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NEUROPHYSIOLOGICAL EFFECTS OF CITALOPRAM IN A RODENT MODEL OF ACTH-INDUCED HPA AXIS DYSREGULATION

by

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DECLARATION

I, Andréa Lubbe, declare that this dissertation is my own, except to the extent indicated in the contribution and acknowledgments sections. It is being submitted for the degree of Master of Science in Medicine in the School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this or any other University.

I hereby certify that the studies contained in this dissertation have been approved by the Animal Ethics Screening Committee, University of the Witwatersrand, Johannesburg. The ethics approval number is 2022/03/06/C.

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Signed on the 5th day of June 2024

Prof Sooraj Baijnath

Prof Aletta Millen

Abstract

Background. Major depressive disorder (MDD) is a debilitating disorder that affects approximately 300 million people worldwide. Despite multiple theories being implicated in the pathogenesis of MDD, most antidepressant drugs primarily target the monoaminergic theory of depression. Selective serotonin reuptake inhibitors (SSRIs) form a part of the most prescribed antidepressants that target the monoaminergic system. Following treatment with antidepressants, around 30% of patients with MDD continue to experience depressive symptoms, rendering them treatment resistant. The most widely accepted definition of treatment resistant depression is the resistance to at least two antidepressant regimens following the proper dose for an adequate amount of time. Treatment resistance in MDD has been attributed to dysregulation of the hypothalamic-pituitary-adrenal (HPA). HPA axis dysregulation leads to chronically high levels of glucocorticoids and failure of the regulatory negative feedback system. Consequently, there is a need for the development of novel drugs and validated animal models to study the antidepressive mechanism of these drugs. The aim of this study was to validate a rodent model of depression via chronic administration of adrenocorticotropic hormone (ACTH), and to investigate the effects of citalopram (a SSRI) on depressive-like symptoms using neurobehavioural tests and the regional brain expression of molecular markers associated with depression.

Methods. Thirty-nine Sprague-Dawley rats (n=20 female; n=19 male) were randomly divided into a control group (n = 10 female, n= 9 male) and a depressed group (n=10 female; n=10 male). Rats in the control group received daily subcutaneous injections of saline (100µg/day) for two weeks. The depressed group received ACTH (100µg/day) subcutaneously daily for two weeks. Following the 2-week administration of ACTH or saline, all animals received citalopram (10mg/kg bw) for four weeks intraperitoneally, concurrently with ACTH or saline. The open-field test was performed to determine anxiety-like behaviour by assessing locomotor activity and time spent in central versus peripheral zones. The OFT was performed prior to the initiation of ACTH or saline administration. Following the 2-week administration of ACTH, the OFT was performed again to determine the effects of ACTH. The OFT was performed for a final time following citalopram treatment. The sucrose preference test (SPT) was performed to determine the presence of anhedonia. During the test, the rats were placed in the SPT apparatus with two water bottles for 12 hours. Water bottles were weighed before and after the test. After the four weeks of drug administration, rats were terminated by decapitation using a rodent guillotine. Brains were surgically removed, and the prefrontal cortex, striatum, hippocampus, and midbrain were surgically dissected and frozen. The RNA was isolated, and cDNA synthesised. Molecular gene expression analysis was performed for BDNF, CREB, TrkB, Akt, mTOR, and eEF2 using real-time polymerase chain reaction.

Results. During the OFT, immobility increased in male and female saline + citalopram and ACTH + citalopram rats after 6 weeks (all $p<0.05$). During the SPT, females that received ACTH + citalopram had a higher preference for regular water consumption ($p=0.004$). In males, sucrose preference ratio was lower in ACTH + citalopram males than in saline + citalopram males ($p=0.007$). Regional mRNA expression of BDNF was significantly different between males and females ($p<0.003$). In the striatum, ACTH + citalopram females had higher expression of BDNF compared to ACTH + citalopram males ($p=0.007$). In the midbrain, ACTH +citalopram males had higher expression of BDNF compared to ACTH + citalopram females ($p=0.01$). Regional expression of

mRNA of CREB resulted in a sex ($p<0.0001$) and treatment effect in the prefrontal cortex and midbrain ($p<0.05$). In the prefrontal cortex and midbrain, saline + citalopram and ACTH + citalopram males had higher mRNA expression of CREB than females (all $p<0.001$). There was a sex effect in the *TrkB* mRNA expression in the prefrontal cortex ($p=0.005$), as ACTH + citalopram females had a higher mRNA expression than ACTH + citalopram males ($p=0.02$). In the striatum, ACTH administration led to a higher *TrkB* mRNA expression in females compared to males ($p=0.04$). No significant differences were observed in *mTOR* mRNA expression between any of the groups (all $p>0.05$). In the midbrain, ACTH + citalopram females had a lower *eEF2* mRNA expression than ACTH + citalopram males ($p=0.02$).

Conclusion. This study suggest that male animals may exhibit greater susceptibility to neurobehavioural effects of ACTH, as oestrogen in females may be protective in regulating HPA axis function. Neurobehavioural changes in male rats may be linked to ACTH-induced effects on the dopamine system, affecting locomotor activity and reward processing. These results aid our understanding of the pathophysiology of depression related to HPA axis dysregulation. Nevertheless, the effects of ACTH induced dysregulation of the HPA axis on monoamine signalling, neuroinflammation, and GABA/glutamate neurotransmission, warrant further investigation.

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STATEMENT OF CONTRIBUTION TO DATA COLLECTION AND ANALYSIS

I declare that I designed this study in conjunction with my supervisors Prof Sooraj Baijnath and Prof Aletta Millen. I was part of a team of researchers that included myself, my supervisors, and postgraduate students responsible for collecting data for a larger study, which this dissertation forms a part of. I was responsible for collection of the data for all the outcome variables presented in this study. I performed the data analysis for this dissertation with the assistance of Prof Sooraj Baijnath and Prof Aletta Millen. I wrote this dissertation which was reviewed by Prof Sooraj Baijnath and Prof Aletta Millen.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
Akt	Protein kinase B
BDNF	Brain-derived neurotrophic factor
CREB	cAMP response element-binding protein
CRH	Corticotropic releasing hormone
CUMS	Chronic unpredictable mild stress
eEF2	Eukaryotic elongation factor 2
FST	Forced swim test.
HPA	Hypothalamic-pituitary-adrenal
MDD	Major depressive disorder
MAOI	Monoamine oxidase inhibitor
MTOR	Mammalian target of rapamycin
OFT	Open field test
PCR	Polymerase chain reaction
SNRI	Serotonin-norepinephrine reuptake inhibitor
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TRD	Treatment resistant depression
TrkB	Tropomyosin receptor kinase B
TST	Tail suspension test

CHAPTER 1:

INTRODUCTION

Major depressive disorder (MDD) is a highly prevalent and widespread mental health disorder, which significantly affects behaviour and quality of life (Becker *et al.*, 2021). The disorder results in significant impairments in mental, emotional and physical well-being, which is marked by feelings of sadness, low energy, sleep disturbances, and an absence of joy. In extreme cases MDD can result in substantial psychological disabilities and is associated with high rates of suicidality (Patel *et al*., 2016).

MDD affects approximately 300 million people worldwide (Patel *et al*., 2016; World Health Organization, 2022), with this number climbing rapidly. Recent statistics have suggested a 50% increase in the global prevalence of depression between 1990 and 2017, leading to the WHO expecting MDD to be the leading cause of morbidity and mortality by 2030 (World Health Organisation, 2022; Liu *et al.*, 2018). Despite these global statistics, the prevalence of MDD and other mental health disorders are severely underestimated in low-middle income countries, such as South Africa (Meyer *et al.*, 2019). Furthermore, the devastating effects of MDD places a significant strain on public healthcare resources (Meyer *et al*., 2019). The high prevalence of MDD worldwide emphasises the need for the effective treatment and management of the MDD.

Currently the primary management of MDD is via psychotherapy and/or pharmaceutical interventions (Marwaha *et al.*, 2023). Nevertheless, the treatment and management of MDD remains challenging, as evidenced by the increasing prevalence rates. The lack of adequate management of MDD could be attributed to the heterogeneity of the disorder and its complex pathophysiology. Several theories have attempted to explain the development of depression, including the monoaminergic theory, GABAergic theory, neurotrophic theory, neuroinflammation, and hypothalamic-pituitary-adrenal (HPA) axis dysregulation (Mlyniec, 2015; Machado-Vieira *et al.*, 2017). Despite the heterogenous pathophysiology of MDD, most current antidepressant regimens mainly target the monoaminergic system. Despite the widespread use of monoaminergic antidepressants, approximately 30% of MDD patients continue to experience depressive symptoms, rendering them treatment resistant (Trivedi *et al*., 2006). Treatment-resistant depression (TRD) is defined as resistance to at least two different classes of antidepressants of adequate dose and duration (Gaynes *et al*., 2020). The consequences of TRD are dire in these patients who experience a lower quality of life, frequent hospitalisations, and are at a higher

risk of suicide (Bergfeld *et al*., 2018; Gibson *et al*., 2010; Hantouche *et al*., 2010). Therefore, patients with TRD remain the most severely affected by the disorder, experiencing the most severe symptoms and who have limited access to alternative therapeutic approaches (Papp *et al*., 2021).

Treatment resistance in MDD has been attributed to dysregulation of the HPA axis and hypercortisolaemia (Papp *et al.,* 2021, Kitamura *et al.*, 2002). The HPA axis regulates the physiological stress response via the release of corticotropic releasing hormone (CRH) from the hypothalamus, which in turn leads to secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, subsequently stimulating the release of glucocorticoids from the adrenal cortex (Boku *et al.*, 2018). Under normal conditions, following psychological or physiological stress, negative feedback inhibits the HPA axis where elevated glucocorticoid levels suppresses the release of CRH and ACTH, through its binding to glucocorticoid and mineralocorticoid receptors (Rothe *et al.*, 2020). In HPA axis dysregulation, chronic HPA axis activation leads to consistently elevated glucocorticoid levels leading to decreased expression and desensitisation of glucocorticoid receptors and ultimately failure of the negative feedback system (Pariante, 2017; Halaris *et al.*, 2021). HPA axis dysregulation and failure of the physiological stress response has been proposed, not only as a possible cause of MDD, but as a contributor of resistance to major antidepressants (Bondy, 2002). Consequently, there is a need for the development of validated animal models, which focus on dysregulation of the HPA axis, which will also allow for the study of the antidepressive mechanism of novel and traditional antidepressant drugs.

Animal models induce HPA axis dysregulation and depressive-like symptoms through exposure to stress or via the administration of exogenous hormones involved in the HPA axis, specifically ACTH and corticosterone. The present study focused on the induction of HPA axis dysregulation through chronic administration of ACTH. Chronic administration of ACTH for at least 7 days has shown to induce a depressive-like phenotype in rats (Kitamura *et al*., 2002; Petrović *et al*., 2018). However, this model has not been fully characterized as previous studies have failed to report on neurobehavioural outcomes in conjunction with multiple molecular outcomes in an additional class of antidepressant, specifically a selective serotonin reuptake inhibitor. Therefore, this study aims to determine the effects of ACTH-induced HPA axis dysregulation on neurobehavioural and molecular outcomes. In this study, face validity

of this model will be determined by investigating depressive-like behaviours displayed by experimental animals in neurobehavioural tests. Construct validity will be determined through the evaluation of changes in the molecular expression of neurotrophic factors and intracellular signalling markers associated with MDD. Finally, predictive validity will be determined through treatment with citalopram and the evaluation of drug efficacy in this model.

The present study is part of a larger study in our lab investigating the mechanisms of action of novel antidepressants. However, at first, we aimed to validate a rodent model of ACTH-induced HPA axis dysregulation as treatment-resistant through investigating the neurophysiological effects of a widely used antidepressants, specifically the selective serotonin reuptake inhibitor (SSRI), citalopram.

CHAPTER 2:

LITERATURE REVIEW

2.1 The pathophysiology of depression

The pathophysiology of depression is complex and has been linked to multiple theories, including the monoaminergic theory, GABAergic, and glutamatergic theory, dysregulation of the HPA axis, dysregulation in neurotrophic factors, and dysregulation of immunological factors leading to neuroinflammation (Mlyniec, 2015; Machado-Vieira *et al*., 2017) (Figure 2.1).

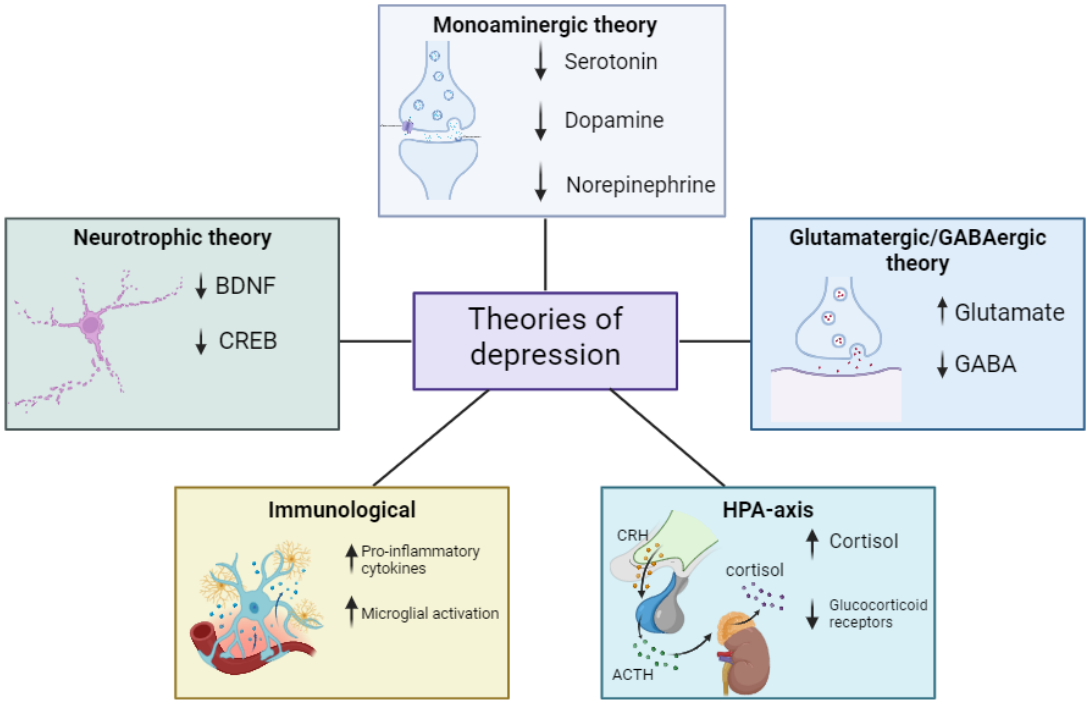

Figure 2.1: A summary of the theories of depression. Several theories implicated in the development of MDD. These include decreases in monoamine neurotransmitters, decreased neurotrophins, increased neuroinflammation, HPA axis dysregulation and glutamate/GABA imbalances.

2.1.1 Monoaminergic theory

The monoamine theory is the most widely accepted theory of depression. Hence, most antidepressant drugs primarily target monoamine imbalances in the brain (Machado-Vieira *et al*., 2017). The monoaminergic theory of depression suggests dysregulation of monoaminergic neurotransmission in the development of depression, specifically of noradrenaline, dopamine, and serotonin (Perez-Caballero *et al.*, 2019). The monoaminergic theory became prominent as drugs that alter monoamine metabolism, such as tricyclic antidepressants, monoamine oxidase inhibitors and selective serotonin reuptake inhibitors displayed antidepressant effects (Hamon & Blier, 2013)

Several preclinical and clinical studies have demonstrated monoamine alterations in depressive disorders. In preclinical models of depression chronic stress is often used as a stimulus for depression. In these models it has been shown that chronic stress dysregulates serotonin and dopamine levels, as well as impair neurotransmission, in the hippocampus and prefrontal cortex of rats (Lu *et al.*, 2019). In maternal separation, a model of early life stress in animals, rats demonstrated decreased expression of serotonin and its transporter in the raphe nuclei and hippocampus in comparison to controls (Lee *et al.*, 2007). This highlights the role of dysfunctional serotonin neurotransmission in a variety of psychiatric disorders, including MDD. Indeed, in a clinical study, MDD patients presented with significantly reduced serum serotonin concentrations when compared to their healthy controls (Obermanns *et al.*, 2021). In addition, monoamine oxidase, an enzyme responsible for the metabolism of monoamines, was found to be significantly higher in multiple brain regions of patients with MDD when compared to age-matched healthy controls (Meyer *et al.*, 2006). Therefore, elevated monoamine oxidase levels may be a contributing factor in monoamine dysregulation in patients with MDD.

Dysregulation of monoamines can manifest as an array of clinical symptoms characteristic of depression. Anhedonia and mood dysregulation are two of the classical symptoms of depression often arising from monoamine dysregulation (McMakin *et al.*, 2012). Anhedonia is described as a failure to experience and/or seek out pleasurable activities and has been associated with resistance to antidepressants targeting monoamine dysregulation (McMakin *et al.,* 2012). Anhedonia has been linked to dysfunctions in the dopamine system and more specifically, the reward system (Belujon & Grace, 2017). In anhedonic depressive patients, there is a significant reduction in dopamine transporter binding when compared with healthy subjects (Belujon & Grace, 2017). Therefore, drugs target the monoamine systems with the aim of alleviating these symptoms of anhedonia and mood dysregulation.

Drugs that target the monoaminergic system primarily act through alterations in the bioavailability of monoamines (Perez-Caballero *et al*., 2019). These include tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), and selective serotonin reuptake inhibitors (SSRIs). TCAs and MAOIs are non-selective and therefore may have substantial neurological side effects (Hamon & Blier, 2013). While, selective drugs,

such as SSRIs and SNRIs, seem to have greater beneficial effects on MDD symptoms, with fewer side effects and a more widespread use. The present study will focus on citalopram, an SSRI, as limited research has focused on the neurophysiological effects of an SSRI in a rodent model of ACTH-induced depression.

2.1.2 HPA axis dysregulation

The dysregulation of neuroendocrine pathways, specifically abnormalities in cortisol production and release by the HPA axis, has also been implicated as a contributor to the development of depression (Charmandari *et al.*, 2004; Bondy, 2002). In situations of physiological and/or psychological stress, CRH is released from the hypothalamus, which stimulates the production and release of ACTH from the anterior pituitary gland. In turn, ACTH stimulates the secretion of glucocorticoids, such as cortisol, from the adrenal gland (Bondy, 2002). Glucocorticoids are responsible for regulating a range of growth, metabolic, developmental, and immune processes, serving a critical function in maintaining both baseline and stress-induced balance within the body (Charmandari *et al.*, 2004). Glucocorticoids mediate the negative feedback mechanism in control of the HPA axis, inhibiting the secretion of CRH and ACTH. Failure of this negative feedback leads to high levels of circulating and brain glucocorticoids, which has been implicated in the development of depression (Boku *et al*., 2017). The relationship between the HPA axis and circulating glucocorticoid levels is evidenced by the high prevalence of MDD among patients with Cushing's syndrome (Pivonello *et al.*, 2015). Cushing's syndrome is characterized by high levels of cortisol, with roughly 80% of cases resulting from ACTH hypersecretion (Pivonello *et al.,* 2015). Dysregulation of the HPA axis has been reported in severe forms of depression, as shown by hypercortisolemia and elevated plasma ACTH levels (Choi *et al*., 2018; Santos *et al.*, 2017). Increased glucocorticoids in the brain deactivate the transcription of CREB via the activation of glucocorticoid receptors. In addition, this leads to a suppression of glutamate activity in the postsynaptic neuron and decreases BDNF expression, which contributes to the classical hallmarks of depression.

In addition, patients with Cushing's syndrome present with a reduced hippocampal volume in comparison to healthy controls, similar to what is observed in depression (Boku *et al*., 2017, Videbech & Ravnkilde, 2004). The hippocampus plays an important role in mediating the HPA axis due to the high expression of glucocorticoid and mineralocorticoid receptors in hippocampal neurons (Boku *et al*., 2017; Nair & Vaidya,

2006). The hippocampal volume loss seen in patients with MDD and Cushing's syndrome may therefore be attributed to dysregulation of the HPA axis. While the exact mechanism underlying hippocampal volume loss remains unclear, it is suggested that it may be due to neuronal atrophy as a result of chronic stress and elevated glucocorticoids, which has also been associated with decreased neurogenesis (Boku *et al*., 2017).

2.1.2.1 The development of treatment resistance

The combined effect of HPA axis dysregulation, subsequent increases in glucocorticoid levels, and decreased hippocampal volume highlights the physiological impact of its dysregulation (Boku *et al*., 2017; Videbech & Ravnkilde, 2015). Further compounding the severity of the resultant MDD from HPA axis dysregulation, is the inability of current drugs to effectively target the HPA axis, which may contribute to TRD. While many definitions of TRD exist, the most widely accepted definition is resistance to at least two regimens of antidepressant therapy of adequate dose and duration (Gaynes *et al*., 2020). Despite this being the most widely accepted definition, only 19% of studies focusing on treatment resistance in depression comply with this definition (Gaynes *et al*., 2020). A study involving MDD patients found that non-remitted patients had significantly higher levels of ACTH than remitted patients; this despite treatment with widely used antidepressants, including SSRIs, SNRIs and specific serotonin antidepressant for 6 months (Choi *et al*., 2018). The high levels of ACTH in non-remitted patients with MDD suggests that dysregulation of the HPA axis is strongly associated with TRD.

In preclinical studies, chronic administration of ACTH in rodents resulted in resistance to two TCAs, namely imipramine and desipramine (Kitamura *et al*., 2002; Petrović *et al*., 2018). While multiple studies have demonstrated that HPA axis dysregulation is associated with resistance to TCAs, few studies have investigated the efficacy of SSRIs in models of HPA axis dysregulation. SSRIs function by primarily inhibiting serotonin reuptake transporters, leading to an immediate increase in extracellular serotonin in the neuronal somatodendritic region (Fasipe, 2018). Therapeutic effects are often only achieved through chronic administration of SSRIs, leading to desensitization of somatodendritic serotonin receptors, which increases the release of serotonin at the terminal presynaptic region (Fasipe, 2018).

Despite being part of the first-line treatment of depression, citalopram is only effective in up to two-thirds of patients with depression (Trivedi *et al*., 2004; Rush *et al*., 2006; Trivedi *et al*., 2006). Standard monoaminergic drugs have remission rates of approximately 40% (Perez-Caballero *et al*., 2019). The partial efficacy of these drugs may be detrimental to patients with MDD as patients are unable to recover or be relieved of debilitating symptoms. Additionally, the effects of monoaminergic drugs have a delayed onset of action, with some patients only experiencing therapeutic effects after weeks (Strasburger *et al.*, 2017). Therefore, the limited efficacy of monoaminergic drugs in patients with MDD highlights the complex aetiology of depression.

The present study focused on the neurophysiological effects of citalopram in a rodent model of HPA axis dysregulation. Developing preclinical models of HPA dysregulation-induced depression will provide a greater understanding of the molecular pathways implicated in depression in addition to neurobehavioural outcomes and provide insight into potential targets for novel therapies.

2.1.3 Dysregulation of neurotrophic factors and intracellular signalling pathways

Another theory that has been implicated in the pathophysiology of depression is the aberrant expression of neurotrophic and transcription factors. Brain-derived neurotrophic factor (BDNF) and cyclic AMP response element binding protein (CREB) play significant roles in the normal functioning and development of the nervous system (Nair & Vaidya, 2006). BDNF supports neuron survival, growth, and differentiation, which are essential for synaptic plasticity, and underlies learning and memory (Nair & Vaidya, 2006). CREB is a transcription factor activated by cellular signals, including BDNF, that promotes the expression of genes required for neuronal survival, plasticity, and growth (Nair & Vaidya, 2006). Together, BDNF and CREB regulate crucial processes for brain development, cognitive function, and nervous system maintenance. As a result, extensive research has been conducted to investigate the expression of BDNF and CREB following chronic antidepressant administration. Dysregulation of these factors severely impacts neuronal health via weakened neurogenesis and hippocampal dysfunction, which have been linked to depressive disorders (Nair & Vaidya, 2006). Reduced neurogenesis can lead to a reduced hippocampal volume, an effect seen in depressed patients as well as animal models of depression (Banasr *et al.*, 2004; Yang *et al.*, 2020; MacQueen *et al.*, 2003).

Indeed, increased BDNF and CREB expression in the hippocampus has been associated with antidepressant-like effects in a model of chronic restraint stress (Nair & Vaidya, 2006; Alfonso *et al.*, 2006). Additionally, postmortem human brain samples showed increased BDNF expression in individuals receiving antidepressant therapy at the time of death, when compared to tissues from individuals who were untreated at the time of death (Chen *et al*, 2001b). Consequently, decreased BDNF and CREB may contribute to the pathophysiology of depression and may serve as potential markers of risk stratification or therapeutic targets in the management of MDD.

In addition, BDNF and CREB regulate multiple intracellular signalling pathways which have been implicated in MDD. Therefore, an improved understanding of these pathways is needed for the identification of potential therapeutic targets of depression. In this regard, tropomyosin receptor kinase B (TrkB) is a BDNF receptor that may elicit antidepressant effects through enhanced neurotransmission (Koike *et al*., 2013). Antidepressant drugs have been shown to enhance TrkB signalling, leading to increased BDNF expression (Saarelainen *et al*., 2003). As a result, TrkB expression may also be reduced in MDD along with a decrease in BDNF. The binding of BDNF activates the TrkB receptor, leading to the activation of downstream signalling pathways including Akt and mTOR, which, in turn, enhance CREB activity and promote eEF2 phosphorylation (Machado-Vieira *et al*., 2017). Decreased mTOR signalling has been observed in the prefrontal cortex of patients with mood and stress-related disorders (Machado-Vieira *et al*., 2017). Akt directly regulates the mTOR pathway, with decreased mRNA expression of Akt and mTOR consistent with mood disorders (Machado-Vieira *et al*., 2017). Furthermore, the inhibition of eEF2 elicits antidepressant effects as it has been associated with increased BDNF expression (Autry *et al*., 2011). Antidepressant drugs that target the TrkB, Akt, mTOR, and eEF2 intracellular signalling pathways to increase BDNF expression have received considerable attention. However, the exact molecular pathways and the factors regulating these molecular markers are uncertain and requires further investigation (Machado-Vieira *et al*., 2017). BDNF, CREB, TrkB, Akt, mTOR, and eEF2 signalling interact with the HPA axis (Figure 2.2). Activation of the HPA axis can have a direct effect on the release of BDNF. Acute stress can lead to increased expression of BDNF, which in turn binds to TrkB and activates the intracellular signalling pathway, including CREB, Akt, mTOR, and eEF2 (Machado-Vieira *et al*., 2017). In chronic stress,

prolonged exposure to cortisol can lead to downregulation of BDNF expression (Machado-Vieira *et al.*, 2017). Therefore, in this study, the regional brain expression of BDNF and CREB, TrkB, Akt, mTOR, and eEF2 will be investigated to gain a better understanding of the molecular mechanisms involved in an ACTH-induced rodent model of HPA dysregulation induced depression and its potential as therapeutic targets.

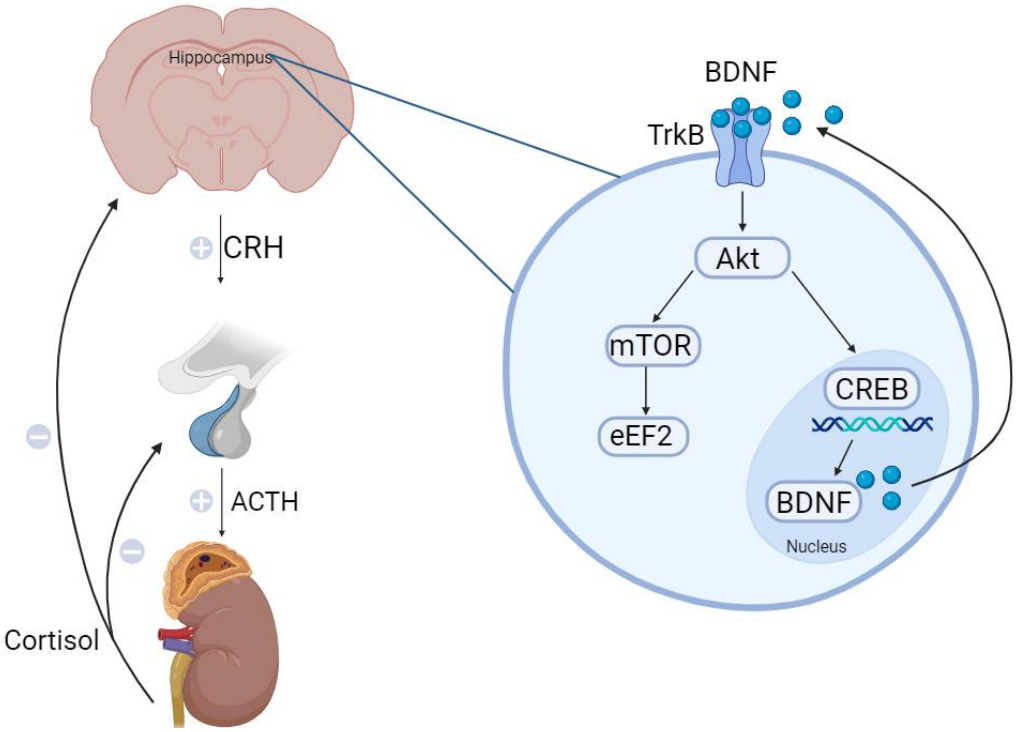

Figure 2.2: Interaction between the HPA axis and synaptogenic pathways in the brain. The HPA axis CRH, corticotropic releasing hormones; ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding protein; Akt, protein-kinase A; mTOR, mammalian target of rapamycin; eEF2, eukaryotic elongation factor 2. Activation of the HPA axis can have a direct effect on the release of BDNF. Acute stress can lead to increased expression of BDNF, which in turn binds to TrkB and activates the intracellular signalling pathway, including CREB, Akt, mTOR, and eEF2. Image created by author.

2.1.4 Neuroinflammation

Neuroinflammation is considered to be an underlying mechanism in the development of multiple neurological disorders including Alzheimer's, multiple sclerosis, and depression (Heneka *et al.*, 2015; Troubat *et al.*, 2021). Recent studies have shifted focus to the role of neuroinflammation in the pathogenesis, and the mechanisms

whereby cytokines and immune markers contribute to the development of depression (Furtado & Katzman, 2015). Indeed, interleukin-6 and C-reactive protein, both pro-inflammatory markers, have been strongly associated with depression (Haapakoski *et al.*, 2015; Troubat *et al*., 2020). Additionally, low-grade chronic inflammation may result in brain structure changes and impaired synaptic plasticity leading to neurodegeneration (Hurley & Tizabi, 2013). Increased neurodegeneration, reduced neuroprotection, and impaired neuronal repair, as a result of increased glucocorticoids, may serve as early markers of depression (Hurley & Tizabi, 2013). Currently, there are no drugs that specifically target neuroinflammation in the treatment and management of depression.

2.1.5 GABA/Glutamatergic theory

Studies have suggested that structural and volume changes in the hippocampus and prefrontal cortex that are associated with depression may be attributed to dysregulation of excitatory glutamate neurons (Duman *et al.*, 2019). In this regard, several studies have shown that chronic stress and depression may lead to dysregulation of inhibitory GABA neurotransmission and dysregulation of glutamate receptor subtypes (Duman *et al*., 2019). Additionally, in models of acute and chronic stress, rodents displayed dysregulated levels of glutamate in the prefrontal cortex and hippocampus (Duman *et al*., 2019). This imbalance in GABA/glutamate signalling leads to an excitation/inhibition imbalance in the brain that is associated with increased excitotoxicity and neurodegeneration (Mlyniec, 2015), which may contribute to the development of depression.

Taken together, the heterogenous theories underlying the development of depression highlight the challenges in finding effective treatment solutions for patients with MDD. Majority of studies investigating the role of these theories study a single contributing factor. Hence, our understanding of the interplay between these theories are limited. Moreover, in human studies, majority of the molecular mechanisms that are investigated in the development of depression can only be done by measuring blood biomarkers. This has major limitations as circulating concentrations of biomarkers may not accurately reflect changes of these biomarkers in the brain. Therefore, animal models investigating several theories of depression, and measuring molecular markers in the brain may improve our understanding of the pathogenesis of depression and highlight the effects of current and novel treatments for depression.

2.2 Animal models of HPA axis dysregulation

Depressive-like symptoms induced by dysregulation of the HPA axis has received considerable attention recently. Animal models of depression have used various experimental procedures to induce HPA axis dysregulation and to establish a depressive-like phenotype.

2.2.1 ACTH induced HPA axis dysregulation

Human studies have shown that baseline ACTH levels are significantly increased in patients with non-remitted MDD when compared to remitted MDD patients (Choi *et al.*, 2018). Therefore, chronic administration of ACTH for a period of at least 7-14 days has been used in rodent models to induce a depressive-like phenotype (Kitamura *et al*., 2002; Petrović *et al*., 2018). Rats chronically treated with ACTH for 14 days displayed longer periods of immobility in the forced swim test (FST), this has been associated with behavioural despair similar to depressive symptoms in humans, and hence an indication of a depressive-like phenotype in rodents (Petrović *et al*., 2018). In another study, healthy rats showed a decrease in immobility in the FST when treated with imipramine, however, this effect was blocked in rats chronically treated with ACTH (Kitamura *et al.,* 2002; Walker *et al*., 2013). These results suggest that ACTH induced HPA axis dysregulation may lead to depressive-like symptoms that are resistant to commonly available antidepressants, which primarily target the monoaminergic system.

Additional evidence suggests a dose-dependent effect of ACTH. One study that administered a low dose of ACTH (10 µg/day) showed changes in neurobehaviour, but no changes in BDNF expression (Petrović *et al*., 2018). However, a higher dose of ACTH (100ug/day) resulted in depressive-like behavioural changes but did not investigate the expression of molecular markers (Kitamura *et al.,* 2002). Therefore, more conclusive evidence about the dose dependent effect of ACTH administration on both neurobehavioural and molecular changes are warranted.

2.2.1.1 Effects of ACTH on depressive-like symptoms using neurobehavioural tests

Neurobehavioural tests are often used in rodent models to assess behavioural phenotypes associated with depression, as well as treatment effects. The FST is commonly used in animal models of depression to measure despair-like behaviour as

determined by time immobile during the swim (Kitamura *et al*., 2002; Rotzinger *et al.*, 2010). Several studies have assessed behaviour using a variety of tests, following ACTH administration, summarised in Table 2.1. Briefly, in rats that received chronic ACTH administration, immobility time during the FST was increased when compared to healthy controls, indicating behavioural despair (Petrović *et al*., 2018). The FST is further used to assess antidepressant efficacy, with antidepressant effects shown by reduction of immobility during the FST. Following administration of magnesium (Mg), a supplement often used for its antidepressant effects, in rats chronically treated with ACTH, immobility time was decreased as compared to rats that received only ACTH (Petrović *et al*., 2018). Thus, the FST has been widely used to assess the antidepressant efficacy of many antidepressants. Similarly, the tail suspension test (TST) is also commonly used to assess immobility time when the animal is suspended from the tail. In rats treated with chronic ACTH, immobility during the TST was increased, suggesting a depressive phenotype and behavioural despair in rats that received ACTH (Song *et al.*, 2019).

The sucrose preference test (SPT) is a well-known neurobehavioural test often used to investigate anhedonia in rodent models of depression (Song *et al*., 2018). In the SPT, rats treated with ACTH had a lower preference for sugar water when compared with control rats that received saline (Song *et al*., 2018). Traditionally, a decreased preference for sucrose water is associated with anhedonia-like behaviour in rodents, a sign often associated with a depressive phenotype. Conversely, rats chronically treated with ACTH showed an increase in sucrose preference compared to healthy rats treated with saline (Kale *et al.*, 2021). Recent studies investigating animal models of depression, the two-bottle method most used in the SPT screens for sucrose-associated carbohydrate preference, rather than anhedonia (Kale *et al*., 2021). To screen for anhedonia, it is more often agreed that the animal should have to work to obtain the sucrose reward (Felger *et al.*, 2013; Kale *et al.*, 2021). In addition, it is suggested that experimental animals could be separated into active and passive copers, a phenomenon also observed in clinical depression where some patients seek out high-caloric foods while others are averse to them.

The open field test (OFT) is used as a measure of locomotor activity and anxiety-like behaviour in animals. During the OFT, time spent in the periphery of the open field maze versus time spent in the centre are frequently measured (Choleris *et al*., 2001).

Anxiety-like behaviour is most often defined as an increase in time spent in the periphery, while more time spent in the central zone is interpreted as healthy exploratory behaviour (Choleris *et al*., 2001). Following chronic ACTH administration, time spent in the central zone and distance travelled in the central zone was significantly decreased in comparison to healthy controls (Petrović *et al*., 2018). The OFT is used during depression studies as anxiety-related disorders are often co-morbid with depressive disorders (Kalin, 2020). By using the OFT in animal models of depression, a wide range of symptoms associated with depression can be investigated.

Taken together, several neurobehavioural tests are available and have been used to assess the depressive like behaviour following ACTH administration. However, the variable results emphasise the importance of using multiple neurobehavioural paradigms to evaluate depressive-like phenotypes in rodents, allowing for the investigation of different translational aspects of depressive symptomology.

2.2.1.2 Effects of ACTH on brain regional expression of molecular markers associated with depression

Changes in the expression of several molecular markers have been implicated in the development and treatment of depression. However, no single clinical diagnostic marker for MDD has been identified. Studies investigating changes in the expression of molecular markers in animal models of ACTH induced HPA axis dysregulation are limited (Table 2.1). BDNF is known to play a key role in the development of depression. BDNF expression has been shown to be decreased in stress induced models of depression, particularly in the hippocampus (Yang *et al.*, 2020). Despite this, the effect of chronic ACTH administration on BDNF expression, as well as expression of other molecular markers, remains limited.

BDNF is a neurotrophic factor responsible for neuron survival and support of synaptogenesis in multiple brain neurons including hippocampal, cortical, cholinergic and serotonergic neurons (Wu *et al.*, 2020; Meshkat *et al.*, 2022). Serum BDNF levels have been shown to be decreased in patients with MDD when compared to healthy controls (Meshkat *et al*., 2022; Jiang *et al*., 2017). Similarly, in an animal model of chronic stress, rats subjected to daily acute stress showed decreased expression of BDNF in the hippocampus and frontal cortex (Xu *et al.*, 2006). Another study showed that stress -induced dysregulation of BDNF expression is dependent on the specific

brain region. In this regard, the authors showed BDNF expression was decreased in the hippocampus, neocortex and amygdala, while increased in the hypothalamus (Russo-Neustadt *et al.*, 2001; Lippmann *et al.*, 2007). While few studies have investigated the mRNA expression of BDNF following chronic administration of ACTH, in rats treated with ACTH for 14 days, no effect on BDNF protein expression was found in the hippocampus of these animals (Li *et al*., 2006). This highlights the need for further research to elucidate the role of BDNF expression in MDD.

CREB is a transcription factor responsible for regulating many genes through binding to gene promoters. BDNF is one of the genes regulated by CREB (Gass & Riva, 2007). Many antidepressants lead to the activation of CREB to enhance levels of BDNF (Krishnan & Nestler, 2011). Chronic antidepressant treatment has been shown to upregulate CREB, especially in the hippocampus (Gass & Riva, 2007). However, in rats subjected to chronic stress, CREB expression in the hippocampus or frontal cortex did not differ between stressed rats and healthy rats. However, the ratio of phosphorylated CREB (pCREB) to CREB was reduced in the hippocampus and frontal cortex of stressed rats, suggesting activation of CREB (Xu *et al*., 2006). Similarly, to BDNF, limited knowledge exists on the effect of ACTH induced HPA axis dysregulation on CREB expression.

TrkB is a receptor for BDNF that may elicit antidepressant effects through enhanced neurotransmission (Koike *et al*., 2013). Antidepressant drugs have been shown to enhance TrkB signalling in a chronic social defeat stress model of depression, leading to increased BDNF expression (Jiang *et al*., 2015). In addition, several other related intracellular signalling molecules are involved in pathways mediated by BDNF and CREB. Decreased mammalian target of rapamycin (mTOR) signalling has been shown in the prefrontal cortex of patients with mood and stress-related disorders (Machado-Vieira *et al*., 2017). Akt directly affects the mTOR pathway, with decreased mRNA expression of Akt and mTOR being expressed in mood disorders (Machado-Vieira *et al*., 2017). eEF2 inhibition elicits antidepressant effects and leads to increased BDNF expression (Autry *et al*., 2011). TrkB, Akt, mTOR, and eEF2 intracellular signalling pathways to increase BDNF expression have received considerable attention as potential therapeutic targets for antidepressants and as potential markers of surveillance for depression (Machado-Vieira *et al*., 2017). However, the effect of

ACTH induced HPA axis dysregulation on these molecular pathways are currently unknown.

Table 2.1: A summary of ATCH-induced neurobehavioural and molecular outcomes in rodent models

Animal/ Strain/ Sex	**Dose and duration of ACTH**	**Neurobehavioural outcomes**	**Molecular outcomes**	**Reference**
Male Wistar rats	100µg/day, 15 days	No significant differences in behaviour between ACTH treated rats and control rats in the OFT and FST. SPT: rats treated with ACTH displayed greater sucrose preference by the tenth day of a 3-week SPT protocol.	Levels of pmTOR were elevated in animals that underwent antidepressant deep brain stimulation in comparison to animals that received ACTH treatment.	Kale *et al*., 2021
Male Wistar rats	100 µg/day, 14 days	No significant differences in behaviour between ACTH treated rats and control rats in the OFT and FST. Significant differences in neurobehaviour were shown in rats that received ACTH and antidepressant treatment in comparison to rats either receiving only saline or only ACTH	Plasma corticosterone was elevated in ACTH treated rats in comparison to control rats following exposure to the FST. Plasma ACTH was significantly higher in ACTH treated rats than in control rats following FST exposure.	Pereira *et al*., 2019
Male Wistar rats	100µg/day, 14 days	Compared with the control group in the SPT, rats in the ACTH group showed a significant reduction in sugar preference. Rats treated with ACTH displayed lower locomotor and increased rearing than control rats. Immobility during the FST and TST was increased in rats treated with ACTH in comparison to control rats.	Concentration of serum serotonin and dopamine were decreased in rats treated with ACTH than control rats. Serum IL-6 and TNF-α were increased in rats treated with ACTH than control rats.	Song *et al*., 2019

Male Sprague-Dawley rats	100 µg/day, 15 days	OFT: Rearing time was greater in ACTH treated animals versus control animals. FST: No differences between ACTH treated animals and control animals.	Dopamine was greatly decreased in rats treated with ACTH than in control rats following FST exposure. Serotonin, noradrenalin, and adrenalin were increased in ACTH treated rats than in control rats following FST exposure	Walker *et al*., 2013
Male Wistar rats	10 µg/day, 28 days	OFT: Time spent, and distance travelled in the central zone was reduced, while locomotor activity was unaffected. FST: Immobility was increased in ACTH treated animals	In stressed rats that underwent the FST, plasma ACTH was higher in ACTH rats than in control rats. Plasma corticosterone was increased in ACTH treated rats in comparison to control rats.	Petrović *et al*., 2018
Male Wistar rats	100 µg/day, 14 days	No significant differences in behaviour between ACTH treated rats and control rats in the OFT and FST. Significant differences in neurobehaviour were shown in rats that received ACTH and antidepressant treatment in comparison to rats either receiving only saline or only ACTH	Protein quantification found that expression of CaMKII, GSK3 an AMPK is associated with lower immobility time, in rats that received high frequency deep brain stimulation.	Kim *et al*., 2016
Male ddy mice	0.45 mg/kg, 14 days	No significant differences in behaviour between ACTH treated rats and control rats in the FST.	Serum corticosterone was greatly increased in ACTH treated mice than in control mice.	Iwai *et al*., 2013

Male Sprague-Dawley rats	100 g/day, 15 days	No significant differences in behaviour between ACTH treated rats and control rats in the OFT and FST. Significant differences in neurobehaviour were shown in rats that received ACTH and antidepressant treatment in comparison to rats either receiving only saline or only ACTH	Akt levels were elevated in ACTH treated rats than in control rats. Levels of mTOR were elevated in ACTH treated rats in comparison to control rats.	Walker *et al*., 2019
Male Wistar rats	100 g/day, 14 days	Immobility time in ACTH treated was higher than that of control rats.	Glutamine, glycine, and the ratio of glutamate and glutamine in the frontal cortex was elevated in ACTH treated rats than in control rats. In the hippocampus, glutamine, glycine, L-Serine, D-Serine, and glutamate/glutamine ratio was increased in ACTH treated rats than in control rats. In the striatum, glutamine glycine and glutamate/glutamine ratio were higher in ACTH treated rats than controls. In the cerebellum, glutamate, glutamine, glycine, L-Serine, and D-Serine were all elevated in ACTH treated in comparison to controls.	Tokita *et al*., 2012
Male Wistar rats	50-100µg/day, 14 days	No significant differences in behaviour between ACTH treated rats and control rats in the OFT and FST. Significant differences in neurobehaviour were shown in rats that received ACTH and antidepressant treatment in comparison to rats either receiving only saline or only ACTH	Plasma corticosterone levels were higher in ACTH treated rats than in control rats.	Kitamura *et al*., 2002

2.2.2 Corticotropin-releasing hormone overexpressing mice

Another model of HPA-axis dysregulation that has been established is a transgenic mouse strain that overexpresses CRH (Vale *et al*., 1991; Kang *et al.*, 2020). These CRH over-expressing transgenic mice were originally developed to investigate the physiological effects of high levels of CRH that may lead to Cushing's syndrome (Kang *et al*., 2020). CRH over-expressing mice displayed anxiety-like behaviours, shown by decreased locomotor activity and decreased time spent in central zones of the open-field test, and higher levels of plasma corticosterone, indicative of chronic HPA axis activation (Stenzel-Poore *et al.*, 1992; Kang *et al.*, 2020). In addition, during the elevated plus maze test, CRH over-expressing transgenic mice spent more time in the enclosed arms in comparison to control mice that spent more time in the open arms of the maze, also suggesting an anxious phenotype (Stenzel-Poore *et* al., 1994). The hypersecretion of CRH in mice mimics the HPA axis dysregulation often observed in MDD, therefore these mice are frequently used to study the physiological effects of the HPA axis dysregulation. However, a well-researched side effect of CRH overexpressing mice is that of muscle atrophy (Kang *et al*., 2020). Muscle atrophy in transgenic mice may limit depression and anxiety studies by introducing a confounding factor in the analysis of neurobehavioural tests, influencing mobility and locomotor activity parameters.

2.2.3 Early life stress

Stressful early life events, such as maternal separation, are associated with the development of behavioural and neuroendocrine abnormalities and therefore serve as a model of depression in rodents (Lee *et al.*, 2007). Maternal separation is the most used preclinical model of early life stress. Maternal separation occurs when pups are separated from the mother during the first hours to first few days of life (Lee *et al., 2007)*. Maternal separation affects the HPA axis response, which may become dysregulated due to high levels of corticosterone, especially in the later stages of life. Studies have found that following maternal separation, serum levels of ACTH and corticosterone were increased in the first 2 weeks of life (Meaney & Szyf, 2005; Wang *et al*., 2017). Additionally, the expression of BDNF was significantly decreased in the hippocampus and prefrontal cortex of rats that experienced maternal separation (Meaney & Szyf, 2005; Wang *et al*., 2017). Therefore, it is suggested that maternal

separation is a model of dysregulation of the HPA axis in adult life with changes in circulating glucocorticoids and the expression of neurotrophic factors.

2.2.4 Chronic unpredictable mild stress (CUMS)

The chronic unpredictable mild stress (CUMS) model of depression induces HPA axis dysregulation through the repeated exposure of rats or mice to multiple stressors over several weeks (Wang *et al*., 2017). Multiple stressors are used in the CUMS model with cage tilting, food and water deprivation and soiled bedding being as commonly used stressors (Wang *et al.,* 2017). The CUMS model aims to increase circulating glucocorticoids through activation of the HPA axis, further leading to dysregulation via inhibition of negative feedback (Boyle *et al.*, 2005).

Taken together, several models of HPA axis dysregulation in animals exist. However, majority of these models introduce confounding factors that may limit the ability to study changes in neurobehaviour and molecular markers of depression. The administration of exogenous ACTH as a model of HPA axis dysregulation has received considerable attention as a suitable model for studying neurobehavioural and molecular changes, with minimal discomfort to the animal. However, limited evidence exists that relate a comprehensive set of neurobehavioural outcomes to molecular changes in this model, and hence require further investigation.

2.3 Problem statement

In summary, several theories and pathophysiological mechanisms have attempted to explain the development of depression. The heterogeneity in these theories partly explains the failure of traditional antidepressant drugs to effectively treat and manage depressive symptoms. Dysregulation of the HPA axis has been implicated in the development of MDD and resistance to commonly prescribed antidepressant drugs. It is therefore imperative that potential novel therapeutics target HPA axis dysregulation, which in turn requires a greater understanding of ACTH-induced depressive symptoms and the pharmacodynamic effects of current antidepressants. In our laboratory, we have established a rodent model of depressive-like symptoms through chronic administration of ACTH (100 µg, s.c.) daily, for at least two weeks (Sallie *et al*., 2024). Chronic administration of ACTH in rodents has previously resulted in resistance to two tricyclic antidepressants (Kitamura *et al*., 2002; Petrović *et al*., 2018). In our rodent model of depression via chronic ACTH administration, the use of the antidepressant

imipramine, a TCA was ineffective at reversing the depressive-like phenotype in rodents (Sallie *et al*., 2024). However, to further validate whether this model is indeed a model of TRD, the efficacy of an additional class of antidepressant needs to be evaluated.

Therefore, the purpose of this study is to validate a rodent model of HPA axis dysregulation induced depression using a second class of antidepressants, i.e., an SSRI, citalopram. Neurobehavioural outcomes will be used to assess face validity where the sucrose preference test and the OFT will be used to assess phenotypic changes. The sucrose preference test will be used to assess anhedonia-like symptoms. While the OFT is used to assess anxiety-like and exploratory behaviour. In addition, construct validity will be assessed via investigating the expression of intracellular signalling molecular markers associated with MDD.

2.4 Aim

The aim of this study was to validate a rodent model of depression via the administration of ACTH, and to investigate the effects of citalopram (an SSRI) on depressive-like symptoms using a neurobehavioural battery of tests and the regional brain expression of molecular markers of depression.

2.4.1 Objectives

In a rodent model of chronic ACTH administrations, the specific aims were to:

- investigate the changes in depressive-like symptoms using neurobehavioural responses in the open-field test as a measure of locomotor activity and anxiety, and the sucrose preference test as a measure of anhedonia.
- determine the effects of chronic treatment with citalopram, a selective serotonin reuptake inhibitor, on depressive-like symptoms using neurobehavioural tests.
- investigate changes in the regional brain mRNA expression of BDNF, CREB, TrkB, Akt, mTOR, and eEF2 in the brain using real-time polymerase chain reaction (PCR), following the administration of ACTH and citalopram.
- determine sex differences in depressive-like symptoms using neurobehavioural tests and molecular markers of depression following ACTH administration and citalopram treatment.

CHAPTER 3:

METHODS

3.1 Ethical clearance

Ethical approval for this study was received from the institutional Animal Research Ethics Committee of the University of the Witwatersrand (approval reference: 2022/03/06/C).

3.2 Animals

Eighty Sprague-Dawley rats (male n=40; female n=40) were obtained from the Wits Research Animal Facility (WRAF) at the University of the Witwatersrand. The animals were housed in a room with a 12-hour light/dark cycle (lights on at 7h00), maintained at ±25°C with a humidity of 30%-40% in polycarbonate rodent cages. Each sawdust-lined cage housed two rats with water and standard rodent pellets *ad libitum*. Male and female rats were housed in separate rooms. Animals were acclimatised for seven days prior to any experimental procedures.

Following exposure to multiple stressors, female rats typically have increased levels of ACTH (due to greater expression of proopiomelanocortin, an ACTH precursor) and corticosterone (Heck & Handa, 2019). In addition, further sex differences exist in the negative feedback regulation of the HPA axis with female rats typically having a delayed return to baseline ACTH and corticosterone, following stress exposure. Therefore, male and female rats were compared to account for hormonal changes and sex-specific changes in the regulation of the HPA axis and in drug metabolism.

3.3 Study design

Following acclimatization and baseline neurobehavioural tests, twenty rats, (females n=10 and males n=10) received administration of ACTH (100µg/day, s.c.) daily for two weeks, to establish a rodent model of HPA axis dysregulation induced depression (Sallie *et al*., 2024; Kitamura *et al*., 2002; Petrović *et al*.,2018) (Figure 3.1). The control animals, (female rats n=10 and male rats n=9) received saline (100µg/day, s.c.) for two weeks. Following the administration of either ACTH or saline for two weeks, all rats received citalopram (10mg/kg.bw) treatment for 4 weeks co-administered with either ACTH or saline. The present study forms part of a larger study in which additional control groups were included (Sallie *et al*., 2024).

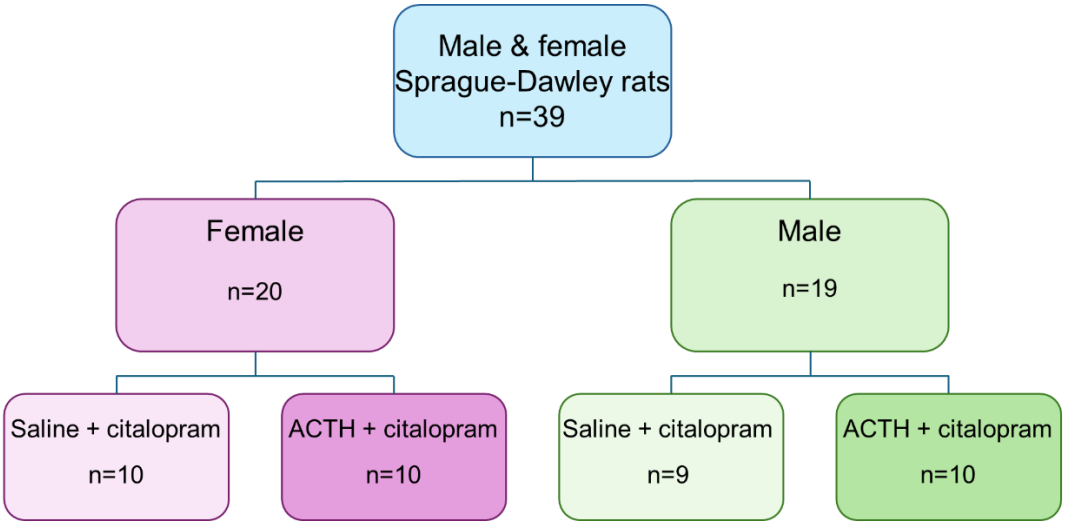

Figure 3.1: Treatment groups used in this study. A total of 39 Sprague-Dawley rats were used. Within the male and female groups, rats were either given saline (100µg/day, s.c.) or ACTH administration (100µg/day, s.c.). All rats received citalopram treatment (10mg/kg.bw, i.p.).

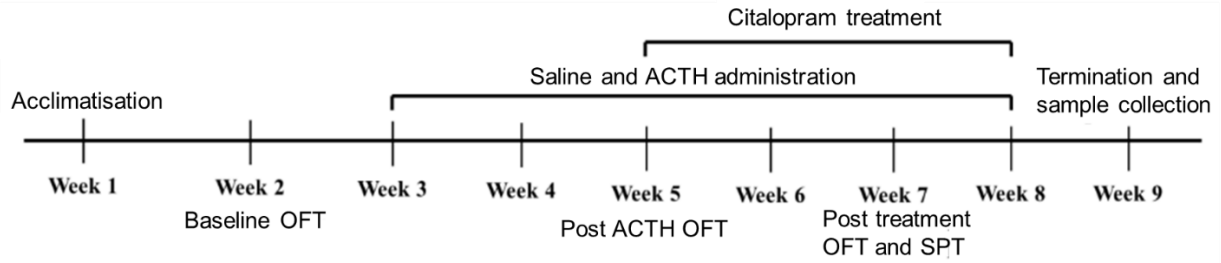

Figure 3.2: Study timeline. The open-field tests (OFT) were done during week 2 following the acclimatization period. During week 3 and 4, the rats only received saline (100µg/day) and ACTH (100µg/day) administration. Between week 5 and week 8, saline and ACTH administration continued, and citalopram (10mg/kg bw) administration began. During week 8, post-treatment OFTs were performed, as well as sucrose preference tests (SPT). During week nine, the rats were terminated, and samples were collected.

3.4 Neurobehavioural analysis

Neurobehavioural tests were performed to establish behaviour at baseline and after treatment with ACTH and citalopram (Figure 3.2). The OFT was performed at 2 weeks to establish behaviour at baseline, at week five to determine changes in behaviour following ACTH administration, and at week seven to determine behavioural changes following citalopram treatment. The SPT was only performed at week 7 to determine sucrose preference following ACTH administration and citalopram treatment. The SPT

is an 8-day protocol and is therefore only done at week 7. Additionally, the protocol cannot be repeated as repeated starvation of rodents would add unnecessary stress experienced by the experimental animals.

3.4.1 Open-field test

The OFT was performed to assess anxiety-like behaviour and locomotor activity. A black Perspex base (90cm x 90cm) with black Perspex walls (20cm) was used as an open field in which the rats were placed. Following acclimatization and prior to any intervention, rats were placed in the open field for 15 minutes to determine baseline performance. The OFT was repeated following two weeks of either ACTH or saline administration, to determine their depressive phenotypes prior to drug treatment. A final OFT was performed following four weeks of citalopram treatment. For all tests, the animal's movement in the open field were recorded. The pre-recorded videos were analysed using EthoVision (Noldus, Wageningen, Netherlands) behavioural tracking software. Analysis parameters included distance travelled, time immobile (Start velocity: 2.00cm/s, stop velocity: 1.75cm/s), average velocity and time spent in the centre vs time spent in the periphery. Healthy rats are expected to show more exploratory type and less anxious behaviour by exploring the centre and periphery of the box. Rats showing a depressive phenotype are expected to show less exploratory behaviour and more anxious behaviour displayed by spending more time in the periphery of the open field than in the centre.

3.4.2 Sucrose preference test

The SPT was used to assess anhedonia in the experimental animals. During the procedure, an adaptation and preference test were performed. During adaption, each cage in the home room contained two bottles for 48 hours. One bottle containing sucrose water and the other regular drinking water. Thereafter, rats were placed in the SPT apparatus for 24 hours for adaptation (Figure 3.3). During the sucrose preference test, the rats were placed in the SPT apparatus with two bottles for 12 hours. Water bottles were weighed before and after the test. The bottles were swapped daily to avoid placement preference behaviour from the rats. The bottles were swapped before apparatus adaptation and the preference test. Anhedonia was defined as a decrease in the sucrose preference ratio in comparison to the control group. Healthy rats are expected to display more pleasure-seeking behaviour by showing a preference

towards sucrose sweetened water. Rats showing a depressive phenotype are expected to have a decreased preference for sucrose, indicating anhedonia. A sucrose preference ratio (represented as a percentage) was obtained to determine the sucrose preference for each animal. Sucrose preference ratio was calculated according to the following formula:

$$\text{Sucrose preference ratio} = \frac{\text{Sucrose intake}}{\text{Sucrose intake + water intake}}$$

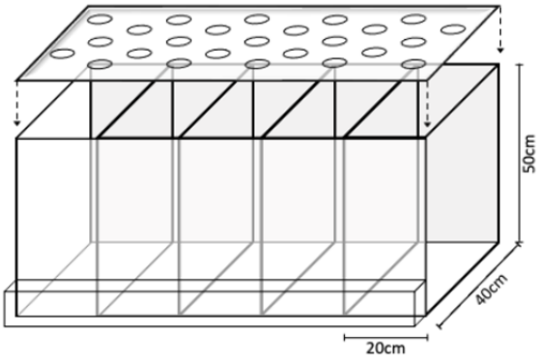

Figure 3.3: Sucrose preference test apparatus. The SPT apparatus consisted of multiple chambers made of Perspex each 20cm wide, 40cm long and 50cm high. Chambers were closed with a Perspex lid with holes once rats are placed into chambers during sucrose tests.

3.5 Termination procedures

Following the four-week treatment period, rats were briefly exposed to isoflurane anaesthesia (2%), prior to being terminated by decapitation using a rodent guillotine. The brain was surgically removed on ice. The prefrontal cortex, cerebral cortex, striatum, hippocampus, hypothalamus, and midbrain were dissected (Figure 3.4). Brain regions were snap-frozen in liquid nitrogen and stored at -80°C for molecular analysis.

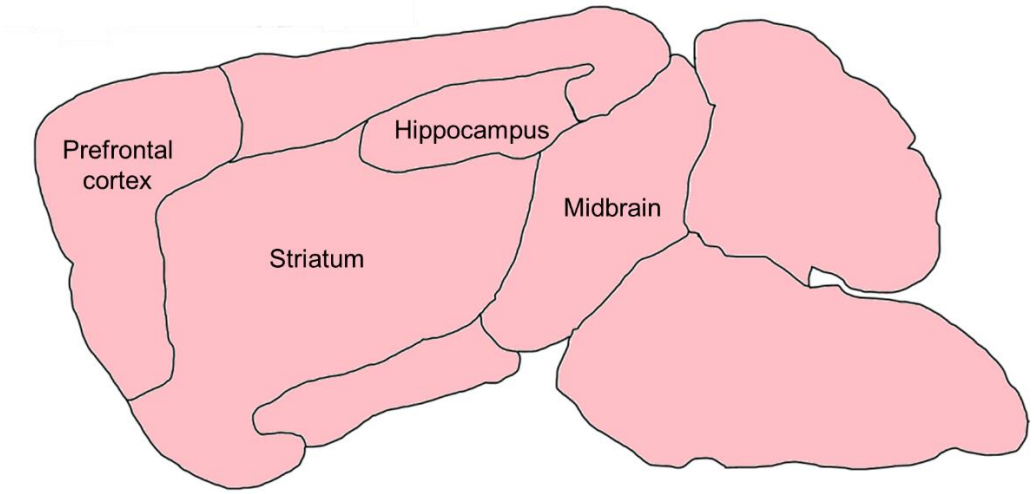

Figure 3.4: Representation of how brain regions were delineated and surgically dissected during termination procedures.

3.6 Tissue analysis

3.6.1 RNA Isolation and cDNA synthesis

Brain regions were homogenized in β-Mercaptoethanol (3.5µl) and lysed in 350µl of lysis buffer, using a probe sonicator. 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 according to manufacturer instructions (ThermoFisher, California, USA) and stored at -80°C prior to gene expression analysis.

3.6.2 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) were determined using real-time PCR. TaqMan assays were used and contained all necessary components to perform PCR of the selected genes of interest. A StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) was used to amplify the genes. Changes in mRNA expression of the genes of interest were evaluated relative to an optimized endogenous control, *TBP*. The $^{\Delta\Delta}$Ct method was used and compared with control animals (Livak & Schmittgen, 2001). In our laboratory, to optimise the housekeeping gene, *TBP,* and *GAPDH* mRNA expression have been compared previously, and we have shown that TBP was the most suitable and stable reference gene for analysis in the brain.

3.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism v9.5 (California, USA) and SAS software (version 9.4). Data are reported as mean ± standard deviation or median and interquartile range, as appropriate. A two-way repeated measures analysis of variance (ANOVA) followed by a Tukey *post hoc* test was used to determine the differences between neurobehavioural outcomes in males and females. A mixed model two-way ANOVA followed by a Tukey *post hoc* test was used to determine differences between gene expressions, of the different treatment groups, and between sexes. Statistical significance was considered at $p<0.05$. An *a priori* power calculation showed that using repeated measures ANOVA, 36 animals will be required to achieve 80% statistical power with an alpha level of 0.05 with a small effect size of 0.23. Therefore, a total of 19 animals were used in the healthy group and 20 rats in the depressed group. Originally, 20 animals were used in the healthy group, however, due to an unforeseen illness one rat was terminated earlier and not included in the analysis.

CHAPTER 4:

RESULTS

4.1 Neurobehavioural analysis

4.1.1 Open-field test

There were no significant differences in the total distance, mean velocity, frequency of central zone entries, duration in the central zone, and total activity between any of the groups (all $p>0.05$) (Figures 4.1 and 4.2). Immobility increased in female saline + citalopram rats after 6 weeks compared to baseline ($p=0.006$). Immobility significantly increased in ACTH + citalopram female rats after 2 weeks ($p=0.04$) and 6 weeks ($p=0.004$) of ACTH administration compared to baseline (Figure 4.1C). Immobility was also increased in saline + citalopram male rats after 6 weeks when compared to baseline ($p=0.05$) (Figure 4.1C). Similarly, immobility was significantly increased in ACTH + citalopram male rats after 6 weeks compared to baseline ($p=0.01$). While no statistical significance was shown, male rats that received ACTH + citalopram showed a downward trend in the duration spent in the central zone between the 2 week and 6-week period ($p=0.22$) (Figure 4.2C).

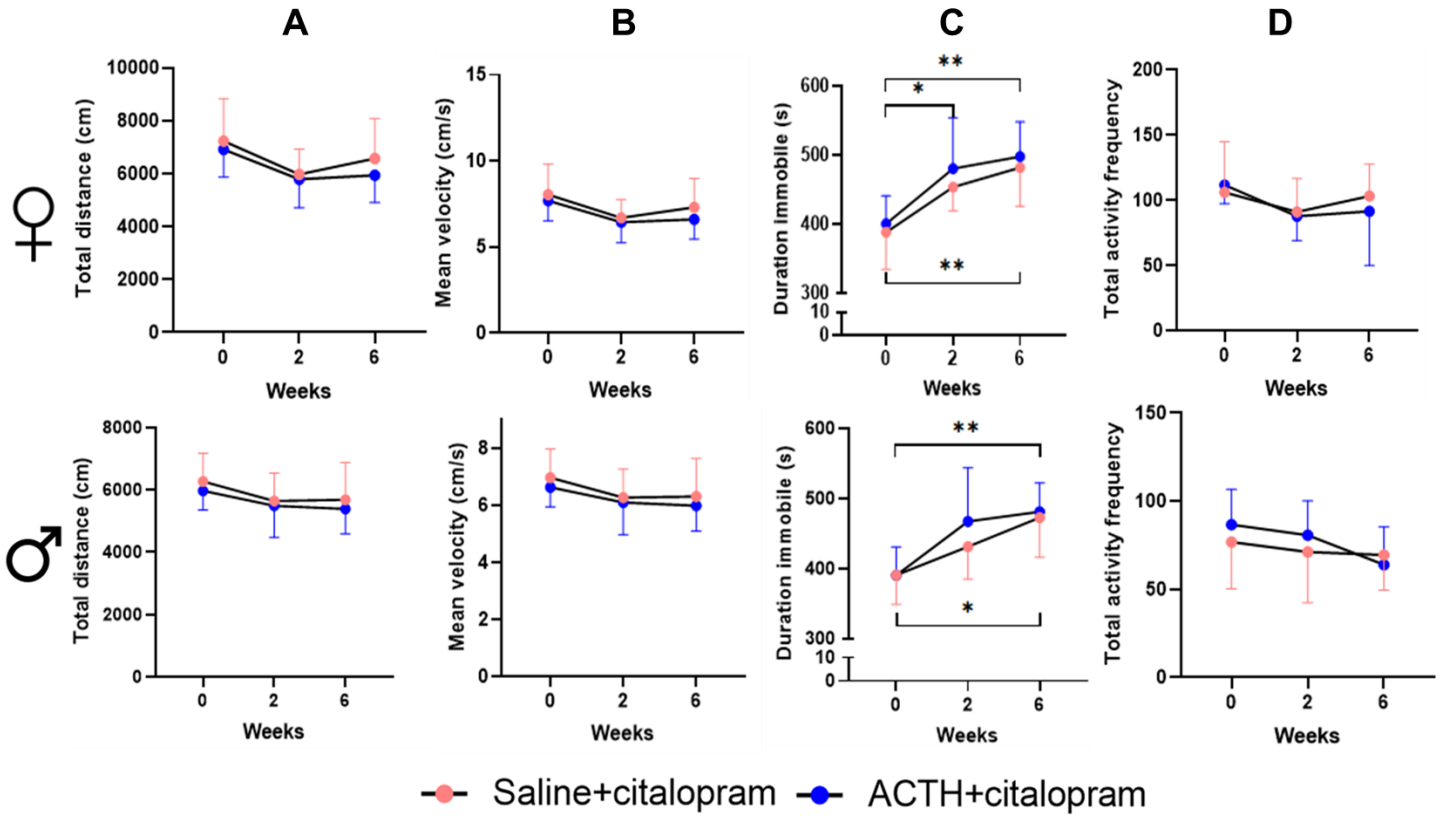

Figure 4.1: Effect of ACTH and citalopram on parameters of locomotor activity during the OFT including total distance travelled (A), mean velocity (B), duration immobile (C), and total activity (D) in male and female rats (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). Measurement time points include 0 weeks (baseline measurement), 2 weeks (post-ACTH administration), and 6 weeks (post-citalopram treatment). Data is represented as mean ± SD. $p<0.05$*, $p<0.01$**. A repeated measures two-way ANOVA was used followed by a Tukey *post-hoc* test.

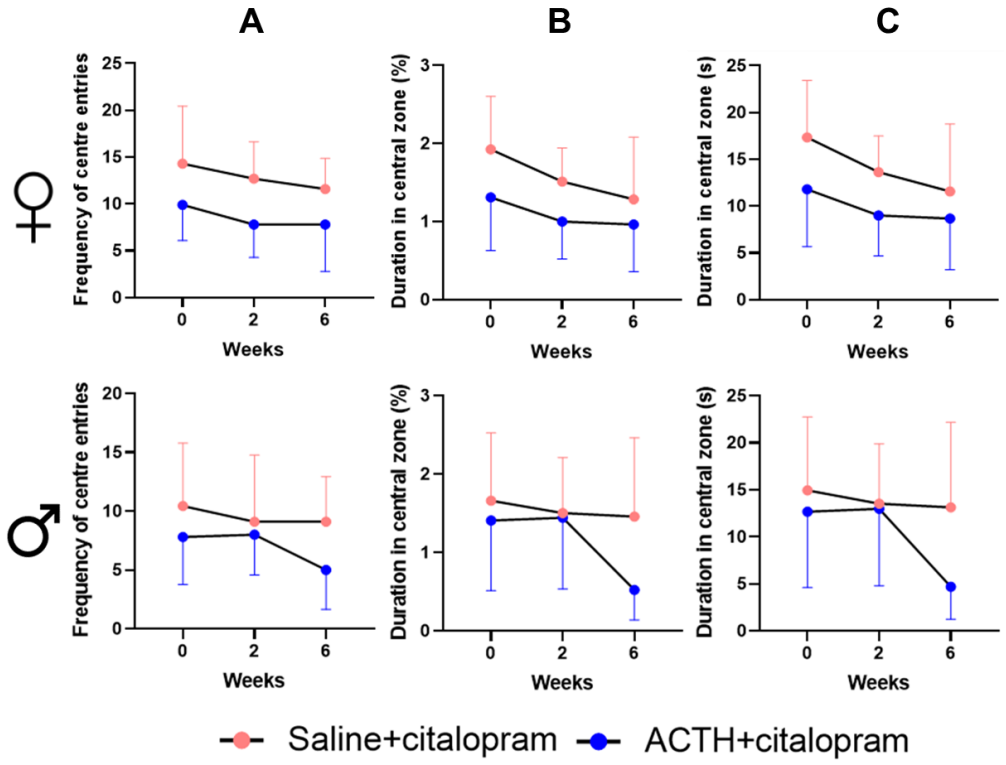

Figure 4.2: Effect of ACTH and citalopram on parameters of anxiety-like behaviour during the OFT. Parameters measured included frequency of central zone entries (A), percentage of total time spent in central zone (B), and duration spent in central zone (C) in male and female rats (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). Measurement time points include 0 weeks (baseline measurement), 2 weeks (post-ACTH administration), and 6 weeks (post-citalopram treatment). Data are presented as mean ± SD. A repeated measures two-way ANOVA was used followed by a Tukey *post-hoc* test.

4.1.2 Sucrose preference test

The SPT showed that normal drinking water consumption was significantly higher in ACTH + citalopram females than in saline + citalopram females ($p=0.004$; Figure 4.3A). Similarly in male rats, there was a trend in which ACTH + citalopram rats drank significantly more regular water than saline +citalopram ($p=0.06$). ACTH administration resulted in a significantly lower sucrose preference ratio in ACTH + citalopram males than saline +citalopram males ($p=0.007$; Figure 4.3D), but not in females. These results suggest a sex-specific effect of ACTH, with ACTH administration having a greater effect in male rats.

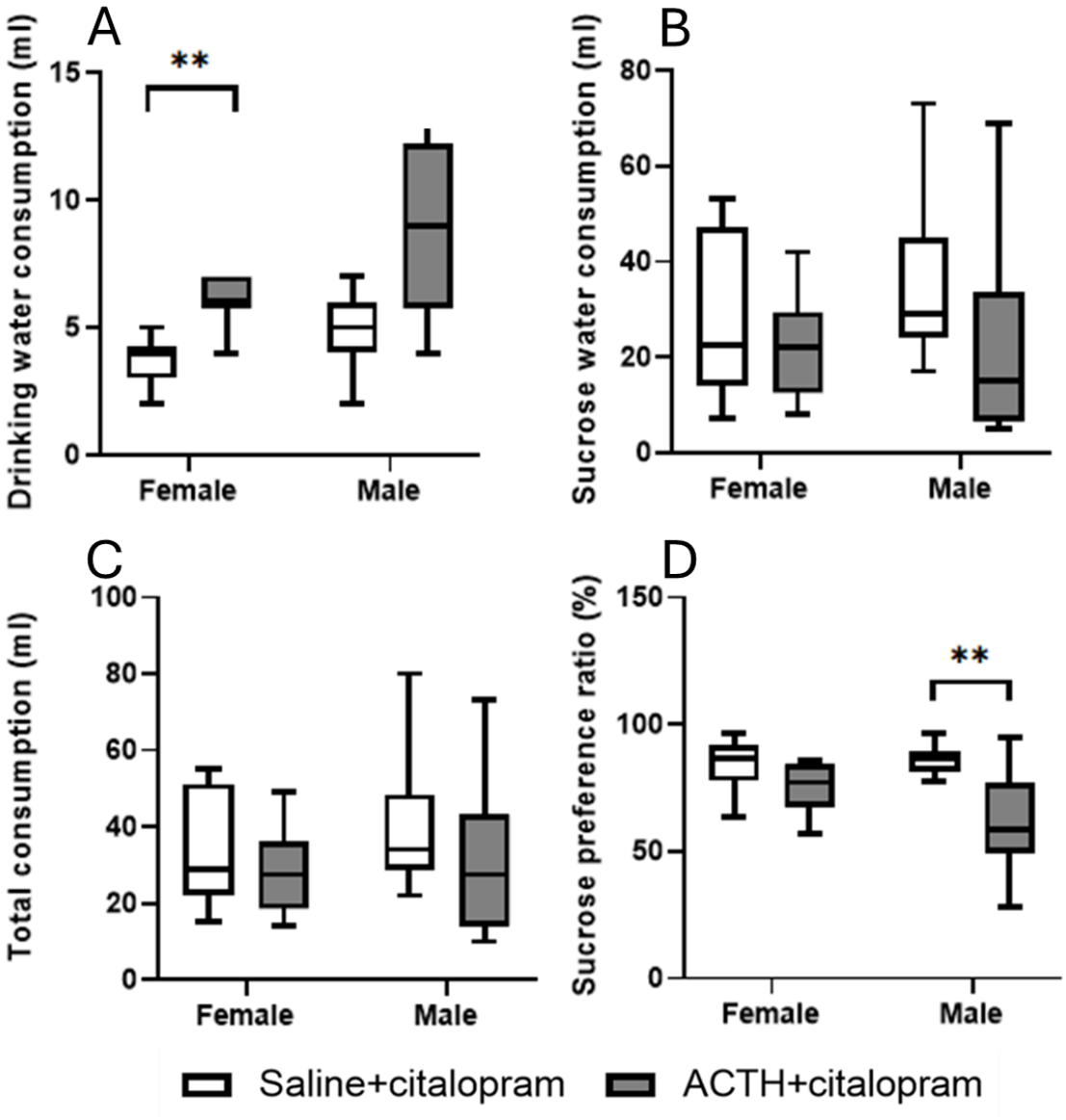

Figure 4.3: Drinking behaviour and sucrose preference measured using the SPT. Drinking water consumption (A), sucrose water consumption (B), total water consumption (C) and the sucrose preference ratio (D) in female and male rats (female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). Data are presented as median and interquartile range (IQR). $p<0.05$*, $p<0.01$**. ACTH, adrenocorticotropic hormone.

4.2 Molecular markers expression in different brain regions

4.2.1 Neurotrophic factors

4.2.1.1 Regional brain mRNA expression of *BDNF*

Gene expression analysis showed a significant overall sex effect for *BDNF* mRNA expression in the striatum ($p=0.03$; Figure 4.4B) and midbrain ($p<0.001$; Figure 4.4D). *Post-hoc* analysis showed that *BDNF* mRNA expression was significantly decreased in the striatum of ACTH + citalopram males compared to ACTH + citalopram females ($p=0.007$). In contrast in the midbrain, ACTH + citalopram females had lower *BDNF* mRNA expression compared to ACTH + citalopram males ($p=0.01$). Administration of ACTH did not result in *BDNF* mRNA expression changes in the prefrontal cortex ($F=0.38$; $p=0.77$) or hippocampus ($F=0.41$; $p=0.75$). These results suggest a sex-specific and region-specific effect in the mRNA expression of *BDNF.*

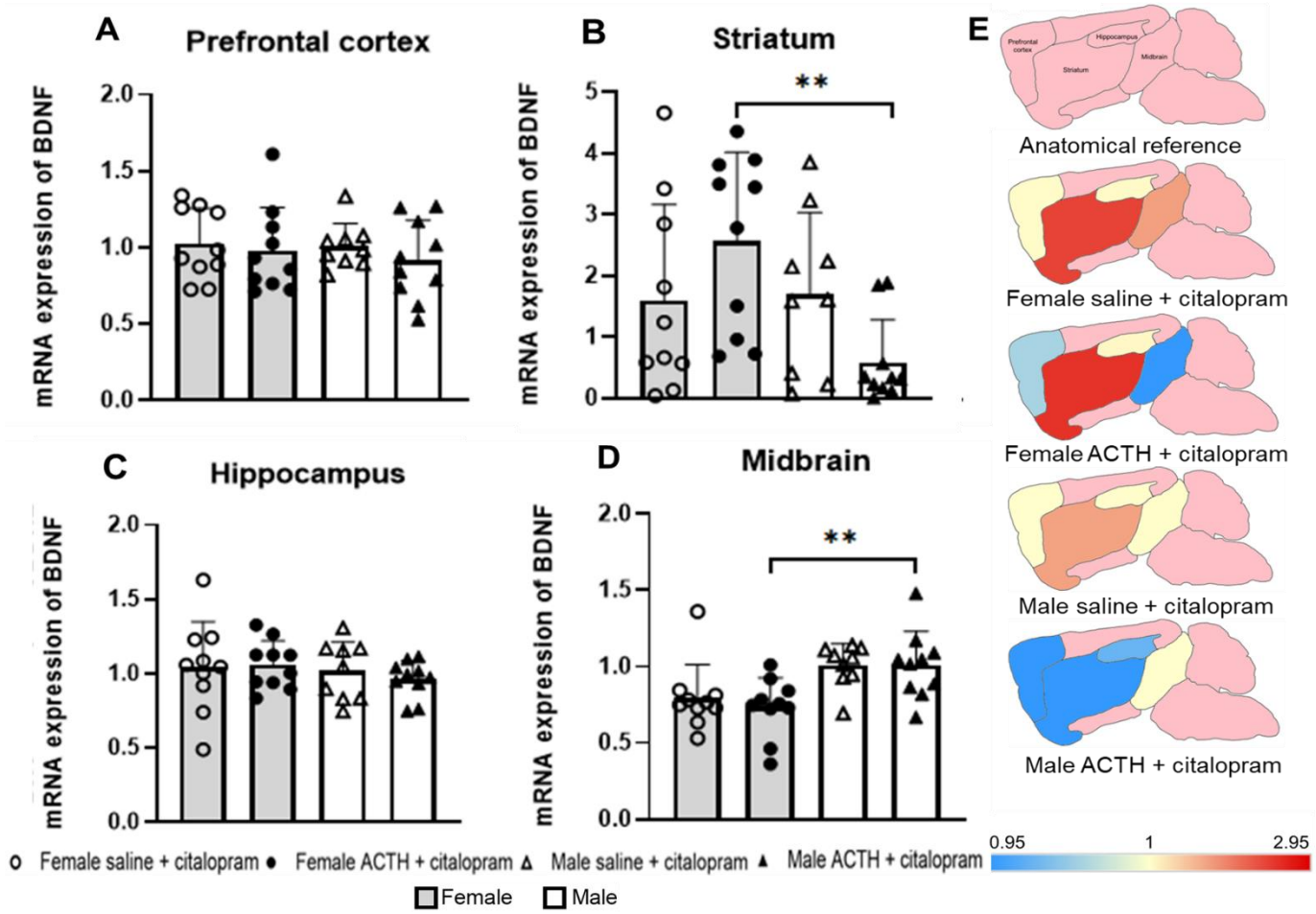

Figure 4.4: Regional mRNA expression of BDNF in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of BDNF are shown in E. Data are presented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). $p<0.01$**. BDNF, brain-derived neurotrophic factor; ACTH, adrenocorticotropic hormone.

4.2.1.2 Regional brain mRNA expression of *CREB*

Administration of ACTH in males and females resulted in a sex (both $p<0.0001$) and treatment group (both $p<0.05$) effect in the prefrontal cortex and midbrain (Figure 4.5). However, there was no effect on *CREB* mRNA expression in the striatum ($F= 1.73$; $p=0.18$) or hippocampus ($F=1.05$; $p=0.05$). *Post-hoc* tests showed that female saline + citalopram rats had significantly lower *CREB* mRNA expression than male saline + citalopram rats in the prefrontal cortex ($p<0.0001$; Figure 4.5A). Similarly, female ACTH +citalopram rats had significantly lower *CREB* mRNA expression than male ACTH + citalopram rats in the prefrontal cortex ($p<0.0001$). *Post-hoc* analysis showed that in the midbrain saline + citalopram females had lower *CREB* mRNA expression than saline + citalopram males ($p<0.0001$; Figure 4.5D). Similarly, in the midbrain, ACTH +citalopram females had significantly lower levels of *CREB* mRNA expression than ACTH + citalopram males ($p<0.0001$). Interestingly, ACTH administration led to increased *CREB* mRNA expression in males compared to saline + citalopram males ($p=0.05$).

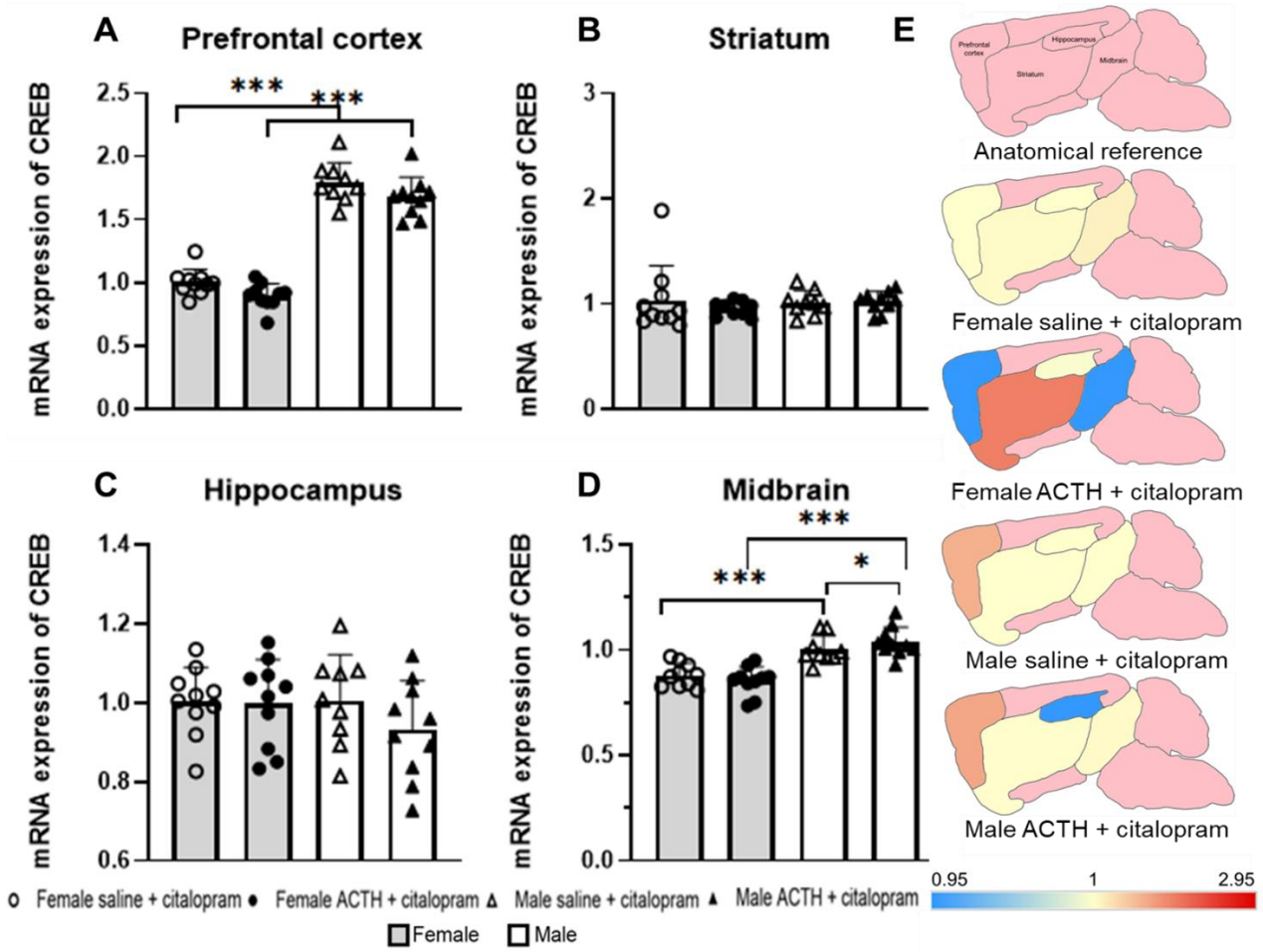

Figure 4.5: Regional mRNA expression of CREB in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of CREB (E). Data is represented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). P<0.05*, p<0.001***. CREB, cAMP response element binding protein; ACTH, adrenocorticotropic hormone.

4.2.2 Intracellular signalling pathways

4.2.2.1 Regional brain expression of *TrkB* mRNA

There was a sex effect in the *TrkB* mRNA expression in the prefrontal cortex (p=0.005; Figure 4.6A). In the prefrontal cortex, ACTH + citalopram females had a higher mRNA expression than ACTH + citalopram males (p=0.02). In the striatum, ACTH administration led to a higher *TrkB* mRNA expression in females (p=0.04; Figure 4.6B). No significant differences in *TrkB* mRNA expression were observed in the hippocampus (F=1.07; p=0.37) or midbrain (F=0.1; p=0.96).

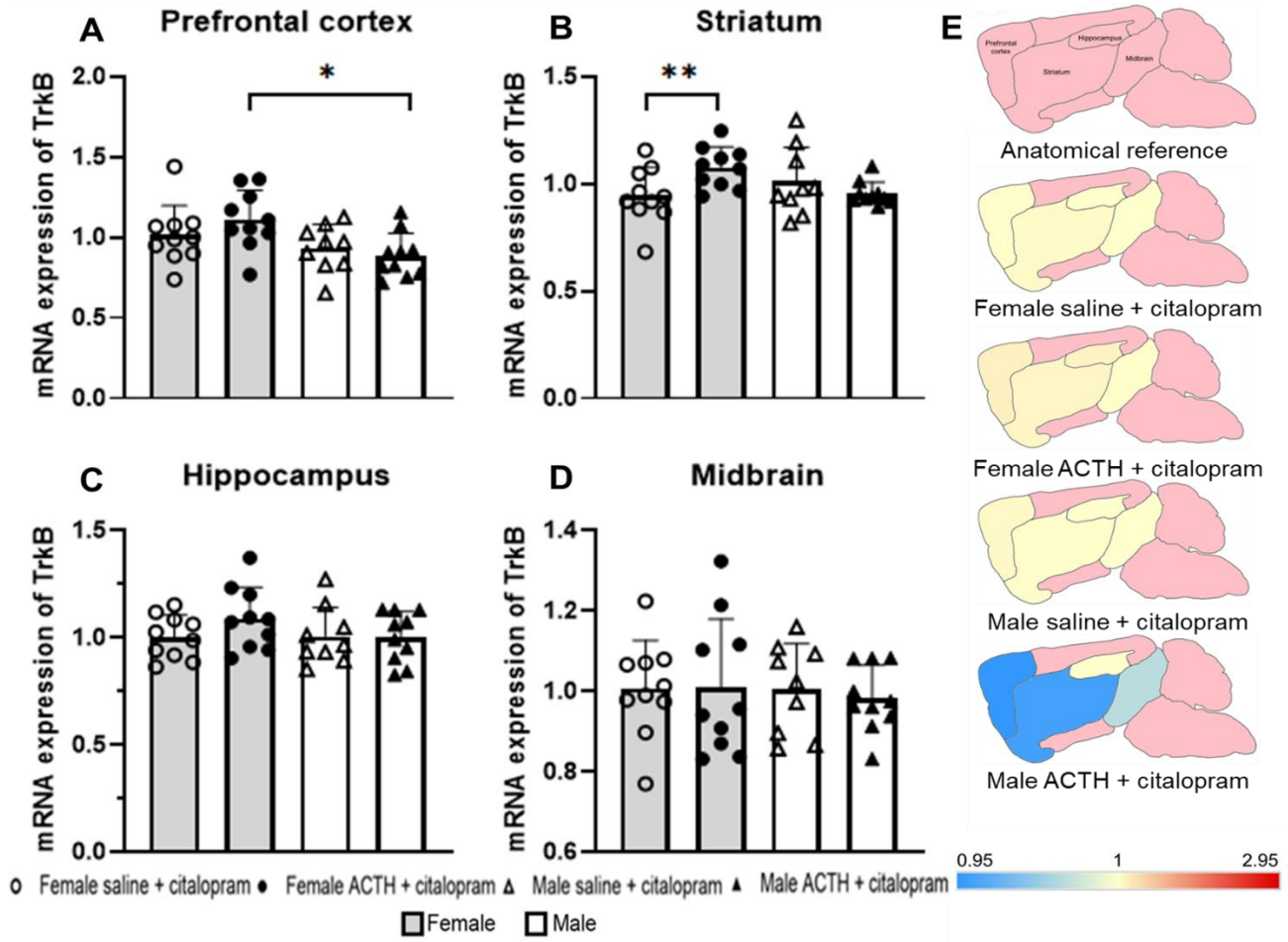

Figure 4.6: Regional mRNA expression of TrkB in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of TrkB (E). Data is represented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). TrkB, tyrosine receptor kinase B; ACTH, adrenocorticotropic hormone.

4.2.2.2 Regional brain expression of *Akt* mRNA

There was a sex effect in the mRNA expression of *Akt* in the prefrontal cortex ($p=0.02$; Figure 4.7A), the striatum ($p=0.03$; Figure 4.7B) and the hippocampus ($p<0.0001$; Figure 4.7C). *Post-hoc* tests showed that in the prefrontal cortex, ACTH +citalopram male rats had higher *Akt* mRNA expression than ACTH + citalopram females ($p=0.05$). In the striatum, *Akt* mRNA expression ACTH + citalopram females were higher than ACTH +citalopram males ($p=0.02$). Similarly in the hippocampus, *Akt* mRNA expression was higher in ACTH + citalopram females than in ACTH +citalopram males ($p=0.001$). No significant changes were observed in *Akt* mRNA expression in the midbrain ($F=1.69$; $p=0.19$).

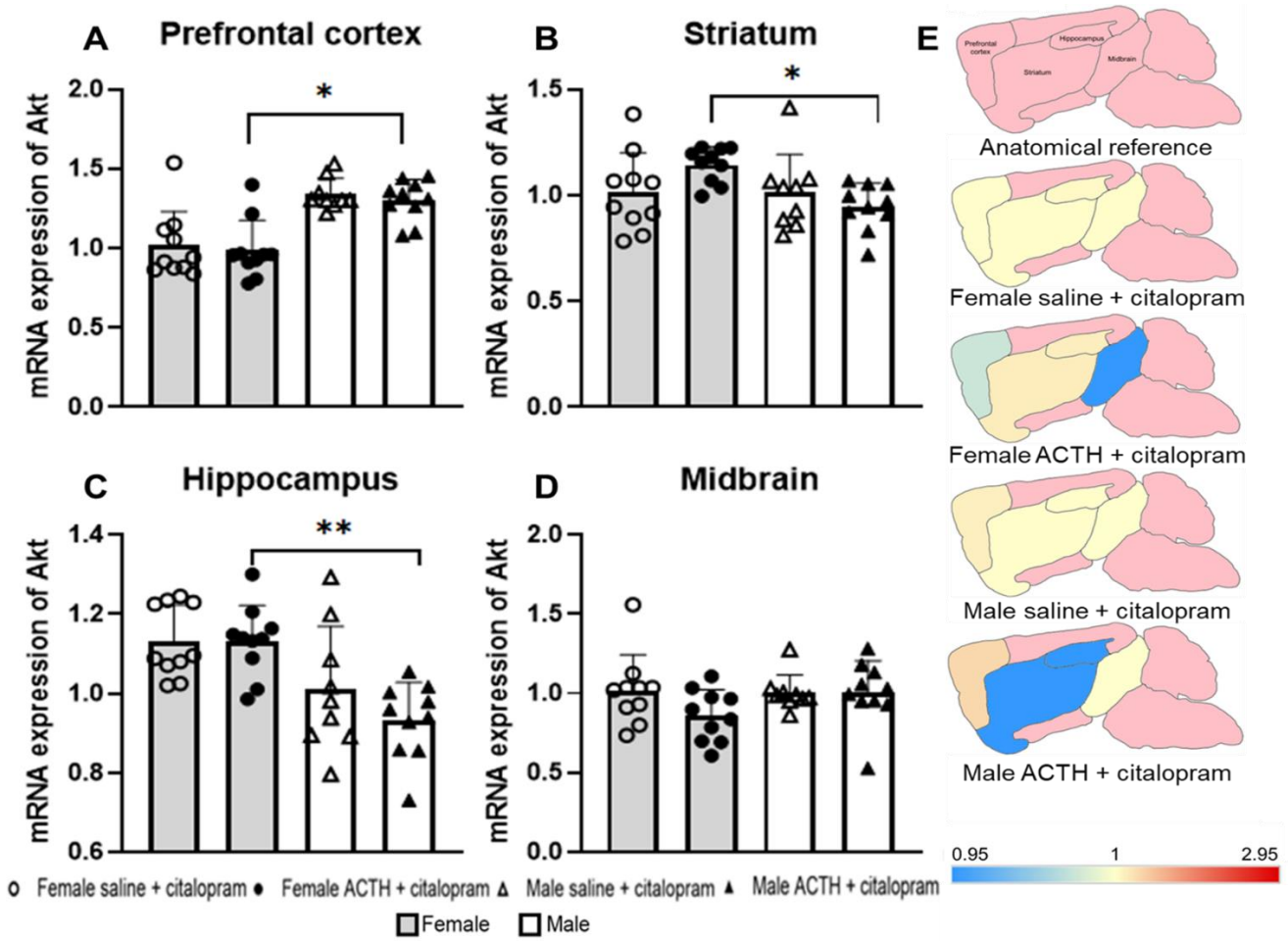

Figure 4.7: Regional mRNA expression of Akt in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of Akt (E). Data are presented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). P<0.05*, p<0.01**. Akt, protein kinase B; ACTH, adrenocorticotropic hormone.

4.2.2.3 **Regional brain expression of mTOR mRNA**

No significant differences were observed in *mTOR* mRNA expression in the prefrontal cortex (F=1.59; p=0.21), striatum (F=0.58; p=0.63), hippocampus (F=0.13; p=0.94), or midbrain (F=0.05; p=0.98) (Figure 4.8).

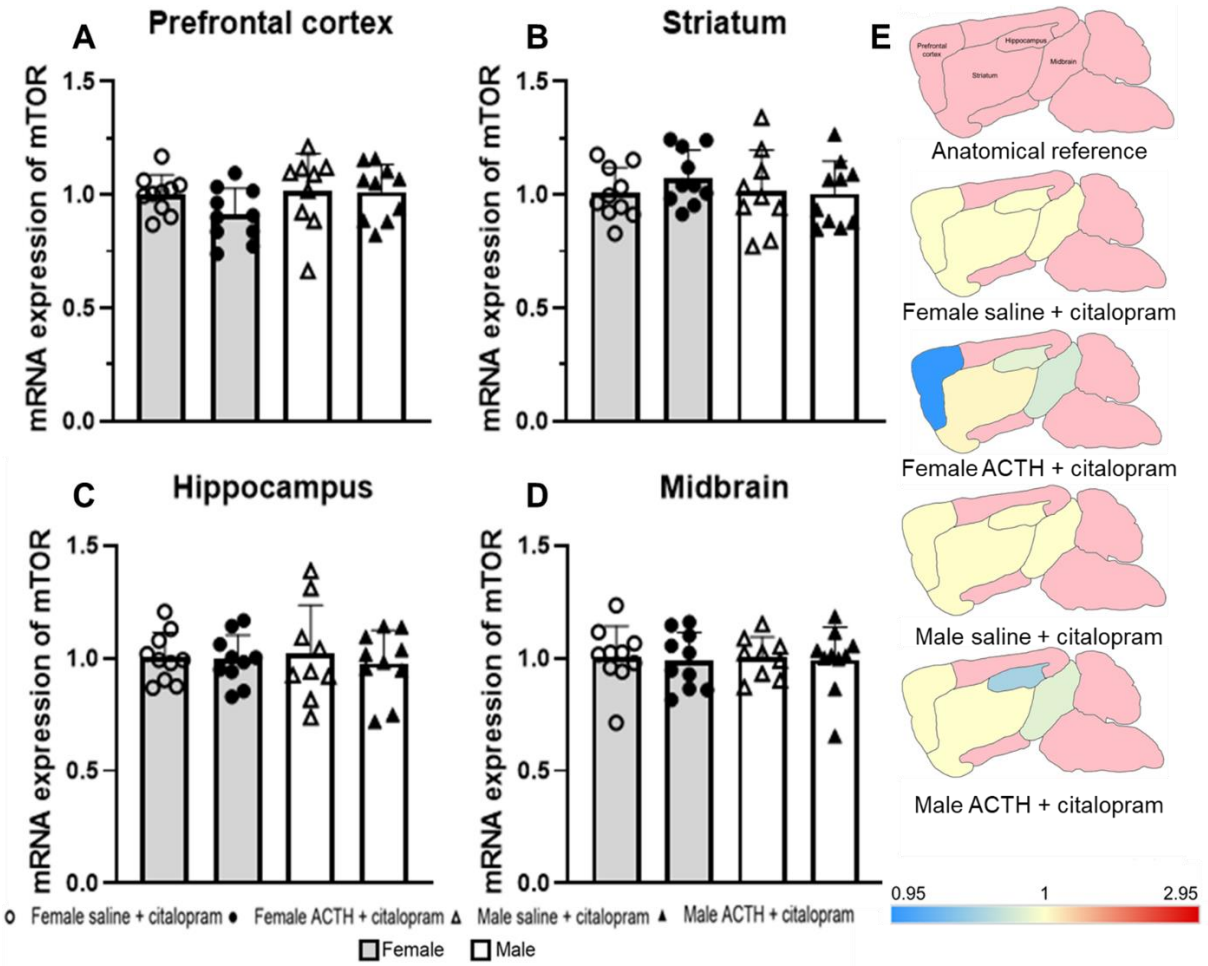

Figure 4.8: Regional mRNA expression of mTOR in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of mTOR (E). Data is represented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). mTOR, mammalian target of rapamycin; ACTH, adrenocorticotropic hormone.

4.2.2.4 Regional brain expression of eEF2

No significant differences were observed in *eEF2* mRNA expression in the prefrontal cortex (F=0.57; p=0.64), striatum (F=1.14; p=0.34), or hippocampus (F=1.04; p=0.39) (Figure 4.10). In the midbrain, ACTH + citalopram females had a lower *eEF2* mRNA expression than ACTH + citalopram males (p=0.02; sex effect p=0.04) (Figure 4.10D).

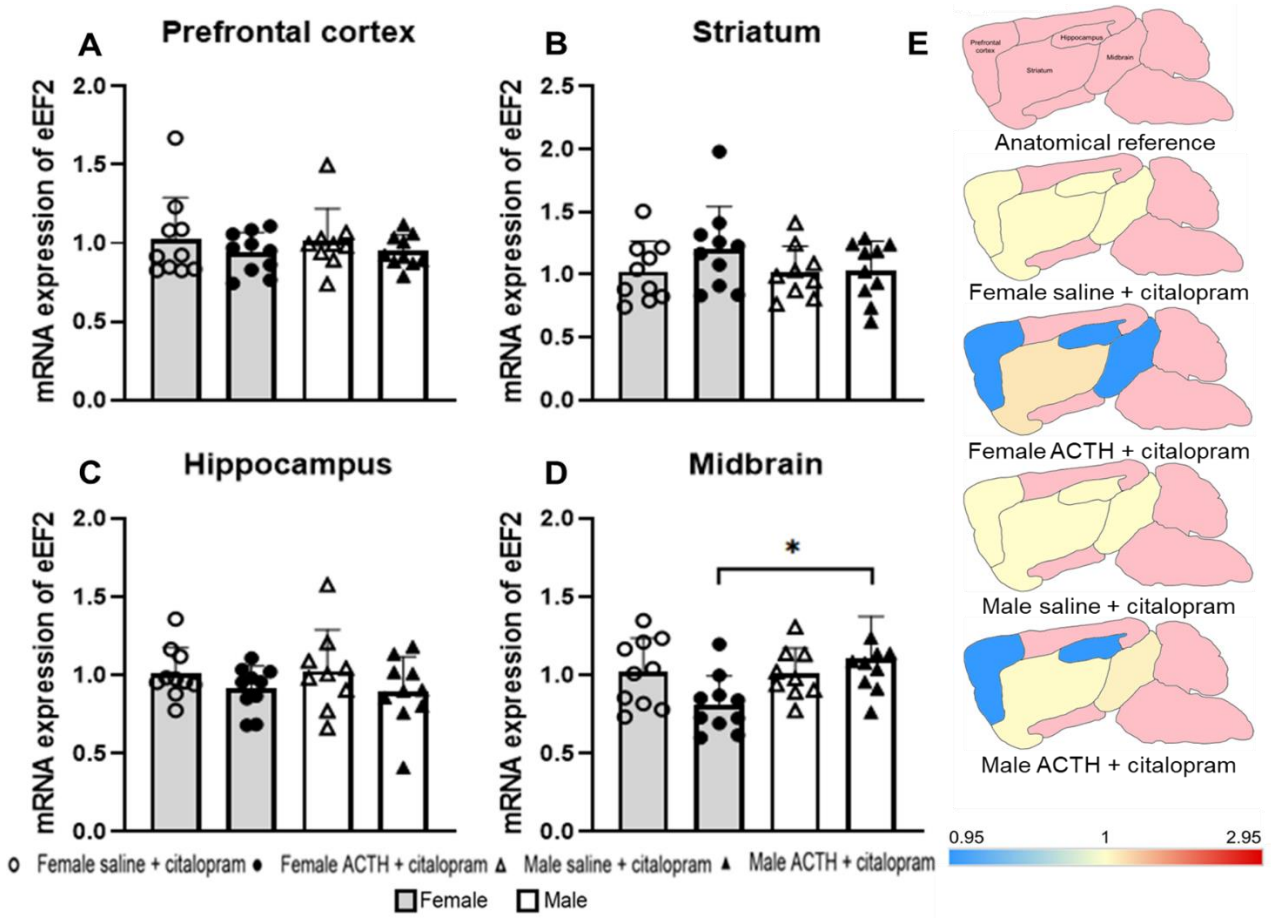

Figure 4.9: Regional mRNA expression of eEF2 in the prefrontal cortex (A), striatum (B), hippocampus(C), and midbrain (D). Sagittal rat brain section heat maps representing the fold change in regional mRNA expression of eEF2 (E). Data is represented as mean ± SD (Female saline + citalopram n=10; female ACTH + citalopram n=10; male saline + citalopram n=9; male ACTH +citalopram n=10). $p<0.05$*. eEF2, eukaryotic elongation factor 2; ACTH, adrenocorticotropic hormone.

CHAPTER 5:

DISCUSSION

The pathophysiology of MDD is complex and multifactorial, with many theories being proposed. Of these theories, dysregulation of the HPA axis has emerged as a leading theory, mainly due to the role of cortisol in regulating the physiological stress response. In this study we used the administration of exogenous ACTH to dysregulate the HPA axis and evaluated the subsequent phenotypic changes using neurobehavioural tests, and the mechanistic effects by evaluating changes in molecular markers in different brain regions using PCR. The main findings of the present study are that ACTH administration resulted in increased immobility in both male and female rats in the open field test, suggesting that HPA axis dysregulation results in a change in locomotor activity. In addition, ACTH administration decreased sucrose preference in male rats only, suggesting anhedonia induced by HPA axis dysregulation. These neurobehavioural changes were not ameliorated by citalopram treatment. In addition, ACTH administration resulted in sex specific changes in *BDNF*, *CREB, Akt,* and *TrkB* mRNA expression in different brain regions. In summary, this study showed that ACTH administration resulted in neurobehavioural, and molecular changes associated with a depressive phenotype, which were not impacted by drugs targeting the monoaminergic system (Sallie *et al*., 2024).

5.1 Neurobehavioural analysis

Neurobehavioural tests such as the OFT and the SPT have been invaluable in assessing depressive-like behaviours in rodent models of depression. In this study we used the OFT to study locomotor activity and anxiety-like behaviour, and the SPT to evaluate anhedonia.

5.1.1 Open-field test

The assessment of locomotor activity is important in gaining insights into the monoaminergic activity in the brain, since dopamine is responsible for mediating locomotor activity (Seibenhener & Wooten, 2015). In addition, the OFT assesses anxiety-like behaviour through determining the duration of time spent in the central versus the peripheral zone of the OFT, as more anxious animals are expected to spend less time in the centre of the open field (Seibenhener & Wooten, 2015). This provides useful insights into the model being studied since anxiety is often comorbid in patients with depression.

With regards to locomotor activity the present study showed that there were no changes in the total distance, mean velocity, or total activity in all groups. However, there were significant differences in the time spent immobile in the arena. Immobility duration increased in all groups over 6 weeks in both males and females. In our laboratory, we have previously shown that immobility duration at 2 weeks and at 6 weeks were increased in rats that received ACTH for 6 weeks (Sallie *et al*., 2024). This immobility may be attributed to the effect of ACTH-induced HPA axis dysregulation on dopamine activity in the brain. Indeed, studies have shown that increased levels of ACTH are associated with decreased dopaminergic activity in the brain, an effect attributed to increased circulating glucocorticoids (Bloomfield *et al*., 2019). In addition, the nigrostriatal dopaminergic pathway is associated with behaviour related to anxiety, including decreased locomotor activity and increased avoidance behaviour (LeBlanc *et al*., 2020). Alteration in the mesolimbic and mesocorticolimbic dopaminergic pathways are implicated in changes in emotional and cognitive processes that may contribute to anxiety-like behaviour (LeBlanc *et al*., 2020). Therefore, the increased immobility seen in this study may be attributed to a decrease in dopamine levels in the brain, leading to decreased in locomotor activity and anxiety-like behaviour. Studies have suggested that glucocorticoids alter dopamine efflux via the activation of glucocorticoid receptors present on dopaminergic neurons in the ventral tegmental area, which affects dopamine production and neuronal excitation (Butts *et al.*, 2011). On the other hand, another possible explanation for this behaviour may be that over time, rats adapt to the open-field apparatus leading to familiarity and reduced exploration. Future studies should assess the effect of ACTH administration on dopamine signalling.

In terms of anxiety-like behaviour, our model showed no changes in the frequency of central zone entries and duration spent in the central zone. Although not statistically significant, male rats that received ACTH + citalopram spent less time in the central zone at 6 weeks compared to baseline. This is interesting since in a previous study chronic ACTH administration resulted in fewer entries into the central zone of the open field and with a shorter duration when compared to the healthy controls only (Sallie *et al*., 2024; Petrović *et al*., 2018). Studies with these findings administered a lower dose of ACTH, however, ACTH administration occurred over a longer period of time (Petrović *et* al., 2018). This may also suggest that citalopram is ineffective in alleviating

anxiety-like behaviour associated with HPA axis dysregulation. Patients with MDD often present with symptoms that include low mood, fatigue, and anhedonia (Belujon & Grace, 2017). These symptoms may be attributed to decreased dopamine, as many of these behaviours are regulated via the dopaminergic pathways, which when dysregulated may manifest as these depressive-like symptoms.

5.1.2 Sucrose preference test

In rodent studies, the SPT is used to assess anhedonia as a measure of symptoms of depression. Anhedonia is a traditional clinical symptom of depression, where patients show a disinterest in normally pleasurable activities (Berrio *et al.*, 2024). In rodent studies, anhedonia is shown by a preference for drinking regular water over sucrose water. The present study showed that male rats that received ACTH + citalopram had a lower sucrose preference ratio than saline + citalopram males, suggesting anhedonia. Previous literature has reported an antidepressive-like effect of citalopram in rats exposed to CUMS, shown by an increased sucrose preference ratio in rats that received citalopram (Roohi-Azizi *et al.*, 2018). In our model, citalopram treatment did not reverse anhedonic behaviour in male rats that received ACTH + citalopram, this suggests that ACTH-induced depressive-like behaviours may be resistant to SSRIs. The differences in results may further be attributed to drinking behaviour by rats as a result of hunger or thirst. Traditionally, the SPT is performed following food and water deprivation. Food and water deprivation may lead to confounding results as drinking behaviour may be from hunger or thirst, rather than anhedonic behaviour (Berrio *et al.*, 2024). In our model, rats were not deprived of food and water prior to the SPT. Therefore, the lower sucrose preference ratio and higher regular water consumption can be attributed to anhedonia, an effect not ameliorated by treatment with citalopram.

Traditionally, SPT data is only represented as a sucrose preference ratio, however, this does not account for other drinking behaviours. Therefore, we investigated the combined effect of ACTH and citalopram on the volume of drinking water, sucrose water, and total water consumed during the SPT. Our findings showed that ACTH administration led to a decreased sucrose preference ratio in male rats that received ACTH. This effect was only seen in male rats, which may indicate a more pronounced effect of ACTH administration on HPA axis dysregulation in males. The higher sucrose preference ratio can be explained by the preference shown for regular drinking water.

Taken together, the neurobehavioural analysis suggests that ACTH administration reduces dopamine signalling in the brain as evidenced by increased immobility in the OFT and reduced sucrose preference in the SPT.

5.2 Expression of molecular markers associated with depression

There are several molecular pathways that have been implicated in the development of MDD. Many of these pathways are associated with the neurotrophic theory and underlie synaptogenic signalling in the brain. As previously outlined in figure 2.2, BDNF, CREB, TrkB, Akt, mTOR and eEF2 are involved in molecular pathways associated the development of depressive-like symptoms in HPA axis dysregulation.

5.2.1 Regional expression of BDNF

In the present study, we investigated the region- and sex-specific effects of ACTH administration and citalopram treatment on *BDNF* mRNA expression. We showed that there was a sex-specific difference in *BDNF* expression in the striatum and midbrain. Interestingly in the striatum *BDNF* expression was higher, while in the midbrain it was lower in females that received chronic ACTH administration compared to males. BDNF is a neurotrophic factor associated with synaptogenesis and synaptic plasticity and is often proposed as a possible diagnostic marker of depression. The increased expression of *BDNF* in females in the striatum may explain why female animals were less susceptible to the neurobehavioural changes seen in males. Importantly, there is a common understanding that BDNF is decreased in depression, however, there have been conflicting reports in ACTH-induced models. In this regard, some have shown that ACTH administration decreases *BDNF* mRNA expression in rodents (Antunes *et al.*, 2015), while others demonstrated no changes in *BDNF* expression following ACTH administration (Kuwatsuka *et al.*, 2013). Moreover, previously in our laboratory, we have shown an increase in *BDNF* mRNA expression in the prefrontal cortex, striatum, hippocampus, and midbrain in rats that received ACTH (Sallie *et al.*, 2024). In addition, a clinical study showed that serum BDNF levels were not decreased in depressed patients but rather dysregulated (Serra-Millàs *et al.*, 2011). Additionally, this study found that 24-weeks of citalopram therapy was able to regulate serum BDNF levels, associated with a reduced severity of depressive symptoms (Serra-Millàs *et al.*, 2011). Therefore, contrary to popular belief, BDNF may not always be decreased in patients with depression, but rather dysregulated. Despite citalopram improving BDNF levels

in previous reports, future studies should prioritise novel drugs capable of swiftly regulating BDNF levels compared to citalopram which requires 24-weeks of treatment. More importantly, our results show that *BDNF* mRNA expression may vary across different brain regions. Furthermore, the sex-specific impact on BDNF expression, signalling, and activity has often been overlooked in BDNF-related research, potentially affecting the investigation into BDNF's functions or effects (Chan & Ye, 2017). Our study found that there were significant sex-specific effects in ACTH-treated animals in the expression of BDNF in the striatum and the midbrain, which may also account for the differences observed during neurobehavioural analyses. These results highlight the lack of understanding of the role of BDNF in the pathophysiology of depression. Interpretations regarding BDNF levels should be made with caution as it may vary significantly based on the sex and species as well as the brain region used in the research model.

5.2.2 Regional expression of CREB

CREB is a nuclear transcription factor implicated in both the adaptive reaction to stress and the physiological and/or pharmacological control of BDNF expression (Alfonso *et al.*, 2006; Alboni *et al.*, 2011). Our results show a significant sex effect on *CREB* mRNA expression in the midbrain and prefrontal cortex. In addition, our study showed no significant changes in *CREB* mRNA expression in the hippocampus and striatum. However, male ACTH + citalopram rats displayed a trend where *CREB* mRNA expression was decreased in the hippocampus, an effect that was not reversed by citalopram treatment. On the other hand, in the midbrain, ACTH + citalopram male rats had increased CREB mRNA expression compared to their saline counterparts. Similarly to BDNF, previous studies have often reported downregulation of *CREB* mRNA expression in patients with depression, while others have reported increases in *CREB* mRNA expression in animal models following chronic stress, specifically in the hippocampus (Böer *et al.*, 2007, 2010). Additionally, it has been shown that citalopram and other SSRIs protect against stress induced CREB activation as part of its antidepressant mechanism (Böer *et al.*, 2010). Thus, the regulatory effect of citalopram may account for the lack of significant changes in CREB mRNA expression in the hippocampus and striatum. This effect may further be explained by the inefficiency of citalopram, as HPA axis dysregulation may have led to activation of

CREB transcription, an effect previously observed in an animal model of chronic stress (Böer *et al*., 2010).

Interestingly, LINC00473, a long non-coding RNA, overexpression in female mice showed behavioural resilience to chronic stress (Kawatake-Kuno *et al.*, 2021). LINC00473 is responsible for the regulation of synaptic plasticity in the prefrontal cortex, an effect only observed in female mice (Chen *et al.*, 2016; Issler *et al.*, 2020). A further effect of LINC00473 is regulation of CREB signalling (Chen *et al*., 2016). Therefore, LINC00473 may play an important regulatory role in the expression of CREB in the prefrontal cortex, however, this effect may only be seen in females. Our study showed no significance in the mRNA expression of CREB in the prefrontal cortex of female rats, an effect that may result from the regulatory effects observed in CREB expression in the prefrontal cortex of animals as previously described. This highlights the regulatory role of CREB in the prefrontal cortex, however, these effects may only be observed in females.

5.2.3 Regional expression of TrkB

TrkB is the main binding receptor for BDNF. Therefore, studies investigating the expression of BDNF often focus on TrkB expression in conjunction with BDNF. In our study, we found that *TrkB* mRNA expression was increased in the striatum of female rats post ACTH administration. We also showed that *TrkB* expression is reduced in the prefrontal cortex of males that received ACTH + citalopram when compared to females that received ACTH + citalopram. Interestingly, these changes were not directly correlated with BNDF expression in these brain regions. However, in there was a trend towards increased striatal expression in female rats that received ACTH + citalopram compared to their saline counterparts in the mRNA expression of *BDNF* and *TrkB*. Therefore, our results demonstrate a possible sex-specific and brain region-specific effect of ACTH on the expression of *TrkB*. Current literature has focused on the expression of BDNF in animal models of depression and postmortem studies of depression, with little focus on TrkB (Reinhart *et al.,* 2015). However, dysregulation of TrkB has been reported in the prefrontal cortex of patients with other neurological disorders, such as schizophrenia (Reinhart *et al*., 2015; Hashimoto *et al*., 2005). Additionally, a decrease in TrkB expression was reported in the striatum of depressed patients and in postmortem hippocampal tissue from suicide victims (Dwivedi *et al*., 2003; Reinhart *et al.*, 2015). These findings highlight the importance of TrkB as a

possible biomarker and therapeutic target of depression. However, further investigations are necessary to understand the relationship between TrkB and BDNF, and its association with depression.

5.2.4 Regional expression of Akt

Akt is a protein kinase that responds to extracellular signals, in this case BDNF, triggering activation through phosphorylation. This in turn prompts activation of numerous downstream targets, including CREB, mTOR, and eEF2 (Yap *et al.*, 2008). Our results showed higher mRNA expression of Akt in ACTH + citalopram females than in ACTH + citalopram males in the striatum and hippocampus. In contrast, in the prefrontal cortex, Akt expression was decreased which may explain the decreased CREB expression in the prefrontal cortex. Previous literature has reported that Akt1 is decreased in the prefrontal cortex of suicide victims (Karege *et al.*, 2007; Jernigan *et al.*, 2011). In animal models of depression, s-citalopram has been shown to elevate levels of phosphorylated mTOR and phosphorylated Akt in the rat hippocampus (Athira *et al*., 2020; Ignácio *et al.*, 2016). In an *in vitro* investigation in rat cortical neurons, administration of 17β-Estradiol led to phosphorylation of Akt after 15 minutes with levels remaining higher than basal levels up to 24 hours following administration (Dhandapani & Brann, 2002; Sheppard *et al*., 2022). Oestrogen may therefore act as a protective factor against inhibition of Akt, as often seen in MDD. Therefore, the observed higher mRNA expression of Akt in females may be explained by oestrogen serving as a protective factor against chronically elevated ACTH. These regional differences highlight the importance of studying regional changes in different molecular markers associated with depression.

5.2.5 Regional expression of mTOR

In the present study there were no significant changes in mRNA expression of *mTOR* in either male or female rats, treated with ACTH. However, it has been proposed that inhibition of the mTOR signalling pathway could serve as a possible mechanism through which BDNF is dysregulated in patients with depression (K V *et al.*, 2020). Previous studies have also reported a decrease in mTOR signalling and lower mRNA expression of *mTOR* in postmortem tissues of depressed patients (K V *et al*., 2020; Hashimoto, 2011). Most *in vivo* studies have indicated a decrease in mTOR brain activation in animal models of depression (Chandran *et al*., 2013). This includes a

decrease in the activity of the PI3K/Akt/mTOR signalling pathway observed in the prefrontal cortex and amygdala of stressed rats (Chandran *et al.*, 2013). Dysregulation of mTOR has been reported in the prefrontal cortex of both male and female rats (Fang *et al.*, 2013), suggesting that there may not be a sex-specific effect. We showed no significant differences in mRNA expression of mTOR in either male or female rats. Therefore, increasing or enhancing mTOR signalling may act as a potential target for the development of novel antidepressants.

5.2.6 Regional expression of eEF2

Recent investigation into novel antidepressants have focused on ketamine and its mechanism of action. One of the main effects of ketamine is the reduction in phosphorylation of eEF2 (Monteggia *et al*., 2013). Eukaryotic elongation factor 2 (eEF2) is a protein that plays a key role in protein synthesis, specifically during the elongation phase of translation (Kaul *et al.*, 2011). eEF2 is part of the downstream pathway that is activated via the binding of BDNF to TrkB and subsequent phosphorylation of Akt (Kaul *et al*., 2011). Our results showed no significant changes in eEF2 in males and females following ACTH administration and citalopram treatment. However, in the midbrain, ACTH + citalopram females had a lower expression of eEF2. This may possibly be explained by the protective effect of oestrogen often observed in rodent models (Sheppard *et al*., 2022). In an LPS-induced model of depression, eEF2 phosphorylation was increased leading to dysregulated of eEF2 signalling (Li *et al*., 2021). Following treatment with an SSRI, dysregulated levels of eEF2 were corrected (Li *et al*., 2021). Our results differ in this regard as no significant effects were found in the mRNA expression of *eEF2*. This may suggest that ACTH induces depressive-like symptoms via a mechanism other than neuroinflammation. Therefore, while SSRIs may be effective in treating depression related to neuroinflammation, the efficacy of such SSRIs may be diminished in a model of HPA axis dysregulation. Investigation into the possible dysregulation of eEF2 in the development of depression remains limited, however, the role of eEF2 in the mechanism of action of antidepressants requires further investigation.

5.3 Limitations and future perspectives

While the current study provides valuable insights, it is important to acknowledge its limitations and consider future perspectives. One of the main limitations of this study

is that we did not measure circulating corticosterone levels to confirm that the HPA axis was indeed dysregulated. Future studies should include measures of circulating levels of cortisol in experimental animals. The present study did not include control groups as part of this portion of the study, however, the current study forms part of a larger study in which control groups have been included (Sallie *et* al., 2024). In addition, most studies involving depressive animal models used the FST as a measure of behavioural despair, which were not included in this study. However, the FST has recently received several criticisms for its use in evaluating despair-like behaviours (Carvalho *et al.*, 2021). Therefore, an alternative neurobehavioural assessment for despair needs to be explored in future studies. In this study, we used PCR to measure changes in gene expression of molecular markers, this may not translate into protein expression. Future studies should investigate the protein expression of these markers and their functionality by measuring the phosphorylated vs unphosphorylated isoforms. Recent literature has also highlighted sex-specific differences in antidepressant drug metabolism; therefore, it would be interesting to correlated drug concentrations as determined by liquid-chromatography mass spectrometry with molecular changes. Interestingly in the current study, females were protected from ACTH-induced neurobehavioral effects. This is often reported in rodent models that oestrogen in females may be protective (Sheppard *et al*., 2022). Future studies may include ovariectomised rats to further investigate the protective effects of oestrogen in depression studies.

5.4 Conclusion

The present study, in combination with published results from our lab (Sallie *et al*., 2024), improves our understanding of the sex-specific changes in depressive-like measures in a rodent model of ACTH-induced HPA axis dysregulation. This study shows that male animals may be more susceptible to neurobehavioural effects of ACTH, while oestrogen in females may have a regulatory effect over the HPA axis. The neurobehavioural changes observed in males may be underlined by HPA axis induced effects on the monoaminergic system, specifically dopamine. The dopaminergic system regulates locomotor activity via the nigrostriatal pathway, and reward and motivation via the mesolimbic pathway, may be responsible for increased immobility and decreased sucrose preference in males. In addition, ACTH administration resulted in sex specific changes in *BDNF*, *CREB, Akt,* and *TrkB* mRNA

expression in different brain regions. These results suggest that other theories involved in the development of depression, including neurotrophin-regulated synaptogenesis may be impacted in HPA axis dysregulation. However, the strong sex specific effects and the differential expression of neurotrophic factors across different brain regions highlight the complexity of studying the pathophysiology of MDD. These insights deepen our understanding of the pathophysiology of depression induced by dysregulation of the HPA axis, providing evidence that the HPA axis also impacts other theories involved in the development of depression. Therefore, further studies are required to provide a comprehensive understanding of how HPA axis dysregulation on monoamine signalling, neuroinflammation and GABA/glutamate neurotransmission.

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APPENDIX

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 70 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) – 2110989 (2110989@students.wits.ac.za)
- Sanelisiwe Xhakaza (PhD) – 1174355 (1174355@students.wits.ac.za)

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). Citalopram will be administered at a dose of 10mg.kg/b.w, daily, intraperitoneally (100µl).

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.

References

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Date: 31/01/2023 Signature:

RECOMMENDATIONS

Approval for an additional 70 animals to be added to the study and the inclusion of the citalopram administered control groups as detailed above.

Approval for the addition of co-workers A. Lubbe and S. Xhakaza to the study. New co-workers must attend the WRAF orientation course prior to handling any of the animals. Please contact Dr. Jardine for details regarding the course (Kimberly.Jardine@wits.ac.za).

Date: 13 February 2023 Signature:

Deputy Chairperson, AREC

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