Unpredictable stress and acute immune stimulation on anxiety measures in adult rats

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DECLARATION

I, **Kholeka Neliswa Vezi,** hereby declare that the dissertation entitled:

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PLAGIARISM DECLARATION

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Abbreviations

Before use, abbreviations are defined when first used in the text throughout the dissertation.

Study Outline

Chapter 1: This chapter consists of a background, aims, objectives, and a brief description of the general methodology of the study. The intention of aims of the study were to answer the gap/lack of research in the literature. The purpose of the objectives depicts how the purposes of the study were answered. The general methodology answers to the specific steps that were taken to answer the aims and objectives.

Chapter 2: This chapter consists of a literature review. It entails the comprehensive foundation of the study and areas where there is a gap/lack of research.

Chapter 3: This chapter consists of the first study in a manuscript format that sought to investigate the effects of acute immune stimulation on chronic unpredictable mild stress on anxiety-like behaviours, interleukin-6, and associated neurochemical changes in adult animals.

Chapter 4: This chapter contains the second study in the manuscript format that evaluates the impact of chronic unpredictable mild stress on the hypothalamic-pituitary axis and anxiety-like behaviour following acute immune stimulation by lipopolysaccharide.

Chapter 5: The last chapter outlines the links between the two manuscripts in reflection on the aims and objectives of the study. Appendices contain additional/supplementary information to support the main text.

Chapter 6: This chapter consists of the conclusion. This is the final chapter that sums up everything and synthesises the key findings, highlighting the significance of the study.

Abstract

Anxiety disorders and neuropsychiatric disorders have a complex and intricate relationship. In several neuropsychiatric disorders, anxiety is both a symptom and a risk factor. Acute and chronic stress exposure can both lead to anxiety disorders, and anxiety is a common neurobehavioral correlate of many stressors. There may be a connection between anxiogenesis and immunomodulation during stress, and research has indicated that the brain and its intricate neurotransmitter networks may affect immunological function. However, the interaction between chronic unpredictable mild stress (CUMS) and immunological stimulation in association with anxiety has been less extensively studied. Therefore, this study aimed to investigate the effect of CUMS on HPA-axis function, associated neurochemical changes, and anxiety-like behaviours following immune-activation by Lipopolysaccharide (LPS) in adult male rats. Rats were assigned to the following groups (n=6/group): control, CUMS, LPS, and CUMS+ LPS. Rats were subjected to CUMS protocol for 12 days and injected once with LPS two days before CUMS ended as per their respective groups. The open field test (OFT) and elevated plus maze (EPM) tests were carried out afterwards. Subsequently, animals were sacrificed to collect plasma, amygdala, hypothalamus, and hippocampus tissues to measure IL-6, 5-HT, BDNF concentrations, corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), glucocorticoid receptor (GR), Nuclear Receptor Subfamily 3 Group C Member 1 (Nr3c1), and corticosterone (CORT) concentrations. Animals subjected to CUMS paradigm exhibited anxiolytic-like behaviour. Whereas LPS did not show overt changes. Additionally, there was a significant reduction in IL-6, GABA, ACTH, and CORT, whereas there was a significant increase in 5-HT and Nr3c1 concentrations following LPS and CUMS treatment, which may indicate suppressed neuro-inflammation. The significant increase in BDNF further supports the suppressed neuroinflammation, which may indicate the activation of a neuroprotective mechanism. Furthermore, a significant decrease in GABA concentration was noted in the amygdala in all the experimental groups. Our study highlights the buffering/ameliorating effect of the combination, highlighting the complex interactions between chronic stress and immune activation.

Keywords: Chronic unpredictable mild stress (CUMS), Lipopolysaccharide (LPS), HPA-axis, Anxiety-like behaviour

Chapter 1: Introduction

1.1 Background

In psychiatry, anxiety disorders are among the most frequently reported mental disorders, which have a significant medical and socio-economic burden (1, 2). Numerous reviews have examined the economic, social, and health care policy ramifications of depression, but far fewer have examined anxiety (1). Diagnosis is ambiguous, and a substantial percentage of underdiagnosed patients (3). Additionally, research has shown that one in fourteen individuals fits the diagnostic criteria for an anxiety disorder (4). The significant variability of anxiety disorders, frequent comorbidities, limited specificity of biomarkers, and inadequate understanding of the etiopathogenesis of anxiety disorders all pose a challenge to the study of anxiety (5, 6).

Research shows that the frequently used questionnaires do not diagnose up to 50% of people with anxiety disorders (7). Over the years, it has been clear that conditions such as anxiety are significantly underdiagnosed and that treating them can be challenging (8). Environmental stressors are perceived and transmitted to different parts of the central nervous system (CNS), including limbic regions like the hippocampus and amygdala, and cortical areas, which are mostly found in the prefrontal cortex (PFC) (9). To support survival and adaptation, stress activates a variety of integrated response systems, such as the immunological, neuroendocrine, autonomic, and behavioural systems (10). It has been shown that chronic exposure to environmental stressors triggers a series of adaptive reactions, such as the activation of the hypothalamic-pituitary-adrenal axis (HPA). Glucocorticoids (GCs) are secreted when the HPA axis is activated by stress (11).

Mineralocorticoid receptors with high affinity and GC receptors with low affinity are the two main corticosteroid receptors in the brain, where GCs can bind (12). According to Karin and colleagues (13) GC receptors regulate the strength of the HPA axis reactions to stress by means of a negative feedback system. For organisms to maintain homeostasis, acute stress must appropriately activate the HPA axis.

Changes in the body, whether physical or psychological, can trigger a complex interplay of immunological, endocrine, and physiological reactions. These reactions support homeostasis, the preservation of the internal environment, and the mitigation of effects at the cellular level. Allostasis, or physiological balance, is maintained by a variety of processes. These processes

combine behavioural, metabolic, neuroendocrine, and autonomic elements (14). Individuals produce an allostatic overload that results in pathological circumstances when an adequate adaptation response is not activated (15). Stress is the reaction to stimuli that cause an imbalance in an organism's homeostasis, and stressors are the stimuli that cause it (16). Environmental factors and stressful events can also contribute to the development of anxiety (17). Stress has been linked to the onset and progression of anxiety disorders (9).

Stressors set off a cascade of regulatory processes that disrupt the immune system's regular operation and promote a swift proliferation of pathogens (18). Understanding the complex two-way contact between the immune system and the neuroendocrine system is essential for both preventing and treating anxiety disorders (19). An example of immune-neuroendocrine communication in animals that has been investigated the most is the connection between the hypothalamus-pituitary-adrenal (HPA) axis components. Cytokines and endotoxins, such as lipopolysaccharide (LPS), which is a substance found in Gram-negative bacteria, both increase HPA anti-inflammatory responses by stimulating adrenal glucocorticoids (20).

Most frequently, bacterial endotoxins such as LPS are used to activate the immune-inflammatory pathway to identify the neurological processes that connect inflammation and anxiety (21). When LPS is administered systemically, the innate immune system is activated, which causes the brain and peripheral regions to generate pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (22), (23), resulting in anxiety-like behaviours (21, 24). Hodes and colleagues discovered that following recurrent social defeat stress, susceptible mice had peripheral levels of IL-6 that were 27 times higher than those of resilient mice (25). Furthermore, via altering brain circuitry, neurotransmitter function, neuroendocrine activity, and neuronal plasticity, LPS-induced cytokine changes may have an impact on behaviour (26).

Moreover, rats subjected to chronic unpredictable stress (CUS) exhibited anxiety-like behaviour on the elevated plus-maze. Both emotional and cognitive abnormalities stimulated by CUS resemble elements of major depressive and anxiety disorders (27). Chronic unpredictable mild stress has been said to suppress the serotonergic function (28). Low serotonin levels may contribute to the increased anxiety symptoms. Stress hormones like cortisol can be released when chronic stress and anxiety trigger the hypothalamic-pituitary-adrenal (HPA) axis (17). Prolonged activation of the HPA axis can cause anxiety disorders and interfere with the immune system's normal stress response. Chronic stress causes tumour necrosis factor alpha (TNF-α), microglial activation, and the release of other cytokines;

additionally, it lowers the production of brain-derived neurotrophic factor (BDNF), which ultimately hinders neurogenesis and compromises neuroplasticity (29).

The impact of a single intraperitoneal injection of LPS (0.1 mg/kg) at various intervals on CUMS-induced anxiety (1 day before, 18 days after, or 35 days after the onset of CUMS) was examined in a previous study. LPS restored brain-derived neurotrophic factor levels and alleviated behaviours associated with anxiety when administered one day before CUMS. Whereas, LPS appeared to enhance the immune response and even marginally worsen neurobehavioral performance when administered 18 days after the onset of CUMS (30). Y Couch, and colleagues (31) evaluated whether lipopolysaccharide (LPS)-induced behavioural and molecular effects are similar to stress-induced behavioural and molecular responses, and whether the combination of these effects is maladaptive or adaptive. It is evident that both stress and LPS challenges caused distinct biochemical and behavioural alterations when applied separately. However, LPS alone does not produce overt behavioural changes, but when combined with stress, it intensifies depression and suppresses aggression. Additionally, a study that aimed at characterising the long-term effects of systemic inflammation on behavioural and brain cytokine alterations, using a single intraperitoneal injection of LPS (5 mg/kg) or vehicle (32) showed that a single peripheral inflammatory insult induces persistent discriminative deficits. Following LPS challenge at two time-points, representing early (7 days) and late (10 months) long-term responses.

In rodent models, lipopolysaccharide (LPS) and chronic unpredictable mild stress (CUMS) induce neurobehavioral alterations; however, it is yet unknown how these two stressors interact to contribute to the pathophysiology of anxiety. Stress-immune interactions have not been extensively explored in terms of immunological dysfunction, neuroplasticity, neurotransmitters, and neuroendocrine alterations concerning anxiety behavioural modifications.

1.2 Justification

Approximately 33.7% of people will experience an anxiety disorder at some point in their lives, according to extensive population-based surveys. (8). Anxiety disorders are significantly underdiagnosed, undertreated, and frequently comorbid with other mental illnesses (8). Research shows that among the commonly conducted questionnaires, anxiety disorders are not identified in up to 50% of affected people (7).

During stress and anxiety, the hypothalamic-pituitary-adrenal (HPA) axis is activated, leading to changes in hormone levels (33, 34). Neuroinflammatory theories in anxiety disorders have not received as much research attention as those in major depression. Whether anxiety is associated with inflammatory activity through a specific anxiety channel or a more general negative emotionality pathway has to be investigated further. Improving the understanding of stress and immune interaction can result in the understanding of anxiety pathophysiology. Therefore, this study aims to add insight and direction as to how we can approach anxiety research and also aims to contribute to the pool of knowledge.

1.3 Aims:

This study investigates the effect of the combination of chronic unpredictable mild stress and immune activation using LPS on anxiety-like behaviours and associated neurochemistry changes in adult male rats.

1.4 Objectives:

To assess the impact of CUMS- and LPS-induced anxiety-like and explorative behaviours using the elevated plus maze and open field test

To assess the potential influence of CUMS and LPS on some neurotransmitters, neuroplasticity, and neuroinflammation

To determine the influence of CUMS and LPS on the HPA-axis by measuring CRH, ACTH, CORT, and Nr3c1 receptors

1.5 Potential Benefits

Understanding how CUMS and LPS interact may be essential for developing effective anxiety treatments. CUMS, as a model for chronic stress, and LPS, as a model for inflammation, can both independently induce anxiety-like behaviours; however, these models do not present the full spectrum of anxiety. Research indicates that combining them may result in adaptive or maladaptive effects, providing valuable insights into potential therapies. This study will give us a new approach to investigating anxiety pathogenesis. Studying this interaction can also help identify biomarkers, therapeutic targets and improve diagnosis.

1.6 Methodology overview

Following the approval of all animal experimentation by the animal research ethics committee of the University of KwaZulu-Natal, male Sprague Dawley rodents (250-300g) were obtained and kept in the Biomedical Research Unit of the University of KwaZulu-Natal under standard laboratory conditions. The 250–300g range guarantees the right size for dependable dosing, handling, and behavioural testing, and males were chosen to minimize the unpredictability related to female oestrous cycles The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content (<5000 p.m.), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and water *ad libitum*. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Ethical clearance number: AREC/00004595/2022). Procedures involving animal care were conducted in conformity with the Institutional Guidelines for Animal Care of the University of KwaZulu-Natal. The animals were randomly assigned to four different groups of 6 animals per cage. After a week of acclimatisation, the rodents were randomly assigned to the following groups (n=6/group): control group, CUMS group, LPS group, and CUMS+ LPS group. Subsequently, the environmental enrichment material from the CUMS group and the CUMS + LPS group was removed to prevent the rehabilitation of animals from stress and maintain the stress effect on the animals. The chronic mild unpredictable stress procedure, as explained below, was administered to the CUMS and CUMS+ LPS groups for 12 days. Stress was induced using a chronic mild unpredictable stress procedure according to the model described by López-López et al (35) and B.M. Cox et al (36) with some modifications. In rodents, both long- and short-term stress paradigms result in strong expression of depressive-like traits, but there has been conflicting evidence about anxiety-like behaviours. Both increased (27, 37) and decreased (38, 39) anxiety-like phenotypes have been observed after 3 or more weeks of CMS therapy; however, only one study has examined anxiety following shorter stress periods, demonstrating increased anxiety 4 days after CUS (40). Since the scientific research on the occurrence of anxiety-like symptoms following CUMS is contradictory, we utilised a 10-day CUMS paradigm to assess anxiety-like phenotypes in conjunction with immune stimulation. The purpose of this study was to evaluate the behavioural, hormonal, and neurochemical alterations that follow a shorter stress paradigm. The control and LPS groups remained undisturbed in their home cages for the duration of the CUMS procedure. The LPS and CUMS+LPS groups were administered a single LPS dose (5 mg/Kg, i.p) after 10 days of CUMS procedure; the control group also received a single 0.9 %

saline dose (5 mg/Kg) intraperitoneally (IP) on the same day as LPS and CUMS+LPS. Three Days after the IP injections, the Elevated Plus-Maze Test was performed. The open field test was also performed 24 hours later.

A day after these behavioural tests, the animals were euthanised to collect blood and harvest the hippocampus and amygdala. Tissue collected was stored at -80 °C in a bio freezer until ready for the analysis of interleukin 6 (IL-6), serotonin (5-HT), BDNF, and GABA, corticotropin-releasing hormone (CRH), Adrenocorticotropic (ACTH), corticosterone (CORT), and Glucocorticoid Receptors (Nr3c1) concentrations using ELISA. Statistical comparisons were performed with GraphPad Prism using appropriate statistical tests to analyse data and determine statistical significance., Statistical comparisons were performed with GraphPad InStat software (version 5.00, GraphPad Software, Inc., San Diego, California, USA), and results were presented as mean ± S.E.M. In both studies, a one-way analysis of variance (ANOVA) was used, followed by the Tukey–Kramer post hoc multiple comparisons test. A value $p< 0.05$ was considered statistically significant.

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Chapter 2: Literature Review

2.1 Anxiety

Anxiety and stress-related disorders are severe psychiatric conditions that affect performance in daily tasks and pose a financial burden on public health. Among the most prevalent mental illnesses, anxiety disorders are characterised by excessive worry, hyperarousal, and fear that is counterproductive and debilitating (1). Anxiety symptoms have a strong correlation to other neuropsychiatric symptoms and have been observed in up to 70% of AD patients (2). Anxiety disorders, including social anxiety disorder, panic disorder/agoraphobia, and generalised anxiety disorder, are the most common psychiatric disorders that are linked to a high burden of disease. In primary care, anxiety disorders are frequently underdiagnosed and undertreated (3). Anxiety disorders are extremely common throughout life and are linked to significantly higher rates of morbidity and early death (4). Anxiety disorders are highly prevalent throughout adulthood (5), and during midlife, their prevalence is highest (6). In modern society, the most common neuropsychiatric disorders are depression and anxiety, which induce loss of ability and even suicide (7). According to multiple studies, more than 90% of people with anxiety disorders have a history of other mental health issues throughout their lives, demonstrating that anxiety disorders are rarely isolated conditions (8).

The exact mechanism is not entirely known in the pathophysiology of anxiety disorders. In an attempt to understand the pathophysiology of anxiety, researchers have proposed a neuroinflammatory theory (9, 10). The question of whether anxiety is linked to inflammatory activity via a particular anxiety pathway, or a broader negative emotionality pathway, is still worth investigating. The pathophysiology of anxiety is closely linked to over-activated microglia (11). The study of neural-endocrine-immune system interactions, or psychoneuroimmunology, has advanced knowledge of the reciprocal interactions between the immune system and the central nervous system (CNS) in neuropsychiatric illnesses over the past three decades (12-15). Pathologic microglial cell activation, dysregulation of the hypothalamic-pituitary-adrenal axis (HPA-axis), altered neurotransmitter metabolism, impaired neuroplasticity, and structural and functional brain alterations impacting cognition and emotional behaviour are all outcomes of neuroinflammatory processes involving the nervous, neuroendocrine, and psychological systems (9).

Research on humans and rodents has shown evidence that the complex etiology of neuropsychiatric illnesses may be associated with the brain's cytokine network, which is

activated by factors such as stress exposure and peripheral immune activation (16, 17). There are potential links between immune function alterations and stressful life events. Chronic stress alters immune system function and causes neuroinflammation, which is characterised, among other things, by an excess of proinflammatory cytokines, which is facilitated by a rise in catecholamine and glucocorticosteroid release (18). Sustained, chronic stress can raise inflammatory levels in the brain and surrounding tissues, triggering the immune system and resulting in an increased cytokine release (19, 20). Activated immune cells release pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumour necrosis factor (TNF)-alpha, in response to pathogen invasion, tissue damage, and psychosocial stress (21) can act either synergistically or antagonistically (22). The release of pro-inflammatory cytokines during an immunological challenge is typically temporary and controlled by anti-inflammatory mechanisms. Therefore, the behavioural effects brought on by the inflammatory response emerge as the central nervous system's (CNS) transient, adaptive, and regulated response to immunological signals (21). However, the inflammatory response and behavioural effects of cytokines may play a role in the development of neuropsychiatric disorders and clinically relevant behavioural symptoms when immune challenge becomes chronic and/or unregulated, as seen in patients undergoing long-term cytokine treatments or those exposed to chronic illness and/or stress (21). Infection, inflammation, and physical or psychological stress can all modulate the expression of cytokines in the central nervous system (23).

Cytokines play crucial functions in signalling the brain in producing neurochemical, neuroendocrine, neuroimmune, and behavioural changes, according to experimental and clinical research (21, 24-27). Our everyday lives frequently involve stress and stressful situations, which cause intricate alterations in our biological systems. The brain, behaviour, neuroendocrine, and immunological systems are all impacted by these stress reactions, which work together to either improve or impair an organism's capacity to manage stressors. However, immunological stimulation and chronic unpredictable mild stress in anxiety disorders have been studied less extensively than in major depression. Therefore, the next section delves into stress.

2.2 Stress

Multiple neuropsychiatric diseases are associated with life stress (28). A wide definition of stress is any actual or predicted disturbance of homeostasis or danger to one's health (29). Stress serves as a defence mechanism that enables an organism to respond to threatening

circumstances (30). The detrimental effects on psychological and physiological performance, known as the "allostatic load," arise if recovery from the acute incident is not followed by a sufficient homeostatic response to stop the acute adaptive response of stress mediators (31). The strain that the body and brain bear when they adjust to physiological and psychological obstacles is known as the allostatic load (30). The adaptive physiological response to acute stress (32), wherein the internal environment changes to meet anticipated and perceived demand (33), Sterling and Eyer first named this process "allostasis." In the short term, reactions to extreme stress that support survival in a life-threatening circumstance might be adaptive. The notions of allostasis and allostatic load establish a connection between the negative outcomes that arise from persisting acute responses to stress and the protective and survival values of the acute response (34).

The "allostatic load," or detrimental effects on psychological and physiological function, arises if the acute incident is not followed by a sufficient homeostatic response to end the acute adaptive response of stress mediators (35). Allostasis and allostatic load are notions that relate the survival and protection values of the acute reaction to stress to the negative outcomes that arise if the acute response continues (36). In the short term, responses to extreme stress that support survival in a life-threatening circumstance might be adaptive (30). Long-term, sustained activation of this response during chronic stress can overwhelm feedback mechanisms and result in structural and functional alterations in the neuroplastic matrix, which may be the cause of altered mood and cognitive functioning (37, 38). A broad definition of stress is any actual or predicted disturbance of homeostasis or danger to one's health. Stress is a defence mechanism that enables an organism to respond to threatening circumstances. The system's equilibrium is maintained by glucocorticoids acting through negative feedback (39, 40). One important subclass of steroid hormones that control immunological, cardiovascular, metabolic, and behavioural functions is glucocorticoids, cortisol, and corticosterone (41, 42). Chronic unpredictable mild stress is known to increase corticosterone levels; thus, we discuss this stress type (43).

2.3 Chronic unpredictable mild stress (CUMS)

Anxiety disorders may develop as a result of chronic stress's significant negative impact on both physiological and psychological well-being (20).

To simulate long-term human stress exposure, several chronic stress methods have been reported that employ various stressors 1-2 times daily for 7-54 days (44). Under stress,

maintaining or restoring homeostasis requires neurochemical and endocrine regulation, which leads to changes in metabolism (45, 46). The hypothalamic-pituitary-adrenal (HPA) axis was triggered by unpredictable chronic stress, which raised levels of the corticosteroid (CORT) hormone, which is a sign of an endocrine stress response (47). Rats exposed to various unpredictable stress methods have displayed heightened anxiety-like behaviour on a variety of behavioural tasks, such as the open field test, elevated plus-maze, the unconditioned and conditioned freezing tests, the social interaction test, and the passive avoidance task (44, 48, 49).

Aversive conditioning, anxiety-related reactions, and the suppression of exploratory activity are only a few of the significant behavioural changes produced by stress exposure. However, there is much ambiguity in the results of research examining anxiety-related responses induced by prior stress exposure. Following exposure to various stressors, certain researchers have documented increases in anxiety, while others have found no effects or even a decrease in anxiety-related reactions (50-57). While reactions to acute or short-term stress are adaptive and prepare the organism to cope with an infection or damage, if the stressor is too severe or persistent, the stress response may be dangerous (58, 59). Neuroinflammation plays an important part in inducing anxiety (60, 61). In animal models, lipopolysaccharide (LPS) is primarily used as a cytokine inducer to explore the link between memory impairment, neuroinflammation, and anxiety (62, 63). Therefore, in the next section, we look at LPS.

2.4 Lipopolysaccharide (LPS)

The glycolipid LPS, a gram-negative bacterial cell wall component, binds to Toll-like receptor 4 (TLR4) to trigger innate inflammatory responses (64). By attaching itself to the TLR-4, LPS imitates a bacterial infection and triggers a sickness response (65).

LPS is a well-established endotoxin since it generates robust systemic inflammation when given in large doses for the first time (64). The first stimulation of high-dose LPS changes microglia into an inflammatory phenotype that encourages the production of proinflammatory mediators; these inflammatory microglia are believed to play a role in the neuropathology caused by LPS (66). LPS is frequently used to stimulate an immunological response in animals, resulting in elevated levels of proinflammatory cytokines and corticosterone in the circulatory system (67, 68). Additionally, during immune challenges, neurons from the pituitary, adrenals, and hypothalamus contribute to the HPA response by releasing peripheral adrenocorticotropic

hormone (ACTH), corticotropin-releasing hormone (CRH), and downstream glucocorticoids (e.g., cortisol, corticosterone) to act on target tissues (69-72).

To regulate the immunological response, mild LPS exposure must be sustained. Thus, it is thought that preconditioning with low-dose LPS is believed to provide neuroprotection by eliciting physiological immune responses as opposed to pathological inflammation (64). Numerous recent studies have begun to reveal the neuroprotective mechanism of LPS, whereby preconditioning with low-dose LPS causes anti-inflammatory microglia to change rather than the inflammatory microglia that are initially created by the administration of high-dose LPS (73, 74). Low-dose LPS preconditioning for immune training (LPS training) presumably helps to maintain brain homeostasis by changing microglia into the neuroprotective phenotype. LPS can cause neuroinflammation, which is an inflammatory reaction in the brain. This may cause the limbic system to malfunction and exacerbate anxiety; hence, next, we look at the limbic system (75).

2.4 Limbic system

An important factor in many neuropsychiatric disease states is the malfunctioning of the limbic system (76). An important factor in many neuropsychiatric disease states is the malfunctioning of the limbic system. The amygdala and the hippocampus, two limbic structures involved in the regulation of stress and emotional responses, may experience functional and neuroanatomical changes as a result of chronic neuroinflammation, which has been shown to disrupt synaptic plasticity, increase neurodegeneration, and decrease neurogenesis (77, 78). Emotional behaviour regulation depends on hippocampal-dependent learning and memory. The ability to distinguish between contextual information about danger and safety is dependent on hippocampus function and is thought to be crucial in reducing anxiety-like behaviours and fear memories (79-81). Infection or psychological stress may lead to behavioural changes through their effects on neurotransmitter function, neuroendocrine activity, and neurogenesis. Hence, we delve into these topics in our next sections.

2.5 Neurogenesis

The neurotrophins (NT) known as brain-derived neurotrophic factor (BDNF) promote proliferation, survival, and differentiation of neurons in both the central and peripheral nervous systems (82). Growth factors, particularly BDNF, have been implicated in the pathophysiology of stress-induced neuropsychiatric diseases in recent years. In animal models, BDNF has been

linked to anxiety-like behaviours, and BDNF expression has been reported to be decreased in response to a variety of stressors (83-85).

The pathophysiology of anxiety has been linked to decreased neurogenesis (86). This has been supported by data that show BDNF is dysregulated in response to stress (87, 88). BDNF has several well-known beneficial impacts, including promoting the longevity of dopaminergic, cholinergic, serotonergic, and noradrenergic neurons (87, 89), weakening the processes of neuronal degeneration (90), causing and enhancing synaptic plasticity and exhibiting a neuroregenerative action (91). In the hippocampus, BDNF infusion improves fear extinction (92). Fear extinction and the control of anxious behaviours may be directly influenced by BDNF signalling in the ventral hippocampus (93, 94). BDNF may promote hippocampus neurogenesis, which could help regulate stress (86).

The neurogenesis of the adult hippocampus is extremely susceptible to glucocorticoids and stress (95). However, there has been conflicting evidence about the level of the BDNF protein in animal models of anxiety disorders. A meta-analysis of the relationship between anxiety and BDNF protein levels has not yet been published. Clarifying these results is crucial to demonstrating the value and practical use of BDNF measurements in the identification and management of anxiety disorders. BDNF appears to support serotonergic neuron development and function. Similar to BDNF, changes in 5-HT signalling have been shown to impact adult neurogenesis and synaptic plasticity in the hippocampus (96, 97). In the next section, we look at other neurotransmitters that may be involved in anxiety disorders.

2.6 Neurotransmitter

Although the pathophysiology of anxiety disorders is complicated, it is accepted that an imbalance of several neurotransmitters is linked to the occurrence of anxiety disorders. The brain's mood and stress reactions are mostly controlled by serotonin (5-HT), norepinephrine (NE), dopamine (DA), glutamate (Glu), and gamma-aminobutyric acid (GABA) (98). Anxiety is one of the many psychiatric disorders linked to dysfunction in serotonin (5-hydroxytryptamine; 5-HT) neurotransmission (99). It has been suggested that the hippocampus's 5-HT neurotransmission plays a role in controlling the activity of the HPA axis throughout life (100).

According to available data, the immunological, affective, and stress systems may interact by influencing common neuromodulatory systems, which may involve serotonergic pathways (101-103). Activation of the peripheral immune system can have significant physiological and behavioural impacts, such as causing fever and illness-related behaviour. Immune modulation or activation may influence physiology and behaviour through influences on brainstem neuromodulatory systems, including serotonergic systems (103). Serotonergic systems have a significant role in regulating mood, motor activity, and behavioural arousal. However, following acute immunological activation, there is an intriguing paradox: serotonergic activity rises but behavioural activity sharply declines, especially in limbic brain regions linked to mood regulation (104).

In a previous study, when the bacterial endotoxin lipopolysaccharide (LPS; 30, 100, and 300 micrograms/kg body weight) was administered intraperitoneally (i.p.), the hippocampus's extracellular concentrations of 5-HT and 5-HIAA increased. This was accompanied by a marked increase in extracellular corticosterone levels and a significant decrease in behavioural activity. As a result, the LPS-treated rats' behavioural activity was not closely correlated with the levels of hippocampus extracellular 5-HT that were seen in control rats (104).

It is well-recognised that 5-HT neurotransmission in the hippocampus plays a role in controlling the activity of the HPA axis. This means that the hippocampus is known to control the activity of the HPA axis through the mediation of glucocorticoid negative feedback (105, 106). An in vitro investigation showed that 5-HT treatment markedly raised the mRNA levels of glucocorticoid receptors in hippocampus neurons (107). Reduced 5-HT levels in rats' hippocampal regions could therefore be linked to abnormalities in the HPA axis. The aetiology of behavioural depression and/or anxiety may be influenced by decreased 5-HT neurotransmission in the hippocampus, potentially in connection with dysfunctional HPA axis activity (102). 5-HT and BDNF are two seemingly different signalling systems that regulate the growth and plasticity of neuronal circuits implicated in mood disorders like anxiety and depression (108). Hypothalamic-pituitary-adrenal axis, 5-HT and BDNF are interconnected systems (108, 109).

2.7 Neuroendocrine activity

The primary neuroendocrine system that governs responses to stress and several bodily functions, such as mood and emotions, is the hypothalamic-pituitary-adrenal (HPA) axis.

Stressful stimuli activate the HPA axis (110). The HPA axis releases corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus in response to stressors. The pituitary gland then responds to stimulate the release of adrenocorticotropic (ACTH) into the bloodstream. The adrenal gland secretes glucocorticoids under the influence of ACTH. The equilibrium of the system is maintained by glucocorticoids acting through a process of negative feedback on the pituitary, hypothalamus, and higher brain areas (39, 40).

This suggests that the hormone plays a more comprehensive and integrative role in the psychological stress response (111). For example, CRH administered centrally alters learning, memory, arousal, anxiety, reward, and locomotion (112-114). It has been demonstrated that stressful situations affect CRH immunoreactivity and release at both hypothalamus and extrahypothalamic locations, in addition to CRH mRNA expression (115). In the hippocampus and hypothalamus, GRs use the glucocorticoid feedback mechanism to regulate the activity of the HPA axis and stress response (116).

An adaptive, transient, and regulated response of the central nervous system is triggered by an acute immunological challenge. However, the resulting chronic inflammatory response plays a role in the development of maladaptive behavioural symptoms and neuropsychiatric disorders when immunological challenge becomes persistent and/or dysregulated due to chronic medical illness, chronic stress, or cytokine therapies. Regarding the immune system's involvement in neuropsychiatric disorders, reciprocal interactions between the brain and immune system have garnered a lot of attention (21). Stress can trigger an inflammatory response, as demonstrated by both clinical and experimental data, as evidenced by an increase in the levels of pro-inflammatory cytokines in the blood. Chronic stress can consequently serve as an anxiety trigger by causing alterations in the immune system and the hypothalamic-pituitary-adrenal axis (14).

Immune challenge and psychological stress interact in intricate ways. There is growing evidence that external immunological challenge can modify immune dysfunction caused by chronic stress, with potentially complex outcomes. An important and completely unexplored question is whether immune system activation, such as occurs during infection, during chronic unpredictable stressors, may influence the development of anxiety-like behaviour in adulthood. Given the substantial overlap between stress and immune system activation, a significant link is not unexpected. Moreover, cytokine responses to a subsequent peripheral immunological challenge can be enhanced by past exposure to stressors (117). Nevertheless, little research has

been done on the relationship between immunological challenge and chronic unexpected mild stress (CUMS) associated with anxiety. Our research demonstrates the intricate relationship between stress and immunological challenge concerning anxiety in adults.

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Chapter 3: Manuscript 1

Prologue

Psychiatric disorders in adults have been associated with immune system activation brought on by stress. Psychological stress significantly increases the symptoms of infection-related illness in animals, indicating that psychological and immunological stressors may work similarly to promote illness-related behaviour. LPS challenges and stress both caused different behavioural and biochemical alterations when administered separately. Our study aimed to evaluate the relationship between stress and the immune system regarding alterations in BDNF, 5-HT, IL-6, and anxiety-like behaviours in adults.

Chronic Unpredictable Mild Stress and Acute Immune Activation Promote Resistance to Anxiety-Like Behaviours and Modulate Neurochemical Changes in Adult Rats

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Chronic Unpredictable Mild Stress and Acute Immune Activation Promote Resistance to Anxiety-Like Behaviours and Modulate Neurochemical Changes in Adult Rats

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Abstract:

Neurodegenerative diseases in adults are often linked to anxiety and depression. Chronic Unpredictable Mild Stress (CUMS) and lipopolysaccharide (LPS) administration challenge the immune system, inducing anxiety- and depression-like behaviours in animals. However, the combined effects of CUMS and acute LPS on behaviour, inflammation, and neurochemistry in adult rats are less studied. Therefore, we decided to investigate the impact of CUMS and acute LPS administration on anxiety-like behaviour, IL-6, and associated neurochemical changes in adult animals.

Adult male rats were subjected to CUMS (10 days), followed by a single intraperitoneal LPS injection (5 mg/kg) and thereafter CUMS (2 days). Behavioural assessments were conducted using the Open Field Test (OFT) and Elevated Plus Maze (EPM). IL-6, 5-HT, and BDNF concentrations in the hippocampus and GABA concentration in the amygdala were measured.

CUMS-exposed rats spent more time in the central zone of the OFT and the open arms of the EPM than CUMS+LPS group. Additionally, LPS and CUMS+LPS rats exhibited decreased IL-6 concentration compared to the control in the hippocampus, which may indicate suppressed neuro-inflammation. In the hippocampus, an increased 5-HT concentration was observed in comparison to the control following LPS and CUMS treatment. The associated higher BDNF concentration further confirms the positive effects. Furthermore, compared to the control, a significant reduction in GABA concentration was noted in the amygdala in all the experimental groups. This suggests that CUMS and low-dose LPS can develop stronger coping mechanisms during stressful situations and suggests that it has inflammatory response-limiting effects.

The combination of CUMS and LPS modulates anxiety-like behaviours and neurochemical changes in adult rats, highlighting the complex interactions between chronic stress and immune activation.

Keywords: Anxiety, chronic unpredictable mild stress (CUMS), lipopolysaccharide (LPS), BDNF, serotonin

3.1 Introduction:

Anxiety Disorders commonly co-occur with mood disorders, particularly Major Depressive Disorder (MDD), which are accompanied by an activated neuro-immune pathway (1). Numerous clinical and experimental findings suggest that anxiety disorder, a prevalent mood condition, is linked to neuro-immune impairment in its aetiology (2, 3). Anxiety may affect up to 20% of adults (4). Anxiety is also involved in neuropsychiatric disorders and can be both a symptom and a risk factor of neuropsychiatric disorders. Anxiety was identified as one of the symptoms in longitudinal research that both indicated a faster progression rate and greatly elevated the risk of conversion from MCI to dementia (5). As a result, anxiety may be a modifiable risk factor for dementia in the early stages (6). Treatment options are significantly impacted by the co-occurrence of anxiety disorders with other mental and physical conditions (7). The degree of convergence and similarity between the underlying molecular profile of anxiety and illness behaviours is still unknown. Chronic unpredictable mild stress (CUMS) is a well-established procedure to induce anxiety-like behaviour in rodents (8, 9). In contrast to chronic stress, the effect of inflammation-induced anxiety-like behaviour measures is frequently overlooked.

The pathophysiology of anxiety might be impacted by the combination of stress and infection, regardless of whether the pathways leading to such abnormal behaviours are separate. One possible evolutionary interpretation of the discovery that stress is linked to the development of inflammation is that it is a logical strategy to improve survival. Infection and damage may result from stressors (10-12). Theoretically, pre-activating the immune system would improve survival and recovery (13). In the present investigation, we aimed to ascertain the extent to which the behaviours linked to chronic mild stress (CMS) could be impacted by a mild, low-dose lipopolysaccharide (LPS) challenge, which typically results in only brief, subtle behavioural changes that last for no more than a few hours. Even though anxiety is common in adults, it is still poorly understood (14).

Research has shown that the complicated aetiology of neuropsychiatric illnesses may be associated with the brain's cytokine network, which is activated by stress exposure and peripheral immune activation (15, 16). Stress and anxiety induce physiological alterations (17) and result in reduced monoamines such as serotonin (5-HT), thus impairing brain plasticity (18). Anxiety is mostly related to the amygdala's GABAergic regulation (19). The amygdala is inhibited by GABAergic neurotransmission, which prohibits humans from exhibiting improper emotional and behavioural reactions (19). Elevated levels of the inflammatory cytokine

interleukin-6 (IL-6) are also linked to anxiety (20). The production of IL-6 is generally strictly regulated; however, this regulation is compromised with a rise in age, as a result, levels of IL-6 tend to rise (21, 22).

A major component of the outer membrane of Gram-negative bacteria is LPS, which causes the synthesis of proinflammatory cytokines such as interleukin 1β (IL-1β) and IL-6 (23). LPS and CUMS both function as cytokine inducers in nature by eliciting both acute and chronic immunological activation (24-26). Conversely, it has been shown that acute stress increases hippocampus neurogenesis and enhances memory function in adult rats (27). Tolerable stress that is moderate in intensity and lasts for the right amount of time can improve health, whereas severe and unmanageable stressors are harmful (28). The intricate relationship between LPS and CUMS is dependent on the nature and/or the duration of exposure (29). Resilience is the capacity of an individual to confront adverse, social, emotional, and biological consequences of chronic stress that would otherwise damage their general well-being. It is the ability to adapt to daily situations in an efficient manner (30). As a result, the overall effect of stress can differ depending on the duration, intensity, length, or predictability, type, and number of the stressors (31). In mice, CUMS (10 days) after low-dose LPS injection led to a depression-like state and inhibited aggressive behaviours (32), whereas predictable chronic mild stress (PCMS) (restraint stress of 5 min for 4 weeks) fostered recovery in low-dose LPS-induced inflammatory alterations in the brain (28). Furthermore, in mice CUMS (4 weeks) followed by LPS (0,5mg/kg) induced depressive-like states to different degrees (33). It is crucial to consider whether CUMS and LPS can elicit reactions other than pathological inflammation, which could be relevant in understanding the mechanisms behind resilience and developing interventions that would promote it, thus managing neuropsychiatric illnesses such as anxiety.

Extreme and unmanageable stressors are harmful, whereas tolerable stress with mild intensity and appropriate duration can promote health (31). Chronic stress also impacts hippocampal neurogenesis, which is itself regulated by 5-HT (34, 35). Numerous studies have addressed the importance of cytokines and the existence of inflammatory responses in severe depression. A "double hit" of stress and infection affects the pathophysiology of depression, regardless of whether the pathways leading to such abnormal behaviours are distinct (36, 37). However, neuroinflammatory theories in anxiety disorders have been studied less extensively, especially in adults. Therefore, in this study, we investigated the combined effects CUMS and LPS on exploratory, anxiety-like behaviours; hippocampal IL-6, 5-HT, and BDNF concentrations as well as GABA concentrations in the amygdala in adult rats.

3.2 Materials and methods:

3.2.1. Animals

24 Male Sprague-Dawley rats (250-300g), bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal, were used in the study. The rats were randomly assigned to four different groups of 6 animals per cage. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content (<5000 ppm), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and water *ad libitum*. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Ethical clearance number: AREC/00004595/2022). Procedures involving animal care were conducted in conformity with the Institutional Guidelines for Animal Care of the University of KwaZulu-Natal.

3.2.2 Experimental design:

After a week of acclimatisation, the rodents were randomly assigned to the following groups (n=6/group): control group, CUMS group, LPS group, and CUMS+ LPS group. Subsequently, the environmental enrichment material from the CUMS group and the CUMS + LPS group was removed to prevent the rehabilitation of animals from stress and maintain the stress effect on the animals. The chronic mild unpredictable stress procedure, as depicted in Fig.3.2.2 below, was administered to the CUMS and CUMS+ LPS groups for 12 days. The control and LPS groups remained undisturbed in their home cages for the duration of the CUMS procedure. The LPS and CUMS+LPS groups were administered a single LPS dose (5 mg/Kg, i.p.) after 10 days of CUMS procedure; the control group also received a single 0.9 % saline dose (5 mg/Kg) intraperitoneally (IP) on the same day as LPS and CUMS+LPS. Three Days after the IP injections, the Elevated Plus-Maze Test was performed. The open field test was also performed 24 hours later. A day after these behavioural tests, the animals were euthanised to collect blood and harvest the hippocampus and amygdala. Tissue collected was stored at -80 °C in a bio freezer until ready for the analysis of interleukin 6 (IL-6), serotonin (5-HT), BDNF, and GABA.

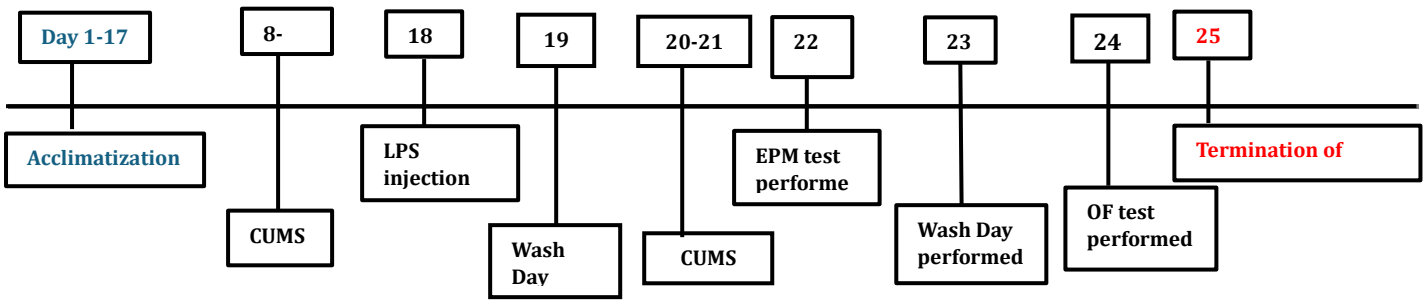

Figure 3.2.1: Experimental timeline

3.2.3 Lipopolysaccharide (LPS):

The animals were injected with LPS 72 hours before commencement of behavioural testing in the elevated plus maze on DAY 18 of the study. LPS (*Escherichia coli*, LPS0111:B4) was made into a stock solution in sterile saline (0.9%) and injected intraperitoneally (i.p) as a single dose of 5mg/kg. The control animals were sham-treated with a single i.p. (5mg/kg) dose of a saline (0.9%) solution to control for injection stress. The single systemic injection of LPS at a dose of 5 mg/kg was selected on the basis of its ability to induce acute and chronic neuroinflammation (38, 39).

3.2.3 Chronic unpredictable mild stress (CUMS) model:

Stress was induced using a chronic mild unpredictable stress procedure according to the model described by López-López et al (40) and B.M. Cox et al (41) with some modifications. Briefly, the stress protocol consisted of daytime darkness (12 HOURS), 45° tilted cage (5 HOURS), cold exposure (5 mins) at 4 °C, and restraint (90 mins) (42). The animals received two random stressors per day over the 12-day continuous period. The stress protocol was as random as possible, occurring at different times of the day. Ten days of CUMS remained continuous (41), while two days of stressors continued 24 HOURS after the Lipopolysaccharide injection. The control and the LPS groups were handled every day, but remained undisturbed in their home cages for the duration of the procedure.

The stress procedure commenced a day after the completion of the acclimatisation period. Once the acclimatisation period was over, environmental enrichment material in the cages containing the animals to be stressed (stressed group and the stressed + LPS group) was removed to prevent stress amelioration. Food and water were available *ad libitum* during the stress protocol.

3.2.4 Behavioural Tests:

The behavioural tests commenced 72 hours post-LPS or saline administration. Two tests were conducted, with a 72-hour recovery period between tests. Tests were performed in all groups and were recorded for analysis post hoc.

3.2.4.1 Elevated plus maze (EPM):

The EPM test is used to assess anxiety-like behaviour. We followed the protocol used by QI et al and Bruijnzeel et al (43, 44). The EPM consisted of a plus-shaped platform, divided into five zones (two open arms, two closed arms, and a centre zone). The arms were made of black polypropylene, with each arm measuring 50×10 cm. The arms radiate from a 10 cm central square. The entire apparatus was elevated by 55 cm on acrylic legs. The two "open" arms had 0,5cm ledges, and the two "closed" arms had 30cm walls. All animals were exposed to the EPM test 72HOURS post-LPS administration. Briefly, a single animal was placed at the junction of the open and closed arms (in the centre) facing an open arm and left to explore the platform for 5 minutes. An entry was defined when all four paws of the animal were in the arm. After each session, the platform was cleaned with 70% ethanol to prevent odour signal interference. Anti-anxiety behaviour was indicated by more time being spent in the open arms than the closed arms, whilst anxiety-like behaviour was indicated by more time spent in the closed arms than the open arms (45). The time spent in the open and closed arms was recorded, and the footage was uploaded to the BORIS software (version 8.21.5) (46). This behaviour was scored by third-party observers blinded to the study protocol.

3.2.4.2 Open field test (OFT):

The Open-field arena (90 cm length x 60 cm width x 35 cm height) was utilised. The animals were moved from their housing room to the testing room and acclimatised for at least 30 minutes before testing. The chambers were wiped with 70% ethanol before use and before subsequent tests to remove any scent clues left by the previous rodent. Each animal was placed in the centre of the arena and given 10 minutes to roam around the arena. The time spent close to the walls and in the open arena was recorded and uploaded to the BORIS software (version 8.21.5).

The exploratory locomotor activity, which is quantified as the number of squares each rodent crossed in a testing chamber, was evaluated. Given that the test measures the rodent's ability and willingness to move about freely and participate in exploratory behaviour, exploratory locomotor activity is the ideal behavioural marker of acute illness behaviour (47). This behaviour was scored by third-party observers blinded to the study protocol.

3.2.5 Tissue sample and blood harvesting:

At the end of the experimental period, all animals were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodepoort, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) for 3mins While rats were unconscious, blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 mins. Plasma was collected and stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until ready for biochemical analysis. Thereafter, animals were decapitated using a sharp guillotine. The brain was removed immediately and placed in frozen 0.9% saline slush to suppress the metabolic processes from continuing post-decapitation. Subsequently, the hippocampus and amygdala were removed and collected into weighed and pre-cooled Eppendorf tubes. The tissue in the tubes was weighed and immediately snap-frozen in liquid nitrogen before storing at -80 °C in a Bio Ultra freezer (Snijders Scientific, Holland) until the day of biochemical analysis.

3.2.6 Biochemical analysis:

Before biochemical analysis using the ELISA technique, the brain samples were thawed and homogenised in PBS (0.01M, pH=7.4) in a ratio of 1:9 (tissue weight (g): PBS (mL) volume)

with an ultrasonic cell disruptor (sonicator). The homogenate was centrifuged for 10 minutes at 5000 xg at 2°C to get the supernatant. Interleukin-6, serotonin, BDNF, and GABA concentrations were measured using their respective ELISA kits (Elabscience Biotechnology Co., Ltd) according to the manufacturer's instructions.

3.2.7 Analysis of data:

All data were expressed as means ± S.E.M. Statistical comparisons were performed with GraphPad InStat software (version 5.00, GraphPad Software, Inc., San Diego, California, USA) using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc multiple comparisons test. A value of $p< 0.05$ was considered statistically significant.

3.3 Results:

3.3.1. Elevated plus maze (EPM)

Time spent in the closed arms (a) and open arms (b) of the elevated plus maze was measured in the Control, CUMS, LPS, and CUMS+LPS groups (n=6 per group). CUMS animals spent less time in closed arms in comparison to the control ($F(3,18) = 12.57$, $P=0.0002$, Fig. 3.3.1a), and the time spent in open arms by CUMS animals was higher than that of the control ($F(3,18)=5.413$, $P=0.0078$, Fig. 1b) . The time spent by CUMS animals in the closed arms was lower than that of the control*(Control vs CUMS, $p < 0.05$, Fig. 3.3.1a). Whereas CUMS spent a higher proportion of time in open arms* (Control vs CUMS, $p < 0.05$, Fig. 3.3.1b). The CUMS group spent less time in the closed arm than the LPS group $^{\alpha}$ (CUMS vs LPS, $P< 0.05$, Fig. 3.3.1a). Multiple comparisons showed that there was a LPS effect in the LPS group and CUMS+LPSα (CUMS vs LPS, CUMS vs CUMS+LPS, $p < 0.05$, Fig 3.3.1b).

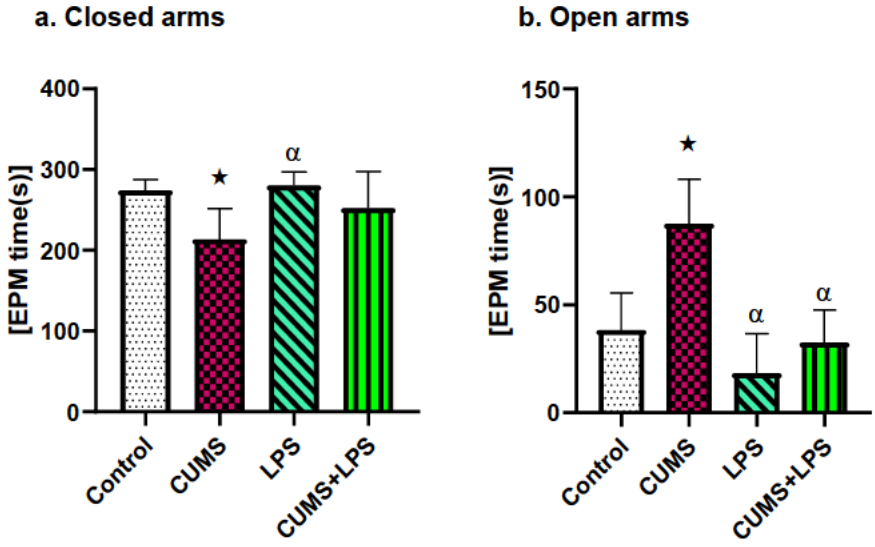

Figure 3.3.1: Time spent in closed (a) and open arms (b) in the Control, LPS, CUMS, and CUMS+LPS (n=6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as the mean ± SEM. $p < 0.05$ is considered statistically significant. *$p < 0.05$ denotes comparison with control, $^{\alpha}p < 0.05$ denotes comparison with CUMS.

3.3.2 Open field test (OFT)

The number of entries close to the walls (a) and in the open zone (b) of the open field apparatus were recorded in the Control, CUMS, LPS, CUMS+LPS groups (n=6 per group). There was an increase in the number of entries towards the wall ($F(3,14) = 10{,}78$, $P = 0{,}0006$, Fig. 3.3.2a) and similarly, an increase was observed in the open arena ($F(3,15) = 12{,}27$, $P = 0.0003$, Fig. 3.3.2b). The number of entries close to the walls was increased in CUMS and LPS compared to the control*(Control vs CUMS, Control vs LPS, $p < 0.05$, Fig 3.3.2a). A LPS effect on the CUMS+LPS groups on entries close to the wall was present, $^{\alpha}$ (CUMS vs CUMS+LPS, $p < 0.05$, Fig. 3.3.2a). A CUMS effect was observed in the open zone *(Control vs CUMS, $p < 0.05$, Fig.3.3.2b). A LPS effect was present in the LPS group and CUMS+LPS in the open zone entries $^{\alpha}$ (CUMS vs LPS, CUMS vs CUMS+LPS, $p < 0.05$, Fig. 3.3.2b).

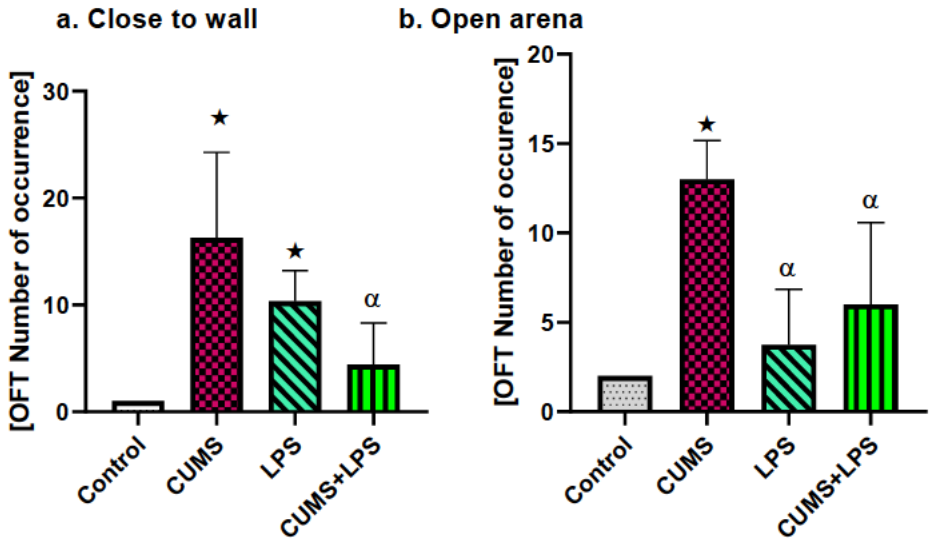

Figure 3.3.2: Number of entries close to the walls (a), in open arena (b), in the control, CUMS, LPS, and CUMS+ LPS groups (n=6, per group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as the mean ± SEM. $p < 0.05$ considered statistically significant *$p < 0.05$ denotes comparison with control, $^{\alpha}p < 0.05$ denotes comparison with CUMS.

3.3.3 Interleukin 6 (IL-6) response

Hippocampal IL-6 concentration was measured in the Control, CUMS, LPS, and CUMS+LPS groups. IL-6 levels were attenuated by CUMS and CUMS+LPS ($F(3,13) = 15, 23$, $P = 0{,}0002$). The post hoc test revealed a decrease in IL-6 in rats subjected to CUMS, and CUMS+LPS effect was observed but not in LPS, compared to control * (Control vs CUMS, Control VS CUMS+LPS, $p < 0.05$, Fig 3.3.3). There was a CUMS effect observed $^{\beta}$ (LPS vs CUMS, LPS vs CUMS +LPS, $p < 0.05$, Fig. 3.3.3).

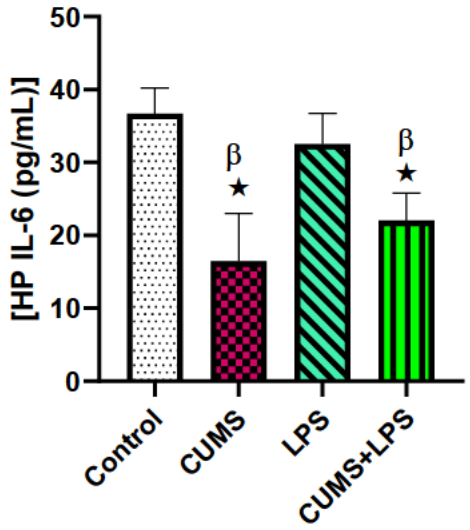

Figure 3.3.3: IL-6 concentration in the hippocampus in the Control, CUMS, LPS, and CUMS+ LPS groups (n=6, per group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ is considered statistically significant. *$p < 0.05$ denotes comparison with control, $^{\beta}$ $p < 0.05$ denotes comparison with LPS.

3.3.4 Neurotransmitter serotonin concentration

Serotonin concentration in the hippocampus was measured in the Control, CUMS, LPS, and CUMS+LPS groups (n=6) and showed a significant difference between the groups ($F(3,18) = 7.036$, $P = 0.0025$). Compared to the controlCUMS, LPS, and CUMS+LPS significantly enhanced 5-HT levels in the hippocampus*(Control vs CUMS, Control vs LPS, Control vs CUMS+LPS, $p < 0.05$, Fig 3.3.4).

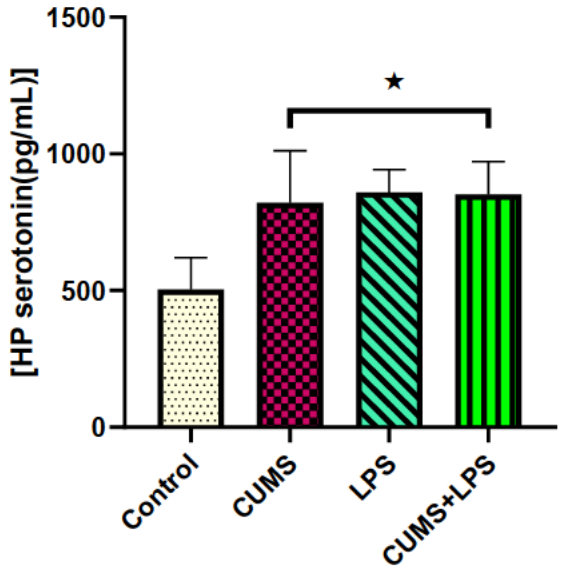

Figure 3.3.4: Serotonin concentration in the hippocampus in the Control, CUMS, LPS, and CUMS+LPS groups (n=6, per group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ is considered statistically significant. *$p < 0,05$ denotes comparison with the control group.

3.3.5 Brain-derived neurotrophic factor (BDNF) concentration

Hippocampal BNDF concentration was measured in the Control, CUMS, LPS, and CUMS+ LPS (n=6) and showed a significant effect ($F(3,18) = 14.46$, $p<0,0001$). There was a CUMS and CUMS+LPS effect on BDNF concentration * (Control vs CUMS, Control vs CUMS+LPS, $p <0.05$, Fig 3.3.5). CUMS+LPS mitigated the stress related effects on BDNF concentration $^{\delta}$(CUMS vs CUMS+LPS, LPS vs CUMS+LPS, $p<0.05$, Fig 3.3.5)

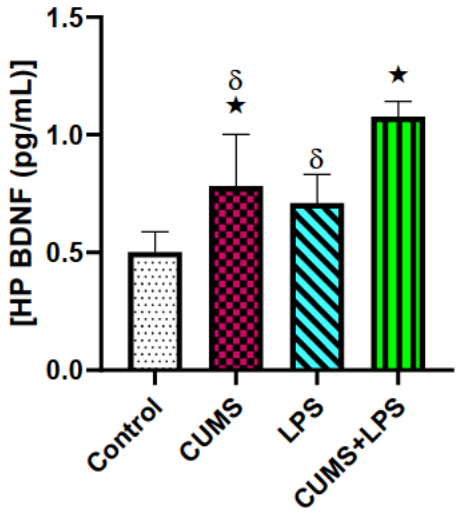

Figure 3.3.5: BDNF concentration in the hippocampus in the control, LPS, CUMS, and CUMS+LPS groups (n =6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ is considered statistically significant. *$p < 0.05$ denotes comparison with the control group. Values are presented as the mean ± SEM. $^{\delta}$ $p < 0.05$ denotes comparison with the CUMS+LPS

3.3.6 GABA concentration

GABA in the amygdala was measured in the Control, CUMS, LPS and CUMS+LPS groups (n=6) (F (3,14) = 7,585, P =0,0030, Fig. 3 .3.6). There was a CUMS, LPS and CUMS+LPS effect on GABA concentration in the amygdala * (Control vs CUMS, $p < 0.05$, Fig 3.3.6).

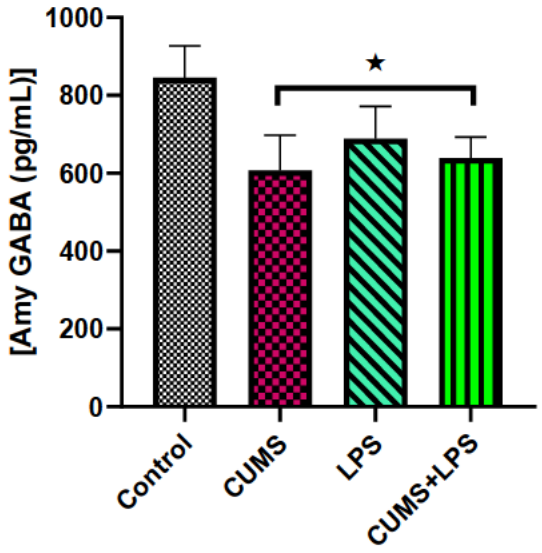

Figure 3.3.6: GABA concentration in the amygdala in the control, LPS, CUMS, and CUMS+LPS groups (n =6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ is considered statistically significant. *p <0.05 denotes comparison with the control group

3.4 Discussion:

Our study sought to determine the effects of CUMS, LPS, and their combination (CUMS+LPS) on behaviour (exploratory and anxiety-like) and whether these changes are supported by the neurochemistry in adult rats. We found evidence that stress for 12 days and low-dose LPS induced positive effects on behaviour and neurochemical changes in adult rats. Our data suggest stress promoted anxiolytic effects, thus protecting against the development of anxiety-like behaviour and modulating the neurochemical changes associated with anxiety.

Our findings from the EPM test demonstrate that rats subjected to CUMS displayed nearly threefold increases in time spent in the open arms. These results indicate an anxiolytic effect, suggesting that CUMS promoted coping mechanisms, thus reducing the chances of developing anxiety-like behaviours in adult life. This may suggest that there was high expectancy and anticipation of stressors, which may have resulted in proactive control, allowing the animals to

deal with stressors and adapt easily. In line with our results, it was shown that predictable chronic mild stress resulted in reduced anxiety-like behaviour, evidenced by increased time spent in the open arms of the EPM (48). Additionally, in line with our findings, another study observed that in adulthood, short predictable stress increased resilience to anxiety-like behaviours analysed in the EPM, indicating a habituation effect of the stress (49). Therefore, this suggests that CUMS promotes adaptive effects, which might be an indication of the protective effects of the stress.

We observed no significant effect of LPS during the EPM. In this study, behavioural tests were performed 72 hours post-LPS injection, which could mean that anxiety-like behaviour associated with a single dose of LPS injection is transient when using this dose. Additionally, the combination of CUMS and LPS did not induce an anxiogenic effect, which may suggest that LPS attenuated the CUMS effect. This may suggest that LPS-induced anxiety behaviour is time-sensitive and transient, typically resolved by 72 hours. Several studies have also unveiled the neuroprotective effects of LPS, where LPS acts as an immunomodulator rather than an inflammatory inducer (50, 51). This might suggest that compensatory responses may have been activated by this time.

Immune training is referred to as changes in the immune response to subsequent stimuli, which can either be an enhancement or a decrease (52). In this study, it can be deduced that CUMS, may have acted as an immune trainer in the CUMS+LPS group for the subsequent stressor, which is LPS. In our study, rats subjected to CUMS displayed a near threefold increase in entries into the open zone in the OFT. This suggests CUMS may have promoted exploratory behaviour. It can be inferred that CUMS stimulated the cues of comfortable exploration of open and vulnerable areas in the environment, indicative of an adaptive coping mechanism. In the LPS group, there were increased entries close to the wall zone and an insignificant number of entries to the open zone. This may suggest that these animals were more comfortable next to the protective wall. This may be an indication that there was still a slight response to the low dose of LPS. However, CUMS increased exploratory behaviour in both the open and walled arena, and this could suggest comfort in exploration while still retaining natural caution.

Our results are consistent with a study that showed that rats subjected to predictable chronic mild stress were more likely to explore the inner zone (48). There was a trend for the combination of CUMS+LPS to increase entries into the open zone. This may suggest that CUMS enhances while LPS attenuates explorative behaviours. Additionally, injecting LPS

seems to attenuate the CUMS effects, suggesting that after repeated exposure to stimuli, changes in the immune response may be decreased after 72 hours.

Several studies have demonstrated that following stress or stimulation, there is an increase in the release of IL-1β or IL-6 (53-55). Our study demonstrates that CUMS reduced IL-6, which may be an indication that there was reduced neuroinflammation in CUMS. This suggests that CUMS may have an adaptive anti-inflammatory response. The combined use of CUMS+LPS also attenuated the IL-6 concentration. This suggests that the release of IL-6 may have been under the regulation of neural and endocrine responses to any stressors. Although acute stress or inflammatory challenges usually cause plasma IL-6 to rise instantly, numerous studies demonstrate that IL-6 then falls, frequently within hours or a few days, signifying homeostatic recovery. (22, 56). This might indicate a protective adaptation to stressors. Our observation is also consistent with previous evidence, showing that predictable chronic mild stress (PCMS) mitigates neuro-inflammation by reducing proinflammatory cytokines, including IL-6 (28).

There was no significance in the LPS group. Despite being comparable, LPS was lower than the control. This further supports the suggestion that LPS effects might have subsided within the 72 hours. According to past studies, the release of proinflammatory cytokines in the blood peaks about two hours following the LPS exposure. (57-61) While we only measured the LPS effect 72HOURS later. This may also imply that by this time, low-dose LPS may have activated neuroprotective compensatory mechanisms that may have decreased or suppressed pro-inflammatory cytokines.

Proinflammatory proteins such as interleukin 1 beta (IL-1β), IL-6, and tumour necrosis factor alpha (TNF-α) are reduced by low-dose LPS (62-67). Further supporting our results. A previous study demonstrated that controlled immune training with low-dose LPS prevents neuronal damage by transforming microglia into a neuroprotective phenotype (52). This suggests that LPS may not be a mere inflammatory inducer, but an immunomodulator that can lead to neuroprotective effects in the brain. Additionally, a prior study showed that regulating the production of proinflammatory IL-6 can improve resilience to chronic stress by correcting stress-induced brain synaptic maladaptation (46). Therefore, it is possible that adaptive responses may have been triggered and may have remained active during testing, which would explain the decline above baseline. IL-6 and tumour necrosis factor-α (TNFα) are two pro-inflammatory cytokines that may be suppressed by extracellular 5-HT concentrations above the baseline physiological levels (68).

Chronic stress impacts hippocampal neurogenesis, which is itself regulated by 5-HT (34). In the current study, 5-HT concentration was increased in CUMS, LPS, and CUMS+LPS groups. The hippocampus has the highest number of 5-HT innervation and expresses a variety of 5-HT receptors (69). This increase may have been due to the potential of 5-HT to normalise hippocampus plasticity under stress conditions to treat mood symptoms. This suggests that CUMS, LPS, and CUMS+LPS may have activated modulatory mechanisms resulting in adaptation. It has been previously demonstrated that adaptation and coping with inescapable stress, to chronic mild stress, was followed by increased serotonin in the hippocampus (70, 71). This suggests that increased 5-HT may have a potential compensatory mechanism that regulates adult hippocampus neurogenesis and thus maintains homeostasis.

In line with our study, a previous study demonstrated that increases in 5-HT concentration in the hippocampus are thought to mitigate anxiety-like behaviours in rats, implying that hippocampal 5-HT levels are associated with adaptive coping (72). Rats that have been bred to exhibit elevated levels of anxiety-like behaviour have lowered levels of stress-related 5-HT release in the hippocampus (73). This might suggest that in the hippocampus, 5-HT was released diffusely by volume transmission and acted more as a neuromodulator whose function might have been to maintain homeostasis.

Evidence suggests that 5-HT plays a significant role in the control of anxiety and fear responses, and several studies have demonstrated a link between 5-HT disruptions and anxiety disorders (74, 75). In contrast to our study, it has been demonstrated that 5-HT was attenuated by 15 days of noise exposure; however, this may have been due to the deleterious effects of the noise on the hippocampus (76-78). It has also been reported that later phases of recovery may see an increase in tissue serotonin levels in the hippocampus due to compensatory mechanisms that result in increased serotonin production or turnover (34, 79-81). In the current study, the 5-HT concentration was elevated, indicating that CUMS, LPS, and CUMS+LPS may raise the extracellular 5-HT, thus regulating anxiety-like behaviour. Microglia can adopt a neuroprotective function when immune responses are controlled, and low doses of LPS are suggested to aid in maintenance by changing microglia into the neuroprotective phenotype (52), which in turn stimulates neurogenesis. It has been suggested that serotonin contributes to the maintenance of a protective state of microglia (82). This may suggest that 5-HT might have been released gradually and served as a neuromodulator to preserve homeostasis. This suggests that the interplay between low-dose LPS as an immune regulator and 5-HT helps foster a

neuroprotective environment, thus contributing to enhanced synaptic plasticity. As a result, these modifications might indicate that 5-HT is concerned with stress adaptive and coping responses.

Another connection between the elevated platform's ongoing stress and habituation was the molecular consequences resulting from the selective release of 5-HT in specific regions of the hippocampal tissue (70), which is a potentially beneficial consequence and one of the probable responses underlying the observed resilience. The positive effects of selective serotonin reuptake inhibitors (SSRIs) are thought to be mediated, at least in part, by increasing the expression of BDNF and neurogenesis in the hippocampus (34). Two seemingly different signalling systems, 5-HT and BDNF, regulate the plasticity involved in mood disorders like anxiety and depression (83).

It has been suggested that BDNF concentration might increase in response to increases in extracellular 5-HT, as would occur upon SSRIs administration (83). BDNF is essential for adult neurogenesis (84). BDNF also prevents the death and degeneration of neurons by enhancing synaptic plasticity and regulating neuronal apoptosis (85). CUMS and CUMS+LPS potentiated BDNF concentrations in the hippocampus, suggesting that neural plasticity, which is an essential component of adult brain function that enables adaptability to environmental changes, was enhanced in the CUMS group. These findings indicate that rapid alterations in BDNF expression could potentially be a component of a compensatory mechanism to maintain hippocampal homeostasis or a type of neural plasticity to adapt to new stimuli (86). This might be due to the regulation of plasticity, which may have promoted cell survival initiated by neuroprotective mechanisms at that time. Inconsistent with our study, previous studies have demonstrated that both acute and chronic forms of unpredictable stress reduce hippocampal neurogenesis (87-90).

It has been shown that hippocampal BDNF mRNA expression is decreased by both acute and chronic stress paradigms (91-95). According to the nature of the stressors and their characteristics (novelty, duration, frequency of exposure, intensity, predictability, and contractability), we propose that the body may adapt to various kinds of stressors (96). Simpkiss and DP Devine showed that stressor-specific adaptations develop even when the acute and chronic stressors are all emotional stressors that favour limbic processing (97). BDNF can stimulate neurogenesis, repair damaged neurons, and exercise neuroprotection (98, 99). In addition, BDNF has been shown to have the ability to protect neurons from injury and

stimulate neurogenesis, which helps alleviate anxiety-like behaviour (100). However, CUMS+LPS displayed a threefold increase in BDNF, suggesting that the CUMS had an augmented neuroprotective effect. These findings support 5-HT findings, which imply a possible link between 5-HT and BDNF, and that they could lead to homeostasis. Furthermore, it has been shown that the interaction between BDNF and serotonin plays a role in the regulation of emotional regulation (101). This further suggests that 5-HT transmission in the hippocampus is likely to engage BDNF in the hippocampus in stressful situations to determine coping outcomes. However, there are different outcomes, depending on the intensity, period of exposure, and the time at which the behaviours and neurochemical changes are tested after exposure.

The extensive GABAergic network inhibits the amygdala during the resting state, resulting in reduced neuronal activity (102). It has been stated that the amygdala maintains a high level of GABAergic inhibitory regulation during the resting state, which helps the organism withstand a variety of physiological and environmental stresses (103). Stress-induced amygdala hyperactivity and hyperresponsiveness are invariably accompanied by the loss of inhibitory control and an increased risk of stress-related neuropsychiatric disorders such as anxiety (104, 105). In this current study CUMS, LPS, and CUMS+LPS decreased GABA in the amygdala, suggesting that the amygdala may have been sensitised. This may indicate that in response to a stressful situation, the amygdala's GABAergic inhibition will be rapidly eliminated. This might increase the activity of excitatory pathways and improve the sensitivity and capacity to react to environmental cues, which will aid in learning and adaptation to novel situations. It has been shown that persistent stress causes the amygdala to become disinhibited and exhibit elevated activation, thus resulting in anxiety-like behaviours (105-107). However, the fact that we had an anxiolytic effect in our study, whilst they had an anxiogenic outcome, could have been caused by the number of stressors. They had eight stressors over ten days, whereas we had four. This could indicate that the more unpredictable the stressors, the higher the chance of developing neuropsychiatric disorders.

In contrast with our study, it has been suggested that GABAergic neurotransmission in the amygdala is reduced by stress and repeated corticotropin-releasing factor receptor agonist administration; this impairment is linked to the intensification of conditioned fear behaviour and anxiety (108, 109). Whereas we had reduced GABA with anxiolytic effects. This may be due to differences in the time of testing of the behaviours. Compared to SSRIs, vortioxetine

treatments, both acute and long-term, significantly raised extracellular 5-HT levels in the rat ventral hippocampal region (79, 80). Given that 5-HT3 receptors are expressed on GABAergic neurons (110, 111), it was proposed that vortioxetine may decrease GABA release and, as a result, mitigate the inhibitory effect that GABA has on 5-HT release in the hippocampus (112). This might suggest that the decreased inhibitory effect was to influence compensatory serotonergic activity to mediate stress adaptation. Zhang X and colleagues suggested that both acute and chronic stress exposure to external stressors can dampen the GABAergic regions (113). The observed decrease in GABA in this study might have been due to improved CNS GABA function and a decreased requirement for GABA release.

In Sprague-Dawley rodents (180-220g), CUMS (7 days) of two different stressors varying from mild to severe intensity, with a total of eleven stressors, significantly decreased 5-HT concentrations (114). Even though CUMS was 12 days, we had four stressors in total. This suggests that the intensity as well as the duration of stressors play a role in stress anticipation and adaptation. In adult Sprague-Dawley rats, using the chronic unpredictable stress (CUS) protocol (two stressors per day for ten days, Cox et al (41) detected behavioural, hormonal, and neurochemical alterations one day after treatment ceased. In support, it has been observed in behavioural and neuroanatomical results that short, predictable stress for 12 days on an elevated platform may have a positive adaptation effect at various stages of life, thus promoting resistance to anxiety-like behaviours (49).

The pattern of CUS-induced changes from the control state immediately after the last stressor and 24 hours was similar, indicating that these changes were due to the chronic exposure to various stressors and are not merely acute effects of the last stressor (114). However, in this paper, we had 12 days of chronic intermittent stress. This may suggest that the duration during which behaviour was tested also plays a role in adaptation. In this current study, behaviours were only tested 72 hours post-injection, which may indicate an adaptation response to stress exposure. In contrast, it was reported that LPS stress caused robust peripheral and central immune activation than CUMS (4 weeks) in adult male C57BL/6J mice, injected intraperitoneally 0.5mg/kg single dose of LPS and subjected to behavioural test 24 hours after LPS injection (33). However, differences in experimental paradigms and procedures concerning pharmacological treatment, the time of analysis after stress, and the behavioural phenotype considered could account for differences between the above-cited studies and ours.

The current study raises the question of what aspects of the stressful experience are beneficial rather than detrimental, as suggested by a large body of literature. As is known, the adversity of chronic stress is highly correlated with the severity of the stressor and the duration of exposure. It has been demonstrated that predictable chronic mild stress improves learning and memory, stimulates neural stem cells, and increases neurogenesis in the hippocampus (48). De Melo and colleagues showed that behavioural results from the EPM suggested that anxiety-like behaviour resistance in adults was promoted by short, predictable stress (49). However, in this study, we used a short chronic unpredictable mild stress, which also resulted in adaptation and habituation. Our data suggests a protective effect, seen in the decrease of anxiety-like behaviour. Dhabhar points out that stress does not necessarily result in morphological changes; in fact, stress may even have beneficial effects (115). These findings suggest that the biological stress response establishes whether its effects are beneficial or harmful. Therefore, the duration, intensity, type, and number of stressors play a role in the development of stress-induced mood disorders.

Furthermore, the biological and psychological stress response's dose and duration also play a role in establishing whether its effects are beneficial or harmful. These protective consequences align with our current study's findings, which indicate that hippocampus plasticity was positively impacted by the short, unpredictable psychological stress and low-dose LPS biological stress. Our research demonstrated that certain chronic mild stress exposure may aid in the development of adaptive capacity and increase resistance to stress-induced behaviours. Additionally, a low dose of LPS is an immunomodulator that can lead to neuroprotective effects. Thus, the interaction between CUMS and LPS influenced the increases in the hippocampal neurotransmitters and neurogenesis.

Conclusion:

The findings of this study indicate that intermittent CUMS in adult rats with a reduced number of stressors and shorter duration have similar responses as PCMS, combined with an immunomodulator, low-dose LPS, modifies neurochemical changes, increases exploratory behaviours, encourages adaptability potentially increases resistance to anxiety-like behaviours. Thus, suggesting that acute LPS and CUMS exposure may be required to control the immune response. This implies that there is a counteracting interaction between CUMS+LPS that enhances homeostasis regulation and triggers neuroprotective effects.

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Declaration of interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None

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Chapter 4: Manuscript 2

Prologue

Behavioural reactions to immunological pathways and innate inflammation can be enhanced by psychological (or heterotypic) stimuli. LPS and Chronic unpredictable mild stress both cause neurobehavioral changes in rodent models, but it is yet unknown how these two factors interact to contribute to the pathogenesis of anxiety. In experimental rodents, LPS and CUMS disrupt the HPA-axis, resulting in increased circulating CORT and impaired glucocorticoid receptors, which leads to neurobehavioral abnormalities. Our study aimed to assess the changes in CRH, ACTH, CORT, and glucocorticoid receptors on anxiety-like behaviour in adult rats.

UNPREDICTABLE STRESS ON HPA AXIS FUNCTION AND ASSOCIATED BEHAVIOURS IN LPS-CHALLENGED RATS

The current manuscript will be submitted for publication in the journal **Brain, Behaviour, & Immunity**. It has been prepared in the style used for papers published in this journal.

UNPREDICTABLE STRESS ON HPA AXIS FUNCTION AND ASSOCIATED BEHAVIOURS IN LPS-CHALLENGED RATS

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Abstract:

Anxiety is among the many neuropsychiatric illnesses that are believed to be triggered by stressful life situations. Furthermore, it is thought that the initiation of anxiety coincides with the activation of the immune system. However, the interaction between chronic unpredictable mild stress (CUMS) and immunological stimulation in association with anxiety has been studied less extensively. Therefore, this study aimed to investigate the effect of CUMS on HPA-axis function and anxiety-like behaviours following immune-activation by LPS. Animals were assigned to the following groups (n=6/group): control, CUMS, LPS, and CUMS+ LPS. Animals were subjected to CUMS protocol for 12 days and injected once with LPS two days before CUMS ended as per their respective groups. The open field test (OFT) and elevated plus maze (EPM) tests were performed afterwards. Subsequently, animals were sacrificed to collect plasma, hypothalamus, and hippocampus tissues to measure corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), glucocorticoid receptor (GR), Nuclear Receptor Subfamily 3 Group C Member 1 (Nr3c1), and corticosterone (CORT) concentrations. Animals subjected to CUMS paradigm exhibited anxiolytic-like behaviour. Whereas LPS did not show overt changes. However, CUMS+LPS animals did not exhibit the anxiolytic effect of CUMS as was observed with CUMS only subjected animals, suggesting a cancellation effect. Our study highlights the buffering/ameliorating effect of the combination of a single, low-dose immune stimulator on short psychological stressors.

Keywords:

Anxiety, Chronic unpredictable mild stress (CUMS), Lipopolysaccharide (LPS), HPA-axis, and glucocorticoid receptors (GRs)

4.1 Introduction:

The health implications of chronic stress exposure are wide-ranging, from immunological response dysregulation to an elevated risk of neuropsychiatric diseases such as anxiety (1-3). Anxiety is among the many neuropsychiatric illnesses that are believed to be triggered by stressful life situations. Chronic unpredictable mild stress (CUMS) is a well-established procedure to induce depressive- and anxiety-like behaviour in animals (4, 5). A growing body of research indicates that the immune system is activated in conjunction with the onset of anxiety (6). There is growing evidence that external immunological challenges can have serious effects by modulating immune dysfunction caused by chronic stress (7).

Lipopolysaccharide (LPS) is the active immunostimulant in the cell wall of gram-negative bacteria, which is known to activate neuroinflammation and secrete proinflammatory cytokines in a dose-dependent manner (8, 9). Depending on its type and duration, stress may strengthen or weaken immune function. However, the interaction between chronic unpredictable mild stress (CUMS) and immunological stimulation in association with anxiety has been studied less extensively than major depression. The hypothalamic–pituitary–adrenal (HPA) axis regulates the levels of glucocorticoid hormones in the bloodstream, such as cortisol in humans and corticosterone in rats (10). The HPA axis is overactivated after stressful life events and may result in mood disorders (11).

The HPA axis, when overactivated, alters the number of functions of synapses in the hippocampus and medial prefrontal cortex (12, 13). These modifications additionally aid in the development of anxiety (14). Glucocorticoids promote proactive coping in adverse situations and are linked to both short-term and long-term adaptation to stress (15). Glucocorticoid receptors (GRs) are highly expressed in the prefrontal cortex, hippocampus, and amygdala, among other brain regions that mediate changes in the HPA axis and are involved in emotional processing (15). An essential function of GRs is to mediate the activity of glucocorticoids. GRs are only bound when there are elevated concentrations of circulating glucocorticoids, as experienced during stress (16).

GRs are activated only during periods of elevated CORT, such as the circadian peak and in stress reaction (17, 18). By binding to glucocorticoids, the glucocorticoid receptor, also referred to as nuclear receptor subfamily 3, group C, member 1 (Nr3c1), provides the HPA axis with negative feedback (19). An organism's adaptive response to stress is to activate the HPA axis, which raises the concentration of glucocorticoids secreted by the adrenal cortex. A persistently

high blood glucocorticoid concentration is the outcome of a reduction in negative feedback caused by GR dysfunction (19, 20). The most well-known functions of CORT are its catabolic role in energy metabolism and its control over immunological and inflammatory responses (21).

Additionally, CORT coordinates the response to stressors to promote resilience and adaptation by providing feedback to the brain. While chronic or more severe stress is immunosuppressive (22), chronic stress sensitises hippocampal microglia to future peripheral and central inflammatory stimuli, resulting in an increased neuroinflammatory response (23). On the other hand, pretreatment with low doses of LPS results in robust neuroprotection by causing hyperresponsiveness to the subsequent immunological challenge and detrimental exposure (24, 25). A short-duration CUMS and a future single low-dose LPS would either result in a synergistic effect leading to a greater effect or an ameliorating effect. However, immunological stimulation in chronic unpredictable mild stress in anxiety disorders has been studied less extensively. Thus, in adult rats, we evaluated the combined effects of CUMS and immunological activation on adaptive behaviours.

4.2 Materials and methods

4.2.1 Animals

24 Male Sprague-Dawley rats (250-300g), bred and housed in the Biomedical Research Unit (BRU) of the University of KwaZulu-Natal, were used in the study. The animals were randomly assigned to four different groups of 6 animals per cage. The animals were maintained under standard laboratory conditions of constant temperature (22±2 °C), CO_2 content (<5000 ppm), relative humidity (55±5%), and illumination (12 h light/dark cycle, lights on at 07h00). The noise level was maintained at less than 65 decibels. The animals were allowed access to food and water *ad libitum*. All animal experimentation was approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal (Ethical clearance number: AREC/00004595/2022). Procedures involving animal care were conducted in conformity with the Institutional Guidelines for Animal Care of the University of KwaZulu-Natal.

4.2.2 Experimental design

After a week of acclimatisation, the rodents were randomly assigned to the following groups (n=6/group): control group, CUMS group, LPS group, and CUMS+ LPS group. Subsequently, the environmental enrichment material from the CUMS group and the CUMS + LPS group was removed to prevent the rehabilitation of animals from stress and maintain the stress effect on the animals. The chronic mild unpredictable stress procedure, as depicted in Fig.4.2.2 below, was administered to the CUMS and CUMS+ LPS groups for 12 days. The control and LPS groups remained undisturbed in their home cages for the duration of the CUMS procedure. The LPS and CUMS+LPS groups were administered a single LPS dose (5 mg/Kg, i.p) after 10 days of CUMS procedure; the control group also received a single 0.9 % saline dose (5 mg/Kg) intraperitoneally (IP) on the same day as LPS and CUMS+LPS. Three days after the IP injections, the Elevated Plus-Maze Test was performed. The open field test was also performed 24 hours later. A day after these behavioural tests, the animals were euthanised to collect blood and harvest the hippocampus and amygdala. Serum and tissue collected were stored at -80^{o} °C in a bio-freezer until ready for the analysis of CRH, ACTH, Nr3c1, and CORT.

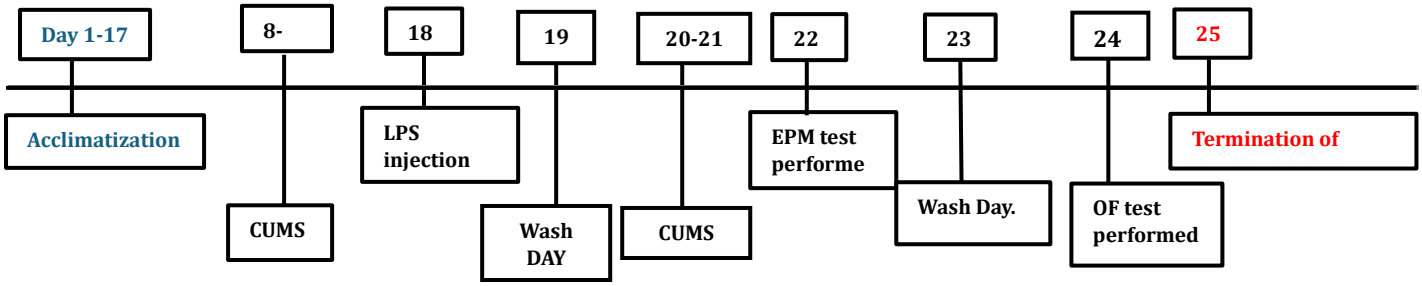

Figure 4.2.2: Experimental Design

4.2.3 Lipopolysaccharide (LPS)

The animals were injected with LPS 72 hours before the commencement of behavioural testing in the elevated plus maze of the study. LPS (*Escherichia coli*, LPS0111:B4) was made into a stock solution in sterile saline (0.9%) and injected intraperitoneally (i.p) as a single dose of 5mg/kg. The control animals were sham-treated with a single (5mg/kg, i.p) dose of a saline (0.9%) solution to control for injection stress.

4.2.4 Chronic unpredictable mild stress (CUMS)

Stress was induced using a chronic mild unpredictable stress procedure according to the model described by López-López et al (26) with some modifications. Briefly, the stress protocol consisted of daytime darkness (12 hrs), 45° tilted cage (5 hrs), cold exposure (5 mins) at 4 ° C, and restraint (90 mins). The animals received two random stressors per day over the 12 days. The stress protocol was as random as possible, occurring at different times of the day. Ten days of CUMS remained continuous, while two days of stressors continued 24 hours after the LPS injection. The control and the LPS groups were handled every day. The stress procedure commenced a day after the completion of the acclimatisation period. Once the acclimatisation period was over, environmental enrichment material in the cages containing the animals to be stressed (stressed group and the stressed + LPS group) was removed to prevent stress amelioration. Food and water were available *ad libitum* during the stress protocol.

4.2.5 Behavioural tests

The behavioural tests commenced 24 hours post-LPS or saline administration. Two tests were conducted, with a 24-hour recovery period between tests. Tests were performed in all groups and were recorded for analysis post hoc.

4.2.5.1 Elevated plus maze (EPM):

The EPM test is used to assess anxiety-like behaviour. We followed the protocol used by QI et al and Bruijnzeel et al (27, 28). The EPM consisted of a plus-shaped platform, divided into five zones (two open arms, two closed arms, and a centre zone). The arms were made of black polypropylene, with each arm measuring 50×10 cm. The arms radiate from a 10 cm central square. The entire apparatus was elevated by 55 cm on acrylic legs. The two "open" arms had 0,5cm ledges, and the two "closed" arms had 30cm walls. All animals were exposed to the EPM test 24 hours post-LPS administration. Briefly, a single animal was placed at the junction of the open and closed arms (in the centre) facing an open arm and left to explore the platform for 5 minutes. An entry was defined as when all four paws of the animal were in the arm. After each session, the platform was cleaned with 70% ethanol to prevent odour signal interference. Anti-anxiety behaviour was indicated by more time being spent in the open arms than in the closed arms, whilst anxiety-like behaviour was indicated by more time spent in the closed arms than in the open arms (29). The time spent in the open and closed arms was recorded, and the footage was uploaded to the BORIS software (version 8.21.5) (30). This behaviour was scored by third-party observers blinded to the study protocol.

4.2.5.2 Open field test (OFT):

The Open-field arena (90 cm length x 60 cm width x 35 cm height) was utilised. The animals were moved from their housing room to the testing room and acclimatised for at least 30 minutes before testing. The chambers were wiped with 70% ethanol before use and before subsequent tests to remove any scent clues left by the previous rodent. Each animal was placed in the centre of the arena and given 10 minutes to roam around the arena. The time spent close to the walls and in the open arena was recorded and uploaded to the BORIS software (version 8.21.5).

The exploratory locomotor activity, which is quantified as the number of squares each rodent crossed in a testing chamber, was evaluated. Given that the test measures the rodent's ability

and willingness to move about freely and participate in exploratory behaviour, exploratory locomotor activity is the ideal behavioural marker of acute illness behaviour (31). This behaviour was scored by third-party observers blinded to the study protocol.

4.2.6 Tissue sample and blood harvesting

At the end of the experimental period, all animals were anaesthetised with Isofor (100 mg/kg) (Safeline Pharmaceuticals (Pty) Ltd, Roodepoort, South Africa) via a gas anaesthetic chamber (Biomedical Resource Unit, University of KwaZulu-Natal, Durban, South Africa) for 3mins While rats were unconscious, blood was collected by cardiac puncture and then injected into individual pre-cooled heparinized containers. The blood was then centrifuged (Eppendorf centrifuge 5403, Germany) at 4°C, 503 g for 15 mins. Plasma was collected and stored at -70 °C in a Bio Ultra freezer (Snijers Scientific, Holland) until ready for biochemical analysis. Thereafter, animals were decapitated using a sharp guillotine. The brain was removed immediately and placed in frozen 0.9% saline slush to suppress the metabolic processes from continuing post-decapitation. Subsequently, the hippocampus and hypothalamus were removed and collected into weighed and pre-cooled Eppendorf tubes. The tissue in the tubes was weighed and immediately snap-frozen in liquid nitrogen before storing at -80 °C in a Bio Ultra freezer (Snijders Scientific, Holland) until the day of biochemical analysis.

4.2.7 Biochemical analysis

Before biochemical analysis using the ELISA technique, the brain samples and plasma were thawed. The brain samples were homogenised in PBS (0.01M, pH=7.4) in a ratio of 1:9 (tissue weight (g): PBS (mL) volume) with an ultrasonic cell disruptor (sonicator). The homogenate was centrifuged for 10 minutes at 5000 xg at 2°C to get the supernatant. CRH, ACTH, Nr3c1, and CORT concentrations were measured using their respective ELISA kits (Elabscience Biotechnology Co., Ltd) according to the manufacturer's instructions.

4.2.8 Analysis of data

All data were expressed as means ± S.E.M. Statistical comparisons were performed with GraphPad InStat software (version 5.00, GraphPad Software, Inc., San Diego, California,

USA) using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc multiple comparisons test. A value of $p< 0.05$ was considered statistically significant.

4.3 Results

4.3.1 Elevated Plus Maze (EPM):

The number of entries into the closed arms (a) and in the open arms (b) of the open field apparatus were recorded in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group). CUMS had a higher number of entries to the closed arms in comparison to the control (F (3, 16) = 6,626, P= 0,0041, Fig 4.3.1a) and similarly in the open arms there was a higher number of entries in comparison to CUMS (F (3, 17) = 13,51, P<0,0001, Fig 4.3.1b). CUMS had an increase in the number of entries into the closed arms compared to the control *(Control vs CUMS, $p <0.05$, Fig 4.3.1a). Similarly, there was an increased number of entries to the open arms observed in CUMS in comparison to the control *(Control vs LPS, $p < 0.05$, Fig 4.3.1b). Additionally, an LPS effect was observed in entries into the open arms in comparison to CUMS $^{\alpha}$ (CUMS vs LPS, and CUMS vs CUMS+LPS, $p < 0.05$, Fig 4.3.1b).

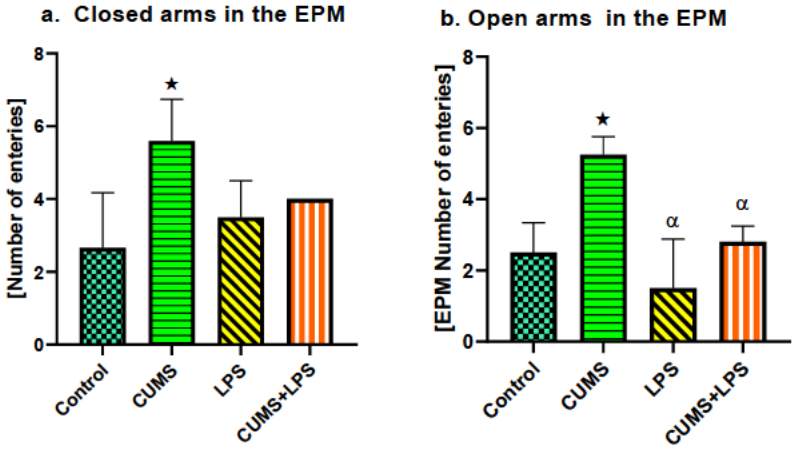

Figure 4.3.1: Number of entries in closed arms (a), and open arms (b), of the Control, CUMS, LPS, and CUMS+LPS groups (n=6/per group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as the standard error of the mean ± SEM. $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$ denotes comparison with control, $^{\alpha}p < 0.05$ denotes comparison with CUMS.

4.3.2 Open Field Test (OFT):

Time spent close to the walls (a) and in the open zone (b) of the open field apparatus was recorded in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group) during the experiment. An overall significant increase in time spent close to the walls occurred between the groups (F (3, 20) = 11,00, P=0.0002) and in time spent in the open zone between the groups (F (3, 14) = 4,534, 0.0202). In the time spent close to the walls, we observed a CUMS effect *(Control vs CUMS, $p < 0.05$, Fig. 4.3.2a). Additionally, an LPS effect was observed in LPS and CUMS+LPS in time spent close to the walls compared to CUMS $^{\alpha}$(CUMS vs LPS, AND CUMS vs CUMS+LPS, $p < 0.05$, Fig. 4.3.2a). Whereas in time spent in the open zone, there was a CUMS effect *(Control vs CUMS, $p < 0.005$, Fig. 4.3.2b).

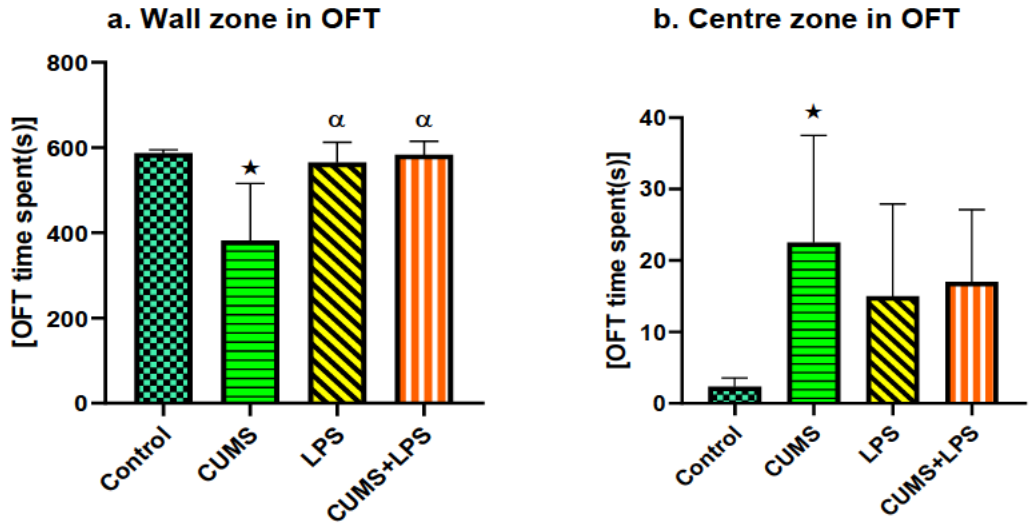

Figure 4.3.2: Time spent in closed arms (a) and open arms in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as the standard error of the mean ± SEM. $p < 0.05$ was considered statistically significant. $^{*}p < 0.05$ denotes comparison with control, $^{\alpha}p < 0.05$ denotes comparison with CUMS.

4.3.3 Corticotropin-releasing hormone (CRH):

Hypothalamus CRH concentration was measured in the Control, CUMS, LPS, and CUMS+ LPS (n=6/group) and showed no significant difference between the groups (F (3, 19) = 2,249, P=0,1156).

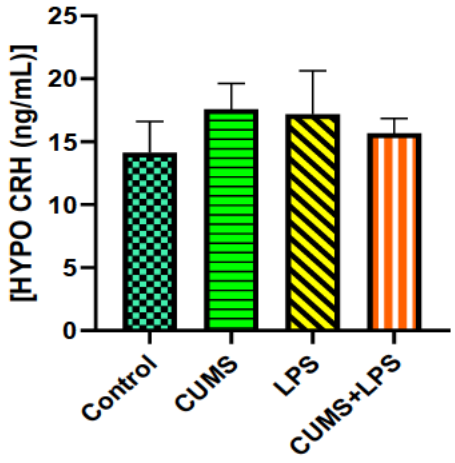

Figure 4.3.3: CRH concentration in the hypothalamus in the control, LPS, CUMS, and CUMS+LPS groups (n =6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ was considered statistically significant.

4.3.4 Adrenocorticotropic hormone (ACTH):

Plasma ACTH concentration was measured in Control, CUMS, LPS, and CUMS+LPS groups (n=6/group). The analysis showed a significant effect on ACTH concentration between the groups (F (3, 12) = 8,369, P=0,0029). A CUMS effect was observed *(Control vs CUMS, $p < 0.05$, Fig 4.3.4). There was an LPS effect observed when compared to CUMS $^{\alpha}$ (CUMS vs LPS, AND CUMS vs CUMS +LPS, $p < 0.05$, Fig 4.3.4).

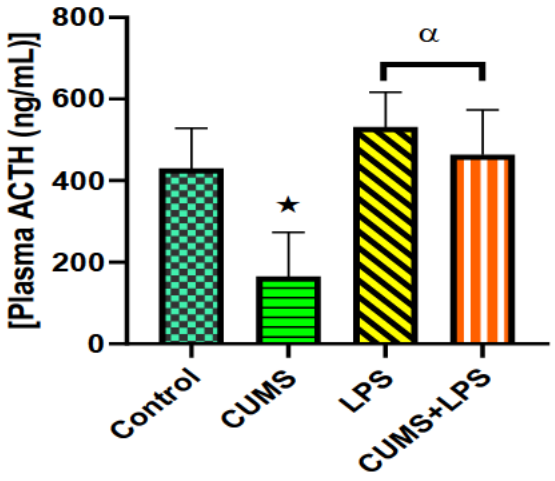

Figure 4.3.4: ACTH concentration in the plasma in the Control, CUMS, LPS, and CUMS+ LPS groups (n= 6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ was considered statistically significant. *$p < 0.05$ denotes comparison with control, $^{\alpha}$ $p < 0.05$ denotes comparison with CUMS.

4.3.5 Corticosterone (CORT):

Plasma and hippocampus corticosterone concentrations in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group) were measured and demonstrated as significant in plasma ($F(3, 20) = 3{,}937$, $P=0.0385$) and hippocampus ($F(3, 18) = 54{,}55$, $P<0.0001$) between the experimental groups. CUMS was reduced in plasma CORT *(Control vs CUMS, $p < 0.05$, Fig. 4.3.5a). Whereas CUMS, LPS, and CUMS+LPS were lower in the HP CORT*(Control vs CUMS, Control vs LPS, Control vs CUMS+LPS, $p < 0.05$, Fig. 4.3.5b). Additionally, an LPS effect was observed in comparison to CUMS $^{\alpha}$ (CUMS vs LPS, CUMS vs CUMS+LPS, $p < 0.05$, Fig. 4.3.5b).

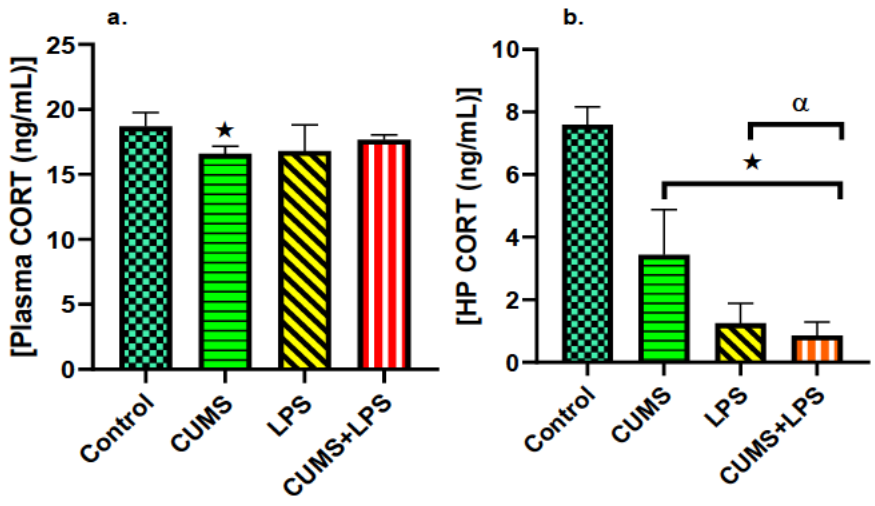

Figure 4.3.5: Plasma corticosterone (a.) and HP corticosterone (b.) concentrations in the control, LPS, CUMS, and CUMS+LPS groups (n =6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ was considered statistically significant. *$p < 0.05$ denotes comparison with the control group, $^{\alpha}p < 0.05$ denotes comparison with CUMS.

4.3.6 Glucocorticoid Receptors (Nr3c1):

Nr3c1 concentration in the hippocampus was measured in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group) and showed an increase in CUMS+LPS in comparison to the control ($F(3, 13) = 3{,}567$, $P=0{,}0443$). There was an increase of CUMS+LPS on Nr3c1 concentration *(Control vs CUMS+LPS, $p < 0.05$, Fig. 4.3.6).

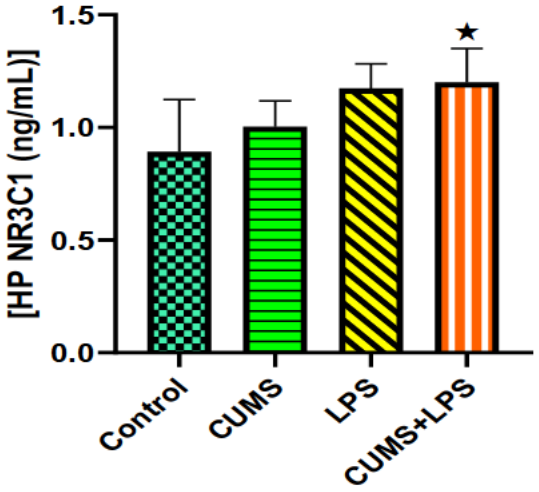

Figure 4.3.6: Glucocorticoid concentration in the hippocampus in the Control, CUMS, LPS, and CUMS+LPS groups (n=6/group). Data were analysed using one-way ANOVA followed by Tukey's multiple comparisons test. Values are presented as mean ± SEM. $p < 0.05$ was considered statistically significant. *$p < 0.05$ denotes a comparison with the control group.

4.4 Discussion

According to the current study, CUMS, which is defined by unpredictability, mild intensity, and an appropriate exposure period to stress, had anxiolytic effects and improved adaptation. Additionally, there were no overt reactions to low-dose LPS. Whereas the combination of CUMS+LPS had an ameliorating effect, resulting in CUMS+LPS cancellation effect. In this investigation, we have shown that CUMS and a single LPS intraperitoneal injection may have activated the regulation of the HPA-axis and GRs, which may have led to an anxiolytic effect and increased exploration. Our findings are supported by other research that used comparable stress paradigms to improve cognitive function, increase adult hippocampus neurogenesis, and alleviate anxiety-like behaviours (32, 33).

Our findings from the EPM test showed a high number of entries to the closed arms in animals exposed to CUMS. However, these same animals similarly showed an equal number of entries to the open arms. This controversy may suggest that there are low anxiety levels, hence the equal number of entries in both arms. In contrast, it has been shown that mice exhibited anxiety-like behaviour when exposed to CUMS (34). This may have been due to the duration of CUMS. Interestingly, it has been shown that three weeks of mild stimulation were enough to cause anxiety-like behaviour (35). Consistent with our findings, short predictable stress promoted resilience to anxiety-like behaviour in adulthood, according to the behavioural data from the EPM (36).

The LPS was comparable to the control, and in CUMS+LPS, there was an LPS effect on CUMS in the open arms; LPS cancelled the CUMS effect. This may indicate that the effect of a single LPS injection and the dose utilised in the present study was transient. In line with our findings, it has been demonstrated that LPS administration did not result in significant alterations between or across treatment groups (37). In parallel, the development of endotoxin tolerance is linked to the neuroprotective effects of low-dose LPS treatment (24, 25). J Xia, Z Lu, and colleagues showed that LPS reversed some of the anxiety-like behaviour when administered at the end of CUMS (7). In this study, CUMS did not cause anxiety-like behaviour; nonetheless, this may indicate that a single low-dose immune stimulator has a neutralising impact on CUMS. According to earlier research, LPS injection causes rats and mice to exhibit anxiety-like behaviours on EPM (38, 39). However, this would suggest that LPS's effects are dose-dependent and contingent on the number of injections. The EPM and OFT exploit the rodents' natural aversion to exposed fields to measure anxiety levels, which are shown by the number of entries into and duration of time spent in the aversive area (40-42).

Animals exposed to CUMS spent less time in the wall zone of the OFT, which may suggest that there was increased exploration. In agreement with these results, we observed that CUMS exposed animals spent more time in the open zone. Similarly, these results are in line with the EPM results, where we observed that CUMS had an anxiolytic effect. This may suggest increased curiosity, indicating that the environment may have been perceived as less threatening, thus increasing exploration and resistance to anxiety-like behaviour. However, in contrast, CUMS exposure has been shown to induce anxiety-like behaviour in the open field test (43-45). Consistent with our results, it has been demonstrated that when compared to the control mice, mice that received a two-week mild stress intervention displayed adaptive behaviours, as seen by more crossings over the centre grid in the open field (46). According to previous findings, the mice's ability to adjust to eustress may be aided by exposure to mild stress, which lasts for a short duration (two weeks) (46). LPS and CUMS+LPS were comparable to the control in time spent close to the walls, whereas there were no significant changes in time spent in the open zone.

It is well accepted that corticotropin-releasing hormone (CRH) mediates stress-induced behaviours and has been linked to both physiological and behavioural stress responses (47). The modulation of the behavioural, affective, and emotional components of the stress response has been strongly linked to CRH (48). However, we did not observe any changes in CRH in this study. There is, however, a slight trend towards an increase. It has been demonstrated previously that the slight increase after cessation of stressors may indicate hypothalamic adaptation by modulating CRH release, which may indicate that the system is compensating to maintain homeostasis (49). The response of CRH at this time had not returned to the baseline after 72 hours, which may be an indication that it may have activated the negative feedback mechanism. A feedback mechanism is crucial for controlling the generation of CRH and reducing the stress response. This is further supported by our previous results of the OFT and EPM that showed exploration and anxiolytic behaviours. The by-product of CRH stimulation is ACTH production from the pituitary gland (50-52).

ACTH ultimately activates the adrenal cortex to release CORT (53). The dynamics of cortisol and ACTH are closely related; ACTH should normalise within minutes to hours of cortisol's normalisation (54). However, we observed a reduced ACTH effect in CUMS, and we speculated that ACTH adaptation was due to exposure to stress. This could be a sign of the intermediate withdrawal that occurs after the HPA axis is resolved (54). A reduction in ACTH expression is linked to slow glucocorticoid-negative feedback (55-57). This is further

supported by the CRH results, which had no significant changes. The pituitary corticotrophs and the adrenal cortex's functional masses enlarge with prolonged HPA activation, and it takes weeks for these functional masses to recover after stress is removed (54). There were no significant changes in LPS, and CUMS+LPS was comparable to the control, due to the effect of LPS. This may indicate that LPS activated an immune response. This may suggest that the low-dose LPS in this regard was an immunomodulator that can lead to neuroprotective effects (58). In line, Mekaouche *et al* demonstrated that 7 days of restraint did not affect the ACTH responses to LPS (59). The adrenal cortex produces and secretes glucocorticoids in response to ACTH, which is released into the bloodstream (60).

Negative feedback mediated by CORT terminates the HPA axis response to stress (10). In this study, CORT was reduced in CUMS. This may suggest that the regulatory mechanisms activated the negative feedback mechanisms to bring homeostasis in animals exposed to CUMS. In line with our results, Grippo and colleagues observed that rats subjected to chronic mild stress (CMS) showed non-significant increases in corticosterone levels, indicating that any abnormalities to the HPA axis after four weeks of CMS are likely to be minimal. More severe HPA axis abnormalities may be linked to a prolonged CMS period (61). There was no significant difference in LPS and CUMS+LPS. The single-dose injection used in this study, which was tested after 72 hours, might have resulted in transient changes. A previous study has demonstrated that LPS stimulated cortisol release in a dose-dependent manner (62). Additionally, it has been shown that the plasma CORT level after CUMS was unaffected by the LPS challenge (7). The hippocampal corticosteroid concentrations were reduced in all experimental groups (LPS, CUMS, and CUMS+LPS). This may suggest increased sensitivity to input from GRs in the hippocampus, which might have prevented further production of corticosterone. Further suggesting activation of neuroprotection mechanisms, thus leading to stress recovery or resilience to stressors. In rodents, lower corticosterone levels in the ventral hippocampus are linked with anxiolytic-like effects after fear exposure (63). This may suggest that the reduced CORT in the hippocampus may have contributed to the anxiolytic effect in this study.

Additionally, the differences between plasma CORT and HP CORT may have been caused by the blood-brain barrier regulation, the rate, and extent of diffusion. After mild stressors, plasma CORT may rise quickly; however, in the HP, CORT levels may rise more slowly and persist longer.

However, despite normal CRH levels, both ACTH and CORT decreased, presumably due to corticotrope inhibition. This may be a result of stressor adaptation, which may have blunted the HPA-axis response. While the pituitary might have been inhibited by higher brain centres (such as the hippocampus) via inhibitory neurotransmitters like GABA.

GRs mediate the vast majority of glucocorticoid negative feedback mechanisms (64). GRs have a role in stressor adaptation and active coping in stressful circumstances (65). CUMS+LPS had increased Nr3c1 concentration. This may suggest an adaptive response of NrC31 that was responsible for activating a negative feedback signal, thus mediating the actions of glucocorticoids. This may suggest that Nr3c1 may have been increased to limit excessive immune activation, possibly as a protective adaptation. In contrast, mice's hippocampus tissue showed a marked reduction in the expression of Nr3c1 after prolonged, unpredictable chronic stress (66). This disrupted the HPA axis's typical negative feedback. Additionally, it has been previously demonstrated that a reduction in the hippocampus's transcription levels of Nr3c1 suggests that the HPA axis was not functioning properly and that the negative feedback loop had damaged the hippocampus (67). Furthermore, this indicates that in the current study, the HPA axis may not have been disrupted, which could have led to the adaptation effects.

However, this this study, we believe the negative feedback system may have been activated. This sensitivity is further supported by CRH and a blunted ACTH response. In line with the current study, GR was triggered to restore glucocorticoid equilibrium (68). Whereas we observed no significant changes in CUMS and LPS. The GR densities are greater in the hippocampus than in other parts of the brain, and a large body of empirical research has shown that activating hippocampus GR significantly reduces the production of ACTH and CORT from stress (69). In line with these, the increased Nr3c1 sensitivity might have mediated this negative feedback mechanism, thus resulting in an adaptive and protective response to CUMS and LPS. Thus, baseline plasma CORT and reduced hippocampal CORT, accompanied by high GRs in CUMS+LPS indicate increased sensitivity to CORT, hence the better adaptation and protective effects. Cortisol suppression has been shown to be associated with GR hypersensitivity and lower cortisol levels (70-72).

Based on the timing of stimulation, it has been shown that activation by LPS challenge has varying impacts on anxiety-like behaviours caused by CUMS (7). Mood disorders are caused

by stressful life experiences, by over-activating the HPA axis (11). Unexpectedly, our study found that molecular changes induced by a short-duration CUMS and single, low-dose LPS are distinct yet result in a cancellation effect.

Conclusion

Our study highlights the buffering/ameliorating effect of acute immune stimulation on short psychological stressors. CUMS+LPS modulated the HPA-axis, attenuated glucocorticoid response, and increased exploration and anxiolytic effects. This further implies that CUMS and LPS, characterised by a short duration, a single and low dose, stimulated an adaptive and protective capacity.

Declarations:

Declaration of interest:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CRediT authorship contribution statement.

Kholeka Neliswa Vezi: Writing — review & editing, Visualisation, Project administration, Methodology, Investigation, Formal analysis, Data Curation, Conceptualisation. **M.V. Mabandla** Writing — review & editing, Validation, Supervision, Resources, Methodology, Conceptualisation. **M. Luvuno**: Writing — review & editing, Project administration, Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualisation.

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Conflict of interest:

None

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Chapter 5: Synopsis

Among the most prevalent psychiatric disorders are anxiety disorders, which are characterised by excessive worry, hyperarousal, and fear that is counterproductive and debilitating. Anxiety disorders are leading contributors to the global disease burden and are linked to significantly low remission rates, poor prognosis, risk of suicide, higher morbidity, and early mortality rates. Compared to the general population, people with anxiety disorders had a higher risk of premature death. In addition to unnatural causes of death like suicide, this extra mortality by the rising incidence of dementia, cardiovascular disease, and other ailments. A higher probability of disability in adults and accelerated biological ageing, which includes early neurodegeneration, is also linked to anxiety disorders.

Less is known about physiological differences between individuals with anxiety disorders and healthy controls, especially in adults. Despite the prevalence of anxiety disorders, especially generalised anxiety disorder, the nature of anxiety in adults is still unknown. In the population of adults, anxiety disorders are underdiagnosed and undertreated. Anxiety disorders are often underrecognized and undertreated in primary care. Anxiety disorders are thought to be caused by a combination of psychosocial stressors, such as stress, trauma, or adversity throughout childhood, and genetic predisposition, which shows up as cognitive and neurobiological dysfunctions. There is ambiguity of diagnosis, and a substantial percentage of patients who go underdiagnosed.

Anxiety is one of the mental illnesses that are brought on and made worse by prolonged or extreme stress. Anxiety is a common neurobehavioral correlate of a variety of stressors, and exposure to stress, whether acute or chronic, can lead to anxiety disorders. Responses to acute stress are adaptive, but chronic stress can cause the brain to dysregulate, making the body more susceptible to physical and mental diseases. The ability to handle stressful situations is a key factor in determining health and illness. Stress is a reaction to unpleasant stimuli, either internal or external, that can change physiological homeostasis. When an organism is exposed to unfavourable circumstances, a sequence of adaptive reactions is set in motion to protect the stability of its internal environment and increase its chances of surviving. We all encounter stress and stressful situations regularly, and these unpleasant experiences cause intricate alterations in our biological systems.

There is a favourable association between anxiety disorders and a range of stressful events, both environmental and emotional. A clear association exists between stress and anxiety. The brain, behaviour, and immune systems are all impacted by these stress reactions, which work together to either improve or impair an organism's capacity to handle stressors. There may be a connection between the development of anxiety and changes in the body's immune system during stress. Research on the relationship between stress and immunity in humans and experimental animals indicates that psychological challenges can alter many aspects of immune response. Both environmental and stressful events can contribute to the development of anxiety. The mechanisms of behavioural changes during anxiety about stress-immune interactions have not been studied extensively.

Our study aimed to investigate the effects of immune system activation influences of the behavioural outcomes of chronic unpredictable mild stress, specifically concerning anxiety-like behaviours. To answer this question, we had four groups with 6 animals/group: the Control, stress, immune stimulator, and stressor+immune stimulator. The stress procedure was administered to the stress group and the stress+immune stimulator group for 12 days. Subsequently, immune stimulation (LPS) was administered to immune-stimulated and stress+ immune-stimulated groups after the stress procedure. We investigate the combined effect of stress and an immune stimulator on brain chemistry and anxiety-like behaviour during behavioural tests.

We found that Rats treated with both the immune stimulator and stress had reduced concentration of one of the known inflammation markers, which could be a sign of reduced neuro-inflammation. Additionally, the combination of the two stressors showed an increase in serotonin, known as one of the happy hormones. The beneficial effects are further supported by the correspondingly elevated neuroprotective biomarker. This implies that a combination of stress and low-dose immune stimulator LPS has inflammatory response-limiting effects and can help people acquire better coping strategies in stressful conditions.

The combination, on the other hand, had a mitigating impact and produced a cancellation effect. In this study, we show that stress and a low-dose immune stimulator challenge were essential for controlling the system that responds to stress, which in turn allowed for greater exploration. Reduced anxiety-like behaviour was displayed by animals exposed to the stress paradigm. The immune stimulator, however, did not exhibit any obvious alterations. The stress-induced stimulated resistance in the development of anxiety-like behaviours, which was seen in mice

subjected to stress alone, was not seen in combination with both stressors, indicating a cancellation effect of the combination.

This study contributes to understanding interactions between stress and an immune stimulator (LPS), their implications on biochemical changes in different brain areas, and anxiety-like behaviours. This study implies that there is a counteracting interaction between the combination of stressors that enhances homeostasis regulation and triggers neuroprotective effects. Improved knowledge of the age-related effects of stress and immune stimulators on anxiety disorders could help develop prevention and intervention strategies that promote healthy ageing.

Recommendations:

It is necessary to have a better understanding of how and when psychological stressors and immunological activation combine to produce subsequent anxiety-like behaviours.

To guarantee that organisational activities operate as intended, resilient people may possess active, adaptive resilience mechanisms. The similar treatment effects of antidepressants for anxiety and depression, in addition to the significant co-morbidity of major depression and anxiety disorders, imply that similar neuronal substrates may be impacted in both illnesses.

Chapter 6: Conclusion

Furthermore, we have discovered that stress and low-grade inflammatory challenges cause different molecular and behavioural changes. CUMS decreased a neuro-inflammatory marker IL-6, and reduced stress reactivity, leading to positive neural adaptation by increasing the 5-HT and BDNF levels in the hippocampus. Additionally, CUMS also reduced ACTH and CORT, suggesting that the HPA-axis may have been activated, which may have led to a neuroprotective outcome and protective adaptation. Whereas LPS did not induce any overt behavioural changes, however, there were overlapping effects on the neurochemistry, suggesting a neurochemical inertia or delayed recovery of stress systems after the immune action. When the stressors were combined (CUMS+LPS), certain behaviours were attenuated, while others were potentiated, potentially due to the simultaneous activation of distinct neural circuits by two stressors with differential sensitivities and modes of action., Low-grade inflammation was unquestionably present, but it appears likely that different systems have developed to allow the body to cope with stress and infection separately.

The current study shows that CUMS could have beneficial effects, and the combination of two stressors could lead to ameliorating effects, thus resulting in neuroprotective mechanisms. These could be used to study adaptation and resilience against anxiety. Future studies could investigate the interplay of immunological activation, stress, and anxiety. These investigations have shed light on how the brain and its intricate network of neurotransmitters may affect immunological activation and suggest a potential connection between stress-induced immunomodulation and anxiety-like behaviour.

APPENDICES

Appendix 1: Ethical Clearance Letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

15 June 2023

Renewal	
Protocol Reference number:	AREC/00004595/2022 (00024334)
Project title:	**Investigating the effects of LPS- induced memory impairments on psycho-social behaviours in pre- stressed animals.**
Main Applicant and School:	**Ms Kholeka Neliswa Vezi (219014889)** **School of Lab Med & Medical Sciences** **Westville**

Dear Ms Vezi,

Regarding your renewal application received on 23 November 2023, the UKZN Animal Research Ethics Committee (AREC) has accepted the documents submitted, and **FULL APPROVAL** for the protocol is granted effective 08 December 2023.

About your approved protocol, please note:

1. All correspondence with the AREC about this protocol must be accompanied by the full reference number.
2. Any alterations to the approved protocol (e.g. title, location, methods, research team) must be reviewed and approved through an official amendment application, via the RIG system, prior to its implementation.
3. Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered veterinarian/para-veterinarian or SAVC authorized person, and their details must be logged with the AREC if it changes during the approval period.
4. The AREC may actively monitor research, ask further questions, request additional information, or require modification on welfare grounds. If animal welfare is compromised in any way, the AREC may suspend the study/withdraw approval.
5. The research team must declare any adverse or serious adverse event(s) to the AREC via the RIG system as soon as possible but no later than **48 hours** after the event.
6. Research data should be securely stored in the discipline/school for a period of 5 years.
7. The approval is valid for one year from the date of issue. If the project is continuing, a renewal application must be submitted to the AREC at least **30 days before the expiration date if a timeline extension is applied for**, and **60-90 days ahead of the expiration date if amendments are included in the renewal application**. If work is completed at the end of the approval period, a close-out report must be submitted. Renewals/close-out reports are submitted via the RIG system.

I take this opportunity to wish you all the best with your study.

Yours faithfully

[redacted]

Dr Dalene Vosloo, PhD, Pr. Nat. Sci.
Chair: Animal Research Ethics Committee
/kr

cc Supervisor: Dr Luvuno Miuleki, Dr Musa Mabandla
cc BRU Manager: Dr Jaca

The UKZN AREC is registered with the SA National Health Research Council (Reg. No. AREC-200111-006; Expires on 31/12/2027) in accordance with the National Health Act (No 61 of 2006) and conforms to the guidelines of the Department of Health (DOH2015) and SANS10386:2021. The UKZN AREC has an active US NIH OLAW assurance (Reg. No. F18-00371; Expires on 31/12/2027).

Animal Research Ethics Committee (AREC)
Ms Karen Reinertsen (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 8850 **Facsimile:** +27 (0) 31 260 4609 **Email:** animalethics@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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Appendix 2: Summary of guidelines to authors – **Elsevier: Pharmacology Biochemistry and Behaviour**

Title page

You are required to include the following details in the title page information:

Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood (e.g., DNA).

Author and Affiliations

Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Affiliations. Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address. Ensure that you provide the full postal address of each affiliation, including the country name and, if available, the email address of each author.

Correspondence:

Corresponding author. Clearly indicate who will handle correspondence for your article at all stages of the refereeing and publication process and post-publication.

Abstract

The author is required to provide a concise and factual abstract which does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results, and major conclusions. Some guidelines:

Keywords

You are required to provide 1 to 7 keywords for indexing purposes.

Article structure

Article sections

The author must divide the article into clearly defined and numbered sections. Number subsections 1.1 (then 1.1.1, 1.1.2, ...), then 1.2, etc. Use the numbering format when cross-referencing your article. Do not just refer to "the text." The author may give subsections a brief heading. Headings should appear on a separate line. Do not include the article abstract within section numbering.

Acknowledgements

Include any individuals who helped you during your research, such as help with language, writing, or proofreading, in the acknowledgements section. Acknowledgements should be placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

Author contributions: CRediT

Corresponding authors are required to acknowledge co-author contributions using CRediT (Contributor Roles Taxonomy) roles:

Appendix 3: Summary of guidelines to authors – **Elsevier: Brain, Behaviour, & Immunity-Health**

Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood (e.g., DNA).

Author and affiliations

Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Affiliations. Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address.

Correspondence

Clearly indicate who will handle correspondence for your article at all stages of the refereeing and publication process and post-publication. It is important that the email address and contact details of your corresponding author are kept up to date during the submission and publication process.

Abstract

The author is required to provide a concise and factual abstract that does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results, and major conclusions. Some guidelines:

Abstracts must be able to stand alone, as abstracts are often presented separately from the article.

Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s). Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

Keywords

The author is required to provide 1 to 7 keywords for indexing purposes. Keywords should be written in English. Please try to avoid keywords consisting of multiple words (using "and" or "of").

Figure captions

All images must have a caption. A caption should consist of a brief title (not displayed on the figure itself) and a description of the image. We advise you to keep the amount of text in any image to a minimum, though any symbols and abbreviations used should be explained.

Provide captions in a separate file.

Article structure

Article sections

Divide your article into clearly defined and numbered sections. Number subsections 1.1 (then 1.1.1, 1.1.2, ...), then 1.2, etc. Use the numbering format when cross-referencing within your article. Do not just refer to "the text." The author may give subsections a brief heading. Headings should appear on a separate line. Do not include the article abstract within section numbering.

Tables

Tables must be submitted as editable text, not as images. Some guidelines:

The author must place tables next to the relevant text or on a separate page(s) at the end of your article and cite all tables in the manuscript text. Number tables consecutively according to their appearance in the text and provide captions along with the tables. Place any table notes below the table body. Avoid vertical rules and shading within table cells.

Glossary

Please provide definitions of field-specific terms used in your article in a separate list.

Acknowledgements

Include any individuals who helped you during your research, such as help with language, writing, or proofreading, in the acknowledgements section. Acknowledgements should be

placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

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