Investigating the molecular and neurobehavioral effects of monoamine antidepressants and ketamine in a rodent model of ACTH-induced HPA axis dysfunction

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DECLARATION

I, Farhanah Sallie, declare that this thesis is my own, except to the extent indicated in the contribution and acknowledgments sections. It is being submitted for the fulfilment of the degree of Doctor of Philosophy in the Department of Physiology, School of Biomedical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this thesis has not been submitted for any degree or examination at this or any other University.

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Signed on the 10th day of November 2025.

ABSTRACT

Major depressive disorder (MDD) is a debilitating mental illness and is among the leading causes of disability worldwide, severely impacting patients' quality of life. The pathophysiology of MDD is multifactorial. Several theories have been proposed, including neuroinflammation, monoamine and neurotrophic dysregulation, hippocampal atrophy, and excitatory/inhibitory imbalances. Of these theories, hypothalamic-pituitary-adrenal (HPA) axis dysregulation is suggested as a frequently pathophysiological mechanism of MDD. HPA axis dysregulation is attributed to chronic stressors, both physiological and emotional, which disrupt glucocorticoid signalling and impair negative feedback mechanisms. Studying the pathophysiology of MDD in humans is limited by confounding factors and access to post-mortem tissues. Therefore, in rodents, administration of exogenous adrenocorticotropic hormone (ACTH) has been used to induce HPA axis dysregulation and produce depressive-like responses. However, there is a lack of comprehensive neurobehavioral and mechanistic evidence to support the validity of this model. In addition, the investigation of the molecular effects of different classes of antidepressants is important to understand the clinical translation of this model.

In **Chapter 2**, I developed and validated the ACTH model using a battery of neurobehavioural tests. Previous studies demonstrated the inefficacy of tricyclic antidepressants (TCAs) in this model suggesting that ACTH-induced HPA axis dysregulation may be associated with treatment resistant depression (TRD), therefore, I also investigated the effect of impramine, a TCA, in this chapter. Male and female Sprague-Dawley rats were randomly assigned to the control or ACTH groups that received saline or ACTH, respectively, for two weeks. Thereafter, rats in the ACTH group were subdivided to receive ACTH plus saline or ACTH plus imipramine for a further four weeks. Neurobehavioral changes were assessed using the forced swim test (FST), open field test (OFT), and sucrose preference test (SPT). The brain regional mRNA expression of brain-derived neurotrophic factor (*BDNF*) and cAMP response element binding protein (*CREB*), neuroplasticity molecules implicated in depression pathology, was determined using real-time polymerase chain reaction (RT-PCR). ACTH administration resulted in a depressive-like phenotype, significantly increasing immobility in the FST, decreasing interaction with the centre of the OFT, and increasing sucrose consumption in male, but not female rats. ACTH administration significantly increased the expression of *BDNF*, a neurotrophin important for synaptic plasticity, in the hippocampus and *CREB*, a transcription factor that regulates neuronal development, in all brain regions in males, but not in female rats. Co-treatment with imipramine did not ameliorate these ACTH-induced neurobehavioral or molecular changes. This chapter showed that ACTH administration resulted in a sex-specific onset of depressive-like symptoms and changes in brain regional expression of neuroplasticity markers, suggesting sex-specific mechanisms in a model of ACTH-induced HPA axis dysregulation. Furthermore, molecular analyses indicate that chronic ACTH alters *BDNF* and *CREB* signalling, suggesting that dysregulated neurotrophic signalling is associated

with HPA axis dysregulation. Although these agents primarily act to regulate neurotransmitters, their effects on neurotrophic signalling, particularly in the ACTH model, remain poorly understood.

Following the development and neurobehavioural evaluation of the ACTH model, in **Chapter 3**, I further validated the model by comparing the neuroplasticity effects of imipramine, a TCA, to citalopram, a selective serotonin reuptake inhibitor (SSRI) and first-line therapy for MDD. The ACTH model was repeated with experimental animals being treated with either citalopram or imipramine for four weeks, respectively. I determined the brain regional mRNA expression of intracellular markers of neurotrophic signalling, including *BDNF*, *CREB*, tropomyosin receptor kinase B (*TrkB*), protein kinase B (*Akt*), mechanistic target of rapamycin (*mTOR*), and eukaryotic elongation factor 2 (*eEF2*) using RT-PCR. In addition, the brain distribution of citalopram and imipramine was determined using atmospheric pressure matrix-assisted laser desorption/ionisation mass spectrometry imaging (AP-MALDI-MSI). ACTH administration increased hippocampal *BDNF* mRNA expression, coupled with decreased mRNA expression of intermediate neurogenesis signalling factors, *TrkB*, *Akt*, *mTOR*, and *eEF2*. Citalopram showed a greater capacity to ameliorate ACTH-induced neurotrophic dysregulation compared to imipramine, particularly by enhancing BDNF-TrkB-mTOR signalling, independent of brain drug distribution. These findings highlight the distinct pharmacodynamic effects of different classes of antidepressants in targeting dysregulated neurotrophic signalling.

Despite the widespread use of monoamine-based antidepressants, approximately one-third of individuals diagnosed with MDD have TRD, where patients do not respond to two or more classes of conventional antidepressants. Ketamine has emerged as a novel, rapid-acting therapeutic agent for the management of TRD. Unlike monoamine antidepressants that require months to reach their full therapeutic effect, ketamine provides antidepressant relief following a single dose. However, the respective effects of acute and chronic ketamine treatment on neurobehavior, as well as on neurotrophic and monoamine neurotransmitter signalling in an ACTH model of depressive-like symptoms, remain unclear.

In **Chapter 4**, I investigated the behavioural, molecular, and neurochemical effects of acute and chronic ketamine treatment in ACTH-treated rats. In the acute group, following two weeks of ACTH administration, rats received a single dose of ketamine. In the chronic group, after two weeks of ACTH administration, rats received a ketamine injection once a week for four weeks. Neurobehavioural changes were assessed using the FST, OFT, and SPT. Following termination, brain regional mRNA expression of *BDNF, TrkB, Akt, mTOR, eEF2,* and *CREB* was measured using RT-PCR. Brain regional protein expression of phosphorylated-CREB (p-CREB) and corticosterone were assessed using an enzyme-linked immunosorbent assay (ELISA). In addition, serotonin, dopamine, and norepinephrine neurotransmitter abundance were determined at various bregma levels using MALDI-MSI. ACTH administration increased behavioural despair in the FST and altered corticosterone and p-CREB protein

expression, mainly in the prefrontal cortex. However, by week six, neurobehaviour and gene and protein expression remained largely unchanged, suggesting a state of homeostatic adaptation. At week six, ACTH-treated rats showed changes in serotonin and norepinephrine levels in the hippocampus and midbrain. Acute ketamine treatment effectively reversed ACTH-induced behavioural despair in the FST and modulated the region-specific expression of monoamines (serotonin) and increased *CREB* mRNA expression. With chronic ketamine treatment, besides increased striatal *mTOR* mRNA expression, there were limited effects on the expression of neuroplasticity markers. Our findings indicate that when administered acutely, ketamine modulates neurotrophic pathways and monoaminergic neurotransmission, which impacts behavioural outcomes. In contrast, the minimal behavioural and molecular effects of repeated ketamine treatment suggest that prolonged exposure attenuates ketamine's neuroplastic and antidepressant actions in this model. These findings highlight the differential mechanism of action of ketamine following acute and chronic dosing, in a model of HPA axis dysregulation.

In conclusion, the findings from this thesis contribute to our understanding of the molecular mechanisms underlying the neurobehavioural effects of chronic exogenous ACTH administration in rodents as well as the model-specific effects of imipramine, citalopram, and ketamine. I provide evidence that chronic ACTH exposure induces depressive-like behaviours as assessed using the FST, OFT, and SPT. These effects were sex-dependent, with male rats exhibiting both behavioural changes and elevated hippocampal BDNF and CREB mRNA expression, while females were largely unaffected. I further demonstrate that citalopram was more effective than imipramine at ameliorating ACTH-induced disruptions in the mRNA expression of neurogenesis-related signalling factors across brain regions. I also show that acute ketamine treatment has more pronounced effects on ACTH-induced neurobehaviour and molecular changes, than chronic ketamine. Overall, this thesis provides evidence that chronic ACTH administration models HPA-dysregulation induced depressive-like symptoms, resulting in disturbances in neurotrophic and monoaminergic signalling. Moreover, neurogenesis-enhancing agents such as SSRIs and acute ketamine may offer greater therapeutic efficacy than classic antidepressants in depressive disorders where HPA axis dysfunction is a core aetiological feature.

PUBLICATIONS AND PRESENTATIONS

Publications arising from this research

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Manne SR, de la Torre BG, Albericio F, Daniels WMU, Millen AME & Baijnath S (2024). Neurobehavioral and molecular changes in a rodent model of ACTH-induced HPA axis dysfunction. *Brain Research* **1834**, 148913.

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Publications not for degree purposes

Millen AME, Maluleke TT, Pienaar L, **Sallie FN**, Veerappan R, Andrén PE & Baijnath S (2024). Regional Changes in Brain Biomolecular Markers in a Collagen-Induced Arthritis Rat Model. *Biology* **13**, 516.

Williams KC, Nto NJ, van Vuren EJ, **Sallie FN**, Molebatsi K, Kroneberg KS, Roomaney AA, Salie M & Womersley JS (2024). Early biological and psychosocial factors associated with PTSD onset and persistence in youth. *European Journal of Psychotraumatology* **15**, 2432160.

Oral presentations arising from this research

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Daniels WMU, Millen AME, Baijnath S. Neurobehavioural and molecular changes in a rodent model of ACTH-induced HPA axis dysfunction. ISN 5th Flagship School, Naxos, Greece, October 2024.

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Daniels WMU, Millen AME, Baijnath S. Effect of chronic ketamine on neurobehaviour and neurotransmitter expression in a rodent model of ACTH-induced HPA axis dysfunction. Wits Faculty Research Day, Johannesburg, South Africa, September 2024.

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Daniels WMU, Millen AME, Baijnath S. Effect of chronic ketamine on neurobehaviour and neurotransmitter expression in a rodent model of ACTH-induced HPA axis dysfunction. Physiological Society of South Africa, Limpopo, South Africa, August 2024.

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Daniels WMU, Millen AME, Baijnath S. Neurobehavioural and molecular changes in a rodent model of ACTH-induced HPA axis dysfunction. MBRT Research Symposium, Johannesburg, South Africa, December 2023.

Sallie FN, Pienaar L, Lubbe A, Xhakaza SP, Daniels WMU, Millen AME, Baijnath S. Neurobehavioural changes in a rodent model of ACTH-induced HPA axis dysfunction. School of Physiology Research Day, Johannesburg, South Africa, September 2023.

Sallie FN, Daniels WMU, Baijnath S. Neurobehavioural characterisation of a rodent model of treatment-resistant depression. The Biological Psychiatry Congress (SANS Symposium), Cape Town, September 2022.

Sallie FN, Daniels WMU, Baijnath S. Investigating the antidepressive mechanism of action of ketamine in a rodent model of HPA axis dysfunction. The Wits 13th PG Cross Faculty Symposium, Johannesburg, South Africa, July 2022.

STATEMENT OF CONTRIBUTION TO THIS RESEARCH

As part of the declaration of this thesis, I acknowledge contributions by various individuals as detailed below.

All studies were designed and carried out under the supervision of Prof Sooraj Baijnath and Prof Aletta Millen. All experimental data collection was performed by myself, with assistance from my supervisors. Together with a team of researchers from our lab, including Ms Andréa Lubbe, Ms Leandrie Pienaar, and Ms Sanelisiwe Xhakaza, we participated in rat termination procedures where I was responsible for brain removal and dissection. I was solely responsible for RNA extraction, cDNA synthesis, gene expression PCR assays, and ELISA assays that were performed. I helped carry out all the MSI experiments with the help of my supervisors. I performed all data analysis, interpreted the data, and wrote the manuscripts for this thesis with the help of my supervisors. The second manuscript included in this thesis also includes experimental data generated by Ms Andréa Lubbe during her MSc combined with my findings. All co-authors on the individual manuscripts contributed to data interpretation, critical revisions and provided expert opinions.

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ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
ACTH	adrenocorticotropic hormone
ADX	adrenalectomized
Akt	protein kinase B
ANOVA	analysis of variance
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
cDNA	complementary deoxyribonucleic acid
CIT	citalopram
cm	centimetre
cm/s	centimetre per second
CREB	cyclic AMP response-element binding protein
CRH	corticotropin-releasing hormone
CUMS	chronic unpredictable mild stress
DSM	Diagnostic and Statistical Manual of Mental Disorders
EAAT2	excitatory amino acid transporter 2
eEF2	eukaryotic elongation factor 2
ELISA	enzyme-linked immunosorbent assay
FKBP5	FK506 binding protein 5
FST	forced swim test
g	gram
GAD67	glutamic acid decarboxylase 67
Glx/GABA	combined glutamate and glutamine to GABA ratio
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HDRS	Hamilton Depression Rating Scale
HPA	hypothalamic-pituitary-adrenal
IDO	indoleamine 2,3-dioxygenase
IL-6	interleukin-6
IMI	imipramine
IQR	interquartile range
kg	kilogram
KYN	kynurenine
LMIC	lower-middle-income country
MADRS	Montgomery-Åsberg Depression Rating Scale

MDA	malondialdehyde
MDD	major depressive disorder
min	minutes
mg	milligram
ml	millilitre
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OBX	olfactory bulbectomy
OFT	open field test
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PVN	paraventricular nucleus
QUIN	quinolinic acid
r	correlation coefficient
REDD1	regulated in development and DNA damage-response 1
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
SERT	serotonin transporter
SPR	sucrose preference ratio
SPT	sucrose preference test
SNRI	selective serotonin-norepinephrine re-uptake inhibitor
SSRI	selective serotonin reuptake inhibitor
Tbp	TATA-box binding protein
TCA	tricyclic antidepressant
TNF-α	tumour necrosis factor alpha
TrkB	tropomyosin-related kinase receptor type B
WRAF	Wits Research Animal Facility

PREFACE

Major depressive disorder (MDD) remains a global health concern due to its growing public health and socioeconomical impact. The treatment and management of MDD remains challenging due to the disorder's complex aetiology. While several theories have been proposed that underlie MDD, including monoamine dysregulation, excitatory/inhibitory imbalances, neuroinflammation, and neurotrophic deficits, hypothalamic-pituitary-adrenal (HPA) axis dysfunction has emerged as one of the most consistent contributors. Chronic stress-induced HPA axis dysregulation contributes significantly to the onset and persistence of depressive symptoms. Yet, its relationship to the brain molecular mechanisms that underly depression development remains unclear. In addition to HPA axis dysregulation, neurotrophic deficits play a substantial role in MDD development and severity. Neurotrophins, such as BDNF, and other neurogenesis-related intracellular signalling factors, are crucial for the maintenance of neuronal health and resilience against stress-induced neuronal insult. Therefore, it is not surprising that dysregulation in these pathways has been recognised as a key etiological mechanism that contributes to the development of many depressive symptoms. Clinical studies into this complex relationship remain limited by the challenges in obtaining adequate biological samples and ethical concerns around access to human brain tissue. Preclinical studies involving rodents provide a way to study the underlying, pathological changes associated with disorders such as depression. Therefore, in this thesis, we aimed to improve our understanding of the neurobehavioral and molecular changes associated with HPA axis dysregulation and subsequent antidepressant treatment using a preclinical rodent model.

Chronic exogenous adrenocorticotropic hormone (ACTH) administration has been proposed as a method of pharmacologically inducing HPA axis dysregulation and depressive-like symptoms in rodents. Due to the complex nature of MDD symptoms, a battery of neurobehavioral assessments is necessary to capture various depressive-like phenotypes. Despite this, a comprehensive neurobehavioral and molecular validation of the ACTH model is lacking, limiting its translational value. Therefore, we intermittently assessed the effects of ACTH administration on rodent behaviour using the forced swim test (FST), open field test (OFT), and sucrose preference test (SPT) in male and female rats. Due to the involvement of neurotrophic deficits in the development and progression of MDD, we additionally assessed the effect of ACTH administration on neurogenesis-related markers across various brain regions implicated in depression.

Monoamine-based antidepressants are the most widely used treatment for MDD. However, monoamine-based therapeutics target one of several physiological pathways that may contribute to MDD. Although signalling factors involved in the neurogenesis pathway are strongly implicated MDD, the effect of common antidepressants, that primarily modulate monoamine neurotransmission, on these factors, remains unclear. Therefore, we investigated the effects of citalopram, a selective serotonin

reuptake inhibitor (SSRI), and imipramine, a tricyclic antidepressant (TCA), on the expression of neurotrophic signalling factors across depression-related brain regions in a rodent model of ACTH-induced HPA axis dysregulation.

Despite their widespread use, monoamine-based antidepressants fail to alleviate depressive symptoms in roughly 30% of individuals diagnosed with MDD. These individuals show resistance to traditional treatments, which has urged the exploration of novel antidepressant agents. In this regard, ketamine has emerged as a 'silver bullet' in depression management. Owing to its multi-target biochemical properties, ketamine demonstrates rapid antidepressant efficacy in individuals with treatment-resistant depression. However, the neurochemical and neurobehavioral effects of ketamine treatment in the ACTH model require elucidation. Therefore, we investigated the effects of acute and chronic ketamine treatment on rodent behaviour in the FST, OFT, and SPT. We also assessed ketamine's effect on HPA axis function, the expression of neurogenesis-related markers, and monoamine neurotransmitter abundance in the ACTH model.

This thesis is written as a series of semi-independent chapters, each containing an introduction, methods, results, discussion, and conclusion section (Chapters 2 to 4). This thesis begins with a literature review chapter that summarises the current knowledge where I highlight the rationale for conducting the various studies included in this thesis. Chapter 5 summarises the main findings of each chapter and accentuates their relevance and translational potential. Chapter 6 provides the list of references cited in this thesis and Chapter 7 provides the list of appendices.

CHAPTER 1: INTRODUCTION

1.1 Introduction

1.1.1 Major depressive disorder - a global health crisis

Major depressive disorder (MDD) is a complex and heterogenous mental health condition and the global leading cause of disability, affecting more than 280 million individuals worldwide (World Health Organization, 2021). Depression affects approximately 5% of adults and 14% of teenagers (aged 10-19 years), with women accounting for 60-70% of reported cases (Kuehner, 2017; WHO, 2021). Clinically, MDD is characterized by anhedonia, a markedly diminished interest or pleasure in all or almost all activities, impairing daily functioning and quality of life (American Psychiatric Association, 2022). Other MDD symptoms include weight fluctuations, sleep disturbances, psychomotor retardation, worthlessness or guilt, cognitive impairments, fatigue, and suicidal ideation (American Psychiatric Association, 2022). A diagnosis requires at least five of these symptoms persisting for two weeks, with at least one being depressed mood or anhedonia.

Epidemiological studies show that risk factors, which may predispose individuals to the development of depression, include younger age, female gender, and marital status, particularly among divorced individuals (Kessler & Bromet, 2013). Other risk factors include chronic illness, childhood adversity, and low economic status, although patterns vary across cultures (Berk *et al.*, 2023). Having MDD has several devastating consequences, including increased suicide risk, decreased social cohesion, and exacerbated socioeconomic strain through the individual's impaired ability to work effectively and contribute to the economy (Bhardwaj, 2020). Social isolation and stigma strains relationships and fragments communities contributing to higher crime rates and public health crises that create institutional distrust. Together, these factors can lead to civil unrest and political instability (Beauvais & Jenson, Jane, 2002). Furthermore, the global economic impact of depression and anxiety disorders is roughly one trillion dollars annually (World Health Organization, 2024*a*). Additionally, depression contributes to higher medical costs as a causal risk factor for several conditions including cardiovascular disease, diabetes mellitus, and certain cancers (Carnethon *et al.*, 2003; Gross *et al.*, 2010). However, much of the data underpinning these studies originates from western regions. This western emphasis introduces potential bias and emphasises the need for more inclusive global research, particularly from lower-middle-income countries (LMICs).

1.1.2 Major depressive disorder in Africa

MDD in Africa poses a unique challenge due to specific socioeconomic, cultural, and healthcare constraints. In Sub-Saharan Africa, the depression prevalence is estimated to be 29.19 million people, and while statistics vary across the region, estimates indicate a general depression rate consistently above 3% (Gbadamosi *et al.,* 2022). For example, in Uganda, depression affects more than 20% of the population, with disproportionately high rates observed among refugees (67.6%), war victims (36.0%),

and individuals living with human immunodeficiency virus (HIV) (28.2%) (Kaggwa *et al.*, 2022). In South Africa, prevalence estimates range from 14.7% to 38.8% (Craig *et al.*, 2022). However, countries in sub-Saharan Africa contribute little to global depression research output and therefore, many regions still lack epidemiological data on depression (Gbadamosi *et al.,* 2022). These alarming MDD rates in Africa highlight major barriers within the mental health care system in LMICs. Barriers include limited access to mental health services, where countries have fewer than 1.4 mental health workers per 100 000 individuals, leaving primary clinics ill-equipped to diagnose or manage depression (World Health Organization, 2020). Additionally, stigma and limited mental health awareness in communities may lead to the failure to recognize depressive symptoms and acknowledge the need for help. This failure results in underdiagnosed or frequently misdiagnosed cases; therefore, it is likely that depression cases in primary care are severely underestimated (Nyongesa *et al.*, 2017). Consequently, surveys show that in many African countries, over 75% of individuals with severe mental disorders receive no care at all, emphasizing the treatment gap (Thornicroft & Semrau, 2019). Expectedly, in LMICs, the quadruple burden of disease and the high cost of infectious diseases, mean that mental health services and public awareness initiatives are low priority in national health budgets (Lund *et al.*, 2011). Consequently, roughly 77% of all suicides occur in LMICs where various sociopolitical factors contribute to high MDD and suicide rates (World Health Organization, 2024*b*). Additionally, in rural areas of many LMICs, access to lethal means of suicide, such as pesticides, is prevalent (Gunnell *et al.*, 2007). High suicide rates may additionally result from poor treatment outcomes. Even when treatment is available, remission rates for MDD are low, with only one-third of patients achieving full remission after their first course of treatment (Madhukar & Ella, 2008). Poor treatment outcomes in MDD highlight the disorder's inherent heterogeneity as patient symptoms and biological etiologies differ widely (Cui *et al.*, 2024). The significant burden that depression imposes on both individuals and societies emphasizes the need to investigate the underlying environmental and biological factors that contribute to the development of MDD.

1.2 The pathophysiology of major depressive disorder

The aetiology of MDD involves the complex and multifactorial interplay of social, psychological, environmental, and biological factors. Social and psychological factors significantly influence depression onset and severity, with chronic stress, isolation, and early adversity increasing risk (Ye *et al.*, 2023). Traits like neuroticism and negative cognitive patterns further increase vulnerability (Navrady *et al.*, 2017). It has been widely suggested that depression develops due to psychological stressors and additional environmental factors however, several other pathophysiological mechanisms have been implicated in the development of MDD. According to the social signal transduction theory, psychological stressors trigger biological changes, such as inflammation and hypothalamic-pituitary-adrenal (HPA) axis dysfunction that contribute to MDD (Slavich & Irwin, 2014). Various additional

hypotheses for depression pathophysiology include monoamine deficits, genetic and epigenetic mechanisms, and neurotrophic deficits (Figure 1.1) (Cui *et al.*, 2024).

The multiple pathophysiological pathways that contribute to MDD make the disorder exceptionally heterogenous. As a result, MDD patients present with varying phenotypes which may result from maladaptive interactions between two or more of these physiological systems (Krishnan & Nestler, 2011). Thus, to better understand the underlying biological mechanisms, there is an interest in studying depression biomarkers to improve MDD diagnosis and treatment. Current diagnostic approaches rely heavily on subjective symptom reporting which may be inconsistent and influenced by various external factors. Robust biomarkers would improve MDD subtype stratification, personalized treatment strategies, and therapeutic response prediction (Schmidt *et al.*, 2011). However, the heterogeneity of depression and its comorbidities complicate the search for consistent, reliable biomarkers, as inconsistent findings have been reported (Jani *et al.*, 2015). Nonetheless, investigating potential biomarkers linked to distinct depression-related biological pathways remain crucial for advancing personalized treatment strategies, and identifying a point of convergence between these many theories to improve the treatment and management of MDD.

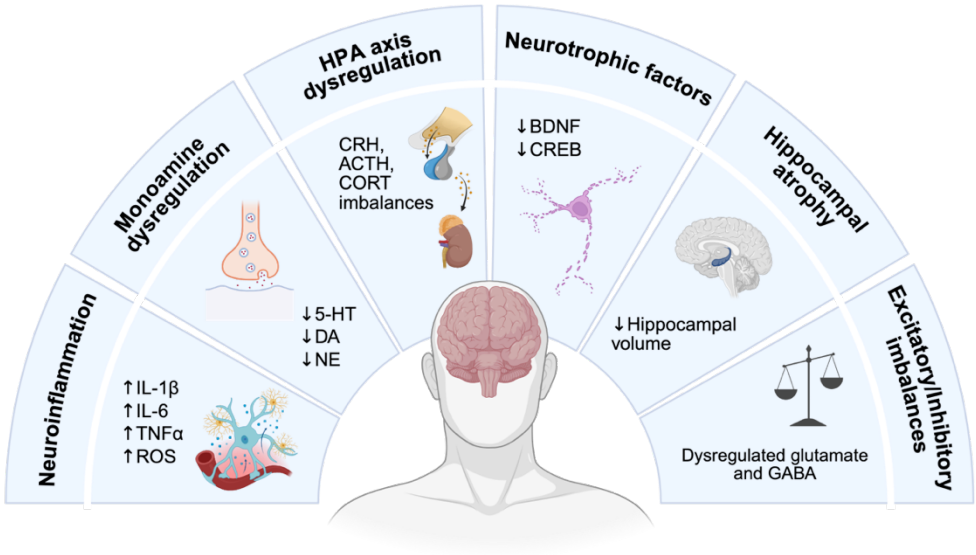


Figure 1.1. Theories regarding the pathophysiology of depression adapted from (Cui *et al.*, 2024).

1.3 Current theories of MDD

The biological theories of depression have evolved over time and have been influenced by emerging research and the development of new technologies that have identified several physiological pathways that contribute to the onset and persistence of MDD.

1.3.1 The monoamine hypothesis

The monoamine hypothesis of depression is one of the oldest and most widely accepted frameworks for understanding the pathophysiology of the disorder. This theory posits that the development of MDD is primarily linked to serotonin (5-HT), dopamine (DA), and norepinephrine (NE) synaptic deficits in the central nervous system (CNS) (Figure 1.2) (Delgado, 2000). Evidence for this hypothesis arises from both clinical and pharmacological studies that have demonstrated the depressive effect of monoamine-depleting agents. Further evidence showed that monoamine-based antidepressants alleviate depressive symptoms by increasing synaptic monoamine levels (Delgado, 2000). However, several features of this hypothesis have attracted scrutiny. The theory fails to explain the variable efficacy of monoamine-based antidepressants and their delayed therapeutic onset despite immediate monoamine increases (Delgado, 2000). Thus, while monoamine dysregulation remains a cornerstone in understanding depression, due to its limitations, it is increasingly viewed as part of a broader neurobiological landscape. Current theories therefore integrate the monoamine hypothesis with downstream effects on receptor sensitivity, gene expression, neural circuit remodelling, neurogenesis, and peripheral and central biochemical signatures (Jesulola *et al.*, 2018).

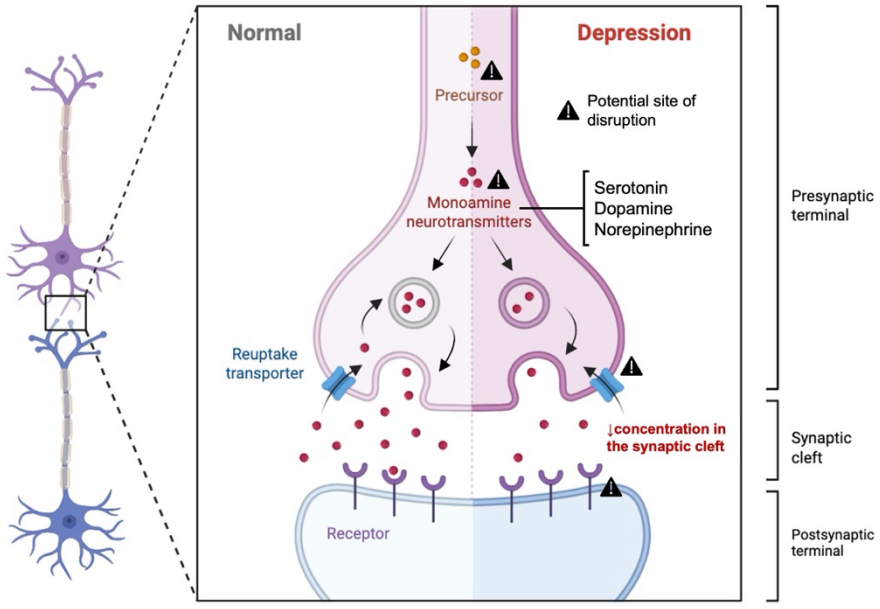


Figure 1.2. Impaired monoamine neurotransmission in major depressive disorder (MDD) adapted from (Chong *et al.,* 2019). According to the monoamine hypothesis, multiple mechanisms contribute to impaired monoaminergic neurotransmission in MDD, including reduced precursor availability, impaired neurotransmitter synthesis, increased re-uptake from the synaptic cleft by transporters, and dysfunctional receptors.

In this context, specific biomarkers offer a measurable representation of monoamine activity in the brain. Monoamine depletion in depression is compounded by increased neurotransmitter metabolism through monoamine oxidase-A (MAO-A) and increased reuptake by the serotonin transporter (SERT)

and the organic cation transporter 3 (OCT3). Indeed, in MDD patients, increased MAO-A expression has been observed in several brain regions, including the limbic areas (Meyer *et al.*, 2006). Similarly, elevated SERT and OCT3 expression is associated with the development of psychiatric disorders (Daws, 2021). Altogether, measuring the abundance of monoamine-metabolising enzymes, along with urinary monoamine metabolites such as 5-hydroxyindoleacetic acid (5HIAA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylglycol (MHPG), may offer a simpler, more accessible biomarker approach.

1.3.2 Excitatory/inhibitory neurotransmitter imbalances

MDD is increasingly associated with neural network dysfunction in which excitatory/inhibitory (E/I) imbalances disrupt neural pathways that govern mood, motivation, and cognition (Sohal & Rubenstein, 2019). Glutamate, the brain's primary excitatory neurotransmitter, and gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter, play critical roles in maintaining synaptic homeostasis and neural network stability (Hu *et al.*, 2023). Topographical studies have demonstrated abnormal brain-wide GABA to glutamate ratios in MDD patients (Hu *et al.*, 2023). Previous studies have shown attenuated GABA levels alongside an increase in glutamate signalling in the cortical-subcortical limbic system (Hu *et al.*, 2023). In these regions, elevated extracellular glutamate causes sustained NMDAR/α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activation that mediates Ca^{2+} influx. This calcium surge triggers further vesicular glutamate release, which impairs astrocytic glutamate re-uptake and produces reactive oxygen species (ROS) (Bai *et al.*, 2024). ROS oxidises and impairs the excitatory amino acid transporter 2 (EAAT2), responsible for glutamate clearance. Reduced EAAT2 contributes to the accumulation of extracellular glutamate (Murrough *et al.*, 2017). ROS further causes DNA damage and lipid peroxidation that impairs the production of plasticity-related proteins and compromises neuronal energy metabolism (Ji *et al.*, 2023). Altogether, reduced inhibitory tone and excessive glutamate activity lead to excitotoxicity and neuronal overstimulation that causes cell damage and cell death (Figure 1.3). Furthermore, E/I imbalances impair the brain's ability to repair damaged neurons via neurogenesis (Ji *et al.*, 2023). Neuronal damage leads to structural and functional impairments in neuronal signalling, particularly in limbic regions. Disruptions in these brain compartments manifest as the clinical symptoms of anhedonia, emotional dysregulation, cognitive impairment, heightened stress responses, and an increased risk of comorbid disorders such as anxiety (Hu *et al.*, 2023).

Several biomarkers have been identified that reflect dysfunctional glutamate/GABA neurotransmission. GABA deficits are reflected by the level of its synthesising enzyme, glutamic acid decarboxylase 67 (GAD67), which is reduced in the prefrontal cortex (PFC) of post-mortem MDD patients. Similarly, reduced EAAT2 levels serve as an additional indicator of excitotoxicity and astrocyte activity and may soon be measured using neuroimaging (Murrough *et al.*, 2017; Fontana *et al.*, 2023). More recently,

reductions in the ratio of combined glutamate and glutamine to GABA (Glx/GABA) have been identified as a potential biomarker for E/I imbalance and antidepressant efficacy (Ohtani *et al.*, 2025). Oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and malondialdehyde (MDA), which are generated by ROS, can serve as a biomarker for glutamate-induced oxidative damage. These markers, which are measurable in blood, urine, and cerebrospinal fluid (CSF), have been associated with neuronal damage and MDD (Ait Tayeb *et al.*, 2023). The normalisation of oxidative stress markers in remitted MDD patients supports their potential as depression biomarkers (Ait Tayeb *et al.*, 2023). Together, these findings highlight a growing biomarker profile targeting E/I imbalances and oxidative stress in MDD.

1.3.3 The neuroinflammatory hypothesis

Immune system dysregulation is one of the main biological alterations leading to depression (Slavich & Irwin, 2014). Studies show that up to 50% of individuals with autoimmune disorders present with depressive-like symptoms highlighting the significant overlap between depression and immune system dysfunction (Pryce & Fontana, 2017). Clinical depression and rodent models of depressive-like symptoms are marked by chronic low-grade inflammation, driven by proinflammatory cytokines such as interleukin-2 (IL-2), IL-6, IL-1β, and tumour necrosis factor α (TNFα) (Woodburn *et al.*, 2021). Peripheral inflammation can compromise blood-brain barrier (BBB) integrity, allowing cytokine entry into the CNS (Huang *et al.*, 2021). Upon entering the brain, pro-inflammatory cytokines activate microglia and astrocytes, leading to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Active NF-κB translocates to the nucleus where it promotes the production of pro-inflammatory genes such as cytokines, chemokines, and enzymes that perpetuate the inflammatory response (Huang *et al.*, 2021). Neuroinflammation contributes to depression by disrupting neurotransmitter systems, converging on several other theories downstream (Figure 1.3). Pro-inflammatory cytokines cause monoamine neurotransmitter deficits by activating indoleamine 2,3-dioxygenase (IDO). IDO diverts tryptophan metabolism away from serotonin synthesis towards the kynurenine (KYN) pathway (Li *et al.*, 2020*a*). This diversion reduces serotonin synthesis and increases neuroactive metabolites, such as quinolinic acid (QUIN) (Murrough *et al.*, 2017). Together with QUIN, pro-inflammatory cytokines impair EAAT2 function, resulting in the accumulation of extracellular glutamate (Murrough *et al.*, 2017). Glutamate and QUIN both activate NMDARs, leading to sustained Ca^{2+} influx and the activation of neurotoxic pathways (Inam *et al.*, 2023). Sustained NMDAR activation, specifically those located extrasynaptically, suppresses cAMP response element-binding protein (CREB) signalling, a transcription factor essential for neuronal survival and plasticity. Inhibited CREB downregulates brain-derived neurotrophic factor (BDNF) production, impairing synaptic integrity and neurogenesis (Hardingham & Bading, 2010; Verdonk *et al.*, 2019). Additionally, Ca^{2+} influx activates proteolytic enzymes that degrade cytoskeletal proteins resulting in neuronal cell death

(Bai *et al.*, 2024). Concurrently, NMDAR overactivation and QUIN increase the production of ROS and reactive nitrogen species (RNS). ROS and RNS cause oxidative damage to DNA, lipids, and proteins and impair adenosine triphosphate (ATP) production and mitochondrial membrane potential, further amplifying neuronal injury (Hu *et al.*, 2023). Together, these pro-death signalling pathways degenerate neurons, particularly in key mood-regulating regions like the PFC and hippocampus (West & Shadel, 2017). Cumulatively, these inflammatory processes result in core depressive symptoms such as emotional dysregulation, anhedonia, and cognitive impairment (Hu *et al.*, 2023). These processes highlight the central role of inflammation in the aetiology of depression and provide a crucial target for more personalised and effective interventions for MDD.

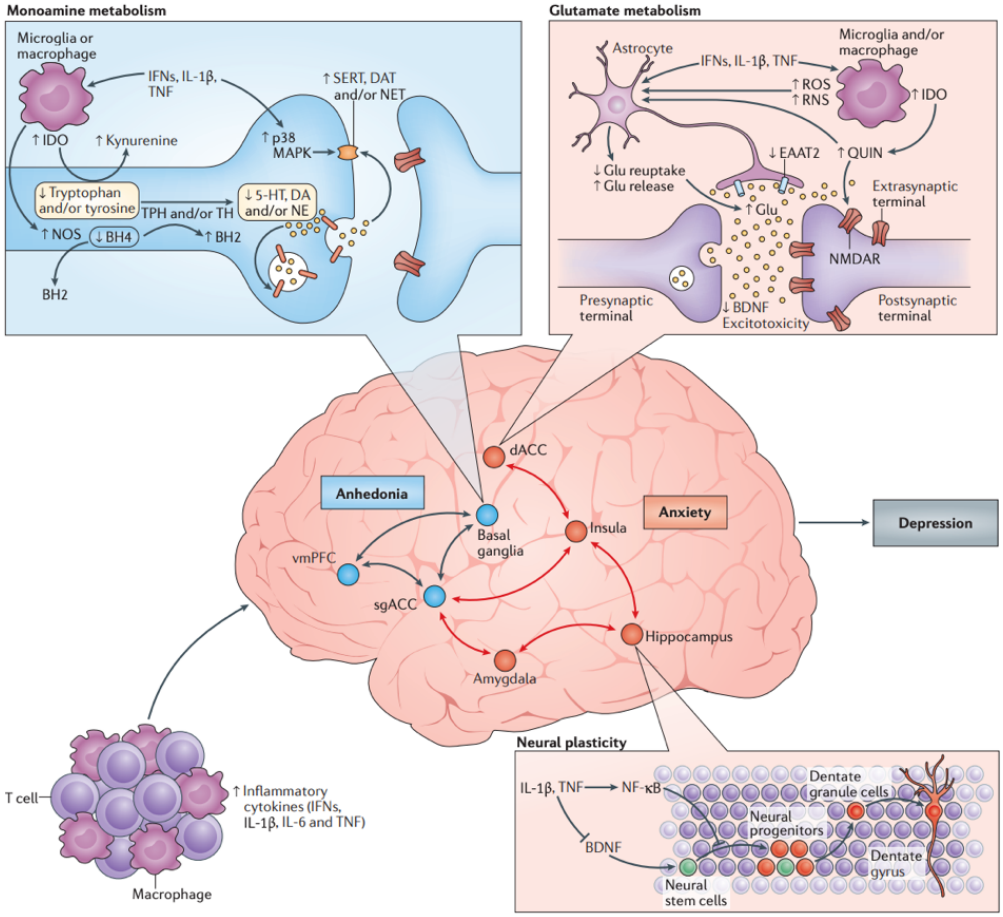


Figure 1.3. The role of neuroinflammation and E/I imbalances in the aetiology of depression (Miller & Raison, 2016). Pro-inflammatory cytokines contribute to depressive pathology by reducing monoamine neurotransmitters availability through the diversion of tryptophan metabolism towards the kynurenine (KYN) pathway. The accumulation of neurotoxic metabolites generated along this pathway, together with inflammatory cytokine activity, promote excitotoxicity and oxidative stress. Together, these processes lead to neuronal atrophy in key limbic regions, thereby contributing to the development of depressive symptoms.

Inflammatory biomarkers have garnered significant attention for their potential role in early MDD detection, treatment response monitoring, and personalised therapies. MDD patients frequently exhibit

marked increases in pro-inflammatory cytokines, including IL-6, IL-1β, TNF-α, and C-reactive protein (CRP) (Macaluso *et al.*, 2012). Although considered pro-inflammatory, IL-6 has pleiotropic effects and can exert anti-inflammatory and neurotrophic effects under certain conditions, complicating its role in depression pathophysiology (Grebenciucova & VanHaerents, 2023). Together, markers of neuroinflammation are not only useful in identifying inflammatory depression subtypes but also have predictive value in treatment planning. Interestingly, clinical trials show that MDD patients with an elevated immune status benefit more from anti-inflammatory therapies, such as non-steroidal anti-inflammatory drugs, when added to standard antidepressant treatment regimens (Raison *et al.*, 2010; Kopschina Feltes *et al.*, 2017). Furthermore, indoleamine 2,3-dioxygenase, kynurenine, and quinolinic acid levels can be measured peripherally in blood and CSF which are correlated with depression symptom severity (Raison *et al.*, 2010). These findings underscore the role of systemic and central inflammation in the pathophysiology of depression, and the utility of targeting the immune system to more effectively treat MDD.

1.3.4 The neurotrophic hypothesis

The neurotrophic hypothesis of depression is based on the correlation between reduced neurotrophins and greater depressive symptomology as a result of neuronal loss and cortical atrophy (Martinowich *et al.*, 2007). BDNF is the most widely studied neurotrophin and is a critical mediator of synaptic plasticity, neurogenesis, and neuronal survival, particularly in mood-regulating regions like the hippocampus and PFC (Neto *et al.*, 2011). Thus, BDNF is also responsible for promoting regeneration and resistance in response to stressful stimuli or insults (Duman *et al.*, 2016). Neuronal health is compromised in depressed patients, due to significant reductions in BDNF and its key transcription factor, CREB (Neto *et al.*, 2011). BDNF and CREB deficits are linked to decreased hippocampal neurogenesis, contributing to the reduced volume consistently observed in depressed patients (Neto *et al.*, 2011). Moreover, downstream disruptions in critical intracellular signalling factors, including mechanistic target of rapamycin (mTOR), protein kinase B (Akt), and eukaryotic elongation factor 2 (eEF2), exacerbate synaptic deficits by impairing protein synthesis and cell survival mechanisms (Figure 1.4) (Ignácio *et al.*, 2016). Consequently, MDD brains are characterized by axonal atrophy, reduced dendritic arborization, and a loss of dendritic spine density (Yao *et al.*, 2024). Dendritic spine loss diminishes signal integration within neurons, attenuating cortical activity, and contributing to the cognitive and memory impairments frequently observed in depression (Yao *et al.*, 2024). These neuroplasticity deficits are driven by various depression-related physiological pathways, most notably

by sustained glucocorticoid (GC) exposure associated with HPA axis dysfunction (Daskalakis *et al.*, 2015).

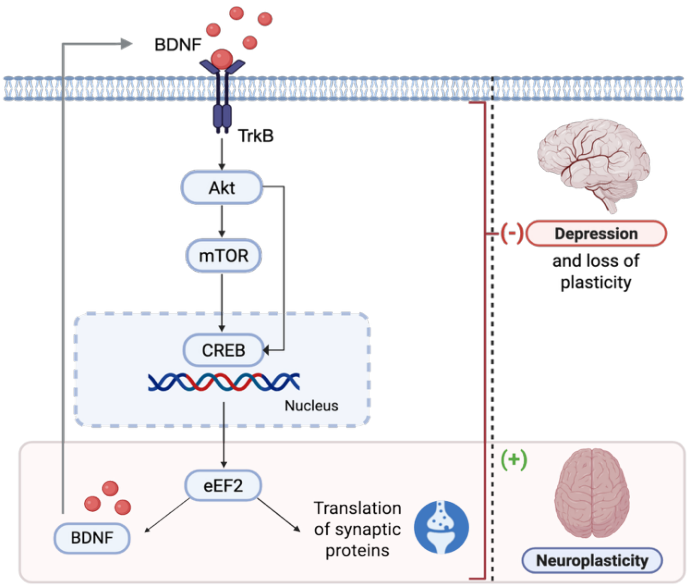


Figure 1.4. Signalling factors involved in the neurogenesis pathway adapted from (Ignácio *et al.*, 2016). Brain-derived neurotrophic factor (BDNF) binds to tropomyosin receptor kinase B (TrkB), triggering receptor dimerization and activating the protein kinase B (Akt) and mechanistic target of rapamycin (mTOR) signalling cascade. Akt and mTOR promote the phosphorylation and activation of cAMP response element-binding protein (CREB), which up-regulates genes essential for synaptogenesis. Eukaryotic elongation factor 2 (eEF2) promotes activity-dependent translation of synaptic proteins, including BDNF, establishing a positive feedback loop that sustains synapse formation.

Given BDNF's critical role in the onset and persistence of MDD, it is one of the most frequently proposed biomarkers (Arosio *et al.*, 2021). Although primarily produced in the brain, BDNF levels are also influenced by signals from peripheral pathways that cross the BBB. Evidence shows that hippocampal BDNF levels may be modulated by peripheral tissues via the vagus nerve, thus influencing adult neurogenesis (Fülling *et al.*, 2019). In support of these findings, researchers have demonstrated a positive correlation between blood and brain tissue BDNF. In both tissues, BDNF and its receptor, tropomyosin receptor kinase B (TrkB), are reduced in MDD, with clinical studies indicating more pronounced aberrations in cases of severe depression (Klein *et al.*, 2011). Additional studies demonstrate that healthy participants exhibit significantly higher serum BDNF compared to untreated MDD patients, but levels are comparable to those on antidepressant treatment (Emon *et al.*, 2020). Similarly, studies show significant reductions in serum BDNF levels between baseline and follow-up in patients who had developed MDD (Ihara *et al.*, 2016). Beyond MDD, BDNF levels may potentially serve as a biomarker for suicidal behaviour. One study found that female MDD patients with a history of suicide attempts also exhibited significantly lower plasma BDNF than those with no attempt history (Kudinova *et al.*, 2019). These studies emphasise the role of BDNF as a crucial MDD biomarker as

well as its role in assessing treatment response and suicidal risk. In contrast to mature BDNF and its downstream molecules, the precursor proBDNF has distinct and opposing functions to mature BDNF. ProBDNF levels are upregulated in MDD and are positively correlated with Hamilton Depression Rating Scale scores (Zhou *et al.*, 2013). Depression disrupts the balance between pro and mature BDNF pathways to favour proBDNF signalling. ProBDNF, in turn, induces apoptosis, dendritic pruning, and neuronal atrophy and a pro-depressant response (Zagrebelsky *et al.*, 2005). Importantly, antidepressant treatments have been shown to reverse these depression-induced alterations, normalizing both pro- and mature BDNF levels, even in the absence of full clinical remission (Gelle *et al.*, 2021).

Downstream of BDNF, additional signalling factors involved in the neurogenesis pathway include Akt, mTOR, eEF2, and CREB. These intracellular signalling factors have garnered attention for their roles in depression and the antidepressant response (Yang *et al.*, 2013; Ignácio *et al.*, 2016). The mTOR pathway, which regulates protein synthesis and synaptic remodelling, is inhibited in chronic stress and depression, contributing to deficits in synaptic plasticity (Li *et al.*, 2010). Rapid-acting antidepressants, such as ketamine, have been shown to activate mTOR, leading to increased synaptic protein synthesis and dendritic spine growth, which are correlated with rapid symptom alleviation (Li *et al.*, 2010). Similarly, Akt, a key regulator of cell survival and plasticity, exhibits reduced activity in depression, particularly in limbic regions (Karege *et al.*, 2007). Antidepressant treatments, including selective serotonin reuptake inhibitors (SSRIs) may restore Akt activity (Guo *et al.*, 2024). eEF2 is a key regulator of protein translation crucial in synaptic plasticity and is dysregulated in depression. Stress-induced eEF2 phosphorylation suppresses the translation of synaptic proteins and impairs plasticity, whereas antidepressants normalize eEF2 signalling to restore protein synthesis (Autry *et al.*, 2011; Yang *et al.*, 2013). CREB is a transcription factor downstream of BDNF that plays a central role in synaptic plasticity and neuronal resilience. CREB is activated via phosphorylation (p-CREB) by BDNF, neurotransmitters, and second messengers leading to binding at gene promotors to initiate transcription of various genes (Blendy, 2006). Depressed individuals exhibit decreased CREB activity that leads to impaired transcription of neuroplasticity-related genes such as *BDNF* and *TrkB* (Sakamoto *et al.*, 2011). This gene downregulation impairs neurogenesis and synaptogenesis, and reduces dendritic arborisation, all contributing to a depressive state (Blendy, 2006). Decreased CREB signalling has therefore been associated with depression risk and with reduced responsiveness to antidepressant therapies. Chronic antidepressant treatments enhance CREB activity and restore the expression of BDNF and other genes essential for synaptic remodelling and mood regulation (Blendy, 2006). However, preclinical models show conflicting results with some showing downregulated p-CREB while others show raised CREB levels. This variability indicates that depressive effects may be more complex, with CREB being dysregulated and not merely reduced (Böer *et al.*, 2007; Yao *et al.*, 2022). Taken together, the dysregulation of the BDNF signalling cascade and its downstream effectors, including mTOR, Akt, eEF2, and CREB offer important insights into the pathophysiology of MDD. These findings underscore

the potential of neuroplasticity biomarkers, not only in diagnostics, but also for monitoring and understanding treatment response mechanisms (Zhou *et al.*, 2013).

1.3.5 HPA axis dysregulation

The HPA axis theory of depression posits that chronic stress and disrupted feedback mechanisms result in HPA axis dysregulation, which plays a critical role in the development and persistence of depression (Keller *et al.*, 2017). In response to environmental stressors such as psychosocial stress, HPA axis activation leads to the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH), which subsequently triggers the adrenal glands to produce cortisol, the body's primary stress hormone (Figure 1.5). Chronically raised cortisol levels result in hippocampal atrophy, cognitive decline, and impaired neurogenesis which all contribute to mood disorders such as anxiety and depression (Keller *et al.*, 2017). Under normal circumstances, homeostatic mechanisms maintain a healthy HPA axis setpoint through a negative feedback system (Gjerstad *et al.*, 2018). The primary target for negative feedback is glucocorticoid receptors (GRs), which are widely expressed in brain regions such as the paraventricular nucleus (PVN), hippocampus, amygdala, and PFC (Sheng *et al.*, 2021). Upon activation, GRs bind to glucocorticoid response elements (GREs) or interact with transcription factors to regulate the expression of genes that modulate HPA axis activity (Sheng *et al.*, 2021). These genomic alterations reduce CRH and ACTH secretion, regulating the duration and magnitude of glucocorticoid release (Sheng *et al.*, 2021). However, during chronic stress, cortisol levels remain persistently high due to impaired negative feedback mechanisms. This maladaptive response often includes reduced GR sensitivity and central hypersecretion of CRH, resulting in chronic HPA axis hyperactivity (Mikulska *et al.*, 2021). Persistently elevated cortisol levels inhibit neurogenesis and alter neurotrophin expression in mood-regulating areas of the brain (Mikulska *et al.*, 2021). The resulting neuronal atrophy in these brain regions leads to emotional instability and heightened anxiety often observed in depression (Menke, 2019). Concerningly, HPA axis dysregulation exhibits a causal and bi-directional relationship with an array of systemic pathologies such as immune system dysregulation, affective disorders, hypertension, and depression (Menke, 2019). Given the HPA axis's central role in MDD and its link to other systems, understanding its mechanisms is key to identifying therapeutic targets and MDD biomarkers.

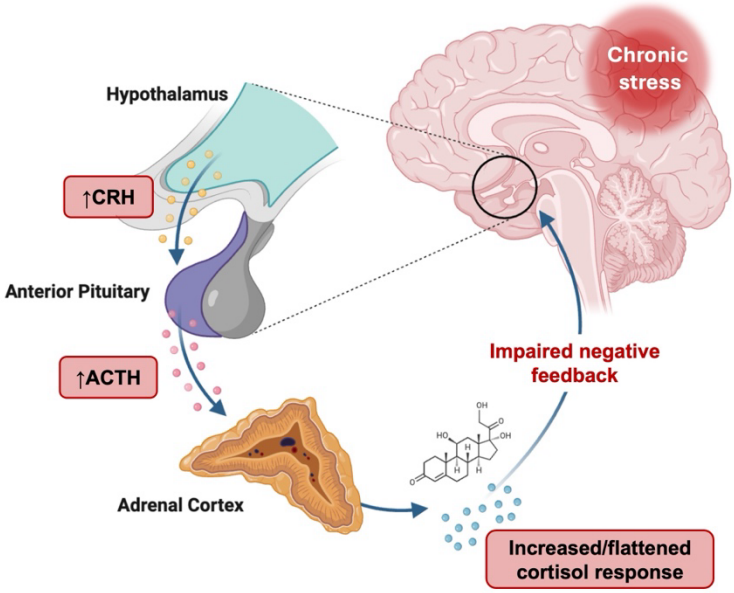


Figure 1.5. Schematic of HPA axis dysregulation. Under conditions of chronic stress, the hypothalamus releases corticotropin-releasing hormone (CRH), triggering the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary and the adrenal release of cortisol. Sustained glucocorticoid exposure leads to impaired negative feedback due to glucocorticoid receptor resistance, resulting in elevated ACTH and cortisol levels that are implicated in major depressive disorder.

Aberrant HPA axis activity is a hallmark of MDD characterized by dysregulated CRH, ACTH, and circulating glucocorticoid levels (Menke, 2019). Transient cortisol increases are a normal and adaptive stress response; however, when chronically dysregulated, cortisol has pathological effects (Ulrich-Lai & Herman, 2009). Studies show increased morning and evening cortisol levels in MDD patients when compared to controls (Vreeburg *et al.*, 2009). Moreover, a higher cortisol awakening response (CAR) is more pronounced in individuals with severe depressive symptoms and is often associated with comorbid anxiety (Vreeburg *et al.*, 2009). Abnormal CAR dynamics, including exaggerated or blunted responses, as well as flattened diurnal cortisol-concentration slopes, have been linked to worse treatment outcomes (Fischer *et al.*, 2017). Elevated baseline cortisol levels in MDD patients are also predictive of poorer responses to psychological therapies, when compared to patients with lower baseline levels (Fischer *et al.*, 2017). Importantly, HPA axis dysfunction is not only characterised by cortisol increases, but also by decreases and blunting of the cortisol response. Evidence shows that early childhood adversity causes a blunted cortisol response which increases the risk for various psychiatric disorders including depression (Williams *et al.*, 2024). These findings highlight the correlation between cortisol and MDD severity. In MDD, a major consequence of HPA axis dysfunction involves GR desensitisation which can be assessed using a dexamethasone suppression test. GR dysfunction is characterised by glucocorticoid resistance, which is associated with poorer treatment outcomes (Cohen *et al.*, 2012). Additionally, polymorphisms in GR modulators, such as FK506 binding protein 5 (FKBP5), serve as potential biomarkers for depression susceptibility and treatment response, as

increased FKBP5 expression is linked to decreased GR sensitivity (Pierscionek *et al.*, 2014). Other receptors such as 5-HT_{1A} and 5-HT_{2C} strongly influence HPA axis function. Sustained GC exposure may downregulate or desensitise these receptors, impairing 5-HT signalling and SSRI efficacy. Assessing 5-HT receptor expression/sensitivity alongside endocrine-related biomarkers may prove to be an additional depression profiling tool (Pierscionek *et al.*, 2014).

To develop more targeted interventions using these biomarkers, a deeper understanding of the biological mechanisms underlying depression is essential. Although it is well established that multiple physiological systems contribute to the aetiology of depression, investigating the underlying disease processes is difficult due to the limited access to human tissues. As a result, researchers increasingly rely on animal models which provide a practical, more ethical means of investigating depression pathophysiology under controlled conditions.

1.4 Preclinical models of depressive-like symptoms

Clinical research is limited due to the ethical consideration of obtaining blood and post-mortem brain tissue samples. Furthermore, these samples are subject to multiple confounding factors such as patient demographics, environmental exposure, medication history, post-mortem intervals, and variability in sample quality (Wang *et al.*, 2017). Rodent models help overcome many of these research limitations and are especially valuable due to their close phylogenetic proximity to humans, which results in comparable brain development and structural organization (Figure 1.6) (Khodosevich & Sellgren, 2023).

Given the heterogeneity of the disorder and the limitations of human studies, understanding the molecular mechanisms underlying depression is difficult. MDD is characterised by a wide array of clinical symptom clusters which can differ significantly between individuals and vary across depression sub-types (Planchez *et al.*, 2019). This variability complicates efforts to establish universal biomarkers or mechanisms for the disorder. Furthermore, while various environmental, social, and psychological factors play an important role in MDD aetiology, they act as confounders that considerably influence the outcome of human molecular studies (Wang *et al.*, 2017). Additionally, limited access to the brain and other human tissues restricts the ability to perform detailed molecular analyses or to investigate the mechanisms of antidepressant action *in vivo*. Animal models provide a practical and powerful approach to delineate the major neurobiological underpinnings of depression in a controlled and reproducible manner.

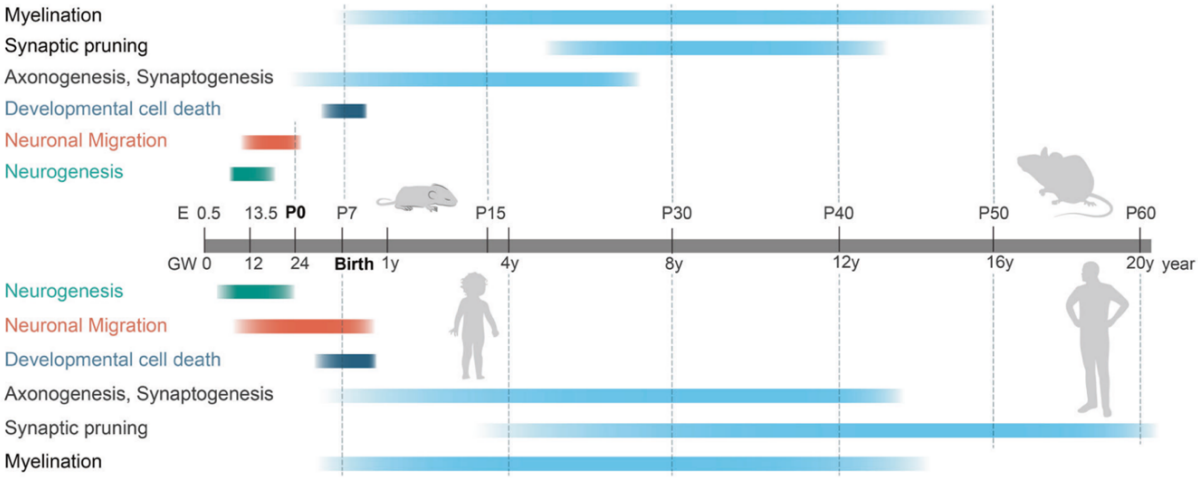


Figure 1.6. An overview of brain development in rodents and humans (Khodosevich & Sellgren, 2023).

Over time, animal models have been refined to capture key features of human depressive behaviours such as anhedonia, despair, and cognitive deficits (Krishnan & Nestler, 2011). Key neurobiological pathways implicated in depression, such as the HPA axis, neurogenic, and monoaminergic systems, exhibit remarkable similarities between rodents and humans. These similarities make rodents an ideal species for translational research (Khodosevich & Sellgren, 2023). Moreover, these models provide ethical ways to study neuronal circuitry and molecular pathways underlying depression, while ensuring adequate sample sizes. To further enhance translatability, animal models are established based on three key constructs: face validity, which requires that the animal phenotype closely resembles the clinical phenotype; predictive validity, which ensures that the phenotype is responsive to interventions effective in humans; and construct validity, which exhibits similar etiological processes between humans and animals that contribute to the pathology (Nestler & Hyman, 2010). In this regard, multiple animal models of depression have been developed by manipulating pathways known to be involved in its aetiology (Table 1.1). While no single model can fully recapitulate the complexity of MDD, specific paradigms are designed to mirror aspects of the disorder.

1.4.1 Classical models

The most widely used animal models for studying depression include the learned helplessness model, the chronic unpredictable mild stress (CUMS) model, and the olfactory bulbectomy (OBX) model (Willner, 2017). The learned helplessness model demonstrates how animals exposed to chronic uncontrollable stressors, such as electric shocks, develop passive reactivity patterns and a generalised expectation of helplessness. Behavioural outcomes of this model include impaired learning, passivity, reduced motivation, and anhedonia which all mimic depression symptomology (Maier & Seligman, 1976). The neurobiological underpinnings of this model have been linked to alterations in monoamine neurotransmitter systems as well as reduced inhibitory control over stress-responsive areas of the brain

by the PFC (Weiss *et al.*, 1981). This model demonstrates how exposure to chronic stressors leads to feelings of helplessness and diminished motivation associated with clinical depression. Similarly, the CUMS model involves exposing rodents to a series of mild stressors over time to induce anhedonia (Willner, 2017). Stressors include chronic changes in housing conditions and exposure to novel environments or social stressors that lead to a gradual decrease in the rodents' ability to experience pleasure or reward. In addition to monoamine dysregulation, the CUMS model is linked to HPA axis dysregulation and increased cortisol levels which, clinically, exacerbate depressive symptoms (Kvarta *et al.*, 2015). This model demonstrates high face validity due to the fact that it replicates anhedonia, a core symptom of depression. The link between chronic stress, HPA axis dysfunction, and anhedonic tendencies in this model also parallel findings in MDD patients (Willner, 2017). The (OBX) model involves surgically removing the rodent olfactory bulb which causes major dysfunction in the cortical-hippocampal-amygdala circuit (Song & Leonard, 2005). In both rodents and humans, this circuit is responsible for emotion and stress regulation as well as memory formation (Song & Leonard, 2005). In rodents, dysfunction in this limbic circuit leads to depression-related behaviours such as impairments in spatial learning and food-motivated behaviours, hyperlocomotion, and disrupted sexual behaviour (Harkin *et al.*, 2003). The OBX model and related symptoms parallel impaired structural and functional connectivity in cortico-limbic structures in humans that result in similar MDD symptoms. This model is notable for its face validity, as rodents respond to chronic but not acute antidepressant treatment, mirroring the time frame observed in the clinical setting (Harkin *et al.*, 2003).

1.4.2 Genetic models

Other depression models induce a depressive-like state by manipulating genes associated with MDD vulnerability. BDNF knockout mice exhibit impaired neurogenesis and synaptic plasticity which mirrors BDNF reductions and related functional impairments in clinical depression (Monteggia *et al.*, 2007). SERT knockout impairs serotonin regulation and alters the stress-response system. Mice with SERT knockout exhibit increased anxious and depressive-like behaviours that parallel the increased depression risk in individuals with SERT polymorphisms (Holmes *et al.*, 2003). GR knockouts alter GR function that disrupts HPA axis regulation, which in turn, triggers a plethora of pathological cascades (Boyle *et al.*, 2005). Together, these genetic alterations produce symptoms that mimic those of clinical MDD (Holmes *et al.*, 2003; Boyle *et al.*, 2005; Monteggia *et al.*, 2007). Conversely, knockouts of epigenetic modifiers such as histone deacetylase (HDAC) yield antidepressant-like effects along with enhanced neuroplasticity (Fukada *et al.*, 2012). Additionally, transgenic models with overexpression of genes such as FKBP5, a regulator of GR sensitivity, have been used to study the impact of stress and HPA axis dysregulation on mood disorders (Binder *et al.*, 2004). Overall, genetic and epigenetic manipulation models provide a targeted framework for understanding the complex interplay of pathways implicated in depression, while highlighting potential therapeutic targets.

1.4.3 Interventional models

Chemical means can be used to activate the immune system, causing inflammation. Inflammation-related models, such as the lipopolysaccharide (LPS) model, have been developed to explore the link between neuroinflammation and depression (Dantzer *et al.*, 2008). In this model, administration of LPS, a component of gram-negative bacteria, induces depressive-like behaviours by triggering systemic immune activation. Systemic inflammation stimulates the release of pro-inflammatory cytokines which activate microglia, resulting in neuroinflammation (Yin *et al.*, 2023). The inflammatory cascade impairs monoamine neurotransmitter synthesis and reduces their synaptic availability through increased re-uptake. Additionally, pro-inflammatory cytokines promote excitotoxicity and oxidative damage and prevent neurogenesis. These processes lead to neuronal degeneration, particularly in mood-regulating regions, contributing to a depressive-like phenotype (Yin *et al.*, 2023). The LPS model thus simulates clinical inflammation-induced depression by promoting neuroinflammation and neuronal damage in rodents, findings that have also been observed in our lab (Pienaar *et al.*, 2024, 2025). This model is especially useful for investigating inflammatory mechanisms involved in MDD and for evaluating the antidepressive efficacy of anti-inflammatory treatments (Dantzer *et al.*, 2008).

Similarly, based on the link between HPA axis dysfunction and depression, the stress-response system can be pharmacologically manipulated to induce a depressive-like phenotype. Cushing's patients that exhibit ACTH hypersecretion and hypercortisolaemia also display resistance to tricyclic antidepressants. Patients with MDD and treatment-resistant depression (TRD) that have Cushing's syndrome respond to steroid-suppressive agents, highlighting the etiological link between these two disorders. Pathological doses of exogenous corticosterone (CORT) or ACTH are used to dysregulate the HPA axis, producing a depressive-like state. ACTH stimulates CORT production via the melanocortin-2 receptor (MC2R) in the adrenal cortex and can cause dysregulation when elevated. CORT is an HPA axis feedback regulator that becomes maladaptive under chronic stress conditions (Gjerstad *et al.*, 2018). Importantly, continuous ACTH administration directly augments GC production without triggering negative feedback mediated by CORT-GR binding (Sterner & Kalynchuk, 2010). Impaired negative feedback results in persistent HPA axis activation. Chronic CORT/ACTH exposure impairs neurogenesis and neuronal repair by down-regulating BDNF. Reduced BDNF compromises neuronal integrity and leads to dendritic pruning and reduced spine density that impairs neuronal connectivity. Compounding on these effects, sustained CORT exposure induces neuroinflammation that further damages neurons (Woodburn *et al.*, 2021). Together, these processes induce atrophic structural remodelling in cortico-limbic regions that manifest as MDD symptoms. Indeed, stress-induced reductions in hippocampal volume are observed in MDD patients and those with Cushing's syndrome (Campbell & MacQueen, 2004). In rats, these pathological brain changes manifest as increased immobility in the forced swim test (FST) and decreased sucrose preference in the sucrose preference

test (SPT), reflecting despair and anhedonia, respectively (Kitamura *et al.*, 2002; Sallie *et al.*, 2024). These behaviours closely mirror symptoms of clinical depression, enhancing the translational validity of this model (Pereira *et al.*, 2019). Several studies that have utilised the model of ACTH-induced HPA axis dysregulation report various neurobehavioral and molecular alterations associated with a depressive phenotype. The ACTH model has further demonstrated resistance to tricyclic antidepressants (TCAs) but has not been validated against other classes of antidepressants. Although the ACTH-induced HPA axis dysregulation model has been used to simulate depressive-like symptoms, its validation as a model for TRD remains incomplete due to its preclinical nature and lack of correspondence with the clinical criteria for TRD (Kitamura *et al.*, 2002; Petrović *et al.*, 2018; Pereira *et al.*, 2019; Sallie *et al.*, 2024). Nevertheless, the ACTH model offers several translational advantages for studying depression. Many stressed-based depression models respond to monoamine-targeting antidepressants, while the ACTH model induces HPA axis dysregulation and reduces responsiveness to these treatments, which more closely mirrors features of TRD (Kitamura *et al.,* 2002). The model demonstrates strong face validity, with both neurobehavioural outcomes (increased despair-like behaviour in the FST and reduced sucrose preference) and endocrine markers (elevated ACTH and corticosterone) mirroring features of clinical TRD (Pereira *et al.,* 2019; Ivanović *et al.*, 2025). The model also demonstrates construct validity by inducing HPA axis dysregulation, which is prevalent in a large portion of individuals with MDD, and shows emerging predictive validity by reliably screening for novel, non-monoamine antidepressants (Menke *et al.,* 2019; Pereira *et al.,* 2019; Ivanović *et al.*, 2025) (Table 1.2).

However, studies typically validate the ACTH model using one or two neurobehavioural tests, limiting the robustness and generalisability of their findings. Therefore, in this thesis, we aimed to validate the ACTH model by using a battery of neurobehavioural tests and by measuring changes in neurotrophic gene expression.

Although these are the most commonly used models, several additional paradigms have been developed to investigate the mechanisms underlying depression (Table 1.1).

Table 1.1. Summary of common rodent models of depressive-like symptoms.

Model	Mechanism of induction	Clinical relevance	Strengths	Limitations	References
Chronic unpredictable mild stress	Exposure to a series of unpredictable, mild stressors over several weeks.	Models chronic stress-induced depression, mimicking environmental stressors in humans.	High translational value; replicates chronic stress effects.	Time-intensive; variability in stress exposure effects.	Antoniuk *et al.*, 2019
Learned helplessness	Subjecting rodents to inescapable stressors until they develop learned helplessness behaviours.	Replicates aspects of learned helplessness and lack of motivation in depression.	Well-validated; responds to antidepressant treatment.	May not fully replicate complex features of depression.	Maier & Seligman, 2016
Chronic social defeat stress	Social stress induced by repeated defeat by an aggressive counterpart.	Mimics social stress and its role in depression, relevant for comorbid anxiety.	Models social stress, which is highly relevant to human depression.	Limited to social defeat aspects; gender bias (mostly male rodents).	Golden *et al.*, 2011
Chronic restraint stress	Physical restraint for extended periods, causing stress-induced depressive-like behaviours.	Induces physiological and behavioural changes associated with acute stress.	Simple and reproducible; effective for acute stress studies.	Models acute stress, less relevant for chronic depression.	Antoniuk *et al.*, 2019
Maternal separation	Early-life stress induced by separating pups from their mothers for specific durations.	Simulates early-life stress, relevant for studying developmental origins of depression.	High relevance to developmental and early-life stress research.	High variability in stress effects; lacks face validity for adult depression.	Lippmann *et al.*, 2007
Olfactory bulbectomy	Surgical removal of the olfactory bulbs, leading to depressive-like symptoms.	Mimics limbic system dysfunction observed in major depressive disorder.	Robust neurochemical and behavioural changes; well-characterized.	Invasive procedure; models specific rather than general depression symptoms.	Song & Leonard, 2005
Genetic manipulation (e.g., BDNF knockout)	Genetic engineering to manipulate depression-relevant genes, such as BDNF.	Explores the genetic basis of depression by investigating specific gene disruptions.	Provides insights into the molecular and genetic underpinnings of depression.	Highly specific to the manipulated gene; lacks generalizability.	Monteggia *et al.*, 2007; Arosio *et al.*, 2021
Interventional models (LPS, corticosterone, and ACTH administration)	Systemic administration of LPS to induce inflammation and cytokine release.	Models inflammation-associated depression by mimicking sickness behaviours and immune dysregulation.	Establishes a clear link between inflammation and depressive behaviours.	May not fully replicate the multifactorial nature of human depression.	Dantzer *et al.*, 2008; Yin *et al.*, 2023; Pienaar *et al.*, 2025
	Chronic administration of corticosterone or ACTH to mimic HPA axis hyperactivity observed in depression.	Replicates stress hormone dysregulation seen in patients with HPA axis abnormalities.	Accurately models physiological stress responses and neuroendocrine dysregulation.	Does not fully capture psychosocial aspects of depression.	Sterner & Kalynchuk, 2010; Sallie *et al.*, 2024; Ivanović *et al.*, 2025

ACTH: adrenocorticotropic hormone; BDNF: brain-derived neurotrophic factor; HPA: hypothalamic-pituitary-adrenal; LPS: lipopolysaccharide.

Table 1.2. Summary of the effects of chronic ACTH administration on physiological and behavioural parameters in rodents.

ACTH dose	Treatment duration	Rat sex/ strain	Key findings in ACTH-treated rats relative to controls	References
10 μg, sc	21 days	Male Wistar rats	Increased immobility in the FST; Elevated plasma superoxide anions, advanced oxidation protein products, malondialdehyde, and total oxidant status; Reduced activity of superoxide dismutase, paraoxonase 1 in plasma; Increased DNA damage in peripheral blood lymphocytes.	Ivanović *et al.*, 2025
100 μg/0.1 ml, sc	42 days	Male SD rats	Increased sucrose consumption in the SPT; Elevated hippocampal BDNF and elevated CREB in the PFC, striatum, hippocampus, and midbrain.	Sallie *et al.*, 2024
100 μg/0.1 ml, sc	14 days	Male SD rats	Elevated plasma CORT and ACTH.	Pereira *et al.*, 2019
10 μg/400 μL, sc	21 days	Male Wistar rats	Increased immobility in the FST; Decreased centre activity in the OFT; Increased Ki-67+ cell count; Elevated plasma CORT and ACTH.	Petrović *et al.*, 2018
100 μg, sc	21 days	Male Wistar rats	Increased immobility in the FST; Decreased ATP-generating capacity.	Kim *et al.*, 2016
0.45 mg/kg, sc	14 days	Female C57BL/6 mice	Elevated CORT, KYN, 5-HIAA, hippocampal IDO; Increased immobility in the FST; Increased latency to feed in the NSFT.	Antunes *et al.*, 2015
100 μg/0.2 ml, sc	14 days	Male Wistar rats	Decreased number of c-Fos+ cells in the CA3 region of the hippocampus.	Li *et al.*, 2013
100 μg/0.1 ml, sc	14 days	Male SD rats	Elevated serotonin and noradrenaline, decreased dopamine in the PFC.	Walker *et al.*, 2013
100 μg/0.2 ml, sc	14 days	Male Wistar rats	Increased hippocampal and cerebellar D-serine, L-serine, glutamine, and glycine and increased glutamate in the cerebellum; Increased glutamate and glycine in the PFC and striatum; Increased immobility in the FST; Decreased locomotor activity.	Tokita *et al.*, 2012
100 μg/0.2 ml, sc	14 days	Male Wistar rats	Increased 5-HT_{2A} receptor mRNA expression.	Kitamura *et al.*, 2008
0.5 mg/kg, ip	7 days	Mice	Elevated serum corticosterone; Decreased hippocampal CRH and CRH-BP mRNA levels.	Wang *et al.*, 2004
100 μg/0.2 ml, sc	14 days	Male Wistar rats	No change in behavioural parameters compared to controls.	Kitamura *et al.*, 2002

5-HIAA: 5-hydroxyindoleacetic acid; BDNF: brain-derived neurotrophic factor; CREB: cAMP response element-binding protein; CRH-BP: corticotropin releasing hormone-binding protein; FST: Forced swim test; IDO: indoleamine 2,3-dioxygenase; KYN: kynurenine; NSFT: novelty supressed feeding test; OFT: open field test; PFC: prefrontal cortex.

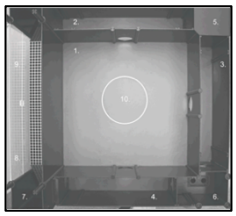
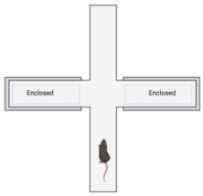
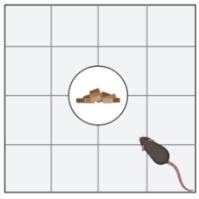
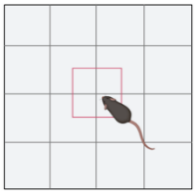
The characteristics outlined in Table 1.2 provide the rationale for selecting the ACTH model to investigate depression pathophysiology in the present study. To establish the translational relevance of these depression-related rodent models, neurobehavioral assessments are necessary to validate depressive-like phenotypes. Since depression is characterised by a broad range of symptoms, multiple symptom-specific tests are needed. By integrating findings from various tests, behavioural outputs not only facilitate the characterization of depressive-like states but also provide critical insights into the neural circuits and molecular pathways driving these behaviours. These behavioural insights enhance the translational relevance of preclinical findings to human conditions.

1.5 Assessment of depressive-like symptoms in preclinical models

Neurobehavioral assessments of depressive phenotypes and antidepressant efficacy are essential for advancing our understanding of MDD. MDD is a multisymptomatic disorder characterised by despair, anhedonia, appetite changes, anxiety, and psychomotor retardation (American Psychiatric Association, 2022). Similarly, in preclinical depressive models, rats exhibit various behavioural changes that translationally mirror symptoms of clinical depression. Rodent behavioural changes are evaluated using an array of established neurobehavioral tests that each assess certain symptomatic features of MDD (Table 1.3). Given the heterogeneity of depressive symptoms, multiple neurobehavioral tests are necessary to sufficiently characterise rodent models of depressive-like symptoms (Sallie *et al.*, 2024).

Table 1.3. Common neurobehavioral tests used in preclinical depression and anxiety research.

Neurobehavioral test	Phenotypic response	Clinical translation	Reference
 Forced swim test	Immobility is measured as an indicator of learned helplessness and despair-like behaviour, serving as a metric for antidepressant screening.	Mimics behavioural despair observed in depression, evaluates antidepressant effectiveness.	Porsolt *et al.*, 1977
 Tail suspension test	Measures the duration of immobility when the animal is suspended by the tail, reflecting passive coping or behavioural despair.	Used to assess depressive-like states and screen antidepressant activity, particularly in mice.	Steru *et al.*, 1985
 Sucrose preference test	Drinking behaviours and sucrose preference are assessed as a measure of anhedonia.	Reflects loss of pleasure, a key depressive symptom.	Willner *et al.*, 1987

 Light-dark box test	Aversion to brightly lit, open spaces and exploratory behaviour is measured as an indicator of anxiety-like behaviour, serving as a metric for anxiolytic drug screening.	Reflects avoidance behaviour related to anxiety disorders.	Bourin & Hascoët, 2003
 Multivariate concentric square field test	Exploratory behaviour, anxiety, and risk assessment behaviours are evaluated for complex behavioural profiling.	Models anxiety and social avoidance behaviours.	Roman *et al.*, 2007
 Elevated plus maze	Shelter-seeking is assessed as a measure of anxiety-like behaviour and anxiolytic drug effectiveness.	Mimics anxiety-related avoidance behaviour.	Walf & Frye, 2007
 Novelty-suppressed feeding test	Feeding motivation in a novel, open arena is measured as an indicator of anxiety-like behaviour and approach-avoidance conflict, serving as a metric for anxiolytic and antidepressant drug screening.	Models anxiety and evaluates approach behaviour.	Samuels & Hen, 2011
 Open field test	General locomotion, exploration, and thigmotaxis are assessed as a measure of anxiety-like behaviour and anxiolytic drug effectiveness.	Mimics anxiety-related avoidance behaviour and psychomotor retardation.	Seibenhener & Wooten, 2015

1.5.1 Despair-like phenotype: escape-based paradigms

The FST has been one of the most widely used tests to assess behavioural despair and helplessness. This test involves placing rodents in a tank of water and analysing their mobility in response to the inescapable stressor for a fixed duration of time. Increased immobility in this test indicates behavioural despair, a common symptom of MDD (Porsolt *et al.*, 1978). The sensitivity of the FST in detecting the reversal of depression-induced immobility by antidepressants, such as selective serotonin and

norepinephrine reuptake inhibitors (SNRIs), is well-documented making it a reliable tool for assessing antidepressant efficacy (Porsolt *et al.*, 1978). Another key feature of the FST includes the test's ability to differentiate between different pharmacological mechanisms linked to climbing and swimming behaviours guiding the development of novel therapies (Detke *et al.*, 1995). Similar to the FST, the tail suspension test (TST) assesses mobility as an indicator of behavioural despair and hopelessness. Rodents are suspended by their tails for a fixed duration and the time active versus immobile is recorded (Steru *et al.*, 1985). The advantage of the TST is that in addition to its sensitivity to a wide range of drug doses, the test is also sensitive to conventional antidepressants as well as psychostimulants (Steru *et al.*, 1985). The effects of antidepressants in the TST show concordance with that of the FST since both tests are based on the concept of the 'waiting-searching strategy' (Steru *et al.*, 1985). Theoretically, the hypothesis suggests that a normal animal, when faced with an inescapable stressor, will alternate between two behaviours namely: agitation (searching strategy) using maximal energy expenditure, and immobility (waiting strategy), aimed at energy conservation (Thierry *et al.*, 1984). Evidence suggests that depression disrupts this balance and that antidepressants shift the balance towards increased 'searching' behaviour as represented by reduced immobility (Thierry *et al.*, 1984).

While neurobehavioral tests are invaluable tools in depression research, their experimental nature, along with limitations such as varying test conditions and species differences, have attracted scrutiny. The FST has faced criticism regarding its interpretation of immobility, which may reflect adaptive, passive coping strategies rather than despair (Krishnan & Nestler, 2011). Non-depressive factors, such as motor function and learning during repeated FST trials can also influence immobility and confound results (Bogdanova *et al.*, 2013). Additionally, the FST exhibits limited predictive validity, as it is only sensitive to certain classes of antidepressants. This limited sensitivity potentially produces false-positive results that misrepresent the clinical efficacy of various agents (Slattery & Cryan, 2012). Another limitation includes the effectiveness of acute monoamine-based antidepressant treatments in reducing immobility. This acute timeframe does not align with the prolonged treatment typically required for therapeutic efficacy in humans (Willner & Mitchell, 2002).

1.5.2 Anhedonic tendencies: reward-based paradigms

The SPT is widely recognised for assessing anhedonia in rodent models of depression. Rats are exposed to a two-bottle choice paradigm, one bottle containing regular water and another containing sucrose solution (Liu *et al.*, 2018). The volume consumed from each bottle is measured and compared, with anhedonia represented as a decrease in sucrose preference in the treated group relative to controls (Liu *et al.*, 2018). In preclinical studies that utilised the SPT, antidepressants have been shown to reverse decreases in sucrose preference induced by stressors or a depressive-like state (Willner *et al.*, 1987). The ability of antidepressants to enhance mood as well as pleasure-related behaviours in the SPT

reinforces their role in modulating reward pathways. In this way, the SPT demonstrates predictive validity for antidepressive efficacy (Willner *et al.*, 1987).

Despite the utility of the SPT, inconsistencies in sucrose consumption across studies, particularly in CUMS models, raise concerns about the reliability of this test. A recent review suggested that these inconsistencies may extend to other rodent models of depressive-like symptoms (Markov, 2022). The review also emphasized the complexity of translating anhedonia across species, as the human experience encompasses physical, social, and intellectual dimensions that are difficult to model in rodents. These differences challenge the translatability of findings from the SPT (Markov, 2022).

1.5.3 Anxiety-like phenotype: approach-avoidance-based paradigms

The open field test (OFT), elevated plus maze (EPM), and novelty suppressed feeding test (NSFT) are the most commonly used behavioural assays that assess anxiety-like behaviour in rodent models. In the OFT, rodents are placed in a large arena, and their movements are recorded to measure time spent in the periphery (thigmotaxis) versus the centre. Increased thigmotaxis, or shelter seeking behaviour, indicates increased anxious behaviour (Seibenhener & Wooten, 2015). Additionally, the OFT assesses locomotor activity, providing insight into potential sedative effects of drugs and the presence of psychomotor retardation (Seibenhener & Wooten, 2015). The EPM involves placing rodents in a plus-shaped arena with closed arms and open arms where increased time spent in the open arms indicates reduced anxiety (Pellow *et al.*, 1985). The NSFT however, was established based on hyponeophagia in which feeding behaviour is suppressed in novel environments (Samuels & Hen, 2011). Food-deprived rodents are placed in a novel environment and the latency to approach the food is measured, with increased latency indicating higher anxiety levels (Samuels & Hen, 2011). All three tests effectively capture the approach-avoidance conflict inherent in anxiety disorders. Since anxiety and depression commonly co-occur, anxiety assays are valuable tools for assessing whether depression models also induce anxiety-related behaviours. These approach-avoidance based paradigms may further evaluate the efficacy of various agents in ameliorating anxiety-like symptoms.

Similar to the FST, the OFT and EPM have been criticised for its susceptibility to external influences. Lighting conditions, prior exposure to the arena, time of day, noise levels, and arena size all contribute to variability during testing. These confounding variables can alter animal behaviour independently of any experimental intervention (Seibenhener & Wooten, 2015). Thigmotaxis, commonly interpreted as anxious behaviour, can also reflect risk aversion or an increased motivation to explore the periphery, further complicating result interpretations (Gromer *et al.*, 2021). These factors compromise standardisation, making it difficult to compare results between labs.

Overall, special consideration should be given to factors known to influence test outputs such as inter-strain variation, age, gender, body weight, environmental preconditioning, and intervention consistency (Bogdanova *et al.*, 2013). Additionally, most neurobehavioral tests measure a single behavioural endpoint, which fails to capture the multifaceted nature of human depression. These limitations highlight the importance of using multiple neurobehavioral tests when characterizing rodent models, particularly newer models such as the ACTH model (Sallie *et al.*, 2024). Robust characterization requires both intra-test cross-validation across different neurobehavioral assessments, and inter-test cross-validation linking behavioural outcomes to molecular biomarkers of depression.

1.6 Pharmacological management of depression

The treatment and management of depression typically involves both pharmacological and psychological approaches. Psychotherapeutic interventions, including behavioural therapy, primarily target cognitive and behavioural disruptions associated with depression. However, pharmacological interventions are often essential due to their direct modulation of neurochemical imbalances underlying depression. Despite their widespread use, many antidepressants show limited antidepressant efficacy and a delayed onset. Therefore, in this thesis, we focused on various common and novel pharmacological interventions used to treat MDD.

1.6.1 Commonly prescribed antidepressants

Despite our enhanced understanding of the various complex pathways that contribute to depression, many of the antidepressants commonly used today still only target a single aetiological pathway. Monoamine-based antidepressants have remained the primary MDD treatment choice since the 1950s, primarily targeting serotonin, dopamine, and norepinephrine pathways (Table 1.4) (López-Muñoz & Alamo, 2009). Initial multi-monoamine targeting agents, such as TCAs, produced severe side effect profiles (Gillman, 2007). Newer agents were developed with greater specificity to minimise off-target effects (Hieronymus *et al.*, 2022). However, while these drugs show clinical utility, their narrow neurotransmitter targets raise questions regarding their effectiveness. Therefore, in this thesis, we aimed to determine, beyond monoamine modulation, the effect of citalopram, an SSRI, and imipramine, a TCA, on the expression of neurogenesis-related intracellular signalling factors across depression-related brain regions in a rodent model of ACTH-induced HPA axis dysregulation.

Table 1.4. Summary of common monoamine-based antidepressants.

Drug class	Mechanism of action	Use cases	Side effects	References
SSRIs	Inhibits serotonin reuptake via the serotonin transporter	MDD, GAD, OCD, PTSD	Nausea, headache, sexual dysfunction, insomnia, and dry mouth	Chu & Wadhwa, 2023
SNRIs	Inhibits reuptake of both serotonin and norepinephrine	MDD, GAD, fibromyalgia, chronic pain	Nausea, increased BP, dry mouth, dizziness, and sexual dysfunction	Sansone & Sansone, 2014
TCAs	Inhibits serotonin and norepinephrine reuptake; block cholinergic, histaminergic, and adrenergic receptors	TRD, chronic pain	Anticholinergic effects, weight gain, sedation, cardiac arrhythmias, and overdose risk	López-Muñoz & Alamo, 2009
MAOIs (monoamine oxidase inhibitors)	Inhibit monoamine oxidase enzyme, reducing breakdown of serotonin, norepinephrine, and dopamine	Atypical or treatment-resistant depression	Hypertensive crisis with tyramine, insomnia, weight gain, and sexual dysfunction	Youdim *et al.*, 2006
Atypical antidepressants	Bupropion: norepinephrine-dopamine reuptake inhibitor (NDRI); mirtazapine: alpha-2 antagonist	MDD, fatigue-related depression, smoking cessation	Bupropion: insomnia, seizures; Mirtazapine: sedation, and weight gain	Sheffler *et al.*, 2025
SARIs (serotonin antagonist and reuptake inhibitors)	Blocks 5-HT_2 receptors and inhibits serotonin reuptake (e.g. trazodone, nefazodone)	MDD with insomnia or agitation	Sedation, dizziness, orthostatic hypotension, and priapism	Santarsieri & Schwartz, 2015
NaSSAs (noradrenergic and specific serotonergic antidepressants)	Alpha-2 antagonism norepinephrine and serotonin; blocks 5-HT_2 and 5-HT_3 receptors	MDD with comorbid anxiety or insomnia	Sedation, weight gain, dry mouth, and increased appetite	Kent, 2000

BP: Blood pressure; GAD: generalised anxiety disorder; OCD: obsessive compulsive disorder; PTSD: post-traumatic stress disorder.

1.6.1.1 Efficacy and limitations of current treatments

The efficacy of monoamine antidepressants in treating MDD and various anxiety disorders is well-documented (Taliaz *et al.*, 2021). Approximately 50-70% of patients respond to these treatments, with only 30-50% achieving remission (Taliaz *et al.*, 2021). Response is defined as a significant reduction (≥50%) in depressive symptoms while remission refers to a near-total alleviation of symptoms (Mendlewicz, 2008). SSRIs and SNRIs are considered first-line treatments due to their effectiveness, safety, and tolerability, while TCAs and monoamine oxidase inhibitors (MAOIs) are typically reserved for more treatment-resistant cases (Cleare *et al.*, 2015). In addition to their widespread use in MDD, monoamine-based antidepressants have also been widely used for the management of other conditions including generalised anxiety disorder, panic disorders, obsessive compulsive disorder, and post-traumatic stress disorder (Chu & Wadhwa, 2023). Maintenance therapy with these antidepressants further reduces relapse and recurrence risk in patients with chronic or recurring depression (Cleare *et al.*, 2015). Despite their efficacy, monoamine antidepressants have notable limitations. One major drawback is that they exhibit a delayed onset of action, typically taking two to six weeks to be effective which can be burdensome for patients in acute distress (Harmer *et al.*, 2017). Even when patients experience symptomatic relief, the varying side effects negatively impact patient adherence to treatment as well as their overall quality of life (Sansone & Sansone, 2014). Monoamine antidepressants are additionally limited by their ability to address certain depression-related symptoms such as cognitive impairment and fatigue (Papakostas, 2014). Furthermore, there is a risk of withdrawal symptoms, antidepressant discontinuation syndrome, and relapse after sudden treatment cessation. These conditions underscore the role of these agents as symptom-management tools rather than curative treatments (Gabriel & Sharma, 2017). Moreover, individual variability in treatment response due to environmental, biological, and genetic factors necessitates more personalised approaches. Approximately 30% of individuals do not respond to common antidepressants, highlighting the prevalence of treatment-resistant depression (McIntyre *et al.*, 2023). This variability has prompted criticism of the monoamine hypothesis for oversimplifying the complex neurobiological underpinnings of depression. The hypothesis does not fully account for other mechanisms that contribute to mood disorders. Given the heterogeneity of depression, it is unsurprising that many individuals show resistance to conventional antidepressant therapies primarily targeting monoamine imbalances.

The failure of common antidepressants to adequately alleviate depressive symptoms has resulted in a growing population of individuals with TRD. TRD is defined as resistance to at least two different antidepressants administered at therapeutic doses for an adequate duration (McIntyre *et al.*, 2023). Treatment resistance significantly increases the risk of suicide and increases the risk of relapsing within a year of achieving remission (Fekadu *et al.*, 2009). Current TRD treatment strategies include augmentation therapy where secondary medications, including lithium, triiodothyronine, and second-

generation anti-psychotics, are combined with first-line antidepressants to enhance efficacy (Voineskos *et al.*, 2020). Other approaches include psychotherapy and brain stimulation techniques. However, these approaches have limited success (Voineskos *et al.*, 2020). The inefficacy of current treatment standards and the risk of suicide in TRD underscores the need for novel therapies as well as more personalised treatment strategies.

1.7 Novel antidepressants

Several novel antidepressants that act on alternative depression-related pathways have been investigated. Among these, psychedelics have shown potential in treating MDD and TRD. Common compounds include psilocybin, lysergic acid diethylamide (LSD), ayahuasca, and alkaloid monoamine oxidase inhibitors (Carhart-Harris & Goodwin, 2017). Psychedelics act primarily through 5-HT_{2A} receptor agonism and glutamatergic modulation, promoting hippocampal neurogenesis, altering amygdala reactivity, and modulating the default mode network (Carhart-Harris *et al.*, 2017). These agents are generally well tolerated, with low addictive potential and transient side effects such as nausea, anxiety, and headaches, although their overall efficacy requires further clinical validation (Muttoni *et al.*, 2019).

Beyond serotonergic modulation, the E/I imbalance theory of depression has garnered increasing attention (Luscher *et al.*, 2011). GABAergic agents, such as brexanolone, zuranolone, and ganaxolone, act as positive allosteric modulators of GABA-A receptors to rapidly improve symptoms and are well tolerated (Abramian *et al.*, 2014). However, brexanolone may cause excessive sedation and is administered under risk evaluation and mitigation strategy protocols (Meltzer-Brody *et al.*, 2018). Similarly, glutamatergic agents, including ketamine, rapastinel, and AV-101, modulate NMDA and AMPA receptors to enhance neuroplasticity and synaptic restoration (Autry *et al.*, 2011). These compounds offer rapid and robust relief, especially in TRD. In this regard, ketamine has shown promise as an antidepressant with evidence of clinical efficacy (Krystal *et al.*, 2013).

1.7.1 Ketamine

Ketamine was first discovered in the 1960s and has since been used as an anaesthetic and analgesic agent due to its dissociative properties (Krystal *et al.*, 2013). Racemic ketamine, also known as (R, S)-ketamine, comprises two stereoisomers: arketamine ((R)-ketamine) and esketamine ((S)-ketamine), which exhibit distinct pharmacological properties. More recently, ketamine and its enantiomers have garnered significant attention for their rapid and robust antidepressant effects, particularly when administered at subanaesthetic doses to patients with MDD and TRD (Correia-Melo *et al.*, 2018).

1.7.1.1 Mechanism of action of ketamine in depression

Based on the anaesthetic and analgesic effects of ketamine, the most widely proposed antidepressant mechanism is via its antagonisation of NMDA receptors (Krystal *et al.*, 2019). Ketamine antagonises NMDARs located on inhibitory parvalbumin and somatostatin interneurons which suppresses tonic GABA release, disinhibiting pyramidal neurons and enhancing synaptic glutamate release . Ketamine further inhibits synaptic and extra-synaptic glutamatergic NMDARs by occupying the receptor pore, reducing calcium influx by competing with Mg^{2+} (Zanos & Gould, 2018). This inhibition prevents excitotoxicity, promotes AMPAR shuttling to the postsynaptic membrane, and redirects glutamate to bind preferentially to AMPARs on postsynaptic neurons (Zanos & Gould, 2018). Increased AMPAR activation enhances the local expression of BDNF which binds to TrkB receptors. Subsequently, TrkB dimerization initiates intracellular signalling pathways involving phosphatidylinositol 3-kinase (PI3K), Akt, and mTOR (Duman *et al.*, 2016). These pathways upregulate eEF2 dephosphorylation and activation, facilitating local protein synthesis (Duman *et al.*, 2016). Concurrently, ketamine increases CREB phosphorylation, which drives the expression of synaptic proteins like PSD-95 and neurotrophic factors such as BDNF itself, creating a positive feedback loop (Duman *et al.*, 2016). This cascade promotes dendritic spine growth, synaptogenesis, and the restoration of synaptic connectivity in mood-regulating brain regions (Duman *et al.*, 2016). Functionally, ketamine enhances connectivity between cortical and limbic networks, correlating with long-lasting symptom relief (Krystal *et al.*, 2019).

Most recently, ketamine has been found to induce homeostatic synaptic plasticity (Kavalali & Monteggia, 2020). Unlike long-term potentiation, which requires NMDAR activation, homeostatic plasticity restores synaptic strength through negative feedback mechanisms that enhance AMPAR-mediated excitation. This mechanism counteracts the reduced miniature excitatory post-synaptic currents (mEPSCs) observed in the PFC, hippocampus, and reward-processing regions such as the ventral tegmental area (VTA) and nucleus accumbens (NAc), of depressed patients (Figure 1.7) (Kavalali & Monteggia, 2020). By rapidly restoring synaptic strength and connectivity in these regions, homeostatic plasticity likely underpins ketamine's fast-acting antidepressant effects, including its ability to alleviate symptoms such as anhedonia (Autry *et al.*, 2011). Furthermore, loss-of-function models that impair synaptic scaling demonstrate diminished ketamine antidepressant efficacy. These

findings highlight the critical role of homeostatic mechanisms in mediating ketamine's therapeutic effects (Autry *et al.*, 2011).

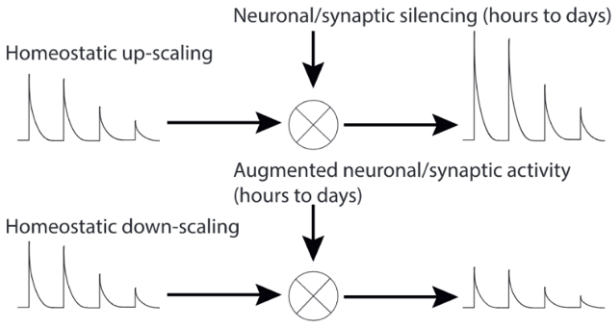


Figure 1.7. Overview of the role of homeostatic plasticity in maintaining neuronal network stability in response to changes in activity levels. Persistent deviations from a circuits set-point firing rate leads to homeostatic, compensatory adjustments in synaptic strength and intrinsic excitability that guide activity back to baseline. This negative feedback augments neuronal activity in response to reduced firing and attenuates heightened neuronal stimulation to prevent over-excitation or network silencing (Kavalali & Monteggia, 2020).

Although NMDAR antagonism is the most widely investigated ketamine antidepressant mechanism, several other hypotheses have been proposed (Figure 1.8). In this regard, preliminary studies suggest that ketamine modulates the monoamine system by increasing neurotransmitter release or inhibiting re-uptake (Tso *et al.*, 2004). However, the relevance of these effects in context of HPA axis dysregulation has not been well characterised. Other suggested effects of ketamine include potentiation of endogenous μ-opioid receptor activity, pro-inflammatory cytokine reduction, and increased insulin-like growth factor-1 (Deyama & Kaneda, 2023). While these findings emphasise ketamine's multi-pronged effect, many of these secondary mechanisms remain poorly understood in rodent models related to HPA axis dysregulation.

Despite growing insight into ketamine's antidepressant mechanisms, key gaps remain. Ketamine's role in restoring neuroendocrine balance in the context of HPA axis dysregulation is poorly understood. This includes ketamine's effects on HPA axis regulation, and on monoaminergic and neurotrophic signalling under ACTH-induced conditions. Given the frequent co-occurrence of HPA axis dysregulation with MDD and TRD, evaluating ketamine in relevant endocrine-based models is essential. Additionally, the lack of reliable molecular or neuroimaging biomarkers limits efforts to personalise treatment. Addressing these challenges will require mechanistic studies in preclinical models of HPA axis

dysregulation, coupled with detailed neurobehavioral profiling, to better define ketamine's therapeutic scope and enhance its clinical usage.

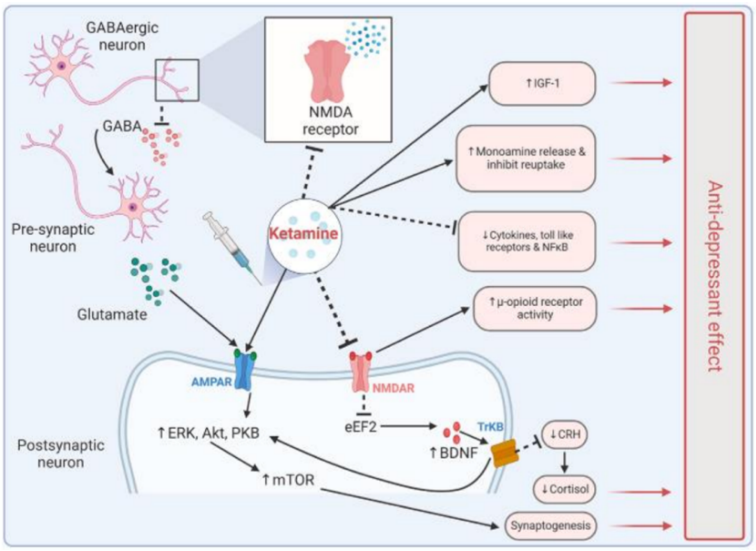


Figure 1.8. Potential mechanisms of action of ketamine's antidepressant effects (Millen *et al.*, 2024). Ketamine's most widely accepted antidepressant mechanism is thought to involve preferential antagonism of N-methyl-D-aspartate receptors (NMDARs), which diverts synaptic glutamate toward post-synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Krystal *et al.,* 2019). Increased AMPAR excitation enhances the expression of brain-derived neurotrophic factor (BDNF) locally (Dunman *et al*., 2016). BDNF binds to TrkB receptors and initiates Akt-mTOR-CREB cascades that up-regulate protein synthesis, promote dendritic spine growth, and foster synaptogenesis (Dunman *et al*., 2016). Other mechanisms include increased monoamine release with reduced reuptake, attenuation of pro-inflammatory signalling, activation of μ-opioid receptors, and reduced cortisol levels.

1.7.1.2 Pharmacokinetics, safety, and adverse effects

While most antidepressants require chronic administration for effective treatment, acute administration of subanaesthetic doses of racemic ketamine (0.5 mg/kg, infused over 40 min iv) produce rapid relief of depressive symptoms (Krystal *et al.*, 2019). The antidepressant effects of ketamine are typically observed within two hours of administration, peak between 24 and 72 hours, and can last up to two weeks (Krystal *et al.*, 2019). Interestingly, racemic ketamine demonstrates similar bioavailability to esketamine when both are administered via the same route, even at low doses (McIntyre *et al.*, 2021). In human studies, a single intravenous bolus of racemic ketamine leads to equal plasma concentrations of (S)- and (R)-ketamine (2 mg/kg; C_{max} ~1800 ng/ml), while a single intravenous dose of esketamine produces higher plasma concentrations at a lower dose (1 mg/kg; C_{max} ~2600 ng/ml) within one minute post-infusion (Geisslinger *et al.*, 1993). Ketamine undergoes rapid metabolism in humans, with a half-life of 2-4 hours for racemic ketamine and approximately 2.5 hours for both (R)- and (S)-ketamine enantiomers, respectively (White *et al.*, 1985).

Some occasionally reported side effects of (R, S)- and (S)-ketamine include hallucinations, depersonalisation, transient hypertension, anxiety, nausea, vomiting, and respiratory depression (Acevedo-Diaz *et al.*, 2020). The high prevalence of psychomimetic side effects induced by (R, S)- and (S)- ketamine may be attributed to their high affinity for the NMDAR (Rafało-Ulińska & Pałucha-Poniewiera, 2022). It is worth noting that the side effects of ketamine are dose-dependent and transient, typically resolving within two hours post-ketamine infusion (Acevedo-Diaz *et al.*, 2020). Although these effects enhance the potential for abuse, studies have demonstrated that low subanaesthetic doses used in the treatment of MDD and TRD have favourable safety and tolerability profiles (Zhu *et al.*, 2016). While the enantiomeric forms of ketamine show increased tolerability, enantioseparation of racemic ketamine into (S)- and (R)-ketamine is often achieved using high-performance liquid chromatography or chiral capillary electrophoresis, both of which are complex and costly processes (Schmidt & Holzgrabe, 2023). Therefore, racemic ketamine remains a cheaper, more widely available and effective antidepressant agent, making the drug more suitable for use in the treatment of MDD and TRD in lower-middle-income countries (Bahji *et al.*, 2021).

1.7.1.3 Clinical eligibility and treatment guidelines

Currently, there is no consensus on the optimal ketamine treatment regimen, and the criterion for initiating treatment is vague and ill-defined. Current diagnostic considerations for the use of ketamine for depression treatment include individuals with MDD or bipolar disorder currently experiencing a major depressive episode, individuals where the rapid reduction of suicidal symptoms is necessary, TRD patients, and patients where improvement of depression symptoms is required to address urgent life circumstances (Andrade, 2017). Some studies have suggested that patients must also present with a Montgomery-Åsberg Depression Rating Scale (MADRS) score of ≥20 before being accepted for a treatment course (Mansoor *et al.*, 2023). However, it is still uncertain which patients are suitable candidates for ketamine treatment. Some clinicians monitor MDD symptom improvement by administering the MADRS questionnaire throughout the treatment course (Ekstrand *et al.*, 2022). Typically, patients are considered responders if their baseline MADRS score decreases by 50% after a patient-specific treatment course (Ekstrand *et al.*, 2022). Treatment courses may range from one to twelve sessions of iv infusions of ketamine depending on patient response, with a general two-hour recovery time after each session (Mansoor *et al.*, 2023). Although the MADRS is commonly used to determine patient eligibility, the rating scale often biases clinicians towards administering a course of SSRIs that may be ineffective for TRD. A more objective approach, such as molecular biomarker profiling, is needed. This profiling will allow clinicians to identify eligible patients for ketamine treatment based on potential response and to recognize potential confounding factors, predicting treatment outcomes. To date, no study has investigated changes in patients' molecular profiles as a measure of ketamine treatment initiation and efficacy. Nevertheless, monitoring depressive symptoms

before and after ketamine infusion is not currently enforced and there is a lack of regulation around ketamine therapy. Therefore, the exact mechanism of action of ketamine requires further scientific investigation to support standardisation of ketamine's clinical use. Hence in the current thesis, in an animal model of ACTH-induced HPA axis dysregulation, I aimed to investigate the acute and chronic effects of ketamine on various neurobehavioral and molecular endpoints.

1.8 Problem statement

MDD remains a growing global health burden. However, the molecular mechanisms underpinning MDD are heterogenous which limits its treatment and management. Among several proposed aetiologies including monoamine deficits, excitatory/inhibitory imbalances, neuroinflammation, and neurotrophic loss, chronic stress-driven HPA axis dysregulation is one of the main contributors to the development and progression of MDD. Specifically, chronic psychosocial stress causes sustained elevations in cortisol, which over time, dysregulate the HPA axis. This dysregulation contributes to the development of MDD both directly, through its effects on stress-related circuitry, and indirectly, by disrupting several other physiological systems. Despite its role in the development of MDD, the interplay between HPA dysregulation, changes in neuroplasticity markers, and altered neurotransmission that may sustain depressive symptoms, remain unclear. Due to the limitations of mental health research in humans, including the influence of confounders and limited access to brain tissue, several questions remain unanswered regarding depression pathophysiology. Therefore, pre-clinical studies remain a valuable tool to investigate the mechanisms of depressive-like symptoms. One of the recently developed models to investigate molecular changes associated with of HPA-axis dysregulation is the administration of exogenous ACTH. Although chronic ACTH has been proposed to induce depressive-like symptoms, important gaps remain, and the model requires thorough behavioural and molecular validation in both sexes. Furthermore, while HPA axis dysregulation is associated with depression, traditional antidepressants primarily act by modulating synaptic 5-HT, dopamine, and norepinephrine levels. Thus, the molecular effects of monoamine-based antidepressants on physiological pathways implicated in MDD, such as the neurotrophic pathway, particularly in the ACTH model, require elucidation. Considering that roughly 30% of patients using monoamine-based therapies do not have symptomatic relief, the need for novel, rapid acting agents is evident. In this regard, ketamine has shown rapid-acting, multi-target antidepressant effects , including in TRD patients. Therefore, ketamine's mechanism of action and its time-dependent antidepressant effects, in a model of HPA axis dysregulation, warrant investigation.

1.9 Study aims

The aim of this study was to investigate the effect of exogenous ACTH on rodent neurobehaviour, neurotrophic signalling, and neurotransmitter outcomes, and to evaluate the effects of monoamine

antidepressants (imipramine and citalopram) and acute versus chronic ketamine on neurobehaviour and brain region-specific molecular changes.

The specific objectives were to:

1. validate the depressive and anxiogenic effects of the ACTH model by assessing behavioural changes using the FST, OFT, and SPT and by measuring the gene expression of neuroplasticity markers such as *BDNF* and *CREB* using real-time polymerase chain reaction (RT-PCR) across depression-related brain regions.
2. evaluate the ability of chronic citalopram and imipramine treatment to modulate the mRNA expression of neurogenic intracellular signalling factors such as *BDNF, TrkB, Akt, mTOR, CREB, and eEF2* using RT-PCR across depression-related brain regions in ACTH-treated rodents.
3. determine the effect of acute and chronic ketamine administration on rodent neurobehavior using the FST, OFT, and SPT, HPA axis function using an enzyme-linked immunosorbent assay (ELISA), neurogenesis-related markers using RT-PCR, and monoamine neurotransmitter abundance (5-HT, DA, NE) using matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI) in the ACTH-induced model of HPA axis dysregulation.

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CHAPTER 2: NEUROBEHAVIORAL AND MOLECULAR CHANGES IN A RODENT MODEL OF ACTH-INDUCED HPA AXIS DYSFUNCTION

The data in this chapter have been published in Brain Research:

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2.1 Abstract

Background: Hypothalamic-pituitary-adrenal (HPA) axis dysregulation is linked to the pathophysiology of depression. Although exogenous adrenocorticotropic hormone (ACTH) is associated with a depressive-like phenotype in rodents, comprehensive neurobehavioral and mechanistic evidence to support these findings are lacking.

Methods: Sprague-Dawley rats (male, n=30; female, n=10) were randomly assigned to the control (male, n=10) or ACTH (male, n=20; female n=10) groups that received saline (0.1ml, sc.) or ACTH (100μg/day, sc.), respectively, for two weeks. Thereafter, rats in the ACTH group were subdivided to receive ACTH plus saline (ACTH_S; male, n=10; female, n=5; 0.2ml, ip.) or ACTH plus imipramine (ACTH_I; male, n=10; female, n=5;10mg/kg, ip.) for a further four weeks. Neurobehavioral changes were assessed using the forced swim test (FST), the sucrose preference test (SPT), and the open field test (OFT). Following termination, the brain regional mRNA expression of brain-derived neurotrophic factor (*BDNF)* and cAMP response element-binding protein (*CREB)* was determined using real-time polymerase chain reaction (RT-PCR).

Results: After two-weeks, ACTH administration significantly increased immobility in the FST ($p=0.03$), decreased interaction with the center of the OFT ($p<0.01$), and increased sucrose consumption ($p=0.03$) in male, but not female rats. ACTH administration significantly increased the expression of *BDNF* in the hippocampus and *CREB* in all brain regions in males ($p<0.05$), but not in female rats. Imipramine treatment did not ameliorate these ACTH-induced neurobehavioral or molecular changes.

Conclusion: In conclusion, ACTH administration resulted in a sex-specific onset of depressive-like symptoms and changes in brain regional expression of neurotrophic factors. These results suggest sex-specific mechanisms underlying the development of depressive-like behavior in a model of ACTH-induced HPA axis dysregulation.

2.2 Introduction

Major depressive disorder (MDD) is a mental health disorder that is characterized by symptoms that include feelings of anxiety, hopelessness and despair, anhedonia, insomnia, chronic fatigue, a sense of worthlessness, and suicidal ideation (American Psychiatric Association, 2022). In severe cases, MDD may lead to cognitive dysfunction, social isolation, hostility, and physical debilitation which affects quality of life (Knight *et al.*, 2020). MDD is one of the leading causes of disability, suicide, and morbidity (World Health Organization, 2014). Currently, MDD affects approximately 300 million people globally, with this number expected to increase. Indeed, the WHO predicts depression to be one of the major contributors to the global disease burden, by 2030 (World Health Organization, 2008).

The pathophysiology of depression is complex and multifactorial. MDD has been associated with changes in hippocampal volume, executive function, and memory, circulating cortisol concentrations, neuroinflammation, neurotransmitter signalling, and expression of neurotrophic and transcription factors such as BDNF and CREB (O'Brien *et al.*, 2004). Dysregulation of the HPA axis is associated with high circulating cortisol levels and has been proposed as a major contributor in the development of MDD (Mlyniec, 2015). Indeed, patients with chronically increased cortisol concentrations are at an increased risk for the development of MDD (Lupien *et al.*, 1999). Clinical evidence shows increased ACTH and cortisol concentrations in individuals diagnosed with MDD (Choi *et al.*, 2018; Posener *et al.*, 2000). Moreover, HPA axis dysregulation is associated with the altered expression of neuroplasticity markers factors, such as BDNF and CREB, which are strongly related to MDD (Kunugi *et al.*, 2010). In this regard, studies have shown that HPA-axis dysregulation and increased glucocorticoid (GC)/corticosterone levels decrease BDNF expression, that directly impacts the expression and function of the BDNF receptor, TrkB (Revest *et al.*, 2014). In turn, decreased BDNF expression results in decreased phosphorylation of CREB, which is associated with synaptic plasticity and neuronal survival (Wang *et al.*, 2019). In addition, increased glucocorticoids have also been linked to decreased CREB phosphorylation and expression (Blendy, 2006; Li *et al.*, 2017). These findings suggest a strong link between HPA axis dysregulation and the clinical presentation of depression (Nandam *et al.*, 2020). However, the exact mechanisms whereby HPA dysregulation contributes to depression are uncertain.

To understand the relationship between dysregulation of the HPA axis and MDD, several animal studies have demonstrated that chronic administration of ACTH resulted in the development of depressive-like symptoms when assessed using neurobehavioral tools (Song *et al.*, 2019). In this regard, symptoms of anhedonia (Liu *et al.*, 2018), helplessness (Porsolt *et al.*, 1977), and anxiety (Kraeuter *et al.*, 2019) have been reported following chronic ACTH administration as assessed by the SPT, FST, and OFT, respectively (Kitamura *et al.*, 2002). In contrast, others have not shown any depressive-like behaviours post-ACTH administration (Pereira *et al.*, 2019). A possible explanation for these discrepancies is

methodological variations and differences in interpretation of neurobehavioral assessments in animal models (Hasanpour *et al.*, 2021).

Although neurobehavioral tests are widely used to identify and quantify depressive-like symptoms in animal models of depression, it remains uncertain whether the reporting, interpretation, and translation of these results are reliable and reproducible, especially when using a single test (Bennabi *et al.*, 2013). In addition, it is important to substantiate neurobehavioral changes with mechanistic molecular data. The relationship between ACTH-induced neurobehavioral changes and the expression of neurotrophic molecular markers, frequently associated with depressive-like symptoms, are limited. Additionally, whether the use of a robust battery of neurobehavioral tests will improve the interpretation and translational ability of depressive-like symptoms in ACTH-induced HPA dysregulation requires investigation. Therefore, this study aimed to assess the effect of ACTH-induced HPA axis dysregulation on depressive-like symptoms using a battery of neurobehavioral tests in rodents, and its effect on the expression of *BDNF* and *CREB* expression.

2.3 Materials and methods

2.3.1 Ethical approval

All experimental protocols were approved by the Animal Research Ethics Committee of the University of the Witwatersrand (ethics number: 2022/03/06/C). All procedures were conducted in accordance with the regulations of the South African National Legislation for animal husbandry, welfare, and experimentation using laboratory animals. All *in vivo* experiments are reported in accordance with ARRIVE guidelines.

2.3.2 Study design

2.3.2.1 Animals

Forty Sprague-Dawley rats (male, n=30; female, n=10) with an initial weight of 250 ± 50 g were obtained from the Wits Research Animal Facility (WRAF) at the University of the Witwatersrand. Rats were allowed to acclimatize for seven days prior to model induction or neurobehavioral analysis. Male and female rats were housed in separate rooms, and neurobehavioral tests were conducted in a dedicated neurobehavioral testing room. Rats were housed two per cage with *ad libitum* access to water and standard rat chow (Epol, Johannesburg, South Africa), except during the SPT. The housing and testing rooms were maintained at an ambient temperature of 25 °C ± 1 °C with relative humidity of 24-26 %. A 12 h:12 h light/dark cycle was followed (lights on at 07 *h*00; testing was conducted at 09 *h*00).

2.3.2.2 Treatment regimen

Rats were randomly divided into the control group (male, n=10) that received saline (0.1 ml/daily, sc) or the ACTH-treated groups (male, n=20; female, n=10) that received ACTH 100 µg/day, (0.1 ml, sc) for two weeks (Kitamura *et al.*, 2002). Thereafter, the control group was subdivided to continue receiving, in addition to daily saline injections (0.1 ml, sc.), either saline (CON; 0.2 ml/day, ip) or imipramine (SAL_I; 10 mg/kg/day, ip). Rats in the ACTH-treated group were subdivided to receive, in addition to daily ACTH injections, either saline (ACTH_S; 0.2 ml/day, ip) or imipramine (ACTH_I; 10 mg/kg/day, ip) for four weeks (Kitamura *et al.*, 2002).

2.3.2.3 Tissue harvest

Two hours following the completion of the neurobehavioral tests, rats were briefly exposed to isoflurane anaesthesia, prior to being terminated by decapitation using a rodent guillotine. Rodent brain tissue was surgically dissected into the prefrontal cortex, striatum, hippocampus, and midbrain. Brain tissues were snap-frozen using liquid nitrogen and stored at −80 °C until molecular analysis.

2.3.3 Neurobehavioral assessments

EthoVision™ XT 17.5 software (Noldus Technologies, Wageningen, Netherlands) was used to automatically score all parameters in the FST and OFT and to manually score climbing behaviour in the FST, as previously reported (Rastoldo *et al.*, 2020; Noldus Information Technology, 2024). The dynamic subtraction detection method was used for both the FST and OFT. For the detection method, video pixel smoothing was set to medium and dropped frames correction was turned on. The minimum and maximum size of each animal was set to half and double the average detected size. Throughout the videos, EthoVision automatically detected the rodents centre point in the FST and the nose, centre and tail-base points of rodents subject to the OFT. Parameters “highly mobile”, “mobile”, and “immobile” were calculated using an average interval of 5 samples and a threshold for highly mobile above 10%, mobile between 10% and 2%, and immobile below the 2% threshold. Parameters for “not moving” (freezing) were calculated using an average interval of 5 samples and a velocity threshold of 2.00 cm/s for start and 1.75 cm/s for stop. Climbing parameters were manually scored by one researcher. During manual scoring, blinding was ensured using trial numbers for video identification with no visible information regarding treatment group. In the OFT, distance travelled, and mean speed were calculated using the rodent centre point, and freezing duration and total activity were calculated using the tail-base point.

2.3.3.1 Forced swim test (FST)

The FST was used to evaluate the presence of depression-associated helplessness and despair behaviours in rodents, as previously reported (Kulkarni & Dhir, 2007; Slattery & Cryan, 2012). At the

end of the acclimatization period, each rat underwent an FST adaptation, which consisted of a one-minute, three-minute, and five-minute habituation swim over three consecutive days, with a rest day, before being subjected to a five-minute FST to assess baseline behaviour (baseline time point, week 0) (Figure 2.1) (Slattery & Cryan, 2012). For the FST, plexiglass cylinders (50 cm high, 20 cm wide), were filled with 30 cm of water that was maintained at a temperature between 23-25^{o}C. Partitions were set up between each cylinder to prevent any effect of social interaction on behaviour during the FST. Black backing was placed behind each cylinder to enhance the colour contrast for improved motion detection by the EthoVision software. The FST was repeated after the 2-week ACTH administration period (week 2) and again following the four-week ACTH plus saline or imipramine treatment period (week 6) (Figure 2.1). The baseline assessment (week 0) for each treatment group served as an individual control for the subsequent FSTs conducted at week 2 and week 6. All animals were allowed to acclimatize for five minutes, prior to the FST being conducted in a separate testing room. Analysis was performed using the standardized FST module in EthoVision software.

2.3.3.2 Open field test (OFT)

The OFT was used to assess locomotor activity and anxiety-like behaviour associated with depressive-like symptoms in a subset of animals (males, n=10; females, n=10) (Seibenhener & Wooten, 2015). Rats were subjected to the OFT after the acclimatization period to determine baseline behaviour (baseline time point, week 0), after the 2-week ACTH induction (week 2), and after the four-week ACTH plus saline or imipramine treatment period (week 6) (Figure 2.1). All animals were allowed to acclimatize for five minutes, prior to the OFT being conducted in a separate testing room, as previously described (Noldus Information Technology, 2024). Briefly, rats were placed in the centre of a square open field arena (90 cm x 90 cm x 30 cm) under dimmed lighting, subsequent behaviours were recorded for five minutes (Rastoldo *et al.*, 2020; Noldus Information Technology, 2024). This was followed by analysis using the standardized OFT module in EthoVision software. Increased time spent in the periphery of the OFT was associated with anxiety-like, thigmotaxic behaviours while increased time spent exploring the centre of the open field was considered healthy or risk-taking behaviour. Repeated exposure of rodents to the open field throughout the study allowed for acclimatisation, enhancing any changes in behaviour as a result of pharmacological intervention.

2.3.3.3 Sucrose preference test (SPT)

The SPT was performed to assess the hedonic tendency of the rats in all groups at the end of the drug treatment period (week 6), adapted from Liu et al. (2018) (Figure 2.1) (Liu *et al.*, 2018). Each SPT included an adaptation period (72 h) and a preference test period (12 h). Each apparatus was made of perspex and contained five chambers each 20 cm wide, 40 cm long, and 50 cm high, adapted from the experimental set-up described by Liu *et al.* (2018). Ethanol (70%) was used to clean each chamber

between testing. All SPTs were performed in a behavioural testing room separate from the housing room. During bottle adaptation, rats were housed two per cage and exposed to two bottles, one containing 1% sucrose solution (w/v) and one with regular drinking water, for 48h in the housing room. Apparatus adaptation was done by transferring the animals to the SPT apparatus for 24h in a separate, temperature-controlled test room. During the adaptation period, rats were also exposed to two bottles, one containing 1% sucrose solution (w/v) and one with regular drinking water. For the preference test, rats were placed into the SPT apparatus for 12h with no food and *ad libitum* access to two bottles, one containing 1% sucrose solution (w/v) and one with regular drinking water. The preference test was performed during the dark phase (19h30-07h30) when animals were expected to be most active and the volume of normal drinking water and sucrose water consumed were recorded. The preference ratio (SPR) was calculated as sucrose intake / (water + sucrose intake) x 100. Following the preference test, rats were transferred to the homeroom with *ad libitum* access to food and regular water.

2.3.4 Molecular analysis

2.3.4.1 Real time-polymerase chain reaction (RT-PCR)

For PCR analyses, brain samples were homogenized using a probe sonicator (Fisher Scientific, Waltham, Massachusetts, USA). RNA was extracted using the Illustra Minispin RNA extraction kit (GE Healthcare, USA), according to the manufacturer's instructions. cDNA was synthesized using the SuperScript VILO cDNA synthesis kit (ThermoFisher, California, USA). RNA and cDNA were quantified using a spectrophotometer (NanoDrop OneC, Thermo Scientific, Waltham, MA, USA) and were stored at −80 °C until needed.

BDNF and *CREB* mRNA expressions were determined using RT-PCR TaqMan assays (ThermoScientific, Waltham, MA, USA), with *Tbp* as the endogenous control. The TaqMan assay IDs of the probes used were: *BDNF* (Rn02531967_s1), *CREB* (Rn01451883_m1), and *Tbp* (Rn01455646_m1). The genes of interest were amplified using a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). Changes in mRNA expression were determined using the $2^{-\Delta\Delta CT}$ method and are represented as a fold change relative to the control (Livak & Schmittgen, 2001).

2.3.5 Data analysis

Data analysis was performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). A Shapiro-Wilk test was used to assess the normality of the data. Molecular data were not normally distributed and were logarithmically transformed for statistical analysis. Data are presented as mean and standard deviation (±SD). A mixed model analysis of variance (ANOVA) (GLM procedure in SAS) was used to assess differences between the groups for the SPT and RT-PCR results with

treatment and sex as the main effects. To determine changes in the FST and OFT in response to the ACTH intervention and determine the effects of imipramine treatment in male and female rats, data were analysed using a repeated measures two-way ANOVA, followed by a Tukey's *post hoc* test. In a repeated measures ANOVA including both within and between factors for four groups, to achieve statistical power at 80 % with a two-sided α value of <0.05 a sample size of 20 was required. Statistical significance was set at $p<0.05$.

2.4 Results

2.4.1 Forced swim test

ACTH administration resulted in a significant change in the time spent highly mobile (model $F=9.68$, $p<0.0001$) in male rats, but not in female rats (sex effect, $p<0.0001$) (Table 2.1). Treatment with ACTH for two weeks resulted in significantly less time spent being highly mobile compared to baseline in male rats ($p=0.0001$). Moreover, after two weeks and six weeks of ACTH administration, male rats spent less time highly mobile compared to female rats ($p=0.003$ and $p=0.01$, respectively). Similarly, the number of episodes spent highly mobile were significantly decreased after ACTH administration for two weeks in males ($p=0.0008$), but not female rats ($p>0.05$) (model $F=4.14$, $p=0.0006$). Male rats treated with ACTH for two weeks had fewer episodes highly mobile when compared to females ($p=0.04$). After treatment with imipramine, time spent highly mobile and highly mobile episodes were not different from baseline in male rats or female rats (all $p>0.05$).

ACTH administration resulted in a significant increase in freezing time ($F=10.58$, $p<0.0001$) and freezing episodes ($F=10.8$, $p<0.0001$) in male, but not female rats (sex effect, $p<0.0001$ for both) (Table 2.1). At baselines, males had a higher freezing duration ($p=0.03$), and freezing episodes ($p=0.002$) compared to females. After two weeks of ACTH administration, male rats had a higher freezing duration compared to baseline ($p=0.003$). After two weeks of ACTH, males had higher freezing duration and episodes compared to females (both $p<0.0001$). After six weeks of ACTH administration, freezing duration and episodes increased compared to week 2 ($p=0.04$ and $p=0.03$) in male rats. After imipramine treatments, there were no differences in freezing duration or episodes compared to baseline or ACTH administration in either male or female rats (all $p>0.05$).

There was an overall significant model effect for time spent climbing ($F=4.4$, $p=0.0004$), but not for climbing episodes ($F=1.97$, $p=0.07$). There was a significant treatment effect ($p=0.03$) and sex effects ($p=0.0001$) for time spent climbing. However, *post hoc* tests did not show significant interaction effects (Table 2.1).

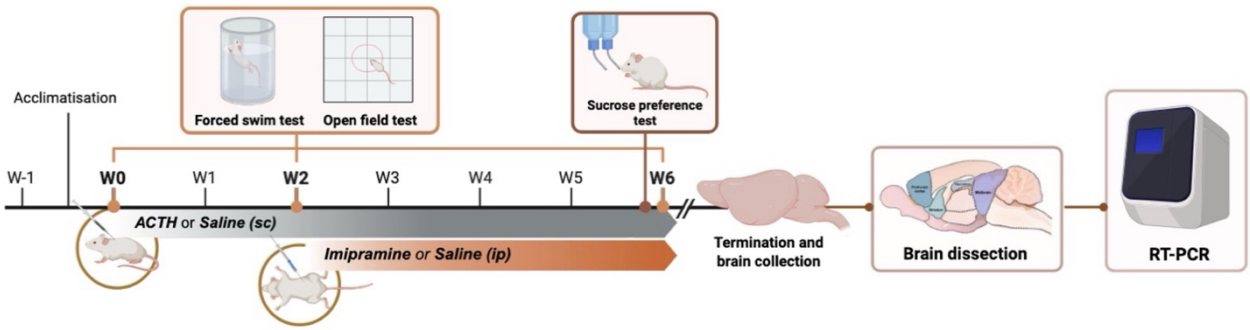

Figure 2.1 Experimental design showing the treatment timeline. Baseline FSTs and OFTs were performed at week zero. Post-ACTH FSTs and OFTs were performed after two weeks of treatment (saline or ACTH). The FST, OFT, and SPT was performed at the end of the treatment intervention period (saline/imipramine) to evaluate the effect of each intervention on neurobehavioral outcomes.

Table 2.1. Summary of results for FST parameters over experimental time points.

FST Parameter		Week 0 Baseline		Week 2 ACTH		Week 6			
						ACTH_S		ACTH_I	
		males (n=19)	females (n=10)	males (n=19)	females (n=10)	males (n=10)	females (n=5)	males (n=9)	females (n=5)
Highly mobile	Time (s)	161.9 ± 47.5	202.0 ± 42.9	84.9 ± 48.0***	162.3 ± 50.5††	114.9 ± 60.1	217.3 ± 45.5$^{\#\#}$	176.6 ± 45.8	196.0 ±54.1
	Episodes (n)	204.3 ± 31.1	190.9 ± 30.3	144.5 ± 51.7***	196.7 ± 18.0†	166.4 ± 68.4	145.8 ± 22.2	181.3 ± 43.5	209.4 ± 35.9
Freezing	Time (s)	42.9 ± 8.5	26.3 ± 2.7*	52.5 ± 11.9**	25.7 ± 6.9†††	38.2 ± 12.6†	23.6 ± 7.2	37.9 ± 12.7	31.0 ± 8.1
	Episodes (n)	400.3 ± 62.7	312.2 ± 25.3**	432.0 ± 57.4	296.9 ± 65.8†††	369.2 ± 72.5†	284.0 ± 63.8	361.7 ± 71.5	344.2 ± 65.9
Climbing	Time (s)	65.4 ± 58.2	13.8 ± 11.3	60.5 ± 45.6	30.7 ± 22.5	85.3 ± 58.2	13.8 ± 11.3	114.1 ± 77.2	59.9 ± 10.5
	Episodes (n)	8.9 ± 3.5	7.8 ± 1.1	7.2 ± 3.3	5.6 ± 2.5	6.3 ± 3.3	4.6 ± 4.6	7.0 ± 2.4	6.4 ± 2.9

FST data was collected at week 0: baseline measurement; week 2: after 2 weeks of ACTH (100 µg/day, sc) treatment; and week 6: after four weeks of ACTH plus saline (0.2ml/day, ip) or imipramine (10 mg/kg/day, ip) treatment. Values are expressed as mean ± SD. Data were analyzed using a repeated measures two-way ANOVA followed by a Tukey's *post hoc* test. ACTH_S, ACTH + saline; ACTH _I, ACTH + imipramine. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus males at week 0; † $p<0.05$, †† $p<0.01$, ††† $p<0.001$ versus males at week 2; ## $p<0.01$ versus ACTH_S males at week 6.

2.4.2 Open field test

Although there were significant differences in the model for the mean speed ($F=7.14$, $p<0.0001$; Figure 2.2C) and the distance traveled ($F=7.12$, $p<0.0001$; Figure 2.2D), the *post hoc* tests show no significant differences between the groups (all $p>0.05$). ACTH administration had a significant effect on the duration of time rats spent immobile in the OFT ($F=10.39$, $p<0.0001$; Figure 2.2E). After two and six weeks of ACTH administration, the immobility duration increased in male rats compared to baseline ($p<0.0001$ and $p=0.0002$, respectively), which were not impacted by imipramine treatment. Similarly in female rats, six weeks of ACTH treatment increased the immobility when compared to baseline ($p=0.01$). ACTH administration for two weeks significantly impacted the time spent in the center ($F=3.72$, $p=0.003$) and percentage time of the total duration spent in the center ($F=3.69$, $p=0.02$) in male, but not female rats. Interestingly, after six weeks of either ACTH plus saline or imipramine treatment, there were no differences in the engagements with the center area of the OFT arena compared to baseline (all $p>0.05$).

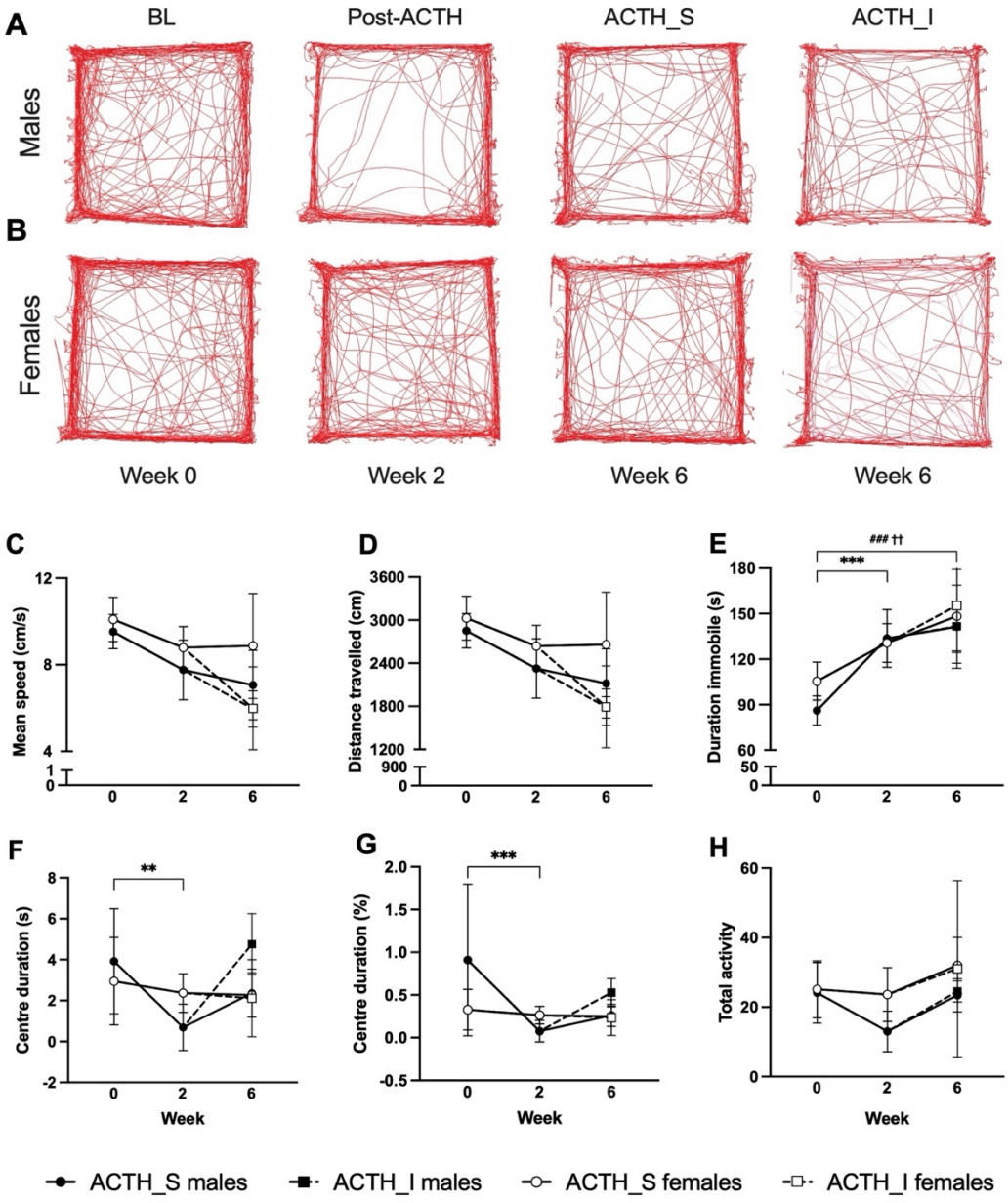

Figure 2.2. Average track plots for all male rats (A) and female rats (B) across each experimental time point. Effect of ACTH and imipramine treatment on (C) mean speed (cm/sec), (D) distance travelled (cm), (E) time immobile (s), (F) center duration (s), (G) center duration (%), and (H) total activity, as assessed by the OFT. OFT measurement time points include week 0: baseline measurement (males, n=19; females, n=10); week 2: after 2 weeks of ACTH (100 µg/day, sc) treatment (males, n=19; females, n=10); and week 6: after four weeks of ACTH plus saline (0.2 ml/day, ip) (males, n=10; females, n=5) or imipramine (10 mg/kg/day, ip) treatment (males, n=9; females, n=5). Values are presented as mean ± SD. Data was analyzed

using a repeated measures two-way ANOVA followed by a Tukey's *post hoc* test. BL, baseline; ACTH_S, ACTH + saline; ACTH _I, ACTH + imipramine. ** p<0.01, *** p<0.001-week 0 vs week 2 between ACTH_S males, ### p<0.001-week 0 vs week 6 between ACTH_S males, †† p<0.01-week 0 vs week 6 between ACTH_S females.

2.4.3 *Sucrose preference test*

Water consumption was significantly different between treatment groups (F=7.36, p=0.0001; treatment effect, p=0.004; sex effect, p<0.0001) (Figure 2.3A). ACTH plus imipramine (ACTH_I) treatment significantly increased water consumption compared to the CON (p=0.03) and the ACTH_S rats (p=0.03) in male rats. In female rats, ACTH plus imipramine treatment resulted in an increased consumption of regular water compared to the control male rats and male rats treated with ACTH_I (p=0.001 and p=0.02, respectively). Interestingly in males, imipramine treatment in the imipramine control group significantly increased water consumption when compared to the controls (p=0.04) (Figure 2.4A). Sucrose solution consumption was significantly different between the male ACTH-treated rats and the controls (F=3.32, p=0.02; Figure 2.3B). Six weeks of ACTH_S treatment in male rats resulted in significantly greater sucrose solution consumption when compared to the controls (p=0.03). Total water and sucrose consumption was significantly different between the male control and ACTH-treated groups (F=3.22, p=0.02; Figure 2.3C). ACTH_S males had greater total water and sucrose consumption compared to control rats (p=0.01). Despite a significant model effect (F=4.01, p=0.01), treatment, and sex effects (both p=0.01), in the sucrose preference ratio, there were no interaction effects (p>0.05) (Figure 2.3D).

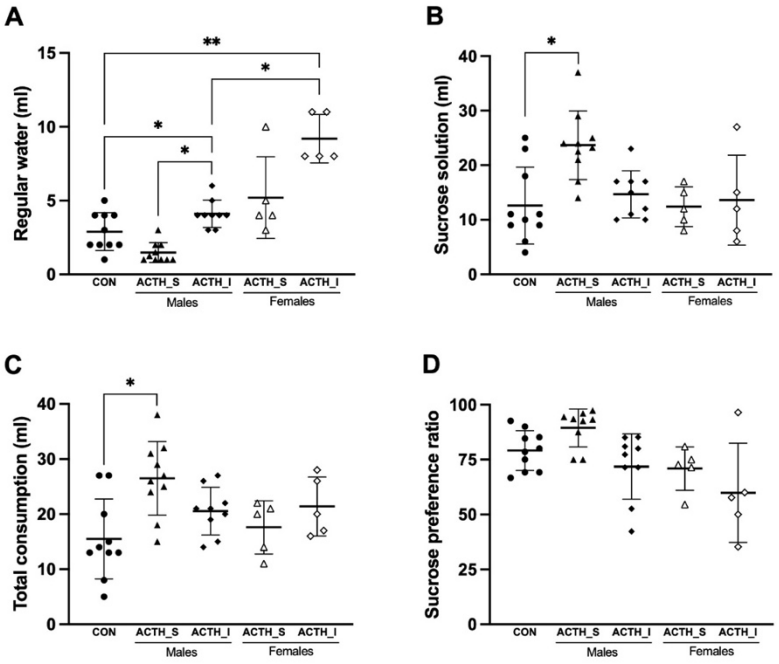

Figure 2.3. Sucrose preference test showing the effect of ACTH and imipramine on (A) regular water consumption (ml), (B) sucrose solution consumption (ml), (C) total volume of water and sucrose consumed (ml), and (D) the sucrose preference ratio. ACTH (100 µg/day, sc) and saline (0.1 ml, sc) were administered once daily for 6 weeks. Imipramine (10 mg/kg/day, ip) and saline (0.2 ml/day, ip) were administered once daily for 4 weeks (weeks 2-6). Values are expressed as mean ± SD. Data was analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (males, n=10); ACTH_S, ACTH + saline (males, n=10; females, n=5); ACTH _I, ACTH + imipramine (males, n=9; females, n=5). * $p<0.05$, ** $p<0.01$.

Figure 2.4 shows that imipramine treatment increased water consumption when compared to the controls ($p=0.04$). No other significant differences were observed between groups (all $p>0.05$).

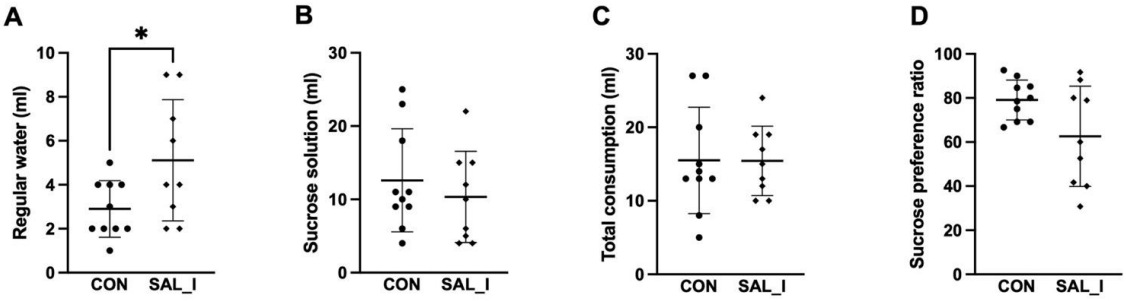

Figure 2.4. Sucrose preference test showing the effect of imipramine on (A) regular water consumption (ml), (B) sucrose solution consumption (ml), (C) total volume of water and sucrose consumed (ml), and (D) the sucrose preference ratio. Saline (0.1 ml, sc) was administered once daily for 6 weeks. Imipramine (10 mg/kg/day, ip) and saline (0.2 ml/day, ip) were administered once daily for 4 weeks. Values are expressed as mean ± SD. Data was analysed using a student's t-test. CON, control (males, n=10); SAL_I, saline + imipramine (males, n=10). * $p<0.05$.

2.4.4 mRNA expression of BDNF and CREB

ACTH administration did not result in changes in *BDNF* mRNA expression in the prefrontal cortex, striatum, or midbrain (sex effect, both p=0.02; Figures 2.5A, 2.5B, and 2.5D). Hippocampal *BDNF* mRNA expression was significantly increased by ACTH administration in males (F=4.68, p=0.004), and was significantly different between males and females (p=0008; Figure 2.5C). Six weeks of ACTH_S treatment in males resulted in significantly higher hippocampal *BDNF* mRNA levels when compared to controls and ACTH_I males (both p=0.04). No differences in *BDNF* mRNA expression were found in females exposed to ACTH or imipramine treatment (all p>0.05).

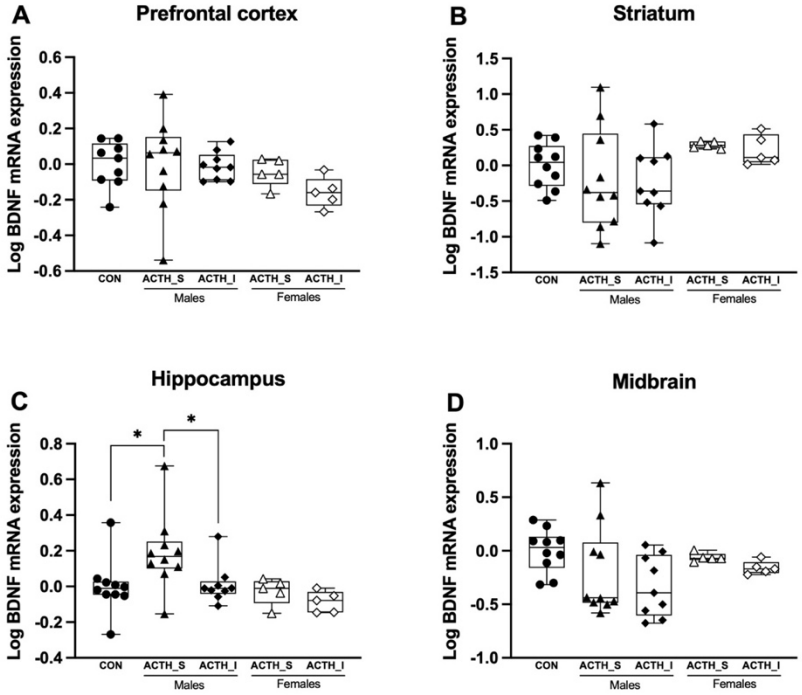

Figure 2.5. RT-PCR results showing the effect of ACTH and imipramine on the log *BDNF* mRNA expression in the (A) prefrontal cortex, (B) striatum, (C) hippocampus, and (D) midbrain. ACTH (100 µg/day, sc) and saline (0.1 ml, sc) were administered once daily for 6 weeks. Imipramine (10 mg/kg/day, ip) and saline (0.2 ml/day, ip) were administered once daily for 4 weeks (weeks 2-6). Data are presented as mean and SD relative to the housekeeping gene *Tbp*. Data was analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (males, n=10); ACTH_S, ACTH + saline (males, n=10; females, n=5); ACTH _I, ACTH + imipramine (males, n=9; females, n=5). * p<0.05.

CREB mRNA expression was significantly increased following ACTH treatment in all brain regions in male rats, but not in female rats (all $p<0.05$). There were no differences in *CREB* mRNA expression between male ACTH_I and male control rats (all $p>0.05$). *CREB* mRNA expression did not differ between female ACTH_S and ACTH_I groups (all $p>0.05$; Figures 2.6A-C).

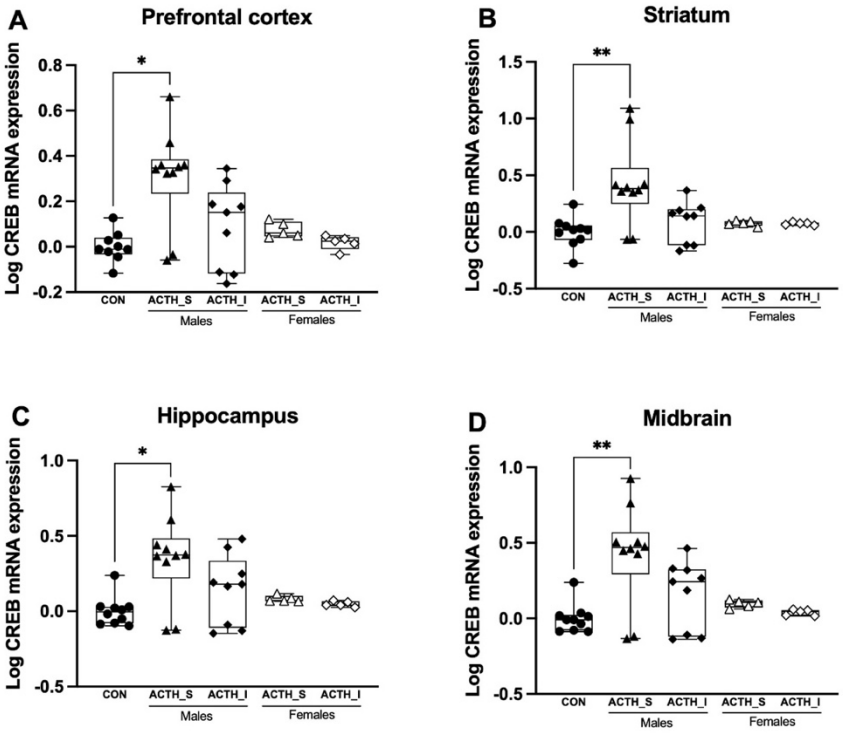

Figure 2.6. RT-PCR results showing the effect of ACTH and imipramine on the log *CREB* mRNA expression in the (A) prefrontal cortex, (B) striatum, (C) hippocampus, and (D) midbrain. ACTH (100 µg/day, sc) and saline (0.1 ml, sc) were administered once daily for 6 weeks. Imipramine (10 mg/kg/day, ip) and saline (0.2 ml/day, ip) were administered once daily for 4 weeks (weeks 2-6). Data are presented as mean and SD relative to the housekeeping gene *Tbp*. Data was analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (males, n=10); ACTH_S, ACTH + saline (males, n=10; females, n=5); ACTH _I, ACTH + imipramine (males, n=9; females, n=5). * $p<0.05$, ** $p<0.01$.

2.5 Discussion

HPA axis dysregulation is strongly associated with the clinical symptoms and pathophysiology of depression (Keller *et al.*, 2017). Animal models of HPA axis dysregulation have shown contrasting neurobehavioral outcomes, largely due to the use of a single neurobehavioral test in the face validation of these models (Kitamura *et al.*, 2008). Moreover, molecular evidence to substantiate these neurobehavioral changes are limited. Hence, this study aimed to determine the effects of ACTH administration on the development of depressive-like symptoms in a rodent model, using a battery of neurobehavioral tests as

well as by assessing associated neurotrophic molecular markers. We showed that in male rats, ACTH administration significantly increased immobility in the FST, which suggests despair-like behaviour. In the OFT, although ACTH administration significantly reduced locomotor behaviour in both males and females, only male rats showed a reduced interaction with the center of the open field, suggesting an increase in anxiety-like behaviour. In addition, ACTH administration resulted in a significant increase in the volume of sucrose solution consumed by males, however, there was no difference in the sucrose preference ratio. There were no differences in any of the neurobehavioral outcomes between rats receiving ACTH plus imipramine and those receiving ACTH plus saline, suggesting imipramine did not impact ACTH-induced changes in neurobehavior. Furthermore, in male, but not female rats, ACTH plus saline administration increased *BDNF* mRNA expression in the hippocampus, while *CREB* mRNA expressions were increased in all brain regions. The expression of these neurotrophic factors was not altered in rats receiving ACTH and imipramine. Taken together, these results show that ACTH administration resulted in depressive-like behaviour in male rats only when assessed using several neurobehavioral tests. These changes were accompanied by increased region-specific expression of neurotrophic factors.

2.5.1 Effects of HPA axis dysregulation on a neurobehavioral measure of despair

Immobility or freezing assessed in the FST is commonly used as a measure of behavioral despair in rodent models (Yankelevitch-Yahav *et al.*, 2015). In the present study, we showed increases in immobility following two weeks of ACTH administration, as shown by a significant increase in freezing duration and episodes. Similarly, we showed decreased time and episodes of mobility during the swim. Interestingly, this phenomenon was observed only in male rats, demonstrating the sex-specific effects of ACTH. Previous studies have shown that chronic administration of ACTH significantly increases immobility in the FST, suggesting behavioral despair (Kim *et al.*, 2016). Other models of HPA axis dysregulation have also demonstrated changes in mobility in the FST. In this regard, in GR knockout rodents, there was reduced immobility (Tronche *et al.*, 1999). Moreover, adrenalectomized (ADX) rats that received a GC antagonist showed a reversal in immobility behavior compared to the controls (Veldhuis *et al.*, 1985).

Interestingly, after six weeks of ACTH administration, there were no differences in freezing or mobility compared to baseline. It has been suggested that the two-week administration of ACTH may align more closely with the acute phase of the stress response; where elevated ACTH levels lead to an increase in corticosterone levels, which, in turn, alters neural circuits involved in mood regulation (Song *et al.*, 2019*b*). These results suggest that the HPA axis may recover with longer treatment durations via an adaptive homeostatic response.

In the present study, despite the ACTH effects on freezing behavior in male rats, there were no effects on behavioural despair in females. The lack of effects in females may be attributed to the suppressive effects of estrogen and progesterone on HPA axis activity (Goel *et al.*, 2011). Progesterone and estrogen have been shown to decrease glucocorticoid-induced expression of hypothalamic CRH and corticosteroid receptors in the hippocampus, leading to suppression of the HPA axis (Patchev & Almeida, 1996). A similar sex-specific effect was seen in a chronic restraint stress model, where an increase in corticosterone was associated with decreased *GR* mRNA expression in the hippocampus of male but not female rats (Karandrea *et al.*, 2000). Taken together, the interplay between the HPA axis and sex hormones in response to ACTH treatment and FST-induced stress plays a crucial role in modulating these sex-specific behavioral responses in the FST.

2.5.2 Effects of HPA axis dysregulation on locomotion and exploration

In the present study, we showed that ACTH administration resulted in a significant decrease in locomotor activity of male rats in the OFT, as measured by mean speed, distance travelled and duration immobile. Similarly, in females, the duration immobile in the OFT increased after ACTH administration. None of the changes in locomotor variables were impacted by imipramine treatment. These changes in locomotor activity may be explained by elevated ACTH levels which strongly correlate with altered dopaminergic activity in the brain via an increase in circulating glucocorticoids (Willner *et al.*, 1991).

Interestingly, in male rats only, ACTH administration resulted in a decreased interaction with the centre zone of the OFT. Similar to the results from the FST, after six weeks of ACTH administration, centre interaction was not different from baseline. Similarly, previous studies have shown that ACTH administration decreases locomotor activity and time in the central zone of the open field arena (Zhao *et al.*, 2018).

It has been proposed that avoidance of the centre of the OFT and more time seeking shelter along the periphery is associated with fear or anxiety-like behaviour (Carola *et al.*, 2002). Previous studies have shown that ACTH administration decreases locomotor activity and time in the central zone of the open field arena (Carola *et al.*, 2002). Our results therefore suggest that ACTH administration may induce anxiety-like behaviour in male but not female rats. This is important considering that chronic anxiety is frequently comorbid with depression and has been associated with the exacerbation of depressive disorders (Vreeburg *et al.*, 2013). These changes in locomotor activity may be explained by elevated ACTH levels which strongly correlate with altered dopaminergic activity in the brain via an increase in circulating glucocorticoids (Willner *et al.*, 1991). This suggests that specific dopaminergic pathways are responsible for neurobehavioral changes observed in ACTH-induced HPA axis dysregulation. In this regard, studies have shown that locomotion and avoidance behaviours related to anxiety are associated with the

nigrostriatal pathway, while cognitive and emotional processes are associated with the mesolimbic and mesocorticolimbic pathways (LeBlanc *et al.*, 2020). Taken together, these results suggest that ACTH administration may alter dopaminergic activity which may lead to changes in locomotor and anxiety-like behaviour in the OFT (Ouakki *et al.*, 2019).

2.5.3 Effects of HPA axis dysregulation on sucrose preference

In rodent studies, anhedonia is associated with a reduced preference for sucrose-sweetened water compared to normal drinking water (Pizzagalli, 2014). Despite these expected outcomes, the results of the SPT in ACTH-treated rodent models are contradicting (Kale *et al.*, 2021). One study has reported an increased sucrose preference ratio, which was the only parameter reported after three weeks of ACTH administration (Kale *et al.*, 2021). Another study reported that male ADX rats consumed more sucrose when treated with either ACTH or corticosterone replacement therapy (Liang *et al.*, 2000). We found that ACTH administration increased the volume of sucrose solution consumed, with no effect on regular water consumption, in males. However, in females, ACTH treatment did not alter any of the parameters measured in the SPT when compared to the male rats.

The contrasting sucrose consumption findings may be explained by inter-individual differences in coping strategies. In this regard, MDD patients often present with varying hunger responses, with some having an increased appetite, while others have decreased appetite (Simmons *et al.*, 2016). Variations in MDD-related appetite may explain why some studies report decreased sucrose preference in ACTH-treated rats while others showed the opposite (Zhao *et al.*, 2018). Moreover, in MDD patients there is also a large variation in the preference for caloric-rich foods, again largely dependent on inter-individual differences in coping mechanisms (Koning *et al.*, 2022). In rodents, these coping mechanisms may manifest as changes in preference for sucrose and may also be explained by altered dopaminergic activity in the mesocorticolimbic pathway (Meye & Adan, 2014). In MDD patients, increased appetite has been linked to hyperactivation of mesocorticolimbic reward pathways while a loss of appetite is associated with hypoactivation of insular regions responsible for homeostatic monitoring (Simmons *et al.*, 2016). Activation of reward circuits increases the hedonic drive to consume substances high in sugar and fat in response to food cues (Adam & Epel, 2007). Indeed, patients with elevated cortisol concentrations have an increased preference for sweet and savoury foods over other foods (Geer, 2016). In our study, chronic ACTH administration in males may have caused a glucocorticoid-related increase in mesocorticolimbic reward signals, resulting in increased sucrose consumption. Due to the extensive adaptation protocol required for the SPT, the present study did not assess sucrose preference at baseline, and hence we could not assess the change in behaviour over time.

Further studies need to assess sucrose preference changes over time to better understand the effects of the dysregulated HPA axis on inter-individual symptoms of anhedonia.

In contrast, imipramine treatment increased the volume of regular water consumed in both male and female rats. Despite an overall significant model effect, there were no differences in the sucrose preference ratio between treatment groups. The increase in water intake subsequent to imipramine treatment has been shown in previous rodent studies (Zabik *et al.*, 1977). This imipramine-induced increase in water consumption may be attributed to the drug's side effects, which include dry mouth and increased thirst (Fayez & Gupta, 2020). Indeed, we have also shown that imipramine treatment alone causes an increase in regular water consumption (Figure 2.4). Taken together, similar to the OFT, imipramine treatment did not reverse the effects of ACTH in the SPT, further illustrating the drug's inefficacy in a model of HPA axis dysregulation.

2.5.4 Effects of HPA axis dysregulation on central BDNF and CREB mRNA expression

This study showed that six weeks of ACTH administration resulted in increased hippocampal *BDNF* and increased *CREB* mRNA expression across all brain regions in male, but not in female rats. These findings differ from previous studies that administered ACTH to rodents for two weeks and found either a decrease (Zabik *et al.*, 1977) or no change in brain *BDNF* and *CREB* expression (Li *et al.*, 2006; Kuwatsuka *et al.*, 2013). The differences in the expression of neurotrophins in a model of ACTH-induced HPA axis dysregulation between studies may be explained by differences in the duration and dose of ACTH administration. The majority of studies investigating the depressive effects of this model administered ACTH for a maximum of three weeks (Li *et al.*, 2006; Kuwatsuka *et al.*, 2013; Antunes *et al.*, 2015). Although a single dose of an ACTH analogue has been shown to significantly increase BDNF and its receptor, TrkB, expression in the hippocampus of male rats (Dolotov *et al.*, 2006), others showed that two weeks of ACTH administration decreases both pro and mature BDNF and CREB expression (Kuwatsuka *et al.*, 2013). During ACTH-induced HPA axis dysregulation, there is impaired negative feedback which results in the hypersecretion of cortisol, adrenal androgens, and mineralocorticoids (Torres *et al.*, 2001). These adrenal androgens, dehydroepiandrosterone, pregnenolone, and their derivatives have been shown to increase BDNF expression in the hippocampus (Naert *et al.*, 2007). Moreover, these neuroactive steroids also increase ACTH and CRH levels in rodents, which supports the suggestion of hypersecretion of adrenal hormones (Naert *et al.*, 2007). GRs, mineralocorticoid receptors (MRs), and TrkB are co-expressed on hippocampal neurons highlighting the interplay between the GC-BDNF pathways (Jeanneteau *et al.*, 2012). In this regard, further studies are required to explore the possible adaptive mechanism through which chronically altered HPA axis function leads to an upregulation of neurotrophins following ACTH administration.

The increased *CREB* mRNA expression in all brain regions in the present study may be explained by a GC-related increase in dopaminergic activity. As previously discussed, increased ACTH and circulating GC levels correlate with increased dopaminergic activity (Willner *et al.*, 1991). CREB regulates genes crucial for the function of dopaminergic neurons (Wang *et al.*, 2018). Dopamine alters the phosphorylation of CREB via G-protein coupled receptors, upregulating its expression (Wang *et al.*, 2018). Consistent with neurobehavioral results, chronic ACTH administration may have resulted in a GC-mediated increase in dopaminergic activity, upregulating CREB expression.

Our findings indicate a strong sex effect in HPA axis activity and the expression of neurotrophins in response to repeated exogenous ACTH administration. ACTH did not alter the mRNA expression of *BDNF* and *CREB* in the female rat brain. This may also be attributed to the suppressive effect of female sex hormones on the HPA axis (Goel *et al.*, 2011). Meanwhile, in the rats treated with ACTH plus imipramine, *BDNF* and *CREB* mRNA expression did not differ from the controls in both males and females. The mechanisms whereby imipramine affects neurotrophic factors in HPA axis dysregulation require further investigation.

2.6 Limitations and future perspectives

Additional limitations should be considered. Differential group numbers may impact the statistical power of the study. The smaller number of female rats when compared to male rats used in this study should be considered when interpreting the effect of sex on neurobehavior and molecular expression. Nevertheless, a *post hoc* power calculation showed adequate statistical power. Changes in the FST and OFT were not reported for control animals. However, since repeated measures were used in the neurobehavioral analysis, animals within each specific treatment group, served as their own control at baseline for which more robust comparisons could be made. In the SPT, due to the extended nature of the protocol (Liu *et al.*, 2018), an assessment could not be performed prior to ACTH administration, hence changes in anhedonia over time could not be assessed and were only assessed prior to termination for group comparisons. Furthermore, although changes were observed in the sucrose preference test between groups, food intake was not recorded in this study. Future studies should include changes in feeding behaviour to better interpret food motivation and anhedonia. Lastly, ACTH and corticosterone levels were not measured in this study and should be measured in future studies to assess HPA axis function.

2.7 Conclusion

This study demonstrates that ACTH administration for two weeks resulted in sex-specific responses, with decreases in overall mobility and locomotion and increases in sucrose consumption, in males. In females,

ACTH had no effect on despair- or anxiety-like behaviours or on sucrose consumption. Additionally, after six weeks, there were no differences in depressive-like behaviour in the FST or OFT between the rats treated with ACTH plus imipramine and those treated with ACTH plus saline. However, imipramine treatment did increase water consumption. ACTH administration increased *BDNF* mRNA in the hippocampus and *CREB* mRNA in the prefrontal cortex, striatum, midbrain, and hippocampus in males, an effect not observed in female rats. Co-administration of ACTH and imipramine did not induce any changes in *BDNF* and *CREB* mRNA expression. Taken together, chronic ACTH administration may result in despair and anxiety-like symptoms as well as depression-like increases in appetite in male rats, which is not altered by imipramine treatment. The contrasting results observed in the neurobehavioral and molecular outcomes between male and female rats underscore the differential sex-associated physiological mechanisms underlying depression. Together our results suggest that drugs targeting the monoaminergic system may not be effective in reversing depressive-like symptoms in a model of HPA axis dysregulation. However, the effects of an alternative class of antidepressants, such as SSRIs have not been tested in the model, and it remains unclear how these monoamine-based antidepressants influence the expression of neurogenesis-related signalling factors implicated in depression pathophysiology.

CHAPTER 3: CITALOPRAM AND IMIPRAMINE MODULATE ACTH-INDUCED NEUROTROPHIC DYSREGULATION

3.1 **Abstract**

Background: One of the central mechanisms implicated in the pathophysiology of major depressive disorder (MDD) is the dysregulation of neurotrophic signalling, which is often associated with hypothalamic-pituitary-adrenal (HPA) axis dysregulation. Monoamine antidepressants are widely used in the management of MDD. Although they primarily act to regulate neurotransmitters, their effects on neurotrophic signalling remain poorly understood. This study investigated the effect of monoamine antidepressant treatment on neurotrophic signalling pathways in a rodent model of adrenocorticotropic hormone (ACTH)-induced HPA axis dysregulation.

Methods: Using male Sprague Dawley rats (n=58), we investigated the brain distribution of citalopram (CIT), a selective serotonin reuptake inhibitor (SSRI), and imipramine (IMI), a tricyclic antidepressant (TCA) via atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-MALDI-MSI). In addition, we determined the brain regional mRNA expression of intracellular markers of neurotrophic signalling, including brain-derived neurotrophic factor (*BDNF),* cAMP-response element binding protein *(CREB),* tropomyosin receptor kinase B *(TrkB),* protein kinase B *(Akt),* mechanistic target of rapamycin *(mTOR),* and eukaryotic elongation factor 2 (*eEF2*) using RT-PCR.

Results: ACTH administration increased hippocampal BDNF mRNA expression, coupled with decreased mRNA expression of intermediate neurogenesis signalling factors, *TrkB, Akt, mTOR,* and *eEF2*. Notably, citalopram showed a greater capacity to ameliorate ACTH-induced neurotrophic dysregulation compared to imipramine, particularly by enhancing *BDNF-TrkB-mTOR* signalling, independent of brain drug distribution.

Conclusion: These findings highlight the distinct regional pharmacodynamic effects of different classes of antidepressants in targeting dysregulated neurotrophic signalling. This study further sheds light on the molecular underpinnings of antidepressant efficacy, which may inform the development of more precise treatments for stress-associated mood disorders.

3.2 Introduction

Neurotrophins play an essential role in regulating neuroplasticity and neuronal growth and survival (Neto *et al.*, 2011). Their dysregulation has been linked to atrophy and functional disruptions in limbic regions, contributing to the pathophysiology of depression and the clinical symptoms associated with the disorder (Neto *et al.*, 2011). Research on MDD has largely focused on the primary markers of neuroplasticity, BDNF and CREB (Blendy, 2006; Martinowich *et al.*, 2007). Although BDNF and CREB represent endpoints in neuroplasticity and neurotrophic pathways, their expression is regulated by several other intracellular signalling molecules, including TrkB, Akt, mTOR, and eEF2 (Tartt *et al.*, 2022). Together these factors play a critical role in neurogenesis and cell survival, hence impacting the brain's structural connectivity and mediating pathophysiological changes in neurological disorders (Koike *et al.*, 2013; Machado-Vieira *et al.*, 2017). As critical promoters of neuronal health, these biomarkers are pivotal in learning, memory, and mood regulation which are often altered in disorders such as MDD (Neto *et al.*, 2011). Our lab and others have demonstrated that chronic ACTH administration alters BDNF and CREB expression, which in turn may be linked to a neurobehavioral depressive-like phenotype (Petrović *et al.*, 2018; Sallie *et al.*, 2024). Although the role of HPA axis dysregulation is well-accepted in the development of depressive symptoms, understanding the interplay between the dysregulated HPA axis and neurotrophic signalling may shed light on the potential pathophysiology underlying MDD (Tartt *et al.*, 2022).

HPA axis dysregulation has gained significant attention as a potential mechanism due to the well-established link between psychosocial stress and depression (Mlyniec, 2015; Machado-Vieira *et al.*, 2017). Chronic stressors may lead to dysregulated HPA axis activity, including enhanced release of feed-forward signals such as corticotropin-releasing hormone (CRH) and ACTH coupled with impaired negative feedback, resulting in abnormal cortisol levels that disrupt the body's stress response system (Keller *et al.*, 2017). Dysregulation of the HPA axis has been linked to compromised neuroplasticity and neurotrophic signalling, which has been linked to the development of depression and its clinical presentation (Price & Duman, 2020). HPA axis dysregulation is associated with a decreased expression of glucocorticoid receptors, leading to altered BDNF expression in the hippocampus and prefrontal cortex (PFC), and dysregulated pro-opiomelanocortin transcription in the pituitary, further promoting HPA axis dysfunction (Kumamaru *et al.*, 2008; Jeanneteau *et al.*, 2012). This provides evidence for the characteristic hippocampal volume reduction and neuronal atrophy seen in MDD patients due to chronically elevated glucocorticoid levels (Boku *et al.*, 2018). The link between glucocorticoid exposure and the progression of depression highlights the importance of preclinical models of HPA axis dysregulation in studying antidepressant efficacy.

Despite the involvement of various biological pathways in the development and progression of depression, current pharmacotherapies largely target monoaminergic neurotransmission (Krishnan and Nestler, 2008). In this regard, SSRIs and TCAs increase the synaptic availability of serotonin and norepinephrine by inhibiting transporter activity and reuptake, contributing to the alleviation of depressive symptoms (Fasipe, 2018). Nonetheless, these drugs show limited efficacy in the treatment and management of depression as evidenced by the high prevalence of TRD (McIntyre *et al.,* 2023). The ACTH model of depressive-like symptoms has been proposed as a model of TRD, although previous studies have only evaluated the model in response to TCAs (Kitamura *et al.,* 2002). According to the most widely accepted criteria for TRD, there must be resistance to at least two different antidepressant classes for a TRD diagnosis (McIntyre *et al.,* 2023). Thus, we evaluated the molecular effects of both a TCA and SSRI and investigated their impact on neurotrophic signalling in the ACTH model. Studies suggest that monoamine antidepressants promote neuroplasticity and neuronal health necessary for mood regulation (Duman and Monteggia, 2006; Thome *et al.,* 2000). However, the modulation of BDNF, CREB, TrkB, Akt, mTOR, and eEF2 by monoamine-based antidepressants in the ACTH model remains unclear.

This study investigated the effect of monoamine antidepressants on ACTH-induced neurotrophic dysregulation by investigating the effect of citalopram (CIT) and imipramine (IMI) treatment on neurotrophic signalling in ACTH-treated rodents. We studied the regional distribution of CIT and IMI using atmospheric pressure matrix-assisted laser desorption ionization (AP-MALDI) mass spectrometry imaging (MSI) as this technique offers high spatial resolution, allowing for visualisation of drug localisation within specific brain regions. In addition, the molecular gene expression of *BDNF, CREB, TrkB, Akt, mTOR,* and *eEF2* was quantified in depression-associated brain regions using RT-PCR. We found that chronic ACTH administration and monoamine antidepressant treatment increased *BDNF* and *CREB* expression, and CIT partially reversed the ACTH-induced decrease in markers of neurotrophic signalling, while imipramine had a minimal effect.

3.3 **Materials and methods**

3.3.1 Study design

3.3.1.1 Animals

This study was approved by the Animal Research Ethics Committee of the University of the Witwatersrand (approval reference: 2022/03/06/C) and all procedures were carried out per the regulations of the South African National Legislation for animal husbandry, welfare, and experimentation using laboratory animals. All *in vivo* experiments are reported per the ARRIVE guidelines.

Male Sprague Dawley rats (n=58) with an initial weight of 250±50 g were obtained from WRAF at the University of the Witwatersrand. The animals were housed in a room with a 12-hour light/dark cycle (lights on at 07*h*00), maintained at ±25°C with a humidity of 30%-40% in polycarbonate rodent cages (Techniplast, Italy). Each sawdust-lined cage housed two rats with water and standard rodent pellets (Epol, Johannesburg, South Africa) *ad libitum*. Animals were acclimated for seven days before any experimental procedures.

3.3.1.2 Drug preparation

Drugs used in this study include citalopram hydrobromide (Sanofi, NSW, Australia), imipramine hydrochloride (Sigma, St. Louis, MO, USA) and adrenocorticotropic hormone-(1-24)-zinc (ACTH). ACTH was prepared by the Peptide Sciences Laboratory at the University of KwaZulu-Natal, South Africa using a solid-phase peptide synthesis and was purified using liquid-chromatography (98% purity) - drug identity was confirmed using nuclear magnetic resonance spectroscopy. Clinical and *in vitro* data show that in diagnostic tests, ACTH(1-24) has equal, if not greater binding potency to melanocortin 2 receptors (MC2Rs) and achieves the same maximum cortisol output and half-life as endogenous ACTH(1-39) when given at steady state doses (Hamilton & Cotton, 2010; Ghaddhab *et al.*, 2017). Furthermore, ACTH(1-24), or Synecthan, is used to test for adrenal insufficiency, and therefore has clinical application (De Kloet *et al.*, 2006). All drugs were freshly prepared and dissolved in saline prior to each administration. Citalopram and imipramine were injected at a volume of 0.2 ml (10 mg/kg/day, ip) and ACTH was injected at a volume of 0.1 ml (100 µg/day, sc).

3.3.1.3 Treatment regimen

Rats were randomly divided into the control group (SAL), (n=29) that received saline (0.1 ml/daily, sc) or the ACTH group (ACTH), (n=29) (100 µg/day, sc) that were treated for two weeks, respectively (Kitamura *et al.*, 2002). The ACTH treatment period consisted of two phases: ACTH was first administered for two weeks to induce a depressive-like phenotype and to establish the model (Kitamura *et al.*, 2002; Sallie *et al.*, 2024), followed by an additional four weeks of ACTH administration with concurrent antidepressant co-administration to assess the treatment effect of citalopram and imipramine in this model. Both the dose and the two-week duration of ACTH was selected based on prior studies that showed that this regimen is sufficient to induce a depressive-like phenotype in rodents (Kitamura *et al.*, 2002; Sallie *et al.*, 2024). Following the two-week saline/ACTH (sc) pretreatment, rats were further subdivided to receive either saline (CON, n=10 and ACTH_S, n=10; 0.2 ml/day, ip), imipramine (SAL_I, n=10 and ACTH_I, n=9; 10 mg/kg/day, ip), or citalopram (SAL_C, n=9 and ACTH_C, n=10; 10 mg/kg/day, ip) for four weeks (treatment timeline shown in Figure 3.1A) (Kitamura *et al.*, 2002). The doses of citalopram and imipramine

were selected based on previous studies that assessed their efficacy in reversing depressive-like behaviours in rodent models (Kitamura *et al.,* 2002; Papp *et al.,* 2002).

3.3.1.4 Tissue harvest

At the completion of the treatment regimen, rats were anesthetized using isoflurane (2%) before being euthanized by decapitation with a rodent guillotine. The use of an anaesthetic prior to termination was part of the humane treatment of animals as suggested by the ethics committee to reduce animal suffering. Brain tissue was removed and bisected; the left hemisphere was surgically dissected into the PFC, striatum, hippocampus, and midbrain, before being snap-frozen in liquid nitrogen (Figure 3.1B). These brain regions were selected due to their established role in mood regulation, depression pathology, and vulnerability to HPA axis dysregulation (Chen *et al.*, 2023). The right hemisphere was gradually frozen in liquid nitrogen vapor for mass spectrometry imaging analysis. All tissues were stored at -80°C until molecular analysis.

3.3.2 Molecular analysis

3.3.2.1 Gene expression of markers associated with neurotrophic signalling

Brain regions were homogenized using a probe sonicator (Fisher Scientific, Waltham, Massachusetts, USA) and total RNA was isolated using the Illustra Minispin RNA isolation kit (GE Healthcare, USA) according to manufacturer instructions. Total RNA was then reverse transcribed into cDNA using the SuperScript IV reverse transcription cDNA kit (ThermoFisher, California, USA) according to manufacturer instructions and stored at -80°C before gene expression analysis. The expression of *BDNF* (Rn02531967_s1), *CREB* (Rn01451883_m1), *TrkB* (Rn01441749_m1), *Akt* (Rn00583646_m1), *mTOR* (Rn00693900_m1), and *eEF2* (Rn00820849_g1) was determined using commercially available TaqMan assays, and a StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) (Figure 3.1B). Changes in mRNA expression of the genes of interest were evaluated relative to *Tbp*. The $2^{-\Delta\Delta Ct}$ method was used and data is represented as a relative fold change compared to controls (Livak & Schmittgen, 2001).

3.3.2.2 Brain drug distribution measured by atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry imaging (AP-MALDI-MSI)

For MSI analysis of brain drug distribution, rodent brains were cryosectioned into 12 µm thick sections at the level of the striatum (bregma +1.92 mm), hippocampus (bregma -3.24 mm), and midbrain (bregma -5.40 mm) (Figure 3.1C). Tissue sections were thaw-mounted onto conductive indium tin oxide (ITO)-coated glass slides (TechnoStar, India) and stored at -80°C until needed for analysis. For detection of citalopram and imipramine, each slide was dried in a vacuum desiccator for 20 minutes after which slides

were then coated with a 2,5-dihydroxybenzoic acid (DHB, 50 mg/ml) matrix, prepared in 70% acetonitrile, 30% H_2O, and 0.1% trifluoroacetic acid (TFA). Matrices were uniformly applied to each slide using a SunCollect Sprayer (SunChrom Industries, Bremen, Germany), with the following settings: Z-distance (25 mm), velocity (1 200 mm/min), flow rate (0.1 ml/min), track spacing (2 mm), and gas pressure (2.5 bar) for 8 passes. MSI was performed using an atmospheric pressure matrix-assisted laser desorption ionization (AP-MALDI) (MassTech, US) coupled to a Thermo Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, US). The mass spectrometer was operated with the following settings: scan range: 150-2000 m/z, resolution: 140 000, AGC target: 5e6, maximum inject time: 100, in positive ion mode. Target Next software (MassTech, USA) was used to operate the AP-MALDI ion source. All experiments were run at a spatial resolution of 20 µm. Mozaic software (SpectroSwiss, Switzerland) was used to normalise and analyse MSI spectra and to visualise drug distributions. The distribution of a citalopram metabolite was determined using a mass of 262.10 m/z (Källback *et al.*, 2020), while the distribution of imipramine was determined at a mass of 281.10 m/z, which represents the parent mass. The mass error threshold for all compounds was set at 5 ppm.

3.3.3 Data analysis

Data analysis was performed using SAS software, version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) and visualized using GraphPad v10. A Shapiro-Wilk test was used to assess the distribution of the data. Data are reported as mean ± standard deviation or median and interquartile range, as appropriate. A mixed model two-way ANOVA followed by a Tukey's *post hoc* test was used to determine differences in gene expression between groups, with group (control or ACTH) and treatment (saline, citalopram, or imipramine) as the main effects A volcano plot was generated to visualize gene expression differences between treatment groups. Log2 fold changes were calculated from the ratio of PCR expression data from treatment groups to controls, and p-values were obtained from a student's t-test comparing each set of experimental groups. Negative log10 p-values were then plotted against the log2 fold changes to highlight markers that were significantly different between groups. In each plot, significant variables are indicated above the $p=0.05$ and $p=0.01$ thresholds. A standardized principal component analysis (PCA) was performed on PCR data from all treatment groups to visualize the relationship between interventions and the corresponding principal components. Statistical significance was considered at $p\leq0.05$.

3.4 **Results**

3.4.1 Differential brain distribution of citalopram and imipramine

MSI analysis showed a distinct region-specific drug distribution of CIT and IMI (Figure 3.1D). Representative images show abundant levels of IMI in the striatum, hippocampus, and midbrain of animals receiving imipramine. Interestingly, MSI analysis showed only moderate levels of a CIT metabolite distributed in the striatum and hippocampus of SAL_C animals, while relatively lower drug levels were observed in the midbrain of SAL_C rats and all regions of ACTH_I rats. These results suggest that the effects of CIT may be mediated by other drug-related metabolites and that ACTH co-administration may further influence its metabolism and distribution of antidepressants in the brain.

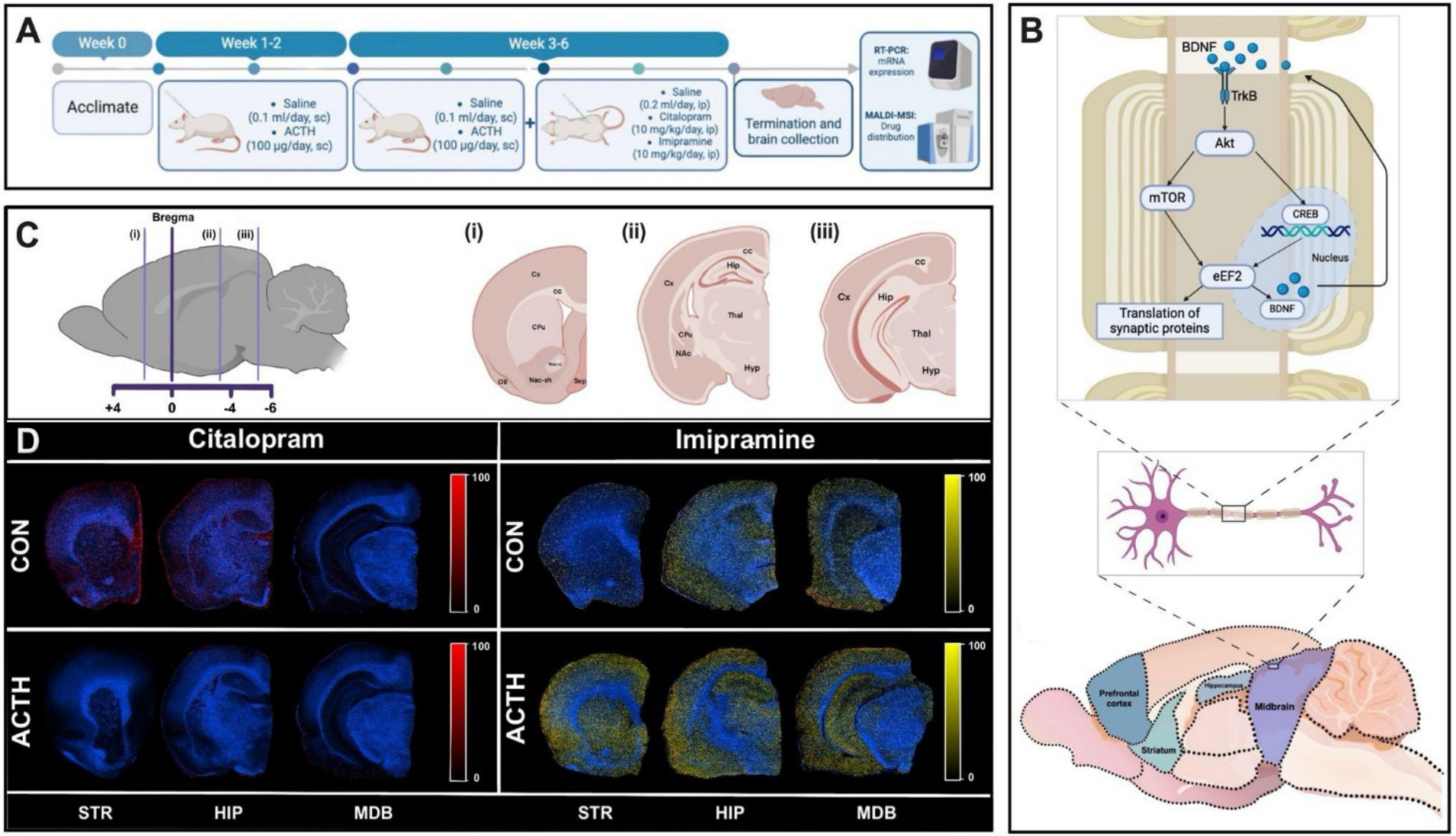

Figure 3.1. Experimental design showing the treatment timeline. Rats received either saline (0.1 ml, sc) or ACTH (100 ug/day, sc) for two weeks (week 1-2), thereafter, in addition to saline or ACTH (sc), animals received either saline (0.2 ml, ip), citalopram (10 mg/kg, ip), or imipramine (10 mg/kg, ip) daily for four weeks (week 3-6) **(A).** Schematic representation of the neurogenesis pathway within a Schwann cell showing the intracellular signalling cascade downstream of BDNF-TrkB binding with a rodent brain that was divided into subregions used in this study; the prefrontal cortex, striatum, hippocampus, and midbrain, were surgically dissected for PCR analyses **(B).** Bregma levels of the striatum (bregma +1.92 mm) **(Ci)**, hippocampus (bregma -3.24 mm) **(Cii)**, and midbrain (bregma -5.40 mm) **(Ciii)** used for MSI analyses. Cx, cortex; cc, corpus callosum; CPu, caudate putamen; Nac-sh, nucleus accumbens shell; NAc, nucleus accumbens; OB, olfactory bulb; Sep, septum; Hip, hippocampus; Thal, thalamus; Hyp, hypothalamus; STR, striatum; MDB, midbrain. Representative MSI images showing the brain distribution of a citalopram metabolite (red; 262.10 *m/z*) (Källback *et al.*, 2020), imipramine (yellow; 281.10 *m/z*) overlaid with a structural lipid (blue; 480.30 *m/z*) in the control and ACTH groups **(D)**. Key: Cx, cortex; cc, corpus callosum; CPu, caudate putamen; Nac-sh, nucleus accumbens shell; NAc, nucleus accumbens; OB, olfactory bulb; Sep, septum; Hip, hippocampus; Thal, thalamus; Hyp, hypothalamus; STR, striatum; MDB, midbrain.

3.4.2 ACTH alters neuroplasticity markers in a region-dependent manner

In the PFC, there was a significant increase in the expression of *CREB* mRNA in the ACTH_S group compared to controls (p=0.0001) (Figure 3.2.1B). However, *TrkB* (p=0.034), *Akt* (p=0.0001), *mTOR* (p<0.0001), and *eEF2* (p<0.0001) mRNA expression decreased in the ACTH_S group compared to CON (Figure 3.2.1C-F). Similarly, ACTH_I rats showed reduced *Akt*, *mTOR,* and *eEF2* mRNA levels compared to the SAL_I group (p=0.013, p<0.001, and p<0.0001) (Figure 3.2.1D-F). In the striatum, similar to the PFC, *CREB* mRNA levels were higher in ACTH_S rats compared to controls (p=0.007) (Figure 3.2.2B), whereas *Akt* mRNA expression was lower in ACTH_S-treated animals relative to CON (p<0.050) (Figure 3.2.2D).

In the hippocampus, *BDNF* and *CREB* mRNA expression increased in the ACTH_S group compared to controls (p=0.003 and p<0.001) (Figure 3.2.3A and B). Rats treated with the ACTH_I combination also showed higher *CREB* mRNA levels than those treated with SAL_I (p<0.050) (Figure 3.2.3B); however, *mTOR* and *eEF2* mRNA was lower in the ACTH_I group compared to SAL_I (p<0.001 and p<0.0001) (Figure 3.2.3E and F). Similarly, ACTH_S rats exhibited significantly lower *Akt* and *eEF2* expression than controls (p<0.050 and <0.0001) (Figure 3.2.3D and F). In the midbrain, *CREB* and *TrkB* mRNA expression was higher in the ACTH_S group compared to CON (p<0.001 and p=0.001) and *CREB* mRNA was higher in the ACTH_I group compared to the SAL_I group (p<0.050) (Figures 3.2.4B and C). Similar to other regions, the expression of *Akt* and *eEF2* mRNA decreased in the ACTH_S group compared to controls (p<0.050 and <0.0001) and *eEF2* mRNA was lower in the ACTH_I group compared to SAL_I rats (p<0.0001) (Figure 3.2.4D and F).

3.4.3 Citalopram restores Akt-mTOR expression across regions

In the PFC, SAL_C animals showed higher *CREB* and *mTOR* mRNA expression than CON (all p<0.0001); however, surprisingly, *Akt* mRNA decreased in the SAL_C group relative to CON (p=0.013) (Figure 3.2.1B, D and E). Animals treated with ACTH_C showed significantly higher *mTOR* mRNA levels relative to the ACTH_S group (p<0.0001) (Figure 3.2.1E). Similarly, striatal *mTOR* expression was higher in SAL_C rats than in the control group (p<0.0001) (Figure 3.2.2E). *Akt* (p<0.010) and *mTOR* (p<0.0001) mRNA expression was also higher in ACTH_C-treated rats than in those treated with ACTH_S (Figure 3.2.2D and E).

In the hippocampus, *BDNF* (p<0.0001), *CREB* (p<0.0001), *TrkB* (p=0.028), and *mTOR* (p<0.0001) mRNA expression was augmented in SAL_C rats relative to CON (Figure 3.2.3A-C and 3.2.3E). ACTH_C rats also exhibited higher *TrkB* (p=0.003), *Akt* (p<0.010), and *mTOR* (p<0.0001) mRNA expression compared

to ACTH_S rats (Figure 3.2.3C-E). In contrast, in the midbrain, rats treated with SAL_C exhibited lower *BDNF* mRNA compared to CON ($p=0.032$), whereas *TrkB* levels were higher in the SAL_C group ($p<0.0001$) (Figure 3.2.4A and C). Citalopram (SAL_C) enhanced *Akt* and *mTOR* mRNA expression compared to CON (all $p<0.0001$) (Figure 3.2.4D and E). ACTH_C rats exhibited significantly higher *TrkB* ($p<0.0001$), *Akt* ($p<0.010$) and *mTOR* ($p<0.0001$) mRNA expression when compared to ACTH_S-treated rats ($p<0.0001$) (Figure 3.2.4C-E).

3.4.4 Imipramine has a limited effect on neuroplasticity markers

In the PFC and striatum, imipramine co-administration (ACTH_I) reversed the ACTH-induced decrease in *TrkB* mRNA ($p=0.001$ and $p=0.022$) (Figures 3.2.1C and 3.2.2C). In the PFC, ACTH_I rats had higher *Akt* expression than those that only received ACTH_S ($p=0.029$) (Figure 3.2.1D) and *eEF2* expression was enhanced in the SAL_I group relative to controls ($p<0.0001$) (Figure 3.2.1F).

In the hippocampus, *BDNF* and *TrkB* mRNA expression was higher in SAL_I rats than in controls ($p=0.002$ and $p=0.001$) (Figure 3.2.3A and C) and ACTH_I rats exhibited significantly higher *Akt* mRNA than ACTH_S rats ($p=0.017$) (Figure 3.2.3D).

3.4.5 Comparative neuroplasticity effects of citalopram and imipramine

In the PFC, the mRNA expression of *TrkB* ($p=0.010$), *Akt* ($p=0.0001$), and *eEF2* ($p<0.0001$) was increased in the SAL_I group compared to the SAL_C group (Figure 3.2.1C, D and F). However, in the PFC and striatum, the CIT groups (SAL_C and ACTH_C) had higher *mTOR* mRNA levels compared to the IMI groups (SAL_I and ACTH_I) (all $p<0.0001$) (Figure 3.2.1E and 3.2.2E).

In the hippocampus, the SAL_C group exhibited significantly higher *CREB* and *mTOR* mRNA levels compared to IMI controls (SAL_I) ($p<0.001$ and $p<0.0001$) (Figure 3.2.3B and E). Whereas the SAL_I group had higher *eEF2* mRNA than the SAL_C group ($p<0.0001$) (Figure 3.2.3F). In the midbrain, SAL_C rats exhibited significantly lower *BDNF* expression relative to SAL_I rats ($p=0.002$) (Figure 3.2.4A) whereas *CREB* and *mTOR* mRNA was higher in SAL_C rats compared to SAL_I ($p<0.001$ and $p<0.0001$) (Figure 3.2.4B and E). The CIT groups (SAL_C and ACTH_C) showed higher *TrkB* mRNA expression compared to the IMI groups (SAL_I and ACTH_I) (all $p<0.0001$) (Figure 3.2.4C). Similarly, *Akt* mRNA levels were higher in the CIT groups (SAL_C and ACTH_C) relative to the IMI groups (SAL_I and ACTH_I) (all $p<0.0001$) (Figure 3.2.4D). However, *eEF2* expression was greater in the SAL_I group relative to the SAL_C group ($p<0.0001$) (Figure 3.2.4F).

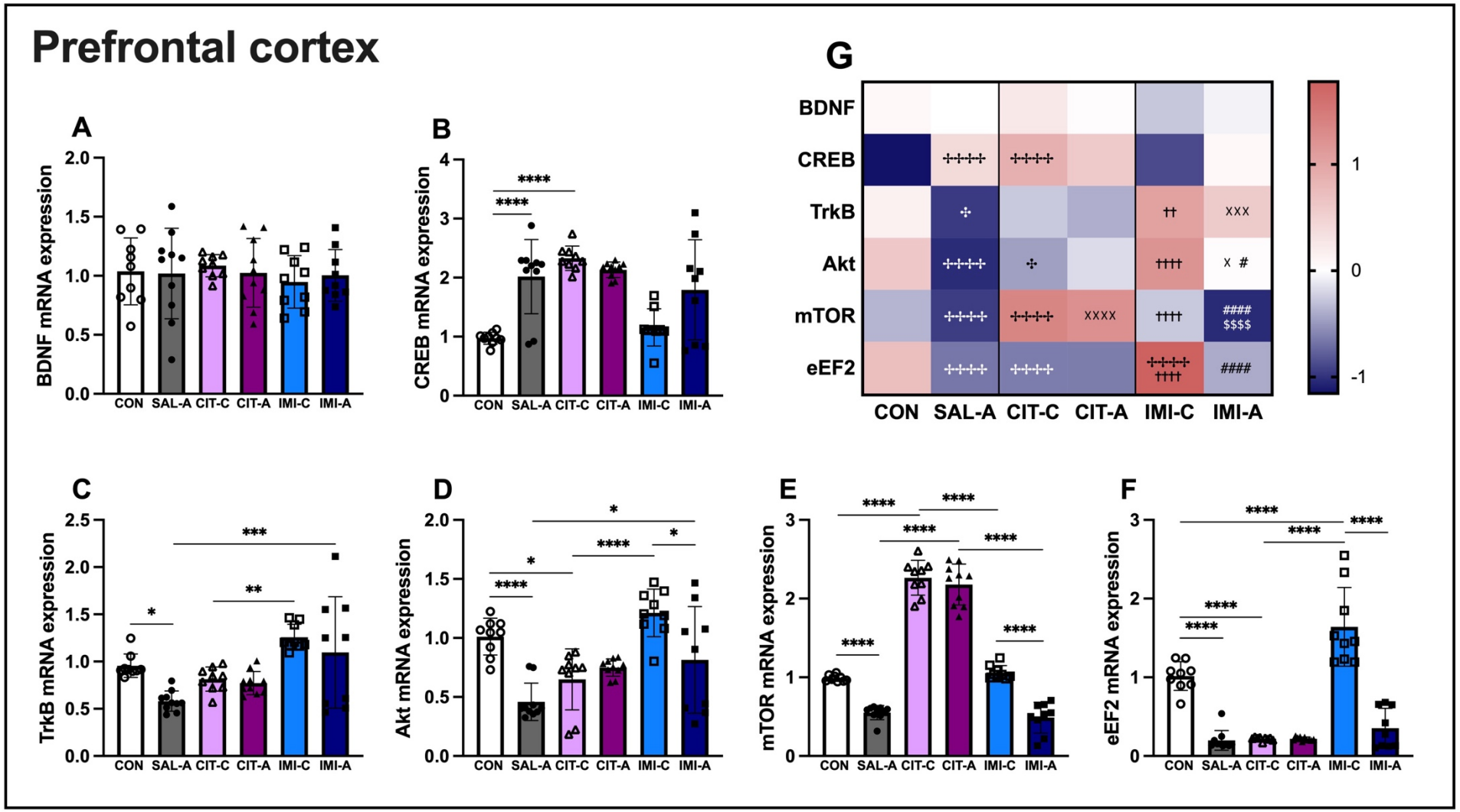

Figure 3.2.1 Brain regional expression of intracellular signalling markers associated with neurogenesis in the prefrontal cortex **(A-F)** relative to the endogenous control *Tbp*. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Heat map representing the z-scores of biomarkers involved in neurogenesis in the prefrontal cortex **(G)**, ✢ p<0.05, ✢✢✢✢ p<0.0001 relative to CON; X p<0.05, XXX p<0.001, XXXX p<0.0001 relative to ACTH_S; †† p<0.01, †††† p<0.0001 relative to SAL_C; $$$$ p<0.0001 relative to ACTH_C; # p<0.05, #### p<0.0001 relative to SAL_I. Data are presented as mean ± standard deviation and were analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (n=10); ACTH_S, ACTH+saline (n=10); SAL_C, saline+citalopram (n=9); ACTH_C, ACTH+citalopram (n=10); SAL_I, saline+imipramine (n=10); ACTH_I, ACTH+imipramine (n=9).

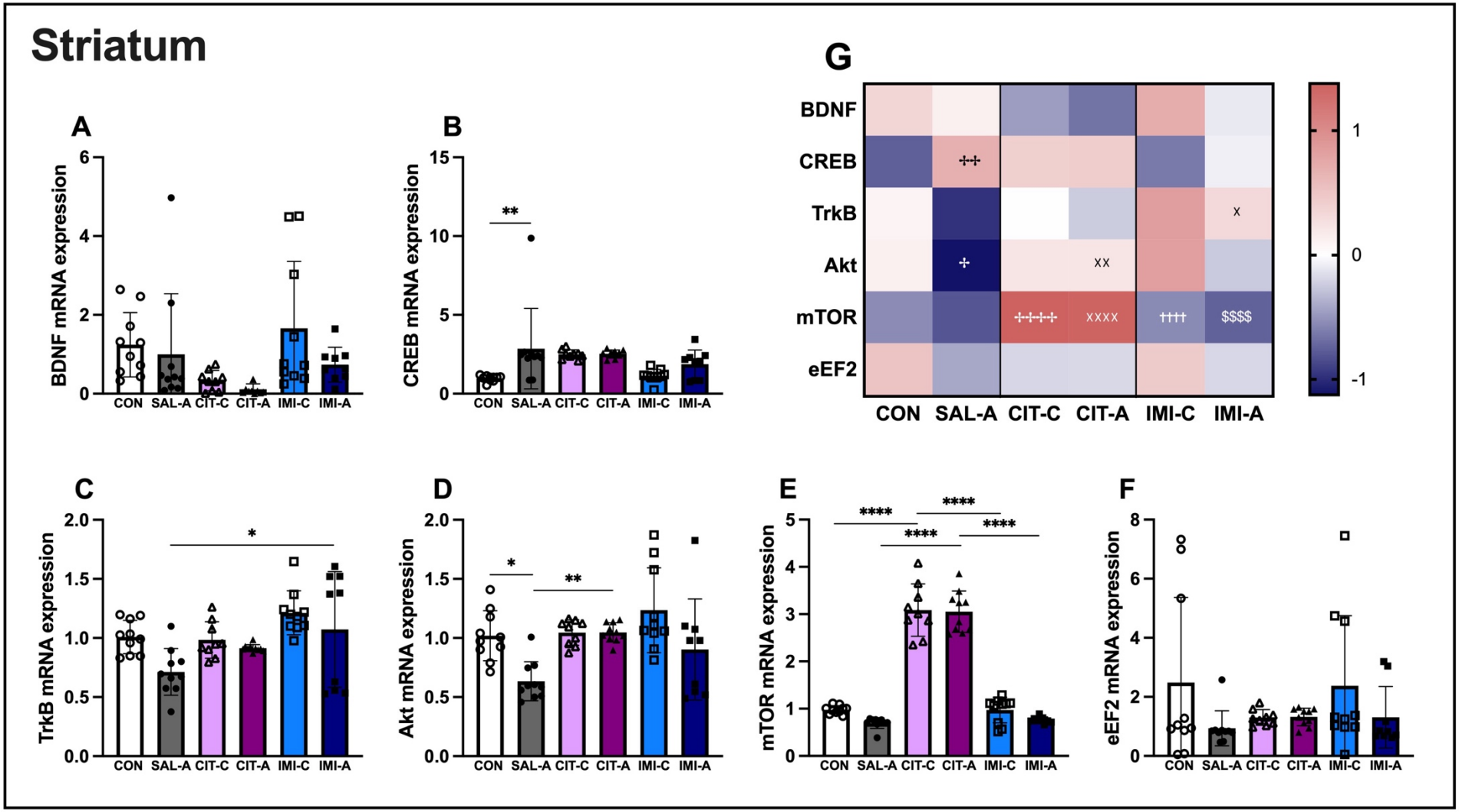

Figure 3.2.2 Brain regional expression of intracellular signalling markers associated with neurogenesis in the striatum **(A-F)** relative to the endogenous control *Tbp*. * p<0.05, ** p<0.01, **** p<0.0001. Heat map representing the z-scores of biomarkers involved in neurogenesis in the striatum **(G)**, ✢ p<0.05, ✢✢ p<0.01, ✢✢✢✢ p<0.0001 relative to CON; X p<0.05, XX p<0.01, XXXX p<0.0001 relative to ACTH_S; †††† p<0.0001 relative to SAL_C; $$$$ p<0.0001 relative to ACTH_C. Data are presented as mean ± standard deviation and were analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (n=10); ACTH_S, ACTH+saline (n=10); SAL_C, saline+citalopram (n=9); ACTH_C, ACTH+citalopram (n=10); SAL_I, saline+imipramine (n=10); ACTH_I, ACTH+imipramine (n=9).

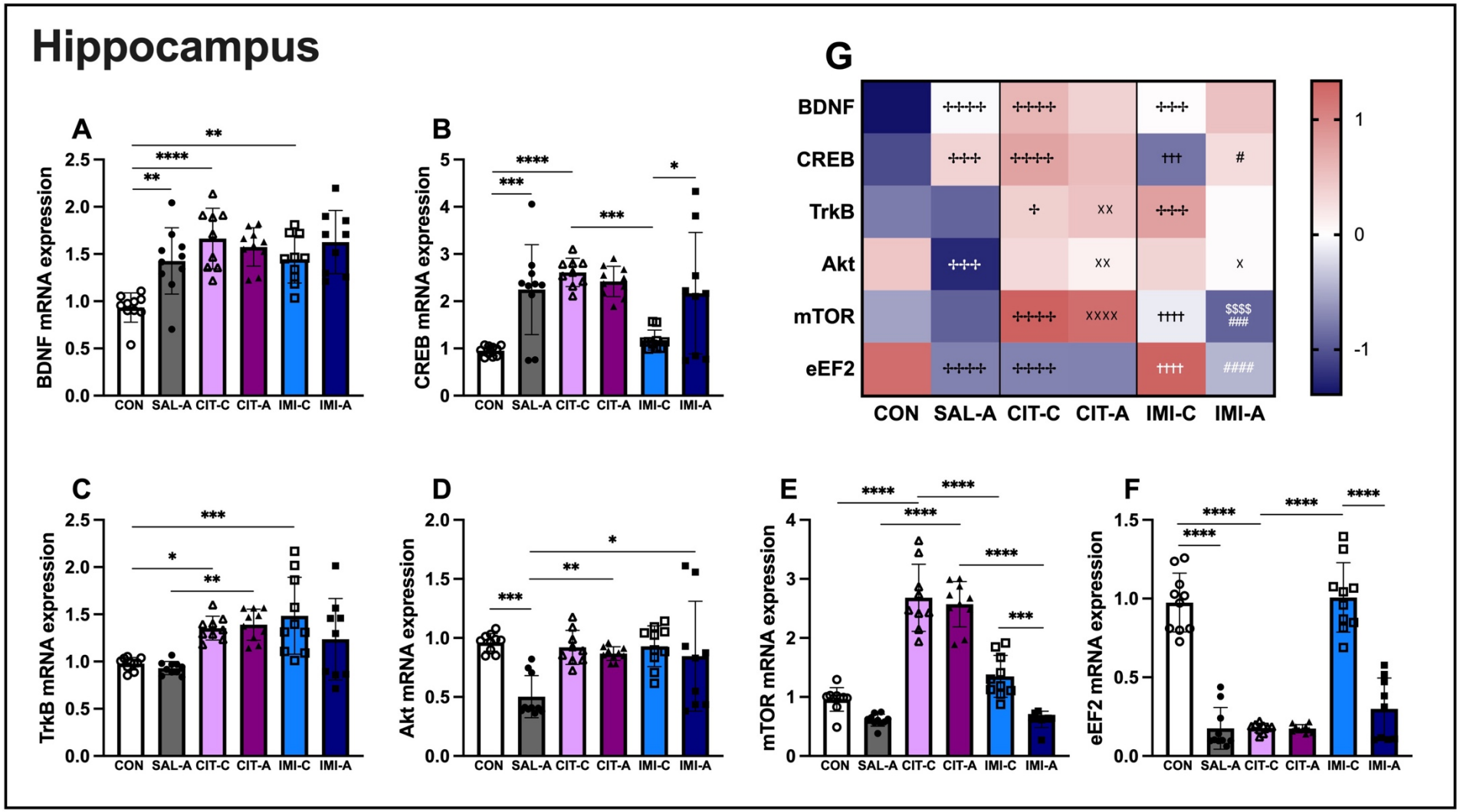

Figure 3.2.3 Brain regional expression of intracellular signalling markers associated with neurogenesis in the hippocampus **(A-F)** relative to the endogenous control *Tbp*. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Heat map representing the z-scores of biomarkers involved in neurogenesis in the hippocampus **(G)**, ✢ p<0.05, ✢✢ p<0.01, ✢✢✢ p<0.001, ✢✢✢✢ p<0.0001 relative to CON; X p<0.05, XX p<0.01, XXXX p<0.0001 relative to ACTH_S; ††† p<0.001, †††† p<0.0001 relative to SAL_C; $$$$ p<0.0001 relative to ACTH_C; # p<0.05, ### p<0.001, #### p<0.0001 relative to SAL_I. Data are presented as mean ± standard deviation and were analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (n=10); ACTH_S, ACTH+saline (n=10); SAL_C, saline+citalopram (n=9); ACTH_C, ACTH+citalopram (n=10); SAL_I, saline+imipramine (n=10); ACTH_I, ACTH+imipramine (n=9).

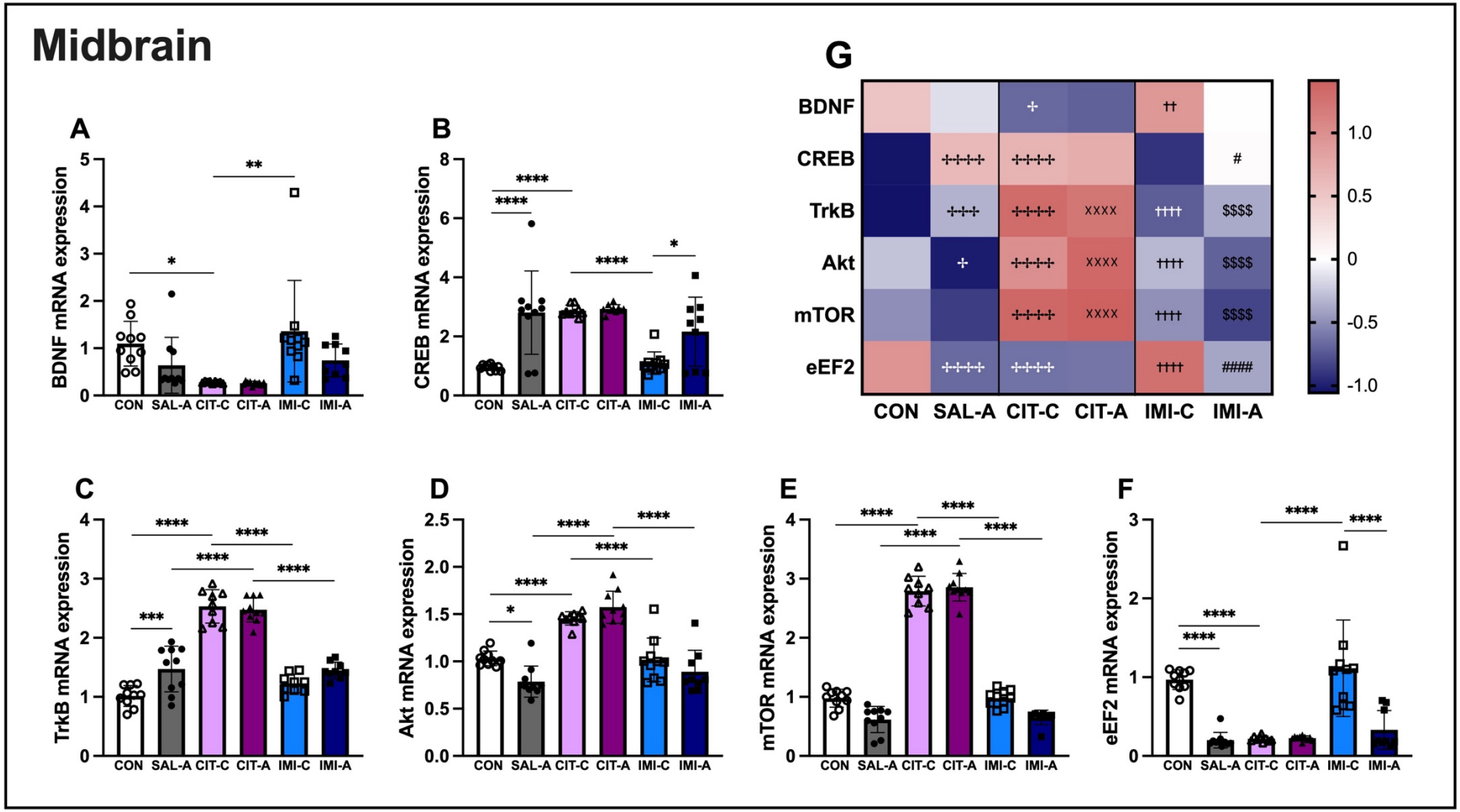

Figure 3.2.4 Brain regional expression of intracellular signalling markers associated with neurogenesis in the midbrain **(A-F)** relative to the endogenous control *Tbp*. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Heat map representing the z-scores of biomarkers involved in neurogenesis in the midbrain **(G)**, ✢ p<0.05, ✢✢✢ p<0.001, ✢✢✢✢ p<0.0001 relative to CON; XXXX p<0.0001 relative to ACTH_S; †† p<0.01, †††† p<0.0001 relative to SAL_C; $$$$ p<0.0001 relative to ACTH_C; # p<0.05, #### p<0.0001 relative to SAL_I. Data are presented as mean ± standard deviation and were analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control (n=10); ACTH_S, ACTH+saline (n=10); SAL_C, saline+citalopram (n=9); ACTH_C, ACTH+citalopram (n=10); SAL_I, saline+imipramine (n=10); ACTH_I, ACTH+imipramine (n=9).

3.4.6 Comparative gene expression profiles among treatment groups

The volcano plot comparing the CON and ACTH_S groups shows that ACTH administration resulted in a significant upregulation of hippocampal *BDNF*, midbrain *TrkB*, and *CREB* mRNA in the PFC, hippocampus, and midbrain ($p<0.01$), while striatal *CREB* mRNA was upregulated at a lower threshold ($p<0.05$) (Figure 3.3A). ACTH also resulted in a downregulation of *Akt* and *mTOR* mRNA globally, along with reductions in *TrkB* in the PFC and striatum and *eEF2* in the PFC, hippocampus, and midbrain ($p<0.01$).

The volcano plot comparing the ACTH_S to ACTH_C and ACTH_I groups shows that ACTH_C differed from ACTH_S in the gene expression of *TrkB* and *mTOR* in all regions and *Akt* in the PFC, striatum, and midbrain ($p<0.01$) (Figure 3.3B). In contrast, the ACTH_I group differed from the ACTH_S group only in the expression of *TrkB* and *Akt* mRNA in the PFC ($p<0.05$).

The PCA shows distinct clustering of treatment groups across PC1 and PC2 (Figure 3.3C). Similar gene expression profiles were seen between the CON and SAL_I groups, the ACTH_S and ACTH_I groups, and the SAL_C and ACTH_C groups, which differ the most from other groups.

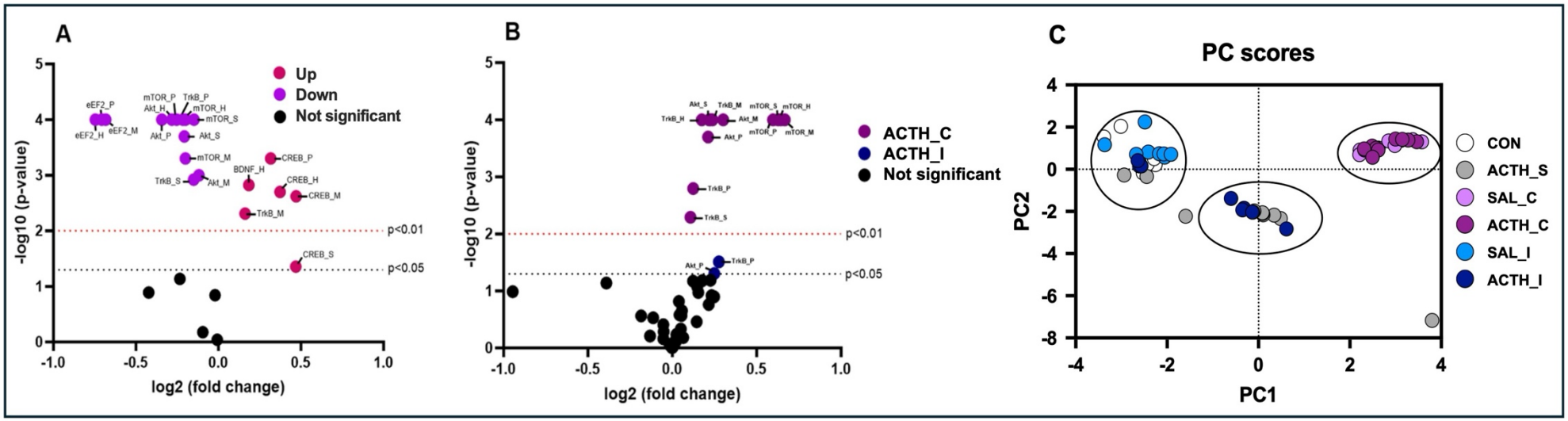

Figure 3.3. Multivariate analyses with a volcano plot comparing CON vs. ACTH_S groups. Upregulated genes (pink), downregulated genes (purple), and non-significant genes (black) are shown **(A)**. Volcano plot comparing ACTH_S vs. ACTH_C and ACTH_I groups. Significant changes in ACTH_C (purple) and ACTH_I (blue) are highlighted. Dashed lines indicate significance thresholds ($p<0.05$, $p<0.01$) **(B)**. Suffix key: _P, prefrontal cortex; _S, striatum; _H, hippocampus; _M, midbrain. A PCA was performed on normalized PCR expression values of neurogenesis markers to visualize variance among the experimental groups **(C)**. CON, control (n=10); ACTH_S, ACTH+saline (n=10); SAL_C, saline+citalopram (n=9); ACTH_C, ACTH+citalopram (n=10); SAL_I, saline+imipramine (n=10); ACTH_I, ACTH+imipramine (n=9).

3.5 Discussion

To determine the brain distribution and molecular effects of monoamine antidepressants in a model of HPA-axis dysregulation, we chronically administered exogenous ACTH to animals, followed by co-administration with monoamine antidepressants, citalopram or imipramine. We showed the widespread distribution of imipramine in the striatum, hippocampus, and midbrain in all animals. The distribution of a citalopram-specific metabolite was moderate in the striatum and hippocampus of SAL_C animals, and much lower in the midbrain of SAL_C and ACTH_C animals. We found that ACTH administration and monoamine antidepressant treatment resulted in differential *BDNF, CREB, TrkB, Akt, mTOR*, and *eEF2* expression in a region-specific manner. ACTH administration dysregulated all intracellular signalling factors in various regions. Following treatment with imipramine and citalopram, these signalling factors also showed differential changes when compared to the effect of ACTH. This study showed that ACTH administration resulted in molecular changes associated with a depressive phenotype, which were more effectively reversed by CIT than by imipramine.

3.5.1 Effect of ACTH administration on neurotrophic signalling

HPA axis dysregulation is strongly associated with depression (Høifødt *et al.*, 2019). Our lab previously demonstrated that exogenous ACTH administration induces neurobehavioral changes associated with a depressive-like phenotype (Sallie *et al.*, 2024). In this study, we showed that ACTH administration resulted in significant neurogenesis-related molecular changes in the PFC, striatum, hippocampus, and midbrain, which are regions implicated in the pathophysiology of depression. ACTH administration led to an upregulation in hippocampal *BDNF* mRNA and a global increase in *CREB* mRNA. *TrkB*, *Akt*, *mTOR*, and *eEF2* expression varied across regions. These transcriptional changes were region-specific suggesting that the neurobiological response to ACTH is heterogenous, possibly reflecting localised vulnerability associated with HPA axis dysregulation and depression pathology.

Clinically, MDD has been strongly associated with altered brain BDNF signalling, with decreased BDNF commonly reported both peripherally and in brain tissue (Tartt *et al.*, 2022; Leschik *et al.*, 2022). In contrast, we found that chronic ACTH administration increased hippocampal *BDNF* expression. The hippocampus is crucial for memory and cognitive function and interacts with other mood-regulating regions such as the PFC and amygdala (Campbell & MacQueen, 2004). Depressed individuals often exhibit stress-induced reductions in hippocampal volume linked to memory and cognitive deficits, additionally affecting limbic regulation of mood (Campbell & MacQueen, 2004). The paradoxical increase in hippocampal *BDNF* mRNA observed in our study suggests that this neurotrophin may play a role in the negative feedback mechanism during HPA axis dysregulation (Jeanneteau *et al.*, 2012). Specifically, chronic ACTH exposure and elevated HPA axis activity may increase glucocorticoid-receptor binding at the BDNF promoter in primary hippocampal neurons (Daskalakis *et al.*, 2015). This

in turn can increase BDNF gene transcription in an attempt to restore homeostasis and negative feedback control of the HPA axis. Indeed, BDNF has been shown to inhibit hypothalamic CRH and adrenal glucocorticoid production to restore basal HPA axis homeostasis, supporting its role as a modulatory agent in the stress-response system (Daskalakis *et al.*, 2015). Therefore, in MDD, BDNF may not always be uniformly decreased, but rather dysregulated. Indeed, in a chronic restraint stress model, basal hippocampal BDNF mRNA and protein were elevated alongside increased CRH, ACTH, and corticosterone (Naert *et al*., 2011). However, other preclinical models show conflicting findings regarding BDNF changes. In this regard, studies have shown that ACTH administration decreases *BDNF* in rodents (Antunes *et al.*, 2015), while others demonstrated no changes (Kuwatsuka *et al.*, 2013). Similarly, previous studies report contradictory findings for *CREB,* a transcription factor that regulates BDNF (Böer *et al.,* 2010; Böer *et al.,* 2007). *CREB* mRNA expression was increased across all brain regions following ACTH administration, which contradicts studies that show CREB reductions in models of depressive-like symptoms. Increased *CREB* gene expression alongside *BDNF* indicates that ACTH induces a compensatory response where CREB regulates BDNF expression and activates pro-survival pathways. This effect is likely triggered by ACTH-induced glucocorticoid release which activates CREB via stress signalling cascades (Lefrancois-Martinez *et al.*, 2011). As a compensatory mechanism to counteract stress and promote survival pathways, CREB may activate a broader set of genes responsible for neuronal survival and resilience against stress (Lefrancois-Martinez *et al.*, 2011).

Despite BDNF being upregulated globally, *TrkB* mRNA, the gene that codes for the primary receptor for BDNF, was decreased in the PFC but increased in the midbrain of ACTH-treated rats. It is well established that chronic HPA axis activation induces transcriptional suppression of *TrkB* via glucocorticoid receptor interactions at its promotor (Tsimpolis *et al.*, 2024). This PFC downregulation aligns with prior findings of TrkB deficits in depressive models and postmortem tissue from MDD patients (Reinhart *et al.*, 2015). Reduced TrkB receptor levels impair BDNFs activation of PI3k-Akt-mTOR pro-plasticity cascades (Sonoyama *et al.*, 2020). Impaired neuroplasticity reduces dendritic spine density and arborisation thus affecting the brains regenerative and functional reorganisation abilities (Puderbaugh & Emmady, 2025). The PFC plays a crucial role in decision making, emotional regulation, and cognitive control. Neuronal atrophy and poor connectivity in fronto-limbic regions impairs mood regulation, leading to learned helplessness, negative processing biases, and anhedonia seen in MDD patients (Reinhart *et al.*, 2015; Duman *et al.*, 2016; Pizzagalli & Roberts, 2022). Increased midbrain *TrkB* expression may be a compensatory mechanism to increase receptor expression due to the low *BDNF* mRNA expression, to maintain neurotrophic signalling. Connectivity in this region is crucial as the midbrain plays a significant role in mood regulation by modulating monoamine neurotransmitter systems. Circuit dysfunction in the midbrain is linked to impaired reward and emotional processing which contribute to anhedonic and amotivational MDD symptoms (Han *et al.*, 2017). *Akt, mTOR,* and *eEF2* mRNA was reduced across multiple brain regions, highlighting supressed

neurogenesis signalling cascades downstream. *Akt* is one of the key downstream intracellular factors that mediate BDNF-TrkB signalling cascades by enhancing CREB activity and eEF2 phosphorylation (Machado-Vieira *et al.*, 2017). Thus, decreased *Akt* mRNA suggests compromised neuroprotection, which may contribute to depressive-like symptoms and brain structural changes (Jernigan *et al.*, 2011). Similarly, reduced PFC *mTOR* mRNA supports evidence showing reduced PFC mTOR signalling and disrupted synaptic function in individuals with mood and stress-related disorders (Machado-Vieira *et al.,* 2017). Reduced *eEF2* expression in the PFC, hippocampus, and midbrain, suggests impaired protein synthesis and neuroplasticity in these regions. Given the vital role of eEF2 in protein translation and its downregulation by ACTH, our results suggest that chronic ACTH disrupts normal translational processes, potentially exacerbating depressive pathology (Karalis & Bateup, 2021). Indeed, ACTH-induced glucocorticoid-receptor binding activates stress proteins that inhibit mTOR and reduce downstream Akt-mTOR-eEF2 activity (Kim *et al.*, 2023). Furthermore, GR activation increases the expression of FK506-binding protein 51 (FKBP51) which inhibits Akt and further supresses downstream neurogenic signalling (Derosa *et al.*, 2024). Reduced neurogenesis, particularly in limbic circuits, disrupts synaptic plasticity and leads to learning impairments, emotional instability, heightened stress sensitivity, and increases depression and anxiety risk (Banasr & Duman, 2007; Hill *et al.*, 2015; Gomes-Leal, 2021). Prolonged suppression of neurogenesis may also lead to the development of neurodegenerative disorders (Abbate, 2023). Importantly, these results align with clinical evidence of reduced mTOR/Akt signalling in depressed patients, particularly in the PFC, thereby strengthening the translation relevance of the ACTH model (Ignácio *et al.*, 2016). Therefore, despite increased *CREB* and *BDNF* expression observed in this study, reduced expression of downstream intracellular signalling factors in various brain regions may more reliably reflect the impact of ACTH on neurogenic capacity in this model. The neurotrophic pathway involving these signalling factors is complex due to the downstream cascades being affected by various regulatory mechanisms including feedback inhibition and crosstalk with other signalling pathways (Hoeffer & Klann, 2010; Park & Poo, 2013).

3.5.2 Citalopram and imipramine's region-specific brain distribution and effects on neurotrophic signalling

MSI analysis showed drug-specific distribution patterns of CIT and IMI. IMI was distributed more diffusely throughout the brain at all bregma levels, whereas the CIT controls showed the highest distribution at the striatal and hippocampal bregma levels, highlighting that drug distribution was not uniform. Interestingly, CIT showed greater localization in the septal region of the striatum, which aligns with previous MSI analyses showing higher CIT intensity in the lateral septal nucleus (Källback *et al.*, 2020). However, when co-administered with ACTH, citalopram distribution decreased at all bregma levels. ACTH may reduce CIT distribution by enhancing the biosynthesis of cytochrome P450

(CYP450) enzymes that accelerate CIT metabolism and reduce the availability of its active metabolites (Ruggiero & Lalli, 2016).

Despite its limited distribution, in all groups, CIT significantly affected all biomarkers analysed. Citalopram selectively inhibits serotonin reuptake by blocking the serotonin transporter (SERT), which increases the bioavailability of synaptic serotonin, thereby improving mood (Chu & Wadhwa, 2023). The dissociation between drug distribution and molecular effect suggests that citalopram's therapeutic effects are less reliant on drug distribution and absolute tissue levels, and more dependent on SERT occupancy and downstream serotonin signalling in regions critical for serotonergic modulation (Sørensen *et al.*, 2022). In these regions, SERT inhibition increases 5-HT residency time in the synaptic cleft, enhancing receptor binding and activation (Kaufman *et al.*, 2016). Over time, increased 5-HT signalling desensitises 5-HT_{1A} autoreceptors, enhancing 5-HT release (Kaufman *et al.*, 2016). Together, these effects promote synaptic remodelling, thus restoring limbic network activity, serotonergic tone, and mood (Martinowich & Lu, 2008).

Imipramine was mainly distributed in the cortical, striatal, hippocampal, and thalamic regions. These regions play a significant role in reward, motivation, and emotional processing and their impairment leads to motivational deficits and blunted affect (Pandya *et al.*, 2012). While imipramine is also metabolized by CYP450 enzymes, it was widely distributed throughout the brain but had a limited impact on neuroplasticity markers, suggesting that drug distribution does not necessarily translate to molecular effectiveness. Mechanistically, TCAs show a lower SERT occupancy than SSRIs on average, likely because these agents engage multiple receptors and transporters (Lundberg *et al.*, 2012). Among these, TCAs antagonise or downregulate histaminergic, muscarinic, adrenergic, and 5-HT receptors while also blocking noreinephrine transporters and Na^+ channels (Khalid & Waseem, 2025). The reduced effect on SERT and therefore on serotonin, may limit the neurotrophic effects of TCAs, even with increased brain abundance. These findings emphasize the importance of correct titration of antidepressant doses and consideration of their varied mechanisms of action.

Both antidepressants displayed distinct effects on the expression of neuroplasticity markers regardless of ACTH administration. Citalopram treatment led to an overall increase in hippocampal *BDNF* expression. This effect is hypothesized to be driven by the SSRI-induced enhancement of serotonin binding to $5\text{-HT}_{4,6,7}$ receptor subtypes, which activate PKA and adenylate cyclase, increasing CREB phosphorylation and BDNF production (Szapacs *et al.*, 2004). Citalopram significantly decreased *BDNF* mRNA in the midbrain which may result from prolonged serotonin upregulation depleting serotonin stores in serotonergic neuron-rich regions such as the raphe nuclei, highlighting the importance of region-specific analysis (Honig *et al.*, 2009). Chronic SSRI treatment has been shown to reduce 5-HT metabolism and production, likely due to the prolonged, indirect activation of serotonin autoreceptors and reduced 5-HT reuptake (Hoeffer & Klann, 2010; Park & Poo, 2013; Hillhouse &

Porter, 2015). Similar to its effect on *BDNF* mRNA, citalopram also increased *CREB* expression in the PFC, hippocampus, and midbrain. These neurogenic effects are thought to be mediated by 5-HT-induced activation of G-protein coupled receptor-PKA pathways that enhance downstream CREB and BDNF expression (Mombereau *et al.*, 2010; Rosas-Sánchez *et al.*, 2024). In contrast, imipramine did not impact *BDNF* or *CREB* expression which may be due to its non-selective action on serotonin and norepinephrine, resulting in a less pronounced effect on 5-HT-BDNF-CREB pathways, limiting its neurogenic effects (Fayez & Gupta, 2020).

Citalopram increased *TrkB* mRNA in the hippocampus, which was associated with increased *BDNF* mRNA. Similarly, citalopram increased midbrain *TrkB* expression, potentially compensating for the reduced *BDNF* mRNA in this region. Additionally, citalopram significantly increased *mTOR* expression across all brain regions. In contrast, imipramine increased hippocampal *TrkB* mRNA but did not affect m*TOR* expression which may indicate the drug's limited ability to improve neuronal function and ameliorate depressive symptoms. The distinct drug effect on *mTOR* expression is significant as the mTOR pathway regulates dendritic development and its reduced expression is linked to depression (Ignácio *et al.*, 2016). Together, these findings suggest that citalopram may exert stronger neurogenic effects via the BDNF-mTOR signalling pathway, supporting its use as a first-line therapy (Ignácio *et al.*, 2016). Although these findings were not assessed alongside neurobehaviour, our lab and others have shown that ACTH induces a depressive-like phenotype in rodents, evidenced by increased immobility in the forced swim test (FST) and reduced centre exploration in the open field test (Bernardus Saayman *et al.,* 2024; Sallie *et al.,* 2024). Imipramine co-administration (15 mg/kg) has consistently failed to improve ACTH-induced increases in rodent immobility in the FST (Kitamura *et al.*, 2002; Kim *et al.*, 2016). While data on citalopram in this model are limited, one study reported that escitalopram (15 mg/kg) and imipramine (15 mg/kg) were ineffective at reversing ACTH-induced immobility in the FST (Bernardus Saayman *et al.*, 2024). These findings suggest that behaviourally, the ACTH model exhibits resistance to monoamine antidepressants, however, additional studies involving citalopram are warranted to validate this conclusion.

3.5.3 Effects of antidepressant therapy on ACTH-induced changes in neurotrophic signalling

When co-administered with ACTH, both antidepressants demonstrated region- and marker-specific effects, reinforcing the complex interplay between antidepressant- and ACTH-induced neurobiological changes in different brain compartments. Interestingly, both antidepressants potentiated the ACTH-induced increase in hippocampal *BDNF* whereas only citalopram impacted *CREB* expression. These ACTH- and monoamine-induced changes reinforce that, in addition to being a possible indicator of therapeutic efficacy, BDNF and CREB also function as mediators of HPA axis modulation (Daskalakis *et al.*, 2015). In the hippocampus, chronic ACTH may enhance BDNF production via increased glucocorticoid-receptor binding and dimerization, enhancing transcriptional activity at the BDNF

promoter which regulates CRH production and HPA axis activity (Daskalakis *et al.*, 2015). Meanwhile, monoamine antidepressants enhance BDNF expression by stimulating serotonergic neurotransmission that promotes neurogenesis pathways (Rosas-Sánchez *et al.*, 2024). These distinct mechanisms may explain the compounding effect of ACTH and antidepressant treatment on *BDNF* mRNA expression.

Both antidepressants prevented ACTH-induced decreases in hippocampal *Akt* expression, although their effects varied regionally. In ACTH-treated rats, citalopram upregulated global *mTOR* expression whilst imipramine increased PFC *eEF2*. Citalopram's effects on the expression of molecular biomarkers were more widespread as evidenced by the significantly higher expression of *TrkB, Akt,* and *mTOR*. Imipramine, however, differed from ACTH only in its effects on PFC *TrkB* and *Akt*. These region- and marker-specific differences highlight imipramine's ineffectiveness in this model which may explain why the ACTH model has been previously described as a model of treatment resistance using only TCAs (Kitamura *et al.*, 2002). In individuals with MDD, citalopram treatment is associated with increased peripheral BDNF levels (Haghighi *et al.,* 2013). Although clinical data on imipramine are limited, post-mortem studies indicate that TCAs may be less effective than SSRIs in stimulating human progenitor cells, which are proportional to BDNF levels (Boldrini *et al.,* 2013). These clinical results are in line with our findings showing that, despite its limited brain distribution, citalopram has a stronger influence on neurotrophic signalling than imipramine, irrespective of exogenous ACTH. However, the differential effects of monoamine antidepressants on the various biomarkers in ACTH-treated rats highlights the limited understanding of the relationship between the HPA axis, neurotransmitter modulation, and neurogenic pathways. Future studies are needed to elucidate the interplay between these systems in order to develop more effective treatment strategies for MDD.

Although mRNA expression of the neurotrophins *BDNF* and *NGF* are strongly associated with its protein expression (Banerjee *et al.*, 2013), in the present study proteomic analyses of neuroplasticity biomarkers may have improved the interpretation of the results. While the phosphorylated levels of Akt, mTOR and eEF2 are an important activity indicator, studies have shown parallel increases in phosphorylated protein levels alongside enhanced mRNA expression (Li *et al.*, 2020*b*; Zhang *et al.*, 2022; Yue *et al.*, 2024). Thus, in the absence of phosphorylation data, the mRNA measurements for these neuroplasticity markers may still provide meaningful mechanistic insight into pathway activity. This study was only performed in male rats; hence future studies should determine the sex-specific effects of citalopram and imipramine on neurotrophic signalling. In one clinical trial, females treated with citalopram had higher remission rates than males, despite women having higher baseline depression severity (Young *et al.,* 2009). Similarly, another study noted that females show more favourable responses to sertraline, an SSRI, while males are more likely to respond to imipramine (Kornstein *et al.,* 2000). While these findings are often heterogenous and show modest sex-related differences, sex-dependent neurogenic effects of monoamine antidepressants are still emphasised. Sex

hormones play a crucial role in modulating adult hippocampal neurogenesis and, in the context of HPA axis dysregulation, females exhibit heightened stress reactivity; both factors affect neuroplasticity and antidepressant efficacy (Mahmoud *et al.,* 2016). Lastly, corticosterone levels were not measured in this study, an important consideration regarding the ACTH model is often characterised by HPA axis dysregulation. Nevertheless, previous studies show that in rats, exogenous ACTH administration increases corticosterone in a time- and dose-dependent manner, reaching peak values within 15 minutes and returning to baseline after around 60 minutes (Han *et al.*, 1998; Spiga *et al.*, 2011). Additionally, chronic citalopram treatment attenuates the ACTH response to acute restrained stress in rats, while imipramine is shown to reduce the ACTH and corticosterone response to CRH challenge in humans (Michelson *et al.*, 1997; Hesketh *et al.*, 2005). These results suggest that monoamine antidepressants play a role in HPA axis modulation which may contribute to its therapeutic effect.

3.6 Conclusion

The ACTH-induced alterations in intracellular neurotrophic signalling marker expression suggest the presence of neurotrophic dysregulation in this model. Increased *BDNF* and *CREB* expression suggest that, beyond neurogenesis, these factors may be actively involved in HPA axis homeostatic regulation. In ACTH-treated rats, citalopram increased the expression of *BDNF, CREB, TrkB, Akt,* and *mTOR* across various brain regions while imipramine had a limited effect on these markers, highlighting the role of the serotonergic system in modulating neurotrophin production and neuroprotection. These findings confirm that beyond neurotransmitter regulation, monoamine antidepressants modulate various intracellular biomarkers involved in neurotrophic signalling pathways, suggesting a pro-neurogenic effect. Future studies should more critically evaluate the region and model-specific changes in these biomarkers to better understand their contribution to depression aetiology and antidepressant drug responses. These insights will help direct more targeted interventions, moving us closer to precision medicine in mental healthcare and better treatment outcomes. In this regard, despite advancements in the pharmacological formulation of these agents, monoamine-based antidepressants still show limited efficacy in treating depression. Thus, novel, rapid-acting agents are needed, particularly for individuals with treatment-resistant depression and those with suicidal ideations where rapid symptom alleviation is crucial.

CHAPTER 4: ACUTE AND CHRONIC KETAMINE DIFFERENTIALLY MODULATE MARKERS OF NEUROPLASTICITY AND MONOAMINERGIC SIGNALLING IN AN ACTH MODEL OF DEPRESSIVE-LIKE SYMPTOMS

4.1 Abstract

Background: Hypothalamic-pituitary-adrenal (HPA) axis dysregulation is associated with depression where traditional antidepressants have limited efficacy. Ketamine shows rapid antidepressant effects; however, the behavioural and molecular effects of the acute and chronic administration of this novel antidepressant, in a rodent model of HPA axis dysregulation, require elucidation.

Methods: After two weeks of adrenocorticotropic hormone (ACTH)/saline administration to establish the model, rats in the acute group received a single dose of ketamine. While in the chronic group, after the two-week ACTH/saline administration period, in addition to receiving ACTH/saline, rats received a single weekly ketamine injection for four weeks. Neurobehavioural changes were assessed using the forced swim test (FST), open field test (OFT), and sucrose preference test (SPT). Following termination, brain regional mRNA expression of *BDNF, TrkB, Akt, mTOR, eEF2,* and *CREB* was measured using RT-PCR. Brain regional protein expression of BDNF, phosphorylated-CREB (p-CREB), and corticosterone was also assessed using an enzyme-linked immunosorbent assay (ELISA). 5-HT, dopamine, and norepinephrine neurotransmitter abundance was investigated at various bregma levels using matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI).

Results: Acute ketamine treatment effectively reversed ACTH-induced behavioural despair in the FST and increased striatal *CREB* mRNA. However, in the ACTH_K group, midbrain *CREB* mRNA was significantly reduced. A single ketamine administration also normalised the ACTH-induced increase in striatal 5-HT, while in the midbrain, the ACTH_K combination increased 5-HT levels compared to other groups. In contrast, chronic ketamine had a limited effect on the expression of neuroplasticity markers in the control group. In ACTH-treated rats, however, chronic ketamine restored *TrkB* and *eEF2* mRNA in the PFC and reversed ACTH-induced increases in hippocampal *CREB* mRNA. Chronic ketamine partially normalised ACTH-induced increases in 5-HT and NE in the hippocampus and NE in the midbrain.

Conclusion: Our findings indicate that acute ketamine treatment modulates neurotrophic pathways and monoaminergic neurotransmission, which impacts behavioural outcomes. In contrast, the minimal behavioural and selective molecular effects of repeated ketamine treatment suggest that prolonged exposure attenuates ketamine's neuroplastic and antidepressant actions in this model. These findings highlight possible differential mechanistic pathways of acute and chronic ketamine treatment, suggesting that acute administration is effective at reversing depressive-like symptoms associated with HPA axis dysregulation.

4.2 Introduction

In the last decade, ketamine has transformed the treatment and management of major depressive disorder (MDD), demonstrating rapid alleviation of depressive symptoms, particularly in individuals with treatment-resistant depression (TRD) (Krystal *et al.*, 2024). Unlike traditional monoaminergic antidepressants, that require several weeks to exhibit therapeutic effects, ketamine has consistently produced significant mood improvements within hours of administration (Zanos & Gould, 2018). One review reported response rates of over 60% within 24 hours of ketamine treatment in patients with MDD, with repeated dosing regimens having achieved response rates of up to 90% in some studies (McMullen *et al.*, 2021). Importantly, clinical trials also show that ketamine rapidly reduces suicidality, within hours to days after administration, in both MDD and TRD patients (Yavi *et al.*, 2022).

Originally used as an anaesthetic and analgesic agent, ketamine functions by competing with Mg^{2+} within the NMDA receptor pore, antagonising its activity (Liu *et al.*, 2001). This antagonistic mechanism is thought to underlie ketamine's rapid antidepressant effects, leading to enhanced synaptic plasticity and neurogenesis (Li *et al.*, 2011; Kavalali & Monteggia, 2020). NMDAR antagonism promotes neurogenesis by disinhibiting eEF2 activity thereby increasing the expression of BDNF, CREB, and various synaptic proteins (Monteggia *et al.*, 2013). Further downstream, ketamine activates the mTOR signalling cascade which promotes synaptogenesis in limbic areas leading to mood improvements (Li *et al*., 2010). Blockade of interneuron NMDARs triggers a glutamate surge that stimulates AMPARs, reinforcing neurogenic capacity through neurotrophin production (Krystal *et al.*, 2013). Together, these growth-promoting molecules stimulate neurite outgrowth and dendritic arborization, enhancing structural and functional connectivity, particularly in limbic structures responsible for mood regulation (Abdallah *et al.*, 2017). Ketamine has also been shown to restore synaptic efficacy by modulating monoaminergic neurotransmission in the CNS (Kavalali & Monteggia, 2020; Krystal *et al.*, 2024). While the precise mechanisms are still unclear, research suggests that ketamine modulates monoamine systems indirectly through glutamatergic disinhibition and downstream signalling (Kavalali & Monteggia, 2020). Through interacting with these systems, ketamine has been shown to effectively reverse functional impairments in both the clinical and preclinical setting (Gass *et al.*, 2014; Abdallah *et al.*, 2017). The growing body of translational neuroscience therefore suggests that ketamine exerts its antidepressant effects through a diverse array of synaptic signalling pathways.

Interestingly, it has been claimed that ketamine's antidepressant mechanism of action may vary based on dosing frequency (McMullen *et al.*, 2021). Acute treatment has been linked mainly to glutamate burst releases and spikes in BDNF levels while repeated ketamine treatment is associated with neuronal circuit stabilisation (Deyama & Duman, 2020; Lullau *et al.*, 2023). However, while the clinical antidepressant efficacy of ketamine is well-documented, the differential, time-dependant molecular

mechanisms underlying its pharmacodynamic effects, particularly within ACTH-induced depressive-like models, remain poorly understood.

This study investigated the effects of acute and chronic ketamine administration in a model of ACTH-induced depressive-like symptoms. Specifically, we assessed the effect of ketamine on various depressive-like behavioural parameters in the FST, OFT, and SPT. Additionally, we quantified corticosterone (CORT), BDNF, and p-CREB protein levels and the brain regional gene expression of neurotrophic signalling factors using ELISA and RT-PCR, respectively. Furthermore, we investigated the impact of ACTH and ketamine on monoamine neurotransmitter abundance using matrix-assisted laser desorption/ionisation mass spectrometry imaging (MALDI-MSI). We found that ketamine exerts region-specific and time-dependent effects, with acute administration reversing ACTH-induced behavioural despair and modulating neuroplasticity and monoaminergic balance, while chronic treatment showed limited neurotrophic modulation, possibly through the engagement of homeostatic mechanisms.

4.3 Methods and materials

4.3.1 Animals and study design

All animal experiments were approved by the Animal Research Ethics Committee of the University of the Witwatersrand (approval reference: 2022/03/06/C). All procedures were carried out in accordance with the South African National Legislation for animal experimentation, welfare, and husbandry, and experiments are reported according to the ARRIVE guidelines for reporting *in vivo* research.

Male Sprague-Dawley rats (n=60, 250 ± 50 g) were obtained from the Wits Research Animal Facility (WRAF) at the University of the Witwatersrand. Rats were housed in polycarbonate cages (Techniplast, Italy) with *ad libitum* access to water and standard rat chow (Epol, Johannesburg, South Africa) in the housing room, with a dedicated room used for neurobehavioral tests. The rooms were maintained at an ambient temperature of 25 °C ± 1 °C and a relative humidity of 24-26 %, with a 12h:12h light/dark cycle (lights on at 07*h*00; testing was conducted at 09h00). Rats were allowed to acclimate for 7 days prior to any experimental procedures.

Rodents were randomly assigned to the control (SAL) (acute n=10, chronic n=20) or ACTH (ACTH) (acute n=10, chronic n=20) group that received either saline (0.1 ml/day, sc) or ACTH (100 µg/day, 0.1 ml, sc) for two weeks, respectively (Kitamura *et al.*, 2002; Sallie *et al.*, 2024). Thereafter, for the acute study, the control and ACTH groups were further subdivided to receive a single injection of either saline (CON, n=5 and ACTH_S, n=5; 0.2 ml, ip) or ketamine (SAL_K, n=5 and ACTH_K, n=5; 15 mg/kg, ip) before being terminated (Figure 4.1A). For the chronic study, in addition to receiving daily saline/ACTH injections (sc), the groups were further subdivided to receive either saline (CON, n=10

and ACTH_S, n=10; 0.2 ml/day, ip) or ketamine (SAL_K, n=10 and ACTH_K, n=10; 15 mg/kg/day, ip) for four weeks, respectively (Kitamura *et al.*, 2002) (Figure 4.1E).

4.3.2 Neurobehavioral analysis

4.3.2.1 Forced swim test

Rats in the acute and chronic groups were subject to the FST to evaluate the effect of each drug on the acquisition of passive-coping behaviours, by assessing rodent immobility and climbing intermittently throughout the study (Slattery & Cryan, 2012) (Figure 4.1A and 1E). Prior to each test, rats were allowed to acclimatise to the dedicated testing room for five minutes, followed by a five-minute test. All rats underwent a baseline assessment (baseline, week 0). Thereafter, the FST was repeated after 2 weeks of saline or ACTH administration (sc) (week 2). For the acute group, the last FST was performed following a single injection of saline or ketamine (ip) (post-tx) and for the chronic group, the final FST was conducted after four weeks of the respective treatments (week 6) as previously described (Sallie *et al.*, 2024). EthoVision™ XT 17.5 software (Noldus Technologies, Wageningen, Netherlands) was used to automatically score mobility parameters and to manually score climbing.

4.3.2.2 Open field test

In a subset of chronically treated animals, the OFT was used to assess anxiety-like behaviour and locomotion by assessing movement velocity, distance travelled, and centre duration in the OFT arena (Seibenhener & Wooten, 2015) (Figure 4.1E). Prior to the OFT, animals were allowed to acclimatise to the dedicated testing room for five minutes before being subject to the OFT as previously described (Sallie *et al.*, 2024). Rats were subject to a baseline OFT assessment (week 0), an assessment after 2 weeks of ACTH or saline administration (sc) (week 2), and a final assessment after the four-week saline/ketamine (ip) treatment period (week 6). EthoVision software was used to automatically score rodent velocity, distance travelled, and centre duration in the OFT.

4.3.2.3 Sucrose preference test

The SPT was performed at the end of the chronic study to assess the effect of drug treatment on hedonic tendency as measured by the SPR, adapted from Liu et al. (2018) (Liu *et al.*, 2018). The SPT included a 72h adaptation period and a 12h test period as previously reported (Sallie *et al.*, 2024). The SPR was calculated as follows: SPR= (sucrose intake/(sucrose+water intake)) x 100.

4.3.3 Tissue harvest and molecular analysis

4.3.3.1 Tissue harvest

Two hours following the final FST, rats were anaesthetised using isoflurane (2%) prior to being decapitated using a rodent guillotine. Trunk blood was collected in heparinised tubes and centrifuged for 10 minutes at 3000 rpm, after which the plasma was separated and aliquoted. Brain tissues were removed and bisected into two hemispheres. The left hemisphere was surgically dissected into the prefrontal cortex, striatum, hippocampus, and midbrain, which were immediately snap frozen in liquid nitrogen. The right hemisphere was kept intact and gradually frozen using liquid nitrogen vapour for MSI analysis. All brain tissue and plasma were stored at -80^{o}C until molecular analysis.

4.3.3.2 Protein quantification of BDNF, p-CREB, and corticosterone

In a subset of animals, BDNF, p-CREB (acute, n=5/group; chronic, n=8/group), and CORT (acute and chronic, n=5/group) levels were measured in the prefrontal cortex and hippocampus, while corticosterone was measured in plasma in duplicate by solid-phase sandwich ELISA according to the manufacturer's instructions. Brain tissues were weighed and homogenised in ice-cold phosphate-buffered saline containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) at a 1:10 (w: v) ratio. Tissue homogenates were centrifuged at 5000 rpm for 7 minutes at 4^{o}C, after which the supernatant was collected, aliquoted, and stored at -80^{o}C until analysis. For BDNF protein quantification, PFC lysates were diluted 1:20 and hippocampal lysates 1:120 (detection range: 31.25-2000 pg/mL; Elabscience Biotechnology Co. Ltd., Houston, Texas, USA, Cat#: E-EL-R1235). For p-CREB, all brain lysates were diluted 1:50 (detection range: 31.20-2000 pg/mL; R&D Systems, Minneapolis, MN, USA, Cat#: DYC2510-5). For CORT, brain lysates were diluted 1:10 and plasma diluted 1:20 (detection range: 0.20-10 ng/mL; R&D Systems, Minneapolis, MN, USA, Cat#: KGE008B).

4.3.3.3 Gene expression of factors involved in neurotrophic signalling

Brain tissue was homogenised using a probe sonicator (Fisher Scientific, Waltham, MA, USA), and total RNA was extracted using the Illustra Minispin RNA extraction kit (GE Healthcare, USA). cDNA was then synthesised using the SuperScript VILO cDNA kit (ThermoFisher, California, USA) and then stored at -80 °C until gene expression analysis. RT-PCR TaqMan assays (ThermoScientific, Waltham, MA, USA) were used to determine mRNA expression of *BDNF* (Rn02531967_s1) and *CREB* (Rn01451883_m1) in the acute and chronic groups and *TrkB* (Rn01441749_m1), *Akt* (Rn00583646_m1), *mTOR* (Rn00693900_m1), and *eEF2* (Rn00820849_g1) in the chronic group, with TATA-box binding protein (*Tbp* (Rn01455646_m1)) as the optimised endogenous control. A StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) was used to amplify the

target genes. Changes in mRNA expression in the various brain regions were calculated using the $2^{-\Delta\Delta CT}$ method and are represented as fold changes relative to the control (Livak & Schmittgen, 2001).

4.3.3.4 Monoamine neurotransmitter concentrations measured by matrix-assisted laser desorption ionisation mass spectrometry imaging (MALDI-MSI)

Coronal rodent brain samples were cryo-sectioned at -18 ℃ with a thickness of 12 µm using a Leica CM1860 UV cryostat (Leica Microsystems, Wetzlar, Germany). Sections were prepared at the striatal (bregma +1.92 mm), hippocampal (bregma -3.24 mm), and midbrain (bregma -5.40 mm) levels and were thaw-mounted onto conductive indium tin oxide (ITO)-coated slides (Bruker Daltonics, Bremen, Germany) and stored at -80°C until MALDI-MSI analysis. Prior to analysis, frozen slides were dried in a vacuum desiccator for 30 minutes at room temperature. For targeted detection of serotonin (5-HT), dopamine (DA), and norepinephrine (NE), in a subset of animals (n=±3 per group), on-tissue derivatisation was performed using a fluoromethylpyridinium-based agent (FMP-10) which selectively targets amines and primary phenolic hydroxyl groups (Shariatgorji *et al.*, 2019). FMP-10 was prepared at a concentration of 0.18 mg/mL in 30% H_2O and 70% acetonitrile. FMP-10 was applied uniformly to the tissue surface using a TM sprayer (HTX Technologies, Chapel Hill, NC, USA) using the following parameters: pump flow rate (80 µl/min), nozzle velocity (1100 mm/min), gas pressure (6 psi), and track spacing (2.0 mm) for 20 passes at 90°C. Samples were then placed in an MTP slide adapter (Bruker Daltonics, Bremen, Germany) and scanned using a Perfection V500 flatbed optical scanner (Epson, Nagano, Japan).

MALDI-MSI was conducted using a solariX 7T 2ω MALDI-Fourier-transform ion cyclotron resonance high-resolution MS system (Bruker Daltonics, Bremen, Germany), equipped with a Smart-beam II 2 kHz laser. The instrument was operated in positive ion mode and calibrated using red phosphorus prior to analyses. For neurotransmitter MSI, tissue slides were imaged at a lateral resolution of 100 µm. Spectra were acquired at a lateral resolution of 100 µm, with 100 laser shots per pixel, a Q1 value of 378 m/z, and a mass range of 150-1500 m/z. Time-of-flight transfer optics were set to 0.7 ms with a frequency of 4 MHz. Calibration was performed online using a lock mass of m/z 555.2231, an abundant FMP-10 ion cluster signal. All data were visualised using FlexImaging v5.0 software; regions of interest and relative abundance were extracted using SCiLS Lab v2019 Pro (Bruker Daltonics, Bremen, Germany).

4.3.4 Statistical analysis

Data analysis was performed using SAS software (version 9.4, SAS Institute Inc., Cary, North Carolina, USA) and visualised using GraphPad v10. Data are presented as mean ± standard deviation (SD) or median and interquartile ranges, as appropriate. Gene expression data were log-transformed to improve normality, then converted to z-scores and visualised in a heatmap. A mixed model two-way ANOVA

was used to assess inter-group differences in the SPT parameters as well as in the gene expression, ELISA, and MSI data with group (saline or ACTH) and treatment (saline or ketamine) as the main effects, followed by a Tukey's *post hoc* test. To determine temporal changes in behaviour in the FST and OFT in response to ACTH and ketamine treatment, data were analysed using a repeated-measures two-way ANOVA followed by a Tukey's *post hoc* test with time and treatment as the main effects. A Spearman's correlation was used to assess the relationship between the protein expression data. Statistical significance was set at $p \leq 0.05$.

4.4 Results

4.4.1 Ketamine shows differential neurobehavioral effects with acute and chronic administration

4.4.1.1 Acute, but not chronic, ketamine reverses ACTH-induced immobility in the FST

In the acute group (week 2), relative to baseline, two weeks of ACTH administration significantly decreased mobility ($p=0.003$) (Figure 4.1B). After two weeks of ACTH, rats given a single dose of ketamine (ACTH_K) were significantly less immobile (increased duration mobile) compared to their own week 2 time point ($p=0.0004$) and compared to ACTH_S rats post-treatment ($p=0.017$) (Figure 4.1B). Consequently, ACTH_K rats also showed significantly less immobility post-treatment than at the week 2 assessment ($p=0.018$) (Figure 4.1C). Interestingly, ACTH_K rats spent significantly more time climbing during the FST compared to both their week 2 assessment ($p=0.003$) and compared to ACTH_S rats post-treatment ($p=0.001$) (Figure 4.1D).

In the chronic group (week 6), mobility in ACTH_S rats significantly decreased from baseline to week 2 ($p<0.0001$) and declined further by week 6 ($p=0.019$) (Figure 4.1F). ACTH_S rats had a higher immobility duration at week 2 compared to baseline ($p<0.0001$). However, at week 6, both ACTH_S and ACTH_K rats showed significant reductions in immobility compared to week 2 ($p<0.0001$ and $p=0.004$, respectively) (Figure 4.1G). After adjusting for between-group differences in OFT locomotor activity, locomotion was not a significant predictor of immobility in the FST ($p=0.221$). Additionally, ACTH_S rats showed a significantly greater climbing duration at week 6 than at week 2 ($p=0.040$) and also had a greater climbing duration compared to ACTH_K rats at week 6 ($p=0.001$) (Figure 4.1H).

4.4.1.2 Chronic ACTH administration reduces rodent activity in the OFT independent of ketamine

In the chronic group, ACTH-treated rats showed a significant decrease in velocity in the OFT after 2 weeks ($p=0.012$) and after 6 weeks ($p=0.036$) when compared to baseline (Figure 4.1L). Similarly, from baseline, there was a significant decrease in the mean distance travelled by ACTH_S rats in the arena after 2 weeks ($p=0.013$) and 6 weeks ($p=0.038$) (Figure 4.1M). Additionally, following two weeks of

ACTH administration (ACTH_S), there was a significant decreased the percentage of time spent in the centre of the arena compared to baseline ($p=0.009$) (Figure 4.1N).

4.4.1.3 Chronic ketamine increases water but not sucrose consumption

ACTH_S rats in the chronic group consumed significantly less water than CON ($p=0.012$) and ACTH_K rats ($p=0.039$). In contrast, chronically treated SAL_K rats consumed significantly more water than CON and ACTH_K rats (all $p<0.0001$) (Figure 4.1I). None of the treatments had any effect on sucrose consumption and SPR (Figures 4.1J and K).

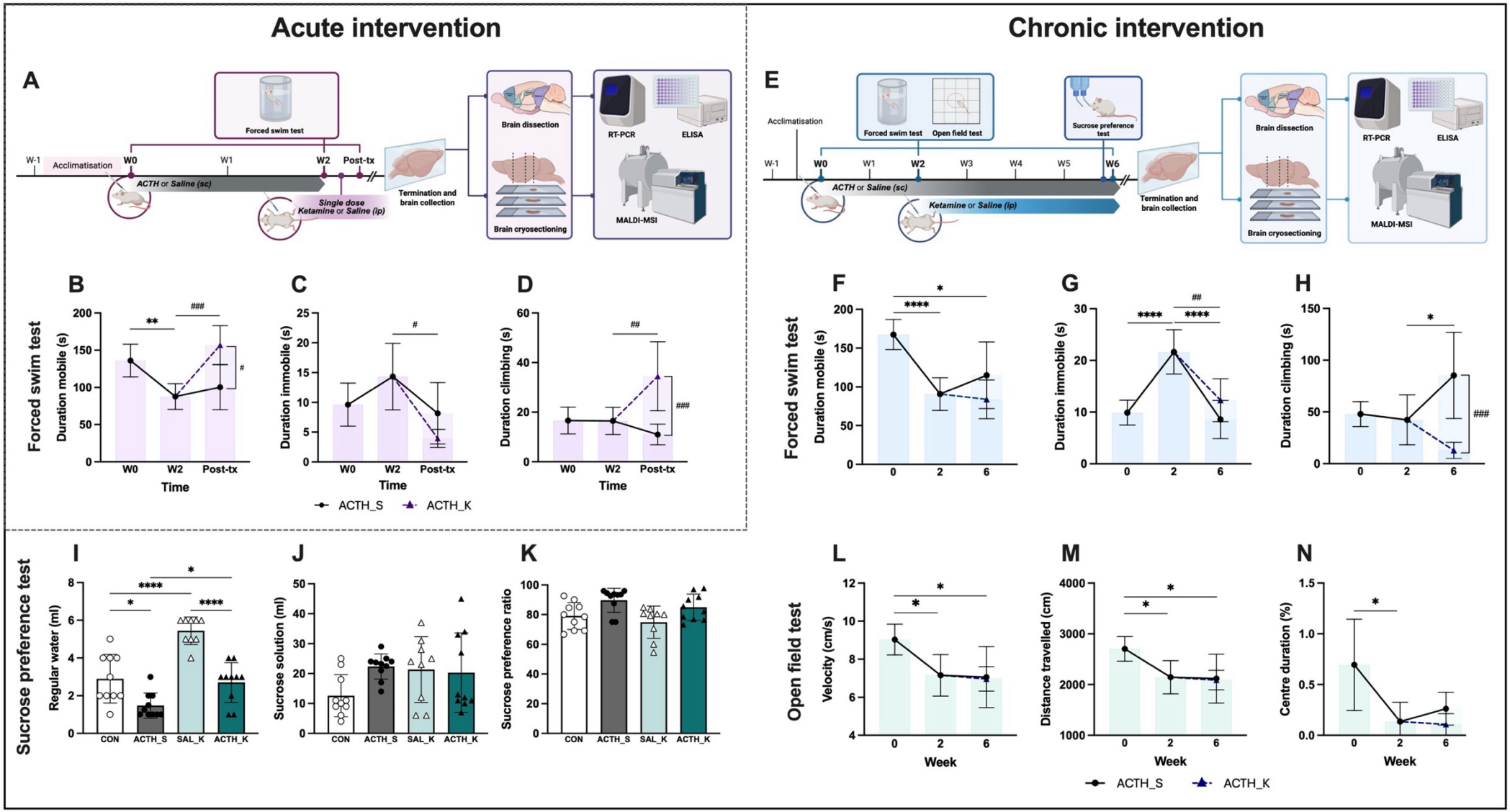

Figure 4.1. Experimental design showing the acute treatment timeline **(A)**, rats received either ACTH (100 µg/day, sc) or saline (0.1 ml, sc) for two weeks (week 0-2) before being terminated in the acute study. The FST was conducted at week 0: baseline measurement; week 2: after 2 weeks of ACTH (sc) administration; and post-tx: after a single dose of ketamine/saline (ip) **(B-D)**, the effect of ACTH and acute ketamine on the duration mobile, immobile, and climbing were assessed (n=5/group); **p<0.01 across time for ACTH_S; #p<0.05, ##p<0.01, ### p<0.001 across time for ACTH_K, and ACTH_S vs ACTH_K post-tx/at W6. Experimental design showing the chronic treatment timeline **(E)**, for the chronic study, after two weeks of administration, rats continued to receive ACTH/saline (sc) and additionally received either ketamine (15 mg/kg, 0.2 ml, ip) or saline (0.2 ml, ip) once a week for four weeks. The FST was conducted at week 0: baseline measurement; week 2: after 2 weeks of ACTH (sc) administration; and week 6: after four weeks of ACTH (sc) plus ketamine/saline (ip) treatment **(F-H)**, the effect of ACTH and chronic ketamine on the duration mobile, immobile, and climbing were assessed (n=10/group); *p<0.05, ****p<0.0001 across time for ACTH_S; ##p<0.01, ### p<0.001 across time for ACTH_K, and ACTH_S vs ACTH_K post-tx/at W6. The SPT was conducted in the last week of the chronic study where the effect of ACTH and chronic ketamine on regular water consumption, sucrose solution consumption, and the sucrose preference ratio were assessed (n=10 per group) **(I-K)**; *p<0.05, ****p<0.0001. In the chronic study, the OFT was conducted at week 0: baseline measurement; week 2: after 2 weeks of ACTH (sc) administration; and week 6: after four weeks of ACTH (sc) plus ketamine/saline (ip) treatment **(L-N)**, the effect of ACTH and chronic ketamine on the velocity, distance travelled, and centre duration were assessed (n=5 per group); *p<0.05 across time for ACTH_S. Data are presented as mean ± SD. FST and OFT data were analysed using a repeated measures two-way ANOVA and SPT data were analysed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control; ACTH_S, ACTH+saline; SAL_K, saline+ketamine; ACTH_K, ACTH+ketamine.

4.4.2 Ketamine shows differential molecular effects with acute and chronic administration

4.4.2.1 ACTH administration has a variable effect on the gene expression of neuroplasticity markers

In the acute group, *post hoc* comparisons revealed that striatal *CREB* mRNA was lower in the ACTH_K group compared to the SAL_K group (p=0.027) (Figure 4.2D).

In the hippocampus, *BDNF* mRNA was significantly reduced in ACTH_S rats compared to controls (p=0.033) and was similarly reduced in ACTH_K rats compared to the SAL_K group (p=0.048) (Figure 4.2J). Similarly, *CREB* mRNA was significantly decreased in rats with the ACTH_K treatment combination compared to SAL_K rats (p=0.027). In the midbrain, a similar pattern was observed where ACTH_K rats exhibited significantly lower *CREB* mRNA than SAL_K rats (p=0.004) (Figure 4.2O).

In the chronic group, in the PFC, *CREB* mRNA expression increased in ACTH_S rats compared to CON (p<0.001) (Figure 4.3A). *BDNF* and *CREB* mRNA levels were also higher in ACTH_K rats compared to SAL_K rats (p=0.004 and p=0.013). However, *TrkB* mRNA levels were reduced the ACTH_S group compared to CON (p=0.0001). The mRNA levels of intracellular signalling factors *Akt, mTOR,* and *eEF2* were reduced in the ACTH_S group relative to CON and in the ACTH_K group relative to SAL_K (all p<0.050). Similar to the PFC, striatal *CREB* expression was significantly higher in ACTH_S rats compared to CON (p<0.001) (Figure 4.3E). However, *TrkB* mRNA levels decreased in ACTH_K rats compared to the SAL_K group (p=0.017) and *Akt* and *mTOR* mRNA levels decreased in the ACTH_S group relative to CON and in the ACTH_K group relative to SAL_K (all p<0.05).

Hippocampal *CREB* expression was augmented in ACTH_S rats compared to CON (p<0.001). Despite this, *Akt, mTOR,* and *eEF2* mRNA levels decreased both in the ACTH_S group relative to CON and in the ACTH_K group relative to SAL_K (all p<0.05) (Figure 4.3J). In the midbrain, ACTH_S treatment significantly reduced *BDNF* and increased *CREB* mRNA relative to CON (p=0.033 and p<0.001) (Figure 4.3O). ACTH_K-treated rats also exhibited significantly higher *CREB* mRNA compared to SAL_K rats (p=0.018), while ACTH_S rats displayed significantly elevated *TrkB* mRNA relative to CON (p=0.006). Consistent with other regions, *Akt, mTOR,* and *eEF2* mRNA levels decreased in the ACTH_S group relative to CON and in the ACTH_K group relative to SAL_K (all p<0.05).

4.4.2.2 Ketamine has a time-dependent effect on the gene expression of neuroplasticity markers

Acute ketamine treatment (SAL_K) enhanced striatal *CREB* expression relative to CON (p=0.033) (Figure 4.2D). In contrast, in the midbrain, ACTH_K rats exhibited significantly lower *CREB* mRNA than ACTH_S rats (p=0.046) (Figure 4.2O).

In chronically treated rats, in the PFC, the ACTH_K group exhibited significantly higher *TrkB* mRNA levels compared to the ACTH_S group (p=0.006) (Figure 4.3A). In this same region, ketamine co-administration (ACTH_K) increased *eEF2* mRNA levels compared to ACTH_S rats (p=0.020). Notably, in the striatum, ketamine treatment alone (SAL_K) enhanced *mTOR* mRNA significantly compared to CON (p=0.012) (Figure 4.3E). Interestingly, hippocampal *CREB* mRNA levels were reduced following the administration of the ACTH_K treatment combination when compared to ACTH_S rats (p=0.019) (Figure 4.3J).

4.4.2.3 Region-specific monoamine alterations following ACTH and ketamine treatment

MSI results from the acute groups show that at the striatal level, following ACTH administration, ACTH_S rats showed significantly lower 5-HT relative to CON (p=0.001) (Figure 4.2F). At the hippocampal level, a single dose of ketamine (SAL_K) resulted in a significant decrease in 5-HT compared to both CON (p=0.0002) and ACTH_K-treated rats (p=0.015) (Figure 4.2L). At this level, ACTH administration (ACTH_S) significantly increased hippocampal DA relative to CON (p=0.002) (Figure 4.2M). Interestingly, at the midbrain level, ACTH_S rats exhibited a significant 5-HT increase relative to CON (p=0.004) (Figure 4.2Q). However, when ketamine was co-administered after the two-week ACTH period (ACTH_K), there was a further increase in 5-HT compared to ACTH_S (p=0.001) and SAL_K rats (p<0.0001).

In the chronic group, at the striatal level, ACTH_K-treated rats exhibited significantly higher 5-HT levels compared to both ACTH_S (p=0.037) and SAL_K (p<0.0001) rats (Figure 4.3G). In contrast, SAL_K rats expressed significantly higher NE levels than CON rats (p=0.001) (Figure 4.3I). At the level of the hippocampus, 5-HT levels were elevated in ACTH_S rats relative to CON (p=0.034) but were significantly lower with ketamine co-administration (ACTH_K) compared to the ACTH_S group (p<0.0001) (Figure 4.3L). ACTH_S-treated rats also showed increased NE levels relative to both CON (p=0.005) and ACTH_K (p=0.002) groups (Figure 4.3N). Lastly, at the midbrain level, ACTH_S rats had significantly higher 5-HT than CON (p<0.0001) and ACTH_K rats exhibited significantly higher 5-HT than the SAL_K group (p<0.0001) (Figure 4.3Q). Similarly, ACTH_S rats also displayed significantly higher NE levels compared to CON (p=0.032) (Figure 4.3R).

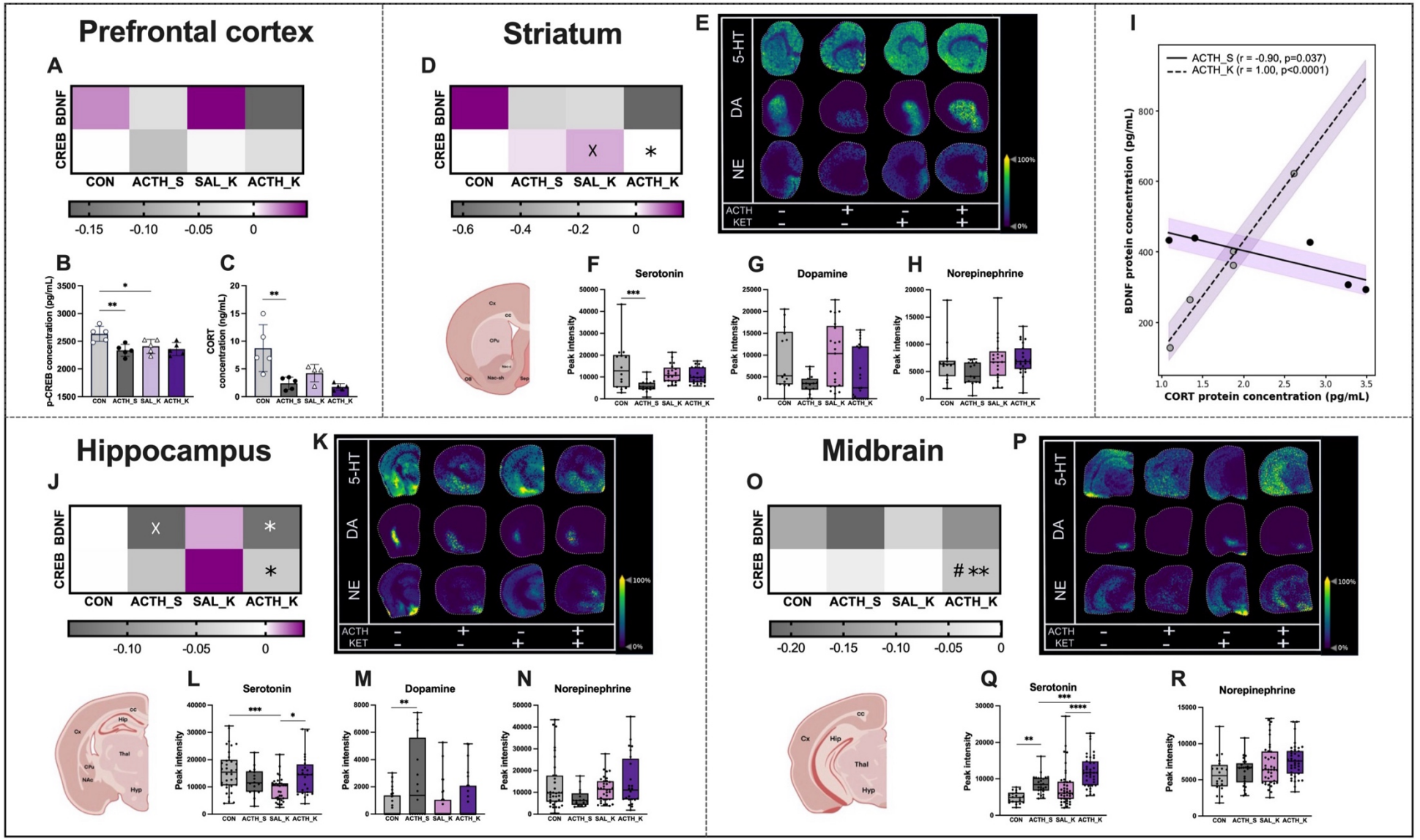

Figure 4.2. PFC: Heatmap of acute *BDNF* and *CREB* mRNA z-scores (n=5/group) **(A)**; Protein expression of p-CREB **(B)** and CORT **(C)** in acutely treated rats (n=5/group); *p<0.05, **p<0.01. **Striatum:** Heatmap of acute *BDNF* and *CREB* mRNA z-scores (n=5/group) **(D)** Χp<0.05 vs CON**,** *p<0.05 vs SAL_K. Representative brain images showing ACTH and acute ketamine effects on 5-HT, DA (double derivatized), and NE (double derivatized) distribution at the striatal level **(E)**. MSI-derived monoamine expression across subregions at the striatal level **(F-H)** (n=±3 per group), ***p<0.001. Correlation plot showing the relationship between CORT and BDNF protein expression in the PFC in the ACTH_S and ACTH_K groups in the acute study **(I)**. **Hippocampus:** Heatmap of acute *BDNF* and *CREB* mRNA z-scores (n=5/group) **(J)** Χp<0.05 vs CON**,** *p<0.05 vs SAL_K. Representative brain images showing ACTH and acute ketamine effects on 5-HT, DA, and NE distribution at the hippocampal level **(K)**. MSI-derived monoamine expression across subregions at the hippocampal level **(L-N)** (n=±3 per group), *p<0.05, **p<0.01, ***p<0.001. **Midbrain:** Heatmap of acute *BDNF* and *CREB* mRNA z-scores (n=5/group) **(O)** **p<0.01 vs SAL_K, #p<0.05 vs ACTH_S. Representative brain images showing ACTH and acute ketamine effects on 5-HT, DA, and NE distribution at the midbrain level **(P)**. MSI-derived monoamine expression across subregions at the midbrain level **(Q-R)** (n=±3 per group), **p<0.01, ***p<0.001, ***p<0.001. Key: Cx, cortex; cc, corpus callosum; CPu, caudate putamen; Hip, hippocampus; Hy, hypothalamus**;** NAc-c, nucleus accumbens core; NAc-sh, nucleus accumbens shell; OB, olfactory bulb; Sep, septum; Thal, thalamus. Data are presented as mean ± SD. Data were analyzed using a two-way ANOVA followed by a Tukey's *post hoc* test. The relationship between CORT and BDNF in the PFC was determined using a Spearman's correlation. CON, control; ACTH_S, ACTH+saline; SAL_K, saline+ketamine; ACTH_K, ACTH+ketamine.

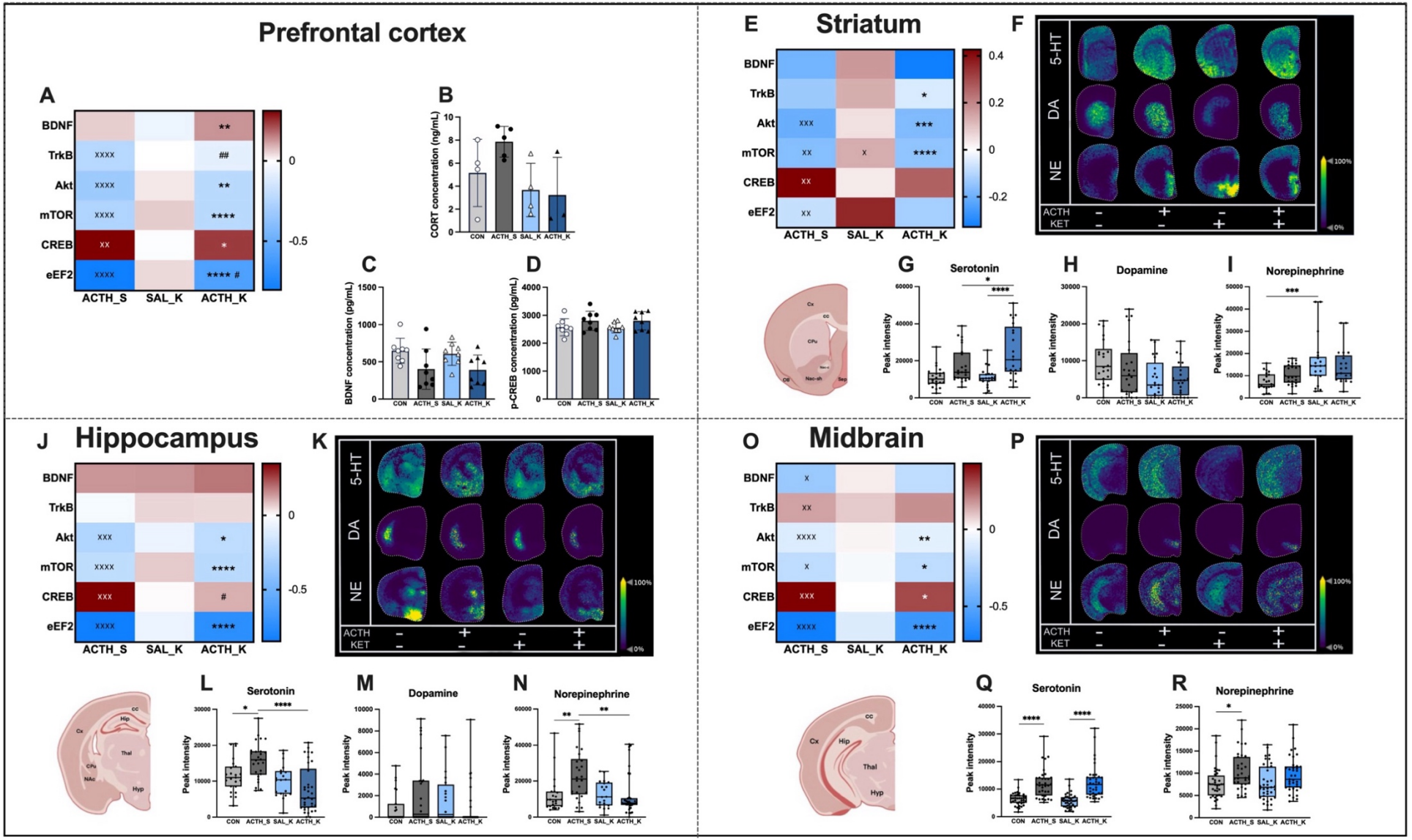

Figure 4.3. PFC: Heatmap showing mRNA z-scores for neurogenesis-related factors in the chronic group (n=±9/group) **(A)** XXp<0.01, XXXXp<0.0001 vs CON, *p<0.05, **p<0.01, ****p<0.0001 vs SAL_K, #p<0.05, ##p<0.01 vs ACTH_S. Protein expression of CORT (n=5/group) **(B)**, BDNF **(C)**, and p-CREB (n=8/group) **(D)** in chronically treated rats. **Striatum:** Heatmap showing mRNA z-scores for neurogenesis-related factors in the chronic group (n=±9/group) **(E)** Xp<0.05, XXp<0.01, XXXp<0.001 vs CON, *p<0.05, ***p<0.001, ****p<0.0001 vs SAL_K. Representative brain images showing the effect of ACTH and chronic ketamine on the distribution of 5-HT, DA (double derivatized), and NE (double derivatized) at the striatal level **(F)**. MSI-derived monoamine expression across subregions at the striatal level **(G-I)** (n=±3 per group), *p<0.05, ****p<0.0001. **Hippocampus:** Heatmap showing mRNA z-scores for neurogenesis-related factors in the chronic group (n=±9/group) **(J)** XXXp<0.001, XXXXp<0.0001 vs CON, *p<0.05, ****p<0.0001 vs SAL_K, #p<0.05 vs ACTH_S. Representative brain images showing the effect of ACTH and chronic ketamine on the distribution of 5-HT, DA, and NE at the hippocampal level **(K)**. MSI-derived monoamine expression across subregions at the hippocampal level **(L-N)** (n=±3 per group), *p<0.05, **p<0.01, ****p<0.0001. **Midbrain:** Heatmap showing mRNA z-scores for neurogenesis-related factors in the chronic group (n=±9/group) **(O)** Xp<0.05, XXp<0.01, XXXp<0.001, XXXXp<0.0001 vs CON, *p<0.05, **p<0.01, ****p<0.0001 vs SAL_K. Representative brain images showing the effect of ACTH and chronic ketamine on the distribution of 5-HT, DA, and NE at the midbrain level **(P)**. MSI-derived monoamine expression across subregions at the midbrain level **(Q-R)** (n=±3 per group), *p<0.05, ****p<0.0001. Key: Cx, cortex; cc, corpus callosum; CPu, caudate putamen; Hip, hippocampus; Hy, hypothalamus**;** NAc-c, nucleus accumbens core; NAc-sh, nucleus accumbens shell; OB, olfactory bulb; Sep, septum; Thal, thalamus. Data are presented as mean ± SD. Data were analyzed using a two-way ANOVA followed by a Tukey's *post hoc* test. CON, control; ACTH_S, ACTH+saline; SAL_K, saline+ketamine; ACTH_K, ACTH+ketamine.

4.5 Discussion

In this study, we investigated the behavioural, molecular, and neurotransmitter effects of ketamine treatment in a rodent model of ACTH-induced HPA-axis dysfunction. ACTH administration resulted in despair and anxiety-like behaviours, which are common preclinical markers of depressive-like changes in rodents. Experimental animals were then administered either a single dose of ketamine or treated once weekly for four weeks. Acute ketamine treatment reversed ACTH-induced immobility, a marker of behavioural despair, and increased climbing, a marker of active coping. In contrast, chronic ketamine treatment did not affect immobility in the FST but prevented the ACTH-induced increase in climbing at week 6. In the acute study, two weeks of ACTH administration reduced the protein expression of p-CREB and CORT in the PFC, while acute ketamine treatment similarly reduced p-CREB in this region. Two weeks of ACTH administration reduced hippocampal *BDNF* mRNA expression, while acute ketamine treatment resulted in increased *CREB* expression in the striatum. However, in the chronic study, ACTH differentially altered gene expression in a region-dependent manner. When co administered with ACTH, repeated ketamine treatment resulted in an increase in PFC *TrkB* and *eEF2* mRNA and hippocampal *CREB* expression compared to the ACTH controls. We then investigated the effect of ketamine on monoamine abundance in the brain using MSI, which showed that neurotransmitter changes were both region-specific and time dependent. When administered for two weeks, ACTH administration reduced striatal 5-HT and increased hippocampal DA and midbrain 5-HT. By week six, ACTH-treated rats exhibited augmented 5-HT and NE levels in the hippocampus and midbrain. Both acute and chronic ketamine treatment attenuated the ACTH-induced effects on monoamine abundance. Ketamine treatment ameliorated the behavioural and neurobiological effects of prolonged ACTH-induced HPA axis dysfunction. Overall, these findings highlight ketamine's rapid and sustained effects on neurogenic and monoaminergic systems, as well as its ability to counteract disruptions induced by HPA axis dysregulation. However, ketamine's effect on other systems implicated in depression pathology remains poorly understood.

4.5.1 Acute ketamine ameliorates ACTH-induced behavioural despair

Acute and chronic ACTH administration resulted in a significant increase in immobility in the FST, suggesting a despair- and depressive-like phenotype. These findings have also been observed in similar studies, following two weeks of ACTH administration (Kitamura *et al.*, 2002; Song *et al.*, 2019*a*). Our study is the first to investigate the effect of six weeks of ACTH administration on neurobehavioral and molecular endpoints. Sustained ACTH elevations are associated with chronic HPA axis activation and increased CORT, which contributes to the development of MDD and helplessness symptoms (Petrović *et al.*, 2018). Interestingly, in the chronic study, immobility returned to baseline levels after 6 weeks of ACTH administration. The initial increase in behavioural despair after 2 weeks, followed by resolution at week 6, suggests that while chronic ACTH administration initially disrupts the HPA axis, adaptive

negative feedback mechanisms may help restore balance over time (Song *et al.*, 2019*a*; Sallie *et al.*, 2024). When interpreting FST behaviour, the reduction in locomotion observed in the ACTH-treated groups was considered. However, by week 6 the pattern of activity in the OFT did not correspond to FST immobility, suggesting that the latter reflects despair-like behaviour rather than reduced locomotion alone. After six weeks, ACTH-treated rats spent significantly more time climbing in the FST compared to week 2. This change aligns with the theory that chronically elevated ACTH activates adaptive feedback mechanisms, shifting rodent behaviour from a depressive-like phenotype to active coping behaviours. Similarly, previous studies in rodents have shown that repeated, predictable stressors cause HPA axis habituation while more severe stressors chronically activate the HPA axis (Herman *et al.,* 2016). In humans, the transition from a hyperactive to hypo-responsive HPA axis may shift the symptom pattern towards a more chronic, atypical MDD subtype (Menke, 2019). Clinically, these data suggest that short-term stress may cause depressive episodes, while prolonged stress may either lead to adaptation or chronic MDD, depending on individual patient responses.

In the OFT, ACTH reduced rodent velocity, distance travelled, and centre duration, indicating persistent hypolocomotion and anxiety-like behaviours, consistent with the high comorbidity between anxiety and depression, which are both linked to HPA axis dysfunction (Vreeburg *et al.*, 2013). Our OFT data suggests possible ACTH-induced GC alterations, which attenuate the activity of neural circuits associated with locomotion and anxiety (Sallie *et al.*, 2024). Chronic GC elevation can dampen dopaminergic neurotransmission in nigrostriatal pathways, which are responsible for motor control (Hensleigh & Pritchard, 2013). Increased GCs disrupt limbic regions, primarily the medial PFC (mPFC) and hippocampus, resulting in anxiety-like behaviours associated with thigmotaxis, or shelter-seeking behaviour (Zhao *et al.*, 2018). Other studies have also found increased anxiety-like behaviours in the OFT following two weeks of ACTH administration (Song *et al.*, 2019*b*). These findings highlight the role of chronic HPA axis activation in the overlapping clinical symptomology seen in anxiety and depressive disorders.

Preclinical models often use the FST to evaluate the efficacy of novel antidepressants. In this study, acute ketamine treatment decreased immobility and increased climbing in the FST. The rapid improvement in behavioural responses in ACTH animals reflects ketamine's ability to target helplessness-like behaviours driven by HPA axis dysregulation which parallels findings from MDD and TRD clinical studies (Ostroff & Kothari, 2015; López-Gil *et al.*, 2019). Similarly, in an ACTH and chronic unpredictable mild stress-induced depressive mouse model, researchers found that ketamine reversed behavioural despair within one hour of administration (Ma *et al.*, 2022). These rapid improvements in symptomology emphasise ketamine's clinical utility in managing severe depressive episodes where immediate intervention is required.

In the SPT, chronic ACTH exposure did not affect the SPR, suggesting that HPA axis dysregulation does not mediate anhedonia-like responses. Overall, chronic ketamine did not alter neurobehavioral responses in the SPT, FST, or OFT.

4.5.2 ACTH administration alters protein expression in the PFC

The PFC is strongly linked to stress regulation, mood, and MDD development. In this study, two weeks of ACTH administration caused a significant CORT reduction in the PFC. Pathologically low CORT levels have been linked to specific atypical depressive phenotypes characterised by hypoarousal, fatigue, and apathy (Harald & Gordon, 2012). This localised hypocortisolaemia may reflect a compensatory mechanism triggered by chronic adrenal stimulation (Menke, 2019). Mechanistically, initial sustained ACTH-induced increases in PFC CORT increase the activity of CORT-inactivating enzymes such as 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), and 11β-HSD1 which reduces the concentration of bio-active CORT in the PFC (Wyrwoll *et al.*, 2011). Additionally, the mPFC regulates the HPA axis via GR-mediated activation of GABAergic relays that inhibit CRH neurons in the PVN, lowering ACTH and CORT synthesis (Ulrich-Lai & Herman, 2009; Jones *et al.*, 2011; McKlveen *et al.*, 2013). Despite these brain changes, we did not observe increases in circulating CORT concentrations following two or six weeks of ACTH administration. Thus, circulating CORT levels may not reliably reflect neuroendocrine dysregulation during chronic stress and MDD. Consequently, despite normal circulating CORT levels, local brain CORT changes may still trigger molecular changes in brain regions responsible for stress regulation.

4.5.3 Ketamine induces region-specific changes in intracellular synaptic plasticity signalling pathways

After two weeks, ACTH administration decreased hippocampal *BDNF* mRNA, which is consistent with the well-established link between reduced BDNF expression and depression (Miao *et al.*, 2020). Despite this, hippocampal BDNF protein levels remained unchanged, which could be explained by BDNF intracellular vesicular stores which maintain basal BDNF levels, providing a protective buffer against reduced mRNA expression (Sonoyama *et al.*, 2020). Intracellularly, BDNF production is tightly regulated by CREB (Sakamoto *et al.*, 2011). After two weeks, ACTH did not affect *CREB* mRNA expression but significantly decreased p-CREB protein in the PFC. Reduced p-CREB is an indicator of impaired neuronal health and atrophy, often observed in MDD (Sakamoto *et al.*, 2011). Chronically elevated GCs may reduce p-CREB by activating microglia, which release reactive oxygen species (ROS) (Vyas *et al.*, 2016). ROS inhibits PKA, the main kinase that phosphorylates CREB (Srinivasan *et al.*, 2013). Reduced p-CREB impairs the expression of genes, such as *BDNF*, involved in neuronal plasticity, as reported in preclinical and clinical MDD studies (Ren *et al.*, 2011; Rossetti *et al.*, 2022).

Taken together, ACTH may impair key plasticity signals in the PFC, which contributes to the development of a depressive-like phenotype (Pizzagalli & Roberts, 2022).

In contrast, following six weeks of ACTH exposure, changes in synaptic plasticity shifted from the PFC to the midbrain, where *BDNF* and global *CREB* mRNA expression increased. These changes suggest a stress-induced, region- and time- dependent shift in neuroplasticity pathways, consistent with heterogeneous BDNF responses observed in MDD patients (Molendijk *et al.*, 2014). Despite increased mRNA expression, transcripts did not translate into increased protein expression, which could be explained by post-translational modifications. ACTH-driven GC-GR activation induces the expression of regulated in development and DNA damage-response 1 (REDD1), a stress-induced protein that inhibits mTORC1 and silences the downstream Akt-mTOR-eEF2 pathway (Kim *et al.*, 2023). mTOR inhibition reduces 4E-BP1 phosphorylation that blocks mRNA ribosomal recruitment and inhibits protein translation, which may explain the mRNA-protein disconnect (Kim *et al.*, 2023). Across all regions, chronic ACTH decreased *Akt, mTOR,* and *eEF2* mRNA, key regulators of cellular survival and neuroplasticity (Duman & Monteggia, 2006). Their downregulation is a hallmark of reduced neurogenesis and has been proposed as a biomarker of MDD severity (Monteggia *et al.,* 2013). While BDNF and CREB expression varies, *Akt*, *mTOR*, and *eEF2* mRNA reductions may more clearly reflect ACTH-induced impairments in neurogenic capacity. In addition to REDD1-mediated silencing, GR stimulation further upregulates FK506-binding protein 51 (FKBP51), which antagonises Akt, further supressing mTOR-eEF2 signalling (Derosa *et al.,* 2024). Overall, HPA axis imbalances disrupt several intracellular signalling pathways involved in neurogenesis that lead to neuronal atrophy and depressive symptoms seen in clinical MDD (Vyas *et al.*, 2016).

In control animals, a single dose of ketamine augmented striatal *CREB* mRNA; however, this effect was blocked by ACTH. Additionally, acute ketamine reduced p-CREB levels in the PFC. Although ketamine promotes neurogenesis via NMDAR antagonism and BDNF-TrkB-mTOR activation, in this paradigm, acute treatment was insufficient to normalise the ACTH-induced mRNA changes. These results suggest that ketamine's rapid antidepressive effects may not depend on sustained transcriptional activation of these pathways. This limited effect mirrors ketamine's transient clinical benefits, which have prompted the need for repeat-dosing regimens or maintenance strategies to sustain neurogenesis (Deyama & Duman, 2020).

Chronic ketamine treatment had negligible effects on neurogenesis-related signalling factors in all groups, suggesting a homeostatic response. Homeostatic plasticity may adaptively prevent excessive AMPAR-mediated excitation (Piazza *et al.*, 2024). Chronic ketamine reduces GluA1 subunits in Ca^{2+}-permeable AMPARs (CP-AMPARs), critical for its neurogenic effects, which may normalise CP-AMPAR levels, possibly explaining the lack of change in neurogenesis markers (Ding *et al.*, 2016; Zaytseva *et al.*, 2023). However, striatal *mTOR* mRNA expression selectively increased, which may

reflect a region-specific sensitivity to prolonged ketamine exposure. Although mTOR is tightly autoregulated, the striatum's dense glutamatergic and dopaminergic input may sensitise this region to repeated NMDAR antagonism, allowing sustaining mTOR activity (Martel & Galvan, 2022). Chronic ketamine drug users exhibit striatal hypertrophy linked to fewer depressive symptoms, which aligns with heightened mTOR levels and increased plasticity (Liang *et al.*, 2020). In our study, ketamine ameliorated ACTH-induced increases in *TrkB* and *eEF2* expression in the PFC and *CREB* mRNA in the hippocampus. Clinically, in individuals with TRD, it is well-known that acute ketamine treatment increases peripheral BDNF levels (Haile *et al.,* 2014). However, across studies, repeated ketamine infusions have yielded mixed results regarding BDNF. In several trials, despite clear antidepressant effects, there were no significant changes in peripheral BDNF levels (Zheng *et al.,* 2020; Jiang *et al.,* 2021). In one study, serum BDNF levels increased after the first infusion but did not change with subsequent infusions, suggesting that BDNF elevation may be transient (Allen *et al.,* 2015). An important limitation of these clinical studies is that BDNF was measured in circulation rather than in brain tissue and considering the blood-brain barrier and the fact that BDNF also supports peripheral neurons, circulating changes may not accurately represent changes in the brain. Evidence of ketamine's effect on other neurogenesis-related markers such as TrkB, mTOR, and eEF2 in humans remains limited. Given that in our study, ketamine failed to produce significant changes in classical neurogenesis markers, this suggests that instead of solely enhancing neurogenesis, ketamine's long-term effects may stem from its ability to restore homeostatic balance which may be achieved by engaging alternative signalling pathways not traditionally associated with depression.

4.5.4 Changes in monoaminergic abundance in response to ACTH model induction and ketamine treatment

After two weeks of ACTH administration, MSI analysis showed reduced 5-HT levels in the dorsal and ventral regions of the rostral striatum, associated with impaired reward processing in MDD (Spring & Nautiyal, 2024). Reduced 5-HT in these subregions likely reflects suppression of raphe-striatal projections during HPA axis activation, consistent with blunted striatal 5-HT observed in MDD (Miyanishi *et al.*, 2023). At the midbrain level, 5-HT increased in the hippocampal formation. Six weeks of ACTH exposure further increased 5-HT and NE in the thalamus and caudal hippocampal formation and increased 5-HT in the neocortex at the midbrain level. The caudal hippocampus, comprising ventral CA1-CA3 and dentate gyrus, receives dense serotonergic projections from the median raphe and regulates mood and HPA axis feedback via 5-HT_{1A} receptors (Fanselow & Dong, 2010; Ghasemi *et al.*, 2022). Limbic thalamic nuclei, including the anterior, midline, mediodorsal, and central medial nuclei, connect with limbic structures and mediate cognition, stress integration, and motivation (Reeders *et al.*, 2022). Although hippocampal 5-HT facilitates stress adaptation, sustained increases in 5-HT and NE in these regions are linked to cognitive fatigue and impaired contextual memory (Linthorst *et al.,* 2002;

Barr and Forster, 2011). Together, these monoamine increases may indicate hyperactivation of thalamo-hippocampal circuits, which is a hallmark of maladaptive stress responses (Shin & Liberzon, 2010).

Following the two-week ACTH period, DA increased in the thalamic and striatal regions at the hippocampal level (Tsutsui-Kimura *et al.*, 2025). The paraventricular thalamus receives rich dopaminergic input and amplifies threat monitoring, especially during stress (Tsutsui-Kimura *et al.*, 2025). Striatal DA increases align with clinical data showing basal ganglia dopaminergic upregulation during chronic stress which alters reward sensitivity and increases depression risk (Baik, 2020). These findings indicate that HPA axis activation may induce a hyper-monoaminergic state. MDD is often associated with monoamine depletions, however, the underlying pathology of MDD involves complex changes that can variably alter monoamine abundance (Pizzagalli & Roberts, 2022). Sustained HPA axis activation increases limbic 5-HT, contributing to depressive symptoms partly through GC-mediated inhibition of uptake mechanisms, such as the organic cation transporter 3 (OCT3), which clears catecholamines in neuronal and glial cells (Baganz *et al.*, 2010; Gasser & Lowry, 2018). Chronic CORT reduces OCT3 more rapidly than it upregulates the serotonin transporter (SERT), thereby enhancing 5-HT and NE availability (Shanker *et al.*, 2020). Indeed, in mice, OCT3 is reduced and 5-HT increases in response to repeated FST exposure and HPA axis activation (Baganz *et al.*, 2010). ACTH additionally upregulates post-synaptic 5-HT_2 receptors, which can increase tyrosine hydroxylase via GR activation, further increasing DA and NE synthesis (Chamas *et al.*, 2004).

In control rats, acute ketamine significantly decreased thalamic 5-HT which may reflect a transient depletion following an initial surge. Subanaesthetic ketamine has been shown to inhibit SERT, increasing 5-HT, which may paradoxically deplete 5-HT stores (Yamamoto *et al.*, 2013). Since the thalamus gates emotional and sensory information to the cortex, reduced 5-HT following a surge may represent a feedback mechanism that restores normal thalamo-cortical signal flow and homeostasis (Reeders *et al.*, 2022). Acute ketamine reversed the ACTH-induced reduction in striatal 5-HT as well as the increase in DA in the caudal striatum, suggesting that ketamine regulates reward processing capacity. In the chronic group, ketamine similarly reversed ACTH-induced increases in thalamic 5-HT and NE, which may indicate reduced thalamic hyperarousal signal transmission. Therefore, ketamine likely triggers a homeostatic recalibration of monoaminergic tone by adjusting neuronal excitability to stabilise overall network function (Kavalali & Monteggia, 2020). Chronic ketamine increased NE in the septal and basal forebrain region, possibly enhancing arousal and cognition (Spencer *et al.*, 2015). Ketamine is also known to activate the locus coeruleus, a key source of NE synthesis, which is interconnected both anatomically and functionally with the medial septal region (Caestecker *et al.*, 2025). The monoamine effects of both acute and chronic ketamine suggest widespread regulatory and restorative processes essential for mood regulation.

Overall, compared to the potent effects of acute ketamine, chronic ketamine had a limited impact on behaviour parameters in ACTH-treated rats. This limited impact may be due to tolerance or homeostatic adaptations that attenuate ketamine's effect on motor-related and limbic systems long-term and may point to varied acute and chronic drug mechanisms of action. ACTH decreased CORT in the PFC, thereby alleviating GR-mediated suppression at neurotrophin promoters, such as *BDNF* (Jeanneteau *et al.*, 2012). Although BDNF didn't increase in the PFC, we observed a significant negative relationship between CORT and BDNF in the PFC of ACTH-treated rats. Reduced CORT in the PFC was reversed with ketamine co-administration, suggesting ketamine's ability to regulate HPA axis activity. ACTH also reduced p-CREB in the PFC, an effect that typically indicates neuronal damage and is known to produce depressive effects (Ren *et al.*, 2011; Rossetti *et al.*, 2022). ACTH further reduced the mRNA expression of neurogenesis-related intracellular signalling factors in all brain regions. However, these ACTH-induced mRNA and protein effects were not reversed with ketamine treatment. Lastly, ACTH induced monoaminergic imbalances, supporting the validity of this model in replicating heterogeneous MDD patient neurotransmitter profiles. Acute and chronic ketamine effectively reversed several of these imbalances, suggesting that monoamine modulation may be an important feature of ketamine's antidepressant effect.

While this study contributes to our understanding of ketamine's effect on various systems, this study is limited by the sample size. Increasing the sample sizes in the OFT and including control groups in FST and OFT analyses may improve the robustness of the results. Nevertheless, each animal's initial assessments served as their own baseline behaviour, an important inclusion considering inter-individual differences in these tests. Additionally, given the lengthy SPT protocol (Liu *et al.,* 2018), the test could not be administered to the acute group. Adaptation of the SPT protocol would be necessary to allow fair neurobehavioural comparisons between the acute and chronic regimens. Given the partial disconnect between mRNA and protein, additional proteomic analysis would be useful to confirm the translational effects of ACTH and ketamine on neurogenesis-related intracellular signalling factors. Future studies may also benefit from including female rats to improve generalisability since studies have shown that female rodents respond variably to ketamine (Zhang *et al*., 2021). Notably, to date, clinical studies have found no significant sex differences in antianhedonic response rates or remission rates (Zheng *et al.,* 2022). Meanwhile, a recent review highlighted inconsistent findings across biomarker, efficacy, and tolerability outcomes by sex, suggesting that further sex-stratified research is needed (Feng *et al.,* 2025). Research addressing these limitations will be useful to further elucidate ketamine's antidepressive mechanisms in the ACTH model, which are varied and multi-mechanistic.

4.6 Conclusion

This study showed that ACTH induced region-specific, time-dependent disruptions in endocrine function, neurotrophic signalling, and monoaminergic neurotransmission, which manifested as passive-

coping behaviours. Two weeks of ACTH produced behavioural despair and disruptions in HPA axis function and p-CREB protein expression. Whereas by week 6, homeostatic mechanisms minimised the effect of ACTH, with notable differences only in the brain regional monoamine abundance. Acute ketamine reversed ACTH-induced behavioural despair and modulated 5-HT in a region-dependent manner, mirroring its rapid but short-lived clinical effect. Chronic ketamine had a limited influence on neuroplasticity markers, suggesting activation of homeostatic mechanisms that prevent overactivation of these pathways. This adaptive property may signify a clinically important safety mechanism that prevents excessive excitation during repeat-dose treatment regimens. This study highlights the importance of studying HPA axis systemically and locally in the brain to improve our understanding of the multi-aetiological nature of MDD. Finally, this study demonstrates ketamine's broad impact on multiple neural pathways that are also implicated in comorbid neurological disorders. An improved understanding of these molecular mechanisms have the potential to direct ketamine's clinical applications for a wider range of disorders.

CHAPTER 5: SUMMARY AND CONCLUSION

5.1 Summary

Psychosocial stress, via its dysregulation of the HPA axis, is a significant contributor to the development of MDD. However, the confounding effects of psychosocial stress in humans and the complex, multifactorial aetiology of MDD limits our understanding of the specific effects of HPA axis dysregulation on brain molecular changes associated with depression. Several studies have used exogenous ACTH to dysregulate the HPA axis as a preclinical model to investigate the relationship between HPA axis function and depression, however, this model has not been well characterised. Effective treatments for MDD remain suboptimal for many patients, in part due to the heterogenous nature of the disorder. Furthermore, the effects of classic monoamine-based antidepressants and novel agents, such as ketamine, have not been assessed in the ACTH model. Therefore, in this thesis, a series of studies were performed to advance our understanding of the neurobehavioral presentation and molecular mechanisms associated with the depressive-like effects of ACTH, and to elucidate the effects of monoamine based antidepressants and ketamine on neurobehavioral and molecular outcomes in this model.

5.2 ACTH administration produces a sex-specific depressive-like phenotype resistant to imipramine treatment

HPA axis dysregulation is driven by increases in ACTH or CORT levels and is often implicated in MDD pathogenesis. MDD is characterised by symptoms of despair and helplessness, anxiety and psychomotor retardation, and anhedonia. In preclinical models of depression behavioural changes, such as increased immobility in the FST, reduced exploration of the centre of the open field arena, and reduced sucrose preference in the SPT, are translated to core clinical symptoms of MDD. Assessing each of these behavioural domains allows more comprehensive profiling of the various symptomatic features of MDD and strengths the translational relevance of preclinical models. At the molecular level, neurotrophic dysregulation is a well-accepted molecular pathology of MDD, which provides a quantifiable molecular marker that can validate behavioural findings.

Therefore, in the first study, the effect of chronic exogenous ACTH on rodent neurobehavior and neuroplasticity marker gene expression was investigated. Chronic ACTH exposure for two weeks induced behavioural despair (greater immobility in the FST) and anxiety-like behaviours (reduced centre exploration in the OFT), while increasing hedonic, consummatory behaviours in the SPT, in male but not female rats. Parallel molecular analyses showed upregulation of hippocampal *BDNF* mRNA and a global increase in *CREB* mRNA in males, but not in females, suggesting a sex-specific effect in HPA axis dysregulation. Four weeks of imipramine co-treatment did not impact ACTH-induced behavioural or neuroplasticity gene expression changes. Importantly, these results show that exogenous

ACTH induced depressive-like neurobehavioural changes in the FST, OFT, and SPT, and altered the mRNA expression of key neurogenesis markers.

5.3 Citalopram, but not imipramine, restores *BDNF-TrkB-mTOR* gene expression in ACTH-treated rats

While HPA axis dysregulation precipitates neurogenic perturbations and depressive symptoms, therapeutic approaches mainly rely on monoamine-targeting agents. These antidepressants modulate synaptic 5-HT, dopamine, and norepinephrine levels. This pathway-specific mechanism implies that, depending on the underlying aetiology of MDD, monoamine based antidepressants may be ineffective and fail to provide adequate symptomatic relief in a large percentage of patients. Although these agents are known to primarily regulate neurotransmitters, their effects on neurogenesis-related signalling pathways, particularly in the ACTH model, remain poorly understood.

Therefore, in the second chapter, I investigated the molecular effects of citalopram, an SSRI, and imipramine, a TCA, on neurogenic signalling pathways in the ACTH model. ACTH administration resulted in increased hippocampal *BDNF* mRNA and decreased downstream mRNA expression of *TrkB, Akt, mTOR,* and *eEF2*, suggesting that BDNF may play a role in systems beyond neurogenesis. Four weeks of citalopram treatment ameliorated ACTH-induced changes in *BDNF-TrkB-mTOR* signalling across multiple brain regions, whereas imipramine treatment resulted in less pronounced changes. Mass spectrometry imaging showed a greater imipramine brain distribution compared to citalopram, indicating that the citalopram effects were independent of drug distribution. These findings imply that SSRIs may impact neurotrophic factors in the context of HPA axis dysregulation. However, overall, these results show that monoamine-based antidepressants did not effectively reverse ACTH-induced changes in the gene expression of neurogenesis-related markers.

5.4 Acute, but not chronic, ketamine reverses ACTH-induced behavioural despair and monoaminergic circuit dysregulation

In the previous chapter, I showed that, while monoamine-based antidepressants partially modulated neurogenic signalling in the brain, they did not fully reverse ACTH-induced changes. Indeed, clinical findings show that approximately 30% of MDD patients do not respond to monoamine-based therapies. The prevalence of treatment resistant depression and the suicide risk of severely depressed patients have emphasised the need for novel, rapid acting agents. Therefore, I investigated the brain molecular mechanism of action of ketamine in a model of ACTH-induced HPA axis dysregulation. Ketamine's multi-dimensional mechanism of action allows it to target several underlying pathophysiological pathways involved in the development of depressive symptoms. Additionally, ketamine's rapid pharmacokinetic profile enables rapid, as well as long term relief in those suffering from MDD. The

antidepressant mechanisms of action of acute and chronic ketamine are hypothesised to involve distinct physiological pathways depending on the dosing regimen.

Therefore, in the third chapter, I investigated the effect of acute and chronic ketamine on neurobehavioral, molecular, and monoamine neurotransmitter endpoints in a rodent model of ACTH-induced HPA axis dysfunction. ACTH administration disrupted HPA axis function, neurogenesis signalling pathways, and monoamine neurotransmitter abundance. Acute ketamine treatment reversed ACTH-induced despair in the FST, up-regulated *CREB* mRNA in the striatum, and corrected region-specific shifts in 5-HT. Conversely, beyond increased striatal *mTOR* mRNA, chronic ketamine administration had a limited effect on the expression of neuroplasticity markers. These findings indicate that when administered acutely, ketamine modulates neurotrophic pathways and monoaminergic neurotransmission, which impacts behavioural outcomes. Surprisingly, chronic ketamine treatment did not impact the ACTH-induced behavioural changes, neurotrophic changes, or monoamine changes. These results suggest that the long-term antidepressant effects of ketamine may be via other molecular mechanisms. Indeed, it has been suggested the ketamine may have long term anti-inflammatory effects, which should be investigated in future studies. Taken together, these findings highlight the differential mechanism of action of ketamine following acute and chronic dosing, suggesting that acute administration may be more effective at reversing depressive-like symptoms associated with HPA axis or neurotrophic dysregulation.

5.5 Limitations and future recommendations

The specific limitations of each study in this thesis have been addressed at the end of each chapter. However, additional limitations should be addressed in future studies. First, unequal group sizes, particularly the smaller female groups and the limited sample sizes in the OFT, limits confidence in statistical conclusions, even though *post-hoc* power calculations indicated acceptable statistical strength. Future studies should use balanced sample sizes which will help clarify sex effects on neurobehavior and molecular expression. Although the FST and OFT data were analysed with repeated measures that allowed each animal to serve as its own baseline, control groups were not assessed in parallel. Additionally, the SPT protocol's length did not allow for a baseline measurement. Adaptation of the protocol and the incorporation of baseline assessments for all groups and recording concurrent food intake would improve longitudinal behavioural tracking. At the molecular level, adding circulating and brain tissue measurements of ACTH and CRH may provide more insight into the effect of chronic ACTH administration on HPA axis function, at various levels of signalling. Furthermore, neurogenesis-related intracellular markers were quantified solely at the mRNA level, and adding proteomic analyses could confirm whether the observed transcriptional alterations translate into protein changes. Finally, experiments in the second and third study were conducted only in males, yet the first study demonstrated pronounced sex effects in ACTH-treated rats. In future studies, replicating treatment interventions in

female rats will clarify how endocrine function, neurotransmitter dynamics, and neurogenesis pathways interact and will guide the development of sex-informed, mechanism-based antidepressant strategies.

5.6 Conclusion

In conclusion, the evidence presented in this thesis shows that exogenous ACTH administration produced depressive-like neurobehavioural and molecular effects that are consistent with the underlying pathophysiology of MDD. In this thesis, chronic ACTH exposure was shown to reliably induce a depressive-like phenotype characterised by increased behavioural despair, heightened anxiety-like behaviours, and altered sucrose consumption patterns. These effects were sex-dependent, with male rats exhibiting both behavioural changes and elevated hippocampal *BDNF* and *CREB* mRNA expression, while females were largely unaffected. Interestingly, unlike previous studies reporting reduced BDNF following chronic stress or glucocorticoid exposure, our findings showed an increase in hippocampal *BDNF* mRNA after ACTH administration. This divergence may reflect a compensatory neuroplastic response to sustained HPA axis activation, or differences in model design, duration of exposure, or timing of tissue collection. It may also point to a sex-dependent regulatory mechanism in which males exhibit heightened but potentially maladaptive neurotrophic signalling under chronic stress conditions.

Traditional monoaminergic antidepressants showed differential effects in this model in that imipramine failed to reverse ACTH-induced behavioural and molecular alterations, whereas citalopram partially restored neurotrophic signalling. The ability of citalopram to enhance the expression of neurogenesis-related markers across brain regions supports the view that monoamine-based antidepressants act not only through neurotransmitter regulation, but also by modulating intracellular pathways involved in neuroplasticity. Importantly, this thesis further establishes the temporal and mechanistic specificity of ketamine's antidepressant effects in the context of HPA axis dysregulation. A single ketamine dose reversed ACTH-induced behavioural despair and normalised region-specific serotonin levels and *CREB* mRNA expression, whereas chronic ketamine treatment failed to elicit these effects. Although ketamine is thought to act primarily via NMDA receptor blockade and consequent enhancement of neurotrophic signalling, our findings suggest that its antidepressant efficacy may not be fully explained by neurotrophic mechanisms alone. Further research is needed to elucidate additional pathways, potentially involving glutamatergic, inflammatory, or synaptic plasticity-related processes that may contribute to its rapid antidepressant action.

Together, these findings confirm that chronic ACTH administration models HPA axis dysregulation-induced depressive-like symptoms, which resulted in disturbances in neurotrophic and monoaminergic signalling. Moreover, the comparative treatment outcomes indicate that interventions enhancing neuroplastic and neurogenic signalling such as SSRIs and acute ketamine, may offer greater therapeutic

benefit than classic monoamine-based antidepressants in depressive states driven by HPA axis dysfunction.

The findings from this thesis highlight the importance of developing targeted pharmaceutical interventions tailored to distinct pathophysiological subtypes of depression, particularly those involving HPA axis dysregulation. Going forward, to further validate our preclinical findings, clinical trials should investigate the molecular markers analysed in this thesis, within the context of HPA axis dysfunction and MDD. Future research should additionally prioritise biomarker-driven treatment strategies for patient stratification that account for variability in HPA axis activation and sex-dependent antidepressant responses. Furthermore, the varying effects of acute versus chronic ketamine treatment emphasise the need for improved dosing regimens and augmentation therapies to maximise therapeutic benefit. These considerations may improve clinical practice by advancing the development of more precise and effective antidepressant interventions, thereby improving patient outcomes.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDICES

7.1 Appendix A: Animal ethical clearance certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2022/03/06/C

APPLICANT: Dr S Baijnath
School: School of Physiology; Department: N/A; Location: WRAF

PROJECT TITLE: Investigation of the anti-depressive mechanism of action of ketamine in a Sprague Dawley rodent model of treatment resistant depression
Category: C; Species and Numbers involved: *120X, 250g, Male, Sprague-Dawley Rodents*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 29 Mar 2022. This approval remains valid until 13 Apr 2024 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____ _____ Date: _14/04/2022_____
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: ____ _____ Date: _19 April 2022_____
(Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Wits Research Animal Facility (WRAF): Dr Kim Jardine

7.2 Appendix B: Modification/Extension clearance for animal study

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Sooraj Baijnath

b. Department: Physiology

c. Experiment to be modified / extended **AESC NO**

Original AESC number	**2022**	**03**	**06/C**
Other M&Es :			2

d. Project Title: Investigating the Anti-Depressive Mechanism of Action of Ketamine in a Model of Treatment Resistant Depression.

		No.	**Species**
e.	Number and species of animals originally approved:	120	SD
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	120	SD
h.	Number of animals used to date:	120	SD

i. Specific modification / extension requested:

a. **Additional 25 animals to be grouped as follows:**

- ACTH – saline treated (n=10, 5 males and 5 females) [additional animals added to group]
- ACTH – ketamine treated (n=10, 5 males and 5 females) [additional animals added to group]
- ACTH – imipramine treated (n=10, 5 males and 5 females) [additional animals added to group]
- ACTH – citalopram (n=20, 10 males and 10 females) [new group added to study]
- Healthy – citalopram (n=20, 10 males and 10 females) [new group added to study]

b. Additional co-workers to be added to the study, these students are new postgraduate students who have joined our lab and will assist with animal studies.

- Andrea Lubbe (MSc)
- **Sanelisiwe Xhakaza (PhD)**

c. The addition of the open-field test

j. Motivation for modification / extension:

a. Request for additional animals

We would like additional animals to repeat the ACTH administration in the ACTH-saline, ketamine and imipramine groups using a pharmaceutical grade of ACTH to compare the neurobehavioral outcomes with the lab grade ACTH that was use. We would also like to include female animals since recent studies have shown that female laboratory animals respond differently to ketamine compared to their male counterparts [1].

The addition of the citalopram group will be to fully validate the model, according to the most widely accepted definition of treatment resistant depression, the patient should show resistance to two or more classes of anti-depressants [2]. We have validated the model using imipramine (a tricyclic antidepressant) and now which to complete the validation using citalopram (a selective serotonin reuptake inhibitor).

b. Additional team members

Andrea Lubbe and Sanelisiwe Xhakaza have joined our lab as new postgraduate students studying towards their MSc and PhD, respectively. They will assist on this study, which will also be used as part of their induction and training into neurobehavioral research.

c. Addition of the open field test

The open field test is a widely used to assess locomotor activity, anxiety, and exploratory behaviour in experimental animals [3]. Thus far, we have assessed neurobehavioral function using the forced swim test and the sucrose placement test, the addition of the open field test will strengthen the neurobehavioral characterisation of our model and improve face validation of the model allowing us to publish in the high-impact journals we are targeting.

In order to conduct the test, rodents will be placed in an open field consisting of a black Perspex base ($1.2m^2$), surrounded by black Perspex walls (0.5m). The rats will be placed in the arena and allowed to explore for 10 min, with their movements being recorded using the AnyMaze behavioral tracking software. We will analyse the distance travelled, time immobile, local movements, average velocity, time spent in the centre vs time in the periphery of the open field [3].

The test will be conducted once weekly, on the Thursday before the FST on Friday.

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Date: 31/01/2023 Signature: 

RECOMMENDATIONS

Date: Signature:

Chairman, AESC