dose of 3.0 mg/kg amitriptyline failed to positively bias new learning, but this is likely due to enhanced experience of adverse side effects associated with the drug[349]. It is also possible that the higher dose produced a general amnesic effect as a result of increased muscarinic receptor antagonism.

The attenuation of the FG7142-induced negative bias following amitriptyline treatment (**Figure 2.7, panel A**) contrasts findings in which treatment with conventional antidepressants has no effect on previously established negative biases[263]. However, these results align with previous data showing that drugs with rapid-acting antidepressant effects, such as scopolamine and ketamine, attenuate negative biases in the ABT[263,321]. In addition, this finding validates reports of rapid symptom improvement in depressed patients treated with tricyclic antidepressants[13]. These data suggest a possible link to the muscarinic receptor antagonism exhibited by many TCAs, as drugs such as venlafaxine which act primarily on monoaminergic signalling fail to demonstrate similar effects in the ABT. Acute treatment with HNK was also able to mitigate the negative bias (**Figure 2.10, panel A**), suggesting that, unlike new learning, the mechanisms underlying the attenuation of existing negative biases are not linked to the same underlying mechanism.

Unlike ketamine, amitriptyline (**Figure 2.7, panel B**) failed to induce a positive bias toward the FG7142-paired substrate when tested 24 hours post-treatment. However, HNK was able to

induce a positive bias at the highest dose tested (**Figure 2.10, panel B**). It is known that ketamine acts as a non-competitive antagonist with high affinity for NMDA receptors, while HNK demonstrates a lower affinity level[350-52]. In addition, both amitriptyline and scopolamine have high affinity for M1 muscarinic receptors which are known to modulate NMDA receptor activity[353-54]. It has been theorized that the antidepressant effects of ketamine are linked to increased BNDF release and the subsequent increases in both neurogenesis and neuronal survival (**Figure 1.1**)[355-57]. This aligns with research demonstrating the interaction between NMDA receptors and BDNF signalling in learning and memory consolidation[358] as well as with studies reporting reduced BDNF and NMDA receptor expression in animals subjected to early life adversity[359]. Treatment with both amitriptyline and HNK have been also associated with increased BDNF expression[360-62], indicating a potential role of this mechanism in the acute attenuation of negative affective biases. Ketamine and scopolamine have additionally been found to indirectly stimulate glutamate release[363-64], and similar effects have been reported in relation to amitriptyline and HNK[365-66]. It is possible that, like scopolamine, amitriptyline exerts enough direct influence on each of these mechanisms to mediate negative affective biases acutely but not enough to mimic ketamine and HNK's ability to reverse negative memory-related biases to positive ones at 24 hours post-treatment. Due to HNK's low affinity for the NMDA receptor, it is unlikely that modulation of NMDA receptor activity alone is responsible for ketamine and HNK's shared reversal effect. However, ketamine and HNK have been shown to induce rapid (< 1hr post-treatment) upregulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), and it is believed that stimulation of these ionotropic glutamate receptors is associated with the increase in hippocampal BDNF levels observed 24 hours following treatment with both ketamine and its metabolite[340,367-68]. Although additional research is needed, it is possible that the shared ability of ketamine and HNK to attenuate and/or reverse negative affective biases is, in part, driven by enhanced AMPAR-induced activation of downstream signalling pathways which promote neurogenesis.

The RLA is often used in conjunction with the ABT to test whether a drug's effects are specific to biases induced by affective state manipulations versus biases influenced by changes in reward value. Neither amitriptyline (**Figure 2.9**) nor HNK (**Figure 2.12**) demonstrated an effect on the formation of a reward-induced positive bias in this task, confirming that the effects of both drugs are specifically due to modulation of affective biases rather than to general memory impairment. It would be interesting to test the 3.0 mg/kg amitriptyline dose in this

assay to confirm if the inability to bias new learning at this dose is linked to the general amnesic effects associated with greater activity at muscarinic receptors.

It is also important to note that, in experiments investigating the effects of these drugs on previously learnt negative affective biases, treatment with FG7142 did not influence measures such as the number of trials to criterion or latency to respond. A similar lack of effect was observed following all treatments in experiments investigating effects on new learning. This data indicates that the affective biases (both positive and negative) developed during pairing sessions were influenced solely by changes in the emotional valence of the experience at the time of learning rather than by learning impairments or changes in motivation.

The results discussed in this chapter demonstrate the ability of the ABT to differentiate between the neuropsychological effects of conventional versus rapid-acting antidepressants on affective biases relating to learning and memory. It remains unclear exactly how amitriptyline and HNK influence affective processing, particularly in terms of their shared ability to modulate previously established negative biases in the ABT. One explanation could be that, like other monoaminergic antidepressants such as venlafaxine, amitriptyline biases new learning in the ABT by increasing positive affect, while the acute attenuation effect shared by amitriptyline and HNK results from decreased negative affect. The ability of HNK to reverse negative biases at 24 hours indicates an additional increase in positive affect, an effect distinct from that seen in studies of new learning and one that is unlikely attributable to changes in monoaminergic transmission. Although there is clinical evidence to suggest that the acute positive changes in affective processing are associated with the monoaminergic components of conventional antidepressants[99-101], there is currently no published data regarding the effects of ketamine and other rapid-acting antidepressants on affective biases in humans. Discerning the specific mechanisms by which these and other rapid-acting antidepressants exert their effects on affectively biased memories warrants further detailed investigation, as doing so may have profound implications for our current understanding of the pathology of MDD as well as for future antidepressant drug research and development.

Chapter 3: Measuring olfactory self-recognition in male Long Evans rats

3.1 Chapter Aims and Objectives

The aim of this chapter is to determine whether rodents demonstrate self-recognition in an olfaction-based measure of self-awareness. Specific chapter objectives are as follows:

- To investigate whether rats exhibit a scent preference when presented with both their own scent and that of a conspecific's.

- To determine if a scent preference is influenced by whether the novel scent belongs to a rat of the same strain versus a different strain.

3.2 Introduction

For many animal species, olfaction is the primary sense used to navigate the environment and engage in social behaviors with conspecifics. The first study measuring self-recognition in an olfactory species was conducted by Bekoff and colleagues using a "yellow snow" test. In this study, the test subject (a dog named Jethro) repeatedly demonstrated preference for snow saturated with his own urine compared to snow containing urine from other dogs, regardless of the sex or placement of the novel sample[309]. Bekoff's protocol was adapted by Horowitz in 2017 and modified to look specifically at self-recognition rather than general scent marking. This study found that dogs spent more time investigating the odors of other dogs compared to their own. However, when a sample of their own odor was contaminated using a spleen sample acquired from a canine necropsy, test subjects spent more time investigating the modified sample[310]. The study also reported that dogs spent more time investigating the modified odor compared to the novel odor by itself, suggesting that neophilia alone was not driving the investigative behavior. This "olfactory mirror test" was also used in a study of grey wolves which produced similar results[369]. Although the results of Horowitz's study were met with controversy, the results offered evidence that a species may be more likely to demonstrate self-awareness when presented with stimuli most relevant to their sensory experience.

Olfactory awareness has been reported in non-terrestrial species, with one study finding that male cichlids preferred caves scented with their odor over caves with odors from both familiar and unfamiliar conspecifics[307]. Another study using storm petrels found that chicks were able to discriminate between their own odor and a conspecific's, even in the absence of visual nesting cues[308]. These findings, among others, suggest that olfaction may be a critical component of self-recognition in some species, and tasks using solely visual stimuli may not have enough salience to induce self-directed behavior.

Few studies have examined whether rodents are capable of self-awareness. In addition to the studies discussed in Chapter 1, Section 5.3 (*Measuring Self-Awareness in Rodents*), there are reports suggesting that rodents are capable of, to some extent, recognizing their own behaviors and distinguishing themselves from conspecifics. A study by Beninger *et al*. (1974) used a four-lever operant task in which the lever providing reward was determined by the rat's own behavior at the onset of a tone[370]. During testing, it was found that rats were able to discriminate above chance, and it was argued that these results indicated awareness of internal state and

mimicked verbal discriminations of behavior seen in humans. Another study reported that mice spent more time investigating a transparent cage containing an unfamiliar conspecific compared to a mirror cage[316]. The same study also found that mice with tape on their heads spent more time in front of a mirror than untaped controls but failed to engage in any self-directed behaviors (i.e., attempting to remove the tape).

All of the studies that have been published regarding rodent self-awareness use tasks which rely heavily on vision or tactility. However, it has been well-established that rodents engage with their surroundings primarily through olfaction. Both rats and mice use olfactory cues to navigate their environment, identify conspecifics, and determine their place within social hierarchies[371-73]. Olfaction is also used to initiate mating behavior and promote genetic diversity[374-75]. Despite this knowledge, there has yet to be a study investigating rodent awareness using olfactory stimuli. The aim of the following study is to determine whether rats will demonstrate a preference for their own scent over that of a conspecific's, either from the same strain or a different strain.

3.3 Methods

3.3.1 Animals and Housing

The animals used in the following studies were a cohort of 13 male Long Evans rats that were 12 weeks old and weighing approximately 310 grams at the start of the study (Envigo, UK). Animal weights were recorded weekly, and growth was monitored against a standard growth curve for male Long Evans rats. Animals were housed in pairs in enriched home cages (55 x 35 x 21 cm) containing sawdust bedding, cardboard tubes, wood block chews, cotton rope, and red Perspex platforms (30 x 17 x 10 cm), under temperature-controlled conditions (21±1°C) and a 12-hour reverse light-dark cycle (lights off at 0815h). All behavioral testing was conducted during the dark cycle between 0900 and 1700h. Laboratory chow (Purina, UK) was provided *ad libitum* in the home cage. Water was provided *ad libitum* in the home cage but was not provided during behavioral procedures/testing. All procedures were conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986 as well as with University of Bristol guidelines and were approved by the University's Animal Welfare and Ethical Review Board.

3.3.2 Rodent Olfactory Self-Recognition Task

Apparatus

The experiment was conducted in a circular Perspex arena (∅ = 78cm) with two plastic scent pots (∅ = 9cm) placed equidistant from each other in the center of the arena (**Figure 3.1**). Each sample contained 50µl of urine and was presented on a 1x1cm square of lining paper placed inside each of the pots. A lid with a circular hole in the center (∅ = 1.5cm) was attached to each pot to allow the animal to engage with the scent while preventing them from removing the paper from the pot. A Logitech web camera was suspended above the arena to film each session.

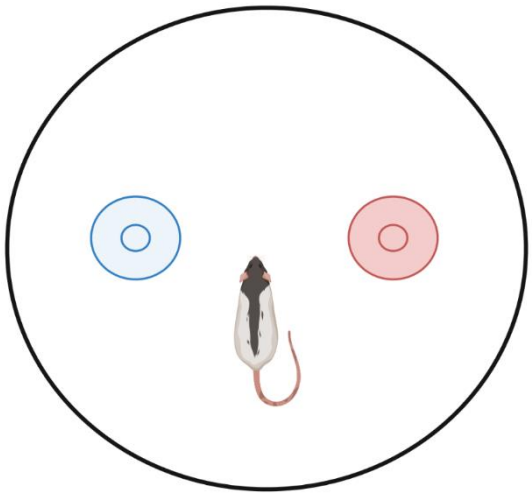

Figure 3.1: Experimental apparatus for olfactory self-recognition task (overhead view).

Sample Collection

Urine samples from three categories from animals were collected for this experiment, with samples being taken from the test subject (SUBJECT), a novel Long Evan's rat (NOVEL – LE STRAIN), and a novel Lister Hooded rat (NOVEL – LH STRAIN). Two Long Evans and two Lister Hooded rats were used as the sources for the novel samples as part of the counterbalanced design (labelled as **LE-1/LE-2** and **LH-1/LH-2**). Both the Long Evans and Lister Hooded rats used for the novel samples were housed in a different room from the test subjects. Urine was collected by placing the rat in an empty, unlined home cage and administering 10ml of a 5%

sucrose water solution to the animal orally. Once the animal urinated, the sample was collected and frozen for later testing. All samples were collected prior to the start of habituation sessions.

Pilot Testing

Prior to the current experiment, a pilot study was conducted based on the protocol outlined in Horowitz (2017)[290]. Using the experimental apparatus seen in **Figure 3.2** (below) and sample collection technique outlined above, animals underwent a single testing session in which they were presented with four bowls containing the following samples: the subject's (SUBJECT), a novel Lister Hooded rat (NOVEL), combined sample of two pair-housed Lister Hooded rats (NOVEL CAGEMATES), and a combined sample of the subject's urine modified with urine from a novel Lister Hooded rat (SUBJECT - MODIFIED). The urine used for the NOVEL, NOVEL CAGEMATES, and SUBJECT MODIFED samples were all collected from different Lister Hooded rats, and sample donors were fully counterbalanced. During testing, animals were placed in the arena containing the four equidistant scent pots with location of scents being fully counterbalanced. Once placed in the arena, the rat was allowed to approach and explore all four pots for 10 min. before being removed by the experimenter. Video footage of the test sessions was collected and manually coded by researchers to determine the amount of time the animals spent sniffing each scent pot as well as the number of times the animal approached each scent pot.

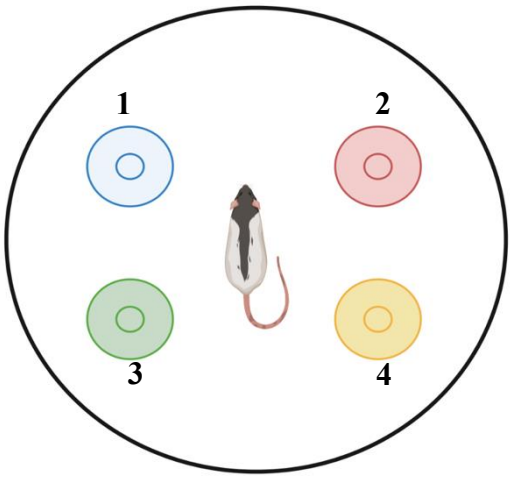

Figure 3.2: Experimental apparatus used in pilot study. Sample key: (1) SUBJECT, (2) NOVEL, (3) NOVEL CAGEMATES, (4) SUBJECT MODIFIED.

As illustrated in **Figure 3.3 (panel A)**, the results of this pilot test show no effect of scent on overall sniffing time for any of the samples (RM ANOVA, $F_{(1.703, 20.43)} = 2.428$, $p = 0.1196$). In addition, there was no effect of scent on the number of approaches to each scent pot (RM ANOVA, $F_{(2.205, 26.46)} = 0.2955$, $p = 0.7672$) **(panel B)**. These preliminary results informed the protocol of the current study (outlined below) in which the primary aim was to determine whether the animals would demonstrate a preference when presented with their own scent versus the scent of a single novel rat. The secondary aim of this experiment was to determine whether the magnitude of the preference would be influenced by the strain of the novel rat.

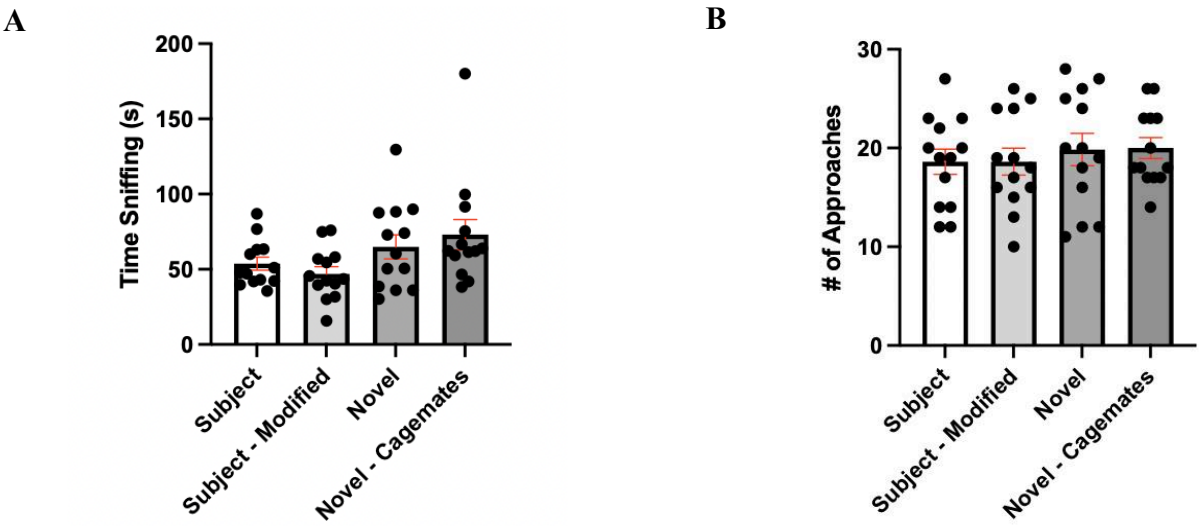

Figure 3.3: SCENT did not affect the number of approaches to each sample pot or the overall time spent sniffing each sample. Previous studies have demonstrated that animals who use olfaction as their main sensory system typically demonstrate a preference for their own scent over that of a conspecific. In this pilot experiment, rats were presented with equidistant pots containing four urine samples – the SUBJECT's, a NOVEL conspecific, a combined sample from two NOVEL CAGEMATES and a sample of the subject's own urine contaminated with that of a conspecific's (SUBJECT MODIFIED). The above figures illustrate that rats spent similar amounts of time sniffing each sample (RM ANOVA, $F_{(1.703, 20.43)} = 2.428$, $p = 0.1196$) (**panel A**) and showed no preference in terms of how many times they approached each scent pot RM ANOVA, $F_{(2.205, 26.46)} = 0.2955$, $p = 0.7672$) (**panel B).** Data shown as mean (n = 13 animals per group) seconds spent sniffing and mean number of approaches ± SEM.

Testing

For the current study, animals were habituated to the arena containing two empty pots for three 10 min. sessions prior to the testing sessions (once per day for three days). Each animal underwent two testing sessions to compare the difference between 'SUBJECT vs. LE strain'

and ‘SUBJECT vs. LH strain,’ with presentation of scents being fully counterbalanced. Each scent was also presented in a counterbalanced order on either the left or right side of the arena as outlined in **Table 3.1**. Once placed in the arena, the rat was allowed to approach and explore both pots for 10 min. before being removed by the experimenter. Once each animal had completed their test session, both the pots and arena were fully sanitized, and new scent samples were prepared for the next animal’s testing session.

Rat ID	Session 1	Session 2
1	LH-1 (left)	LE-1(right)
2	LE-1 (right)	LH-2 (left)
3	LH-2(right)	LE-2 (left)
4	LE-2 (left)	LH-1(right)
5	LH-1 (left)	LE-1(right)

Table 3.1: Presentation of NOVEL scents with fully counterbalanced location/sample #.

Data Analysis

DeepLabCut software[376] was used to extract and analyze data from video footage of each session. Using the scent pots and the rat’s nose as regions of interest, researchers manually labelled images extracted from each video in order to train the software’s neural network and track the animal’s movements in relation to each scent pot. The trained network then analyzed each video, delivering a readout of the number of seconds the animal’s nose spent at each pot. Videos were also visually coded by researchers using a custom novel object video counting software. Data for each scent was then compared using discrimination ratios, calculated as the amount of time spent sniffing the SUBJECT’s scent subtracted from time spent sniffing the NOVEL scent which was then divided over the total sniffing time for both samples. Animals who spent more time sniffing their own scent were given a negative discrimination ratio, and animals who spent more time sniffing the donor scent received positive ratios. Discrimination ratios were analyzed using a paired t-test to determine effect of LE vs. LH strain and a one sample t-test (hypothetical mean of 0) to determine effect of SUBJECT vs. NOVEL scent. To determine the effect of donor strain on sniffing time, data was analyzed using a 2-way analysis of variance (ANOVA) with SCENT and STRAIN as factors. In order to determine the effect of overall time spent in the arena on sniffing time, data from each 10-minute session was recorded at 60 second intervals and analyzed using a 2-way analysis of ANOVA with MINUTE and SCENT as factors via GraphPad Prism 9.0 (GraphPad Software, USA). Post-hoc analysis used Sidak’s test of multiple comparisons.

3.4 Results

When presented with samples of their own urine versus those from a novel donor, rats spent similar amounts of time sniffing both the SUBJECT and NOVEL scents (2-way ANOVA, $F_{(1,12)} = 0.9456$, $p = 0.3500$) (**Figure 3.4**). However, when the donor sample was that of the LH strain, rats spent more time sniffing both the SUBJECT and NOVEL samples than when the donor sample was taken from the LE strain (2-way ANOVA, $F_{(1,12)} = 5.279$, $p = 0.0404$). There was no interaction effect between STRAIN and SCENT (2-way ANOVA, $F_{(1,12)} = 0.1865$, $p = 0.6735$). Analysis of discrimination ratios revealed no effect of SUBJECT vs. NOVEL scent on overall sniffing time, and this effect did not change when the NOVEL scent came from a LE or LH donor rat (paired t-test, $t_{(12)} = 0.5502$, $p = 0.5923$) (**Figure 3.5**).

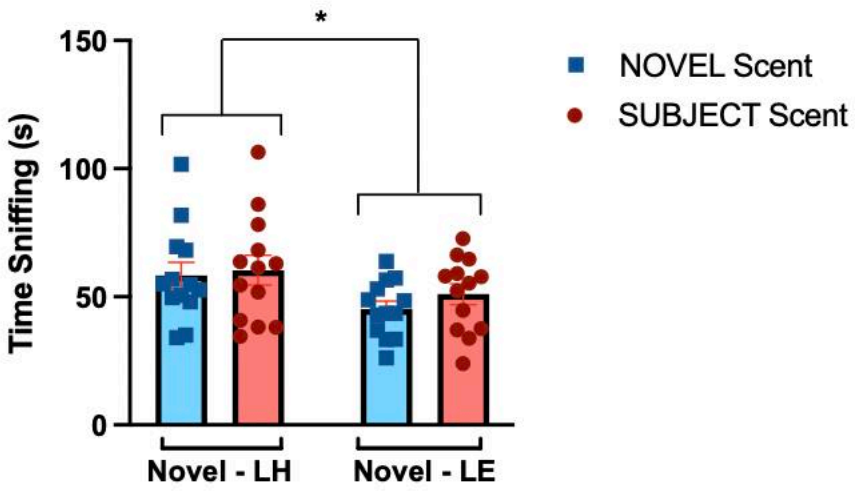

Figure 3.4: Rats spent more time sniffing both the SUBJECT and NOVEL samples when the NOVEL sample was collected from a different strain. Rats demonstrated no preference for either the SUBJECT or NOVEL sample (2-way ANOVA, $F_{(1,12)} = 0.9456$, $p = 0.3500$). Overall time spent sniffing both samples increased when the NOVEL scent was collected from a rat of a different strain (2-way ANOVA, $F_{(1,12)} = 5.279$, $p = 0.0404$). Data shown as mean (n = 13 animals per group) time spent sniffing ± SEM, 2-way ANOVA with LE vs. LH STRAIN and SUBJECT vs. NOVEL SCENT as factors ($^{*}p < 0.05$).

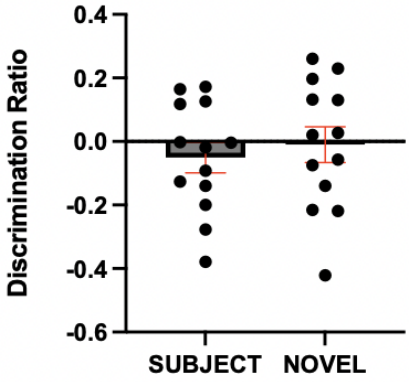

Figure 3.5: Effect of STRAIN and SCENT on overall sniffing time presented as discrimination ratios. Ratios calculated as the amount of time spent sniffing SUBJECT scent subtracted from time spent sniffing NOVEL scent divided over total sniffing time for both samples. No effect of STRAIN on overall sniffing time regardless of whether the NOVEL sample came from the subject's own strain or a different strain (paired t-test, $t_{(12)} = 0.5502$, $p = 0.5923$). Data shown as mean (n = 13 animals per group) time spent sniffing ± SEM.

When analyzing each 60-second interval of the 10-minute testing sessions, results demonstrate an effect of MINUTE on the amount of exploration (2-way ANOVA, $F_{(4.921, 59.05)} = 7.507$, $p < 0.0001$), suggesting that time spent sniffing each sample decreased as the session continued (**Figure 3.6**). There was no effect of SCENT on sniffing time (2-way ANOVA, $F_{(15.14, 18.17)} = 3.458$, $p = 0.0643$), and there was no interaction effect between SCENT and MINUTE (2-way ANOVA, $F_{(7.415, 88.98)} = 1.308$, $p = 0.2532$). However, sniffing time for the NOVEL LE scent was significantly lower at the two-minute timepoint (post hoc Sidak's test, $p = 0.0019$).

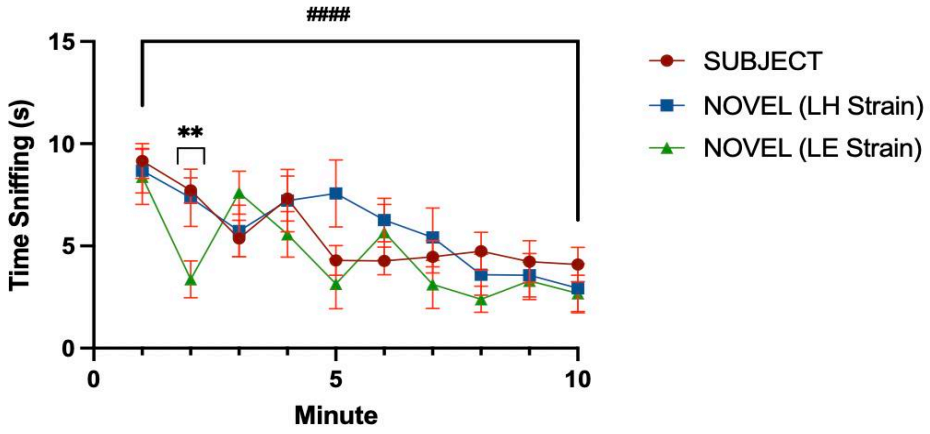

Figure 3.6: Overall sniffing time decreased for all scents as time increased. Data recorded at each 60-second interval of the testing session. Rats spent less time exploring/sniffing all samples as testing continued (2-way ANOVA, $F_{(4.921, 59.05)} = 7.507$, $p < 0.0001$). Rats spent less time sniffing the NOVEL LE scent at the two-minute time point (post hoc Sidak's test, $p = 0.0019$). Data shown as mean (n = 13 animals per group) seconds spent sniffing ± SEM, 2-way ANOVA with MINUTE and SCENT as factors ($^{\#\#\#\#}p < 0.0001$), post hoc Sidak's test ($^{**}p < 0.01$).

3.5 Discussion

The results of the above experiment demonstrate that, when presented with their own scent alongside that of a novel conspecific, rats spent equal time sniffing both samples. However, when the novel scent was taken from a rat of a different strain, sniffing time for both the subject's own sample and the novel sample was greater compared to when the novel sample came from a rat of the same strain.

The effect of MINUTE on the sniffing time for each sample was expected as a result of habituation to the presence of the samples. The decrease in sniffing time for the NOVEL LE sample at the second timepoint (**Figure 3.6**) supports the trend in which rats spent less time sniffing the NOVEL LE (same strain) scent than both their own scent and the NOVEL LH (different strain) scent. When coupled with the data outlined in **Figure 3.4**, the results of this experiment suggest a preference for social novelty in rats. This finding aligns with previous reports of rats demonstrating increased investigative behavior toward novel compared to familiar conspecifics[377-78]. Interestingly, this level of social neophilia contrasts behaviors observed in other rodent species[379-80] and instead mimics findings in studies of species such as canines[381]. Outside of a social context, neophilia has been reported to be greater in laboratory rats than in wild types[382] and is consistent across strains[383]. It is, therefore, likely that the presence of a scent with a higher degree of social novelty prompted greater exploration in this experiment, particularly at the start of the encounter when the animal has yet to encode the olfactory cue and form the relevant social memory. This may also explain why the olfactory presence of a rat from a different strain increased exploration of the subject's own scent as well.

In terms of investigating awareness, it cannot be determined from the results of this experiment whether rats are capable of the level of self-awareness believed to be demonstrated by species who pass Gallup's mirror test. However, it has been theorized that animals may possess different levels of awareness depending on their cognitive capabilities. The results outlined above suggest that rats may possess a level of social self-awareness. This type of awareness extends beyond simple proprioception or the ability to feel physical sensations but also differs from the introspective awareness that Gallup argues is unique to humans and certain primate species[291]. Degrazia (2009)[273] argues that social self-awareness requires awareness of oneself as a member of a larger group as well as an understanding of how one's position within the

group influences behavior. Given what is known about their behavior, the idea that rats possess social self-awareness is very plausible. Rats typically live in large social groups, and to be aware of one's place within the group would be highly advantageous. In the context of this experiment, the ability to recognize the difference between members of familiar versus unfamiliar social groups poses many benefits in terms of protecting resources and identifying intruders[384]. Therefore, the preference for social novelty demonstrated in this experiment may be indicative of social self-awareness in rats.

Whether rats are self-aware cannot be definitively ruled out due to the methodological limitations of this experiment. First and foremost, the only recorded measure was time spent sniffing each sample, and no measures of self-directed behavior were considered. Therefore, it cannot be determined if the test subjects recognized their scent as coming from their individual being. In addition, the scent pots were placed quite close together due the relatively small size of the arena. It is possible that the animals may have indicated a preference for one scent if more effort was required to explore both samples.

Ultimately, it is still unclear whether laboratory rats possess the same level of self-awareness demonstrated by chimpanzees and other species in vision-based tasks. This is in part due to the small number of studies investigating rodent self-awareness as well as high variation between protocols. The results of these studies may also be due to confounding factors such as neophilia or procedural learning of the task. There are some who claim that all sentient animals possess self-awareness[273], while others argue that different species are capable of varying levels of awareness which may or may not include a sense of self[272]. Whether it is even possible to measure self-awareness in animals is still a subject of great debate due to the inability for animals to fully convey their internal experience. Although Gallup's mirror test is considered the gold standard of studying awareness in non-human species, results have been inconsistent and do not account for differences in sensory experiences between species.

Understanding the extent to which laboratory rodents experience awareness is highly important for preclinical research, primarily in terms of maintaining and improving animal welfare practices. The degree to which a rodent is self-aware will influence the nature of distress and suffering experienced by the animal in response to aversive procedures or experiences. Animals with a greater level of cognitive awareness may experience heightened pain or distress in response to changes in their environment, and memories of such distress may hold more

emotional salience for these species[272]. In addition, understanding the cognitive capabilities of different animal species prevents researchers from using human definitions of suffering to ascertain the experience of non-human animals.

Chapter 4: General Discussion

The affective bias studies evaluated two additional pharmacological treatments with either established antidepressant effects (amitriptyline) or potential antidepressant effects (HNK). These studies were the first characterization of a tricyclic antidepressant in the affective bias test and found effects which were similar to previous studies with both conventional, delayed onset antidepressants and rapid-acting antidepressants. Whilst this is not surprising given the pharmacology of amitriptyline, most of the established literature would consider this to be in the class of delayed onset antidepressants. The concept of delayed onset is a complex one, and the majority of studies have focused on second generation antidepressants e.g., SSRIs. Early studies with tricyclic antidepressants would actually support a more rapid onset of clinical benefit than implied by established hypotheses. In the study by Kuhn in 1958[13], imipramine was found to have effects after a few days of treatment not unlike the effects seen clinically with the muscarinic antagonist, scopolamine[389-90]. At this time, no other TCAs or MAOIs have been studied in the ABT, but the findings from these experiments suggest these first generation antidepressants may have different neuropsychological effects in addition to efficacy and rate of onset than the second generation re-uptake inhibitors. Although the side effects and safety issues associated with these drugs limit their use clinically, there may be opportunities to develop novel treatments or combination treatments based on these findings which may yield improved efficacy and more rapid onset of clinical benefits.

HNK was investigated in the ABT due to the body of literature from conventional rodent models of depression suggesting this metabolite of ketamine is the main mediator of its clinical benefits. Although the ABT studies found effects similar to ketamine, thus supporting its potential as a RAAD, the dose required to achieve these effects was higher than that of ketamine in the same assay. At a dose of 1.0 mg/kg, ketamine induces a similar, if not more potent modulation of affective biases[263]. Therefore, HNK is unlikely to be the main mediator of these neuropsychological effects.

This thesis also investigated the extent to which rats are capable of demonstrating olfactory self-recognition. Although rats failed to show a preference for either their own scent or that of a novel conspecific's, there was an increase in exploration time for both scents when the novel scent was taken from a rat of a different strain. Analysis revealed a trend in which rats spent less time sniffing a novel scent from a rat of their own strain compared to their own scent or a

novel scent from a different strain. This area of research requires further development, but the studies piloted here provide a foundation for designing experiments to explore this concept.

4. 1 Validation of the ABT and RLA

Recapitulating symptoms of human mood disorders such as MDD in animals has proven to be a difficult endeavor due to the lack of reliable animal models and poor translation of preclinical findings to a clinical setting. Many of the currently used animal models as well as the assays used to screen antidepressant treatments often fail to demonstrate adequate face, construct, and predictive validity[104-05,179]. The findings outlined in Chapter 2, in combination with findings from previous studies of both rodents and humans[92,101,262-63], provide evidence of the ABT as a translational assay that meet criteria for validity in a number of domains (see **Table 1.2**).

The neuropsychological mechanisms assessed in the ABT are thought to be highly relevant to both the symptomology and treatment of MDD. This is evidenced by findings from human studies in which depressed patients demonstrate negative biases in many different measures of affective processing, and acute treatment with conventional antidepressants may attenuate such biases[92,101]. Previous experiments have shown performance in the ABT is sensitive to pharmacological and psychosocial manipulations of affective state (both positive and negative)[246,262-63], and this is further validated by the negative affective bias demonstrated following treatment with FG7142 as well as the positive bias induced via venlafaxine treatment in the experiments undertaken in Chapter 2 (see **Figures 2.7-2.8** and **Figures 2.10-2.11**).

It has been well-established that depressed patients often exhibit anhedonic tendencies by attributing less value to pleasurable or rewarding experiences compared to healthy controls[385-88]. Inducing a negative affective state may recapitulate this phenomenon in the ABT by decreasing the value of the reward associated with the FG7142-paired substrate, suggesting that the ABT can be used to measure similar deficits in animals in a way that adequately translates to clinical findings. In addition, the task demonstrates mechanistic and predictive validity[104-05] as indicated by its ability to differentiate the effects of delayed versus rapid-acting antidepressants (discussed below).

The RLA is used in these experiments as a control measure for non-specific effects on learning and memory. This assay has previously been used in models of depression where an

impairment in reward learning is observed. In these studies, however, normal animals were used throughout. In the RLA, control animals remain in the same affective state throughout the protocol with a reward-induced bias generated by pairing one of the substrate-reward cues with a higher value reward. To test for non-specific effects, the same treatments which attenuated an affective state-induced bias are given before the preference test. For both amitriptyline and HNK, these treatments had no effect on the reward-induced bias and thus confirmed the specificity of the affective bias modulation observed in the ABT.

4.2 Insights into mechanisms underlying the effects of delayed versus rapid-acting antidepressants

The results of the ABT experiments found that amitriptyline and HNK differ in their ability to modulate affective biases. Different hypotheses have been proposed to explain the temporal differences in the clinical effects of antidepressants. Largely supported by pre-clinical studies and with a number of potential caveats, the most prominent hypothesis in the neurotrophic hypothesis (see Chapter 1, Section 3.2 *The Neurotrophic Hypothesis*). An alternative perspective, referred to as the affective bias hypothesis[391], offers an explanation regarding the mechanistic differences between delayed and rapid-acting antidepressants (see **Figure 4.1**). This theory argues that conventional antidepressants are able to positively bias new learning and reduce behavioral symptoms in depressive individuals, but time is needed for new positively biased memories to accumulate and the benefits to occur. In contrast, rapid-acting antidepressants offer immediate relief from symptoms by attenuating previous negative biases but do not impact on new memories and hence may have less benefit long-term. The results outlined in Chapter 2 offer further validation of this hypothesis in showing how drugs that are able to rapidly attenuate previous negative biases such as HNK are unable to bias learning of new reward-associated stimuli, while drugs with predominantly monoaminergic components such as venlafaxine can positively bias learning but fail to impact on established memories.

The results discussed in Chapter 2 align with this hypothesis as demonstrated by the finding that amitriptyline positively biased new learning (see **Figure 2.8**) while HNK failed to have an effect (see **Figure 2.11**). The lack of effect of HNK on new learning affirms that the effects of delayed-onset versus rapid-acting antidepressants are unlikely to result from the same underlying mechanism. The effect of amitriptyline on new learning aligns with that of the SNRI venlafaxine, suggesting that positively biased learning and memory may arise from acute changes in monoaminergic transmission. Evaluation of other monoaminergic antidepressants

using the affective bias test is needed to confirm this hypothesis. Likewise, the finding that HNK attenuates previously established negative biases is similar to results from ketamine studies[263,321] and suggests that this modulation is linked to the fast-acting effects of these and other rapid-acting antidepressants such as scopolamine[321]. In addition, the ability of amitriptyline to also attenuate such biases aligns with early findings of rapid symptom improvement in depressed patients following treatment with tricyclic antidepressants and offers further validation of the affective bias hypothesis. The exact mechanism by which rapid-acting antidepressants act on negative affective biases remains unclear, and both preclinical research and clinical observations are needed to further validate and refine this theory.

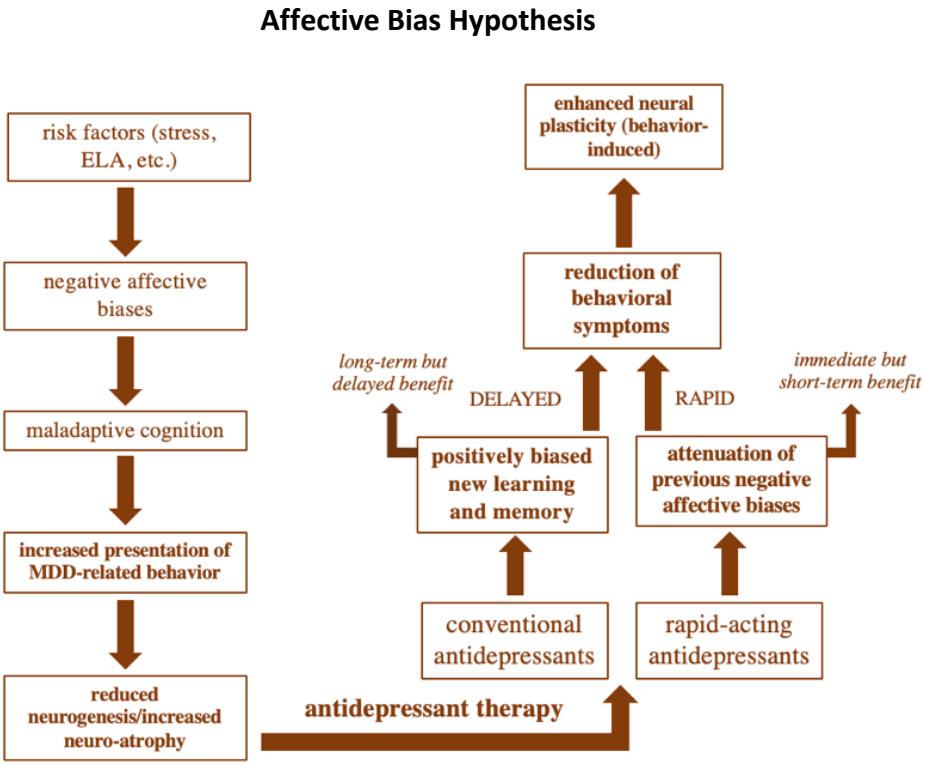

Figure 4.1: Overview of the affective bias hypothesis. The affective bias hypothesis proposes an alternative relationship between behavioral expressions of MDD and morphological changes associated with the disorder as well as how such changes are affected by delayed versus rapid-acting antidepressants. Figure adapted from Hinchcliffe, 2019[321].

4.3 Olfactory self-recognition in rodents

The results of the experiment discussed in Chapter 3 suggest that while laboratory rats may not possess the introspective abilities associated with the idea of self-awareness, they may be capable of the social awareness outlined by Degrazia[253] and similar theorists. This idea is highly plausible given the current knowledge surrounding social behavior in rats. However, as

discussed in Chapter 3, Section 3.5, this data does not definitively rule out the notion that rats are, in fact, capable of the same self-awareness that researchers such as Gallup and Horowitz have attributed to primates and other mammalian species.

In addition to the methodological limitations of the experiment, a major obstacle in studying self-awareness in animals lies in the lack of a standardized definition of self-awareness by which to interpret experimental outcomes. For example, those with similar views to Gallup may argue that the inability of rats to distinguish between their own scent and a novel scent (assuming that increased sniffing is indicative of preference resulting from recognition) is evidence that they do not possess self-awareness. It could also be argued, however, that the increased exploration of both scents when there is a greater degree of social novelty (see **Figure 3.4**) suggests that the animal is, to an extent, aware of the olfactory qualities of its own strain, and this may indicate an awareness of itself as an individual with qualities that can be similar or different to those of conspecifics. Some have even proposed that any animal capable of feeling sensations such as hunger or thirst and understanding their connection to those sensations enough to address them demonstrates self-awareness. Another example put forth by Sommerville is the idea that prey animals such as rats who respond to and avoid predators demonstrate an awareness of themselves via an ability to assess and understand the significance of a predation threat in relation to themselves[272]. Until there is an agreement regarding exactly what distinguishes the level of self-awareness originally thought to be unique to humans, operationalizing and designing definitive, non-linguistic measures of self-awareness will be nearly impossible. While those such as Gallup claim that self-directed behaviors are sufficient evidence of awareness, there is still a cogent lack of consideration for context in regard to such behaviors. Even the touching of the forehead by the chimpanzees in Gallup's original experiment[274] cannot be definitively ruled out as simple kinesthetic matching of social behavior exhibited by what is thought to be a conspecific. Similarly, whether any measure of self-directed behavior exists that can be assessed in multiple species remains an important question. If a fish fails to perform in a task similarly to a primate, it is highly likely that this is due to the fact that both animals experience the world in entirely different ways. Therefore, a valid measure of self-awareness would require a nearly complete knowledge of a species' sensory experience as well as of their behavioral repertoire in order to conclusively interpret a behavior as self-directed. As long as there are gaps in our knowledge of rodent behavior, it will be difficult to determine the validity of any measures which claim to indicate self-directed

behavior in rodents. As a result, the extent to which rats possess self-awareness remains unclear.

4.4 Implications for preclinical animal research

Despite the many difficulties associated with studying the emotional states and experiences of laboratory rodents, continued pursuit of such information is critical to the future of animal research as discussed in Chapter 3, Section 3.5. Understanding the way in which a species experiences their environment will not only inform researchers of which species makes the most appropriate preclinical model (thus improving ethological, construct, and remission validity) but will also aid in improving animal welfare standards as a whole.

However, knowing the extent to which an animal is aware of their own suffering may also have implications for the affective bias research discussed in Chapter 2. For example, if an animal undergoes a negative affective state manipulation and is then later asked to recall the experience as part of an ABT protocol, the bias which forms following the manipulation could be influenced by the degree to which the animal is aware of the negative emotional valence of that experience. If capable of self-awareness, an animal unknowingly treated with a prodepressant drug is going to experience the subsequent negative change in affective state differently than an animal who consciously experiences CUMS or learned helplessness. Likewise, animals with different levels of cognitive awareness may also differentially experience the effects of certain antidepressant drug treatments, and this could potentially influence the outcomes of studies using behavioral assays such as the ABT. This may become a more prevalent issue as research into the therapeutic potential of psychoactive and/or hallucinogenic substances such as ketamine continues to grow and understanding the neuropsychological mechanisms of these drugs becomes increasingly imperative.

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