

**RECOVERY AFTER STRESS ON HPA ACTIVITY, MEMORY AND
ASSOCIATED MOLECULAR CHANGES IN LPS-CHALLENGED
RATS**

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Discipline of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

2025

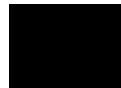


DECLARATION

I, **Esethu Siku**, hereby declare that the dissertation entitled “**Recovery after Stress on HPA Activity, Memory and Associated Molecular Changes in LPS-Challenged Rats** “ is the result of my own investigation and research, and it has not been submitted in part or in full for any other degree or to any other university. Where the use of the work of others was made, it is duly acknowledged in the text.

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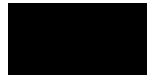
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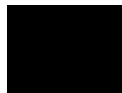
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Master of Medical Sciences, 2025

1. I know that plagiarism is wrong. Plagiarism is to use another's work and pretend that it is one's own.
2. I have used the Vancouver Convention for citation and referencing.
3. Each contribution to and quotation in this dissertation has been attributed, cited and referenced.
4. This dissertation is my work. I have not allowed anyone to copy my work to pass it off as his or her work.

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr Luvuno and Prof Mabandla, for their unwavering support, expert guidance, and constructive feedback throughout this research. Their insight and encouragement were instrumental in shaping the direction and quality of this thesis.

I am also sincerely grateful to the Department of Physiology and the Biomedical Research Unit's technical staff for providing the academic environment and resources necessary to conduct this study. Their technical assistance and kind support greatly facilitated my work.

To my fellow lab mates and colleagues, thank you for your collaboration, stimulating discussions, and for creating a supportive and motivating atmosphere throughout this journey.

I am forever grateful to my family for their unconditional love, patience, and constant encouragement.

Lastly, I would like to dedicate this thesis to everyone who has helped me, directly or indirectly, during this journey. Your contributions, no matter how small, have had a lasting impact on this work.

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ABBREVIATIONS

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

STUDY OUTLINE

Chapter 1: Includes the introduction, which provides a synopsis of the research theme and knowledge gaps. The current study's aims and objectives show how this goal was achieved. The study's overall methodology provides an overview of how the goals and objectives were accomplished.

Chapter 2: presents the literature review, which is an analysis of key findings and methodologies of previous research. It highlights knowledge gaps, thus providing context and justification for further study.

Chapter 3: This chapter consists of the first research study in manuscript format, titled “**post-stress recovery on the HPA-Axis activity and associated neurochemistry in LPS-challenged rats**”.

Chapter 4: presents the second research study in a manuscript format. This study investigated the role of “**Recovery after stress in memory performance and associated molecular changes in LPS-challenged rats.**”

Chapter 5: This chapter provides a synopsis of the research themes covered in two studies, explaining them and their findings to a non-science community. The discourse focuses on the linkages between the content covered by the studies because of the broad study aim(s) and objectives.

The appendix contains supplementary information that might be useful to the reviewers.

MAIN ABSTRACT

The long-term impact of chronic stress and immune activation on hypothalamic-pituitary-adrenal (HPA) axis function, cognition, neuroinflammation, oxidative stress, and neuroprotective proteins like FOXO3 and CDK10 remains poorly understood, especially in the context of post-stress recovery followed by acute immune challenge.

Therefore, this study aimed to investigate the effects of a recovery period following chronic unpredictable mild stress (CUMS) on HPA axis function, memory performance, oxidative stress, neuroinflammation, and the concentration of FOXO3 and CDK10 in lipopolysaccharide (LPS)-challenged rats.

12-week-old male Sprague-Dawley rats were randomly divided into five groups (n=5/group): Control, CUMS, PSR, LPS, and PSR+LPS. After a week of acclimatisation, a 10-day CUMS protocol was applied, and a 7-day recovery period. Thereafter, rats received a single dose of LPS (5 mg/kg, i.p.). After LPS injection, Spatial learning and memory were evaluated with a Morris-Water Maze Test. Blood, hippocampus, and cerebral cortex were then collected for biochemical analyses such as ELISA and RT-qPCR. These biochemical analyses were used to quantify specific proteins and assess gene expression levels.

CUMS elevated CRH, ACTH, and corticosterone concentration, indicating sustained HPA axis activation. LPS increased corticosterone and GR levels, reflecting a compensatory neuroendocrine response. Importantly, the PSR+LPS group demonstrated an adaptive HPA axis profile, marked by increased GR and reduced ACTH and corticosterone in specific brain regions. Recovery following stress partially improved memory performance and reduced neuroinflammation, as indicated by increased time spent in the goal quadrant and lower IL-1 β concentration. However, oxidative stress persisted as evidenced by an increase in the CUMS and LPS groups, and FOXO3 and CDK10 concentrations were only partially restored. Overall, these findings suggest that recovery may promote partial neuroendocrine adaptation to subsequent immune challenges, although residual oxidative and inflammatory disturbances persist.

Keywords: Chronic stress, Stress Recovery, Lipopolysaccharide, Oxidative stress, Inflammation, Cognitive function, HPA axis.

CHAPTER 1

1.1 Background

Stress affects both physical and mental health in people of all ages, genders, ethnicities, and religious backgrounds (1). The body's and mind's response to stress is shaped by several factors, including whether the stress is acute or chronic, the type of stressor involved, and individual characteristics such as life stage, gender, and even species (2). The HPA axis plays a central role in the body's response to stress by regulating the release of cortisol through a cascade involving CRH and ACTH (3). This system operates in a circadian rhythm, and cortisol exerts negative feedback to maintain balance. The hippocampus, with its high density of glucocorticoid and mineralocorticoid receptors, is key to this feedback regulation and is highly sensitive to changes in glucocorticoid levels (3). Cortisol, especially during stress, affects brain regions involved in emotion, cognition, and memory, particularly the cortex and hippocampus. Chronic stress, especially during early adulthood, initiates or accelerates neurodegenerative processes by disrupting HPA axis regulation, promoting neuroinflammation, increasing oxidative damage, impairing neurotrophic support, and damaging synaptic and mitochondrial function (3). These mechanisms collectively increase the brain's vulnerability to neurodegenerative disease later in life. Research has increasingly demonstrated that the immune system, particularly through microglial activation, plays a key role in regulating central nervous system (CNS) function and cognitive processes (4). Microglial activation likely serves as a central mechanism linking chronic stress and LPS-induced immune activation to cognitive impairment (4). Prolonged activation of microglia results in sustained production of pro-inflammatory cytokines such as Interleukin-1 β (IL-1 β) and oxidative mediators, contributing to elevated 4-Hydroxynonenal (4-HNE) and altered antioxidant defence (SOD-1)(4). LPS, an endotoxin derived from gram-negative bacteria, is a potent immune activator capable of inducing systemic and central inflammatory responses(5). When administered peripherally, LPS stimulates the production of pro-inflammatory cytokines such as interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α), both of which are pivotal in mediating neuroimmune signalling within the CNS (6). Notably, LPS crosses the blood-brain barrier and rapidly activates resident immune cells, microglia, initiating a cascade of neuroinflammatory processes (7). Despite substantial evidence in aged populations, the role of neuroinflammation in 12-week-old individuals remains underexplored. Oxidative stress is tightly intertwined with

inflammation because immune activation leads to the production of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), and pro-inflammatory cytokines, all of which contribute to cellular and neuronal processes (8). Oxidative stress and inflammation are central to the pathological processes explored in this study. Chronic stress and LPS-induced immune activation are expected to disrupt redox balance and initiate inflammatory cascades, particularly in the hippocampus and cortex (9). These disruptions are expected to be seen as elevated 4-HNE and IL-1 β levels, along with compromised antioxidant defences such as superoxide dismutase-1, contributing to the cognitive deficits which can be observed in the Morris Water Maze in animals. The combined stress and LPS group are anticipated to show the most severe molecular and behavioural impairments, underscoring the cumulative effect of prolonged stress and inflammation on brain function. LPS is known to reduce antioxidant defences (e.g., SOD-1, Catalase (CAT), Glutathione Peroxidase (GSH-PX)), resulting in a redox imbalance and promoting oxidative stress (10). This oxidative burden activates key transcription factors such as Nrf and NF- κ B, which in turn regulate the expression of cytokines and inflammatory mediators (10). Over time, this process may contribute to mild chronic inflammation and cognitive impairments, especially if recovery mechanisms are inadequate. Importantly, there are certain genes, such as FOXO3, FOXO1, CDK10, and CDK11, which are involved in neuroprotective processes such as oxidative stress resistance, autophagy regulation, and neural progenitor cell differentiation (11). FOXO3 has been shown to protect neurons against oxidative damage, reduce neuroinflammation, and regulate autophagy functions that are critical for maintaining neural health and preventing the progression of neurodegenerative conditions (12). CDK10, on the other hand, is associated with neurodevelopment and may be crucial for brain plasticity (13). Current literature predominantly focuses on aged individuals when studying oxidative stress, neuroinflammation, and neuroprotective gene activity. This then leaves a significant gap in understanding how these processes unfold in the young adult's brain, particularly in the context of immune-induced inflammation and chronic stress. According to literature about the relationship between the ageing brain and neurodegenerative disorders, little attention has been given to 12-week-old brain and its link with chronic stress recovery, inflammation, HPA axis response, oxidative stress and neuroprotective genes in cognitive function. Chronic stress and LPS-induced acute immune activation are expected to interact in ways that profoundly disrupt brain homeostasis. CUMS is anticipated to activate the HPA axis, leading to elevated levels of CRH, ACTH, and corticosterone, while downregulating Glucocorticoid receptor (GR) expression in stress-sensitive brain regions such as the hippocampus and cortex. Although a recovery phase

may allow partial normalisation in some animals, full restoration of HPA axis balance is not expected. Consistent with LPS-induced neuroinflammation, LPS exposure is expected to induce a robust neuroinflammatory response characterised by increased IL-1 β and sustained microglial activation, particularly in animals with prior stress exposure. This immune response would likely contribute to oxidative stress, evidenced by elevated 4-HNE and decreased SOD-1, especially when combined with chronic stress. These processes disrupt neuronal function and plasticity, impairing memory performance. At the molecular level, chronic stress and LPS are also expected to suppress the expression of FOXO3 and CDK10, two neuroprotective genes involved in antioxidant defence, cell survival, and stress response. When stress and inflammation are combined, their effects are predicted to be synergistic, leading to the most severe impairments in spatial memory, neuroinflammation, oxidative damage, and gene dysregulation, reflecting a failure of defence mechanisms and increased vulnerability of the brain to cumulative physiological insults. To assess the functional consequences of chronic stress and immune activation on memory performance, this study will employ the Morris Water Maze (MWM), a well-established behavioural test for evaluating spatial learning and memory, which are primarily hippocampus-dependent functions. Given the hippocampus's vulnerability to both oxidative stress and inflammation, the MWM serves as a sensitive and reliable method for detecting cognitive impairments resulting from these pathological processes. Incorporating the MWM ensures a comprehensive evaluation of how impaired physiological recovery from stress and immune challenges is translated into functional cognitive deficits. Therefore, this study aims to investigate how the physiological recovery process following stress will influence cognitive abilities, inflammatory responses, and oxidative damage in LPS-challenged animals. Furthermore, to explore the potential effects of physiological recovery from CUMS exposure on the HPA axis function in LPS-challenged animals.

1.2 Hypothesis: Exposure to a post-stress recovery period following CUMS will restore spatial learning abilities in LPS-challenged animals by attenuating oxidative stress and inflammation, normalising HPA axis activity, and enhancing neuroprotective gene expression.

1.3 Aims

To investigate how recovery following exposure to chronic unpredictable mild stress influences memory performance, oxidative stress, inflammation, HPA axis function, and the concentration of neuroprotective markers (FOXO3 and CDK10) in LPS-challenged rats.

1.4 Objectives

1. To assess spatial learning and memory performance using the Morris Water Maze test in LPS-challenged male Sprague-Dawley rats following CUMS and subsequent recovery.
2. To assess oxidative stress and inflammation, cortical & hippocampal concentrations of 4-HNE, SOD-1, and IL-1 β were measured using ELISA in LPS-challenged male Sprague-Dawley rats following chronic unpredictable mild stress (CUMS) and subsequent recovery.
3. To assess neuroprotective mechanisms by quantifying the expression of FOXO3 and CDK10 in the hippocampus and cortex in LPS-challenged male Sprague-Dawley rats following CUMS and subsequent recovery, using real-time quantitative polymerase chain reaction (RT-qPCR).
4. To check the HPA axis by measuring concentrations of CRH, ACTH, GR, and corticosterone in the hippocampus and cortex of LPS-challenged male Sprague-Dawley rats following CUMS and subsequent recovery, using Enzyme-Linked Immunosorbent Assay (ELISA).

1.5 Potential Benefits

Findings from this study may provide insights into the potential neurobiological significance of recovery periods in mitigating the adverse effects of stress and inflammation. However, since these findings are based on an animal model, caution is needed when applying them to humans.

1.6 Methodology Overview

Male Sprague-Dawley rats were obtained from the Biomedical Research Unit (BRU) at the University of KwaZulu-Natal, Westville campus. All animals were provided with

environmental enrichment and were given *ad libitum* access to standard rat chow and tap water. All animal experimentations were approved by the animal research ethics committee of the University of KwaZulu-Natal (AREC/00004699/2022).

1.7 Experimental design

Animals were randomly group-housed (n=5/group) with the same mates throughout the acclimation and testing periods. The Morris Water Maze Test was performed on days 23 & 30 of the experiment. This was followed by decapitation using a guillotine on days 24 and 30 of the study. The cerebral cortex was collected and stored at -80°C in a bio-freezer for further analysis. Cytokines, oxidative stress markers, and neuroprotective genes were assessed. Statistical comparisons were performed using GraphPad PRISM version 8 software. Data were expressed as mean $\pm$ SEM. A one-way analysis of variance (ANOVA) was used to compare the five independent groups, namely, Control, CUMS, PSR, LPS and PSR+LPS, followed by a Tukey's post hoc test. A p-value < 0.05 was considered significant. Data were expressed as $\pm$ SEM.

CHAPTER 2

Literature Review

2.1 Stress

Stress has an impact on both physical and mental health across an individual's lifespan (14). The body's response to stress is shaped by several variables, including the duration and nature of the stressor, as well as individual differences such as age and gender (15). Recent data from the American Institute of Stress reports that 33% of Americans experience high stress levels, with notable effects on physical (77%) and mental (73%) health, and sleep disturbances in 48% of individuals (16). Globally, stress affects around 35.1% of people, with a higher prevalence in men (64%) than women (36.1%). Notably, stress levels have increased over time in low- and middle-income countries. According to the WHO, stress-related issues contribute to the loss of 12 billion workdays and cost the global economy about \$1 trillion annually (17).

2.2 Physiological Stress Mechanisms: HPA Axis and Glucocorticoids

Stress activates the release of two primary types of hormones: glucocorticoids (corticosterone in animals and cortisol in humans) and catecholamines (adrenaline and noradrenaline). The rapid release of these hormones in response to a stressor initiates the hormonal cascade of the stress response. Together, they stimulate the fight-or-flight reaction, leading to changes like elevated heart rate and blood pressure (18). This fight or flight response then activates the HPA axis, the HPA axis is essential for maintaining homeostasis (19). When a stressor activates the hypothalamus, it triggers the release of CRH (20). These hormones stimulate the production of ACTH from the anterior pituitary, and ACTH stimulates the adrenal cortex to produce cortisol. Normally, CRH and ACTH levels fluctuate in a regular circadian rhythm, with high cortisol levels providing negative feedback to inhibit their production. This predictable rhythm and response of the HPA axis make it useful for both experimental and clinical studies (20). Cortisol levels rise during stress and affect the cortex, particularly areas involved in higher cognitive functions, like decision-making, working memory, and emotional regulation (21).

2.3 Hippocampal Vulnerability and Neurogenesis

The hippocampus plays a crucial role in the negative feedback loops that ultimately terminate the physiological stress response and is therefore particularly important in the regulation of glucocorticoid levels (22). Because of its high density of mineralocorticoid receptors and glucocorticoid receptors, the hippocampus is the region of the brain that is most susceptible to alterations in glucocorticoid levels (23). Animal studies have demonstrated that prolonged severe stress causes damage to hippocampal CA1 pyramidal neurons in the CA1 region (24), and that neurogenesis in the dentate gyrus is reduced by stress & enhanced by adrenalectomy (25). Prolonged exposure to elevated levels of corticosterone has also been associated with hippocampal neuronal cell death, changes to cell morphology, including atrophy of dendritic processes and reduced neurogenesis in animals (26). Stress-induced memory impairment has been reported in rats (27). The HPA axis helps the body adapt to stress by triggering hormonal responses that mobilise energy to manage challenges and maintain balance (28). To prevent overactivation, glucocorticoids (GC) provide feedback inhibition at all stages of the axis (29). These hormones bind to receptors, including low-affinity glucocorticoid receptors (GR) and high-affinity mineralocorticoid receptors (MR), which are essential for cellular function and brain processes like learning and memory (30). GC release follows a circadian rhythm regulated by MR, but during stress, GC levels rise, activating GR significantly (31). The hippocampus and cortex are essential in regulating the hypothalamic-pituitary-adrenal (HPA) axis through their interactions with glucocorticoids. In the hippocampus, both glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) are highly concentrated, especially in areas such as the ventral hippocampus and subiculum (32). These receptors play a critical role in modulating stress responses; for instance, damage to the ventral hippocampus or its major output pathways can impair the ability of corticosteroids to suppress HPA activation, leading to altered stress responses (32). In the cortex, GRs are primarily found in pyramidal cells, and the genetic deletion of GRs in these cells results in increased circulating glucocorticoid levels, emphasising the key role of GRs in maintaining balance within the HPA axis (32). Glucocorticoids, such as cortisol and corticosterone, play a key role in regulating cognitive functions and stress response through their actions in the hippocampus and prefrontal cortex (PFC) (33).

2.4 Allostatic Load and Chronic Stress

Chronic exposure to stress has been shown to impair cognitive function through mechanisms involving inflammation, oxidative damage, and structural brain changes, particularly in regions such as the hippocampus (34). Persistent activation of stress-regulatory systems, including the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis, may result in delayed recovery and cumulative biological wear and tear, as described by the allostatic load model (35, 36). Prolonged sympathetic and HPA axis activation due to chronic stress may extend the vulnerability to illness and injury (37). Although the benefits of recovery from acute work-related stress are well-documented, the long-term effects of recovery following chronic stress, particularly in young adults, remain poorly understood (38). The gap lies in understanding the effect of recovery after chronic mild stress in young adults on cognitive function and performance.

The chronic unpredictable mild stress (CUMS) model, originally adapted by P. Willner in 1987 from R. Katz's 1982 chronic stress protocol, is widely used to simulate depression-like states in rodents, particularly anhedonia, assessed via sucrose preference tests (39). Chronic stress alters neuronal structure and function in key corticolimbic regions prefrontal cortex, hippocampus, and amygdala, which are critical for emotion regulation and cognition (40). In stress-exposed rodents, neural atrophy occurs in the frontal cortex and hippocampus, while neuronal hypertrophy is observed in the amygdala, reflecting region-specific vulnerability associated with depression-related pathology (41). CUMS involves exposing animals to a series of mild, variable stressors in an unpredictable manner, leading to physiological, behavioural, and neurobiological disruptions that mimic depressive symptoms (42). Despite its widespread adoption, concerns have been raised regarding the model's reproducibility, predictive validity, and inter-laboratory consistency (43). A survey by Willner revealed that 58% of researchers were unwilling to comment on the model's reliability, highlighting hidden issues with replication and unpublished negative findings (44). Additionally, low statistical power in neuroscience studies may contribute to inflated effect sizes and poor reproducibility, calling for improvements and standardisation of the CUMS protocol to ensure more reliable and translatable results. Chronic stress and elevated glucocorticoid levels in the hippocampus can lead to dendritic retraction, impairing memory and learning processes, a phenomenon known as the "glucocorticoid vulnerability hypothesis". In the PFC, glucocorticoids enhance memory

consolidation but can also impair working memory, with these effects being mediated by noradrenergic activity and the activation of membrane-bound steroid receptors (45). Prolonged chronic stress exposure can cause dendritic atrophy in hippocampal neurons, impairing memory formation and spatial navigation. However, studies have shown that these stress-induced changes in the hippocampus can be reversible within 10 days after the termination of chronic stress, suggesting a degree of plasticity and recovery potential in this region (46). Chronic stress and systemic immune activation have profound effects on brain function and structure, leading to cognitive impairments and emotional disturbances. Specifically, clinical research indicates that stress exposure activates interconnected corticolimbic brain regions, such as the prefrontal cortex (PFC), amygdala (AMYG), hippocampal region (HPC), and hypothalamus, which control emotional well-being and cognitive function in animals and humans (47). In humans, it is believed that a combination of stress-resistant genes, temperament and coping mechanisms, and resources/stress buffers, which in some circumstances may promote greater cell longevity, enables psychological "stress resistance" in the face of adversity. Beyond suggestive correlations, it is unknown if psychological stress resistance, which is sometimes referred to as "resilience," is connected to cellular resistance to a variety of stressors or if the terms are only theoretically but not mechanistically related (48). Exogenous glucocorticoids or high levels of stress may harm hippocampus neurons and impair memory, according to animal research. These alterations have been noted in conjunction with the application of exogenous glucocorticoids or stress, and they seem to progress over the course of a lifetime of stress or glucocorticoid excess (49).

2.5 Effects of Stress on Memory and Cognitive Function

The hippocampus is well-positioned to recognise and react to stress due to its abundance of glucocorticoid receptors (50). Numerous reported alterations in hippocampus structure, such as altered neurochemistry, excitability, neurogenesis, neuronal morphology, and even cell death, are brought on by prolonged exposure to stress or glucocorticoids (51). Decades of studies have demonstrated the importance of the hippocampus in spatial learning and memory, prompting researchers to investigate the connection between chronic stress and spatial abilities (52). According to a previous study, Vyas *et al.*, chronic restraint causes hippocampus dendritic retraction, which can be increased by prolonged stress (53). Notably, hippocampus dendritic retraction develops gradually and self-corrects after recovery from long-term stress (46). There

are similarities between the reversibility of stress-induced structural alterations in the hippocampus and mental health problems like depression, which is reassuring. According to (54), depressed people have lower hippocampal activity and impaired hippocampal-dependent declarative memory. In fact, research has shown that prolonged antidepressant therapy, exposure to enriched environments, or physical exercise might decrease depressive-like behaviour and/or improve memory performance with increasing levels of hippocampal neurogenesis (6,14). Likewise, elevated depressive-like episodes and cognitive dysfunction have been associated with circumstances that inhibit hippocampal neurogenesis, such as highly elevated levels of stress hormones or unpredictable chronic stress hormones (UCS) (19, 26, 28). Therefore, research indicates a connection between reduced levels of hippocampal neurogenesis and depression brought on by stress, as well as cognitive failure. In terms of stress, it's evident that a significant amount of the public exhibits varying degrees of chronic stress in their daily lives (33). Yet, in only a fraction of individuals, chronic stress leads to mental illnesses such as depression & anxiety, suggesting that the type, intensity & duration of stress play roles in the development of stress-related mental illnesses.

Brain function also includes the crucial aspect of cognition. A person's ability to learn, make decisions, pay attention, and exercise judgment is referred to as cognitive ability (55). The degree, duration, source, and severity of stress all affect cognition in different ways (55). Expressing memories, the hippocampus, amygdala, and temporal lobe are the primary locations where cognition is generated (56). Stress is generally thought to lower cognition; hence, any behavioural measures performed to lower stress are thought to boost cognition (57). Stress triggers the activation of certain physiological systems, including the hypothalamus-pituitary-adrenal axis, autonomic nervous system, and central neurotransmitter and neuropeptide system. These systems directly impact the neuronal circuits in the brain responsible for processing information (58). Glucocorticosteroids are produced and released when stress is activated. Glucocorticosteroids can penetrate the blood-brain barrier and have long-term impacts on processing and cognition due to their lipophilic characteristics (59). According to (58, 60), stress appears to have the potential to induce pathophysiologic alterations in the brain that can lead to behavioural, cognitive, and mood disorders. Research has indicated that long-term stress can result in negative effects, such as elevated levels of IL-6 and plasma cortisol but decreased levels of brain-derived neurotrophic factor (BDNF) and cAMP-responsive element binding protein. These findings are strikingly similar to those reported in individuals with depression and mood disorders who display a variety of cognitive difficulties (60). Furthermore, a

physiological connection between stress and mood-based cognitive disorders has been demonstrated by the elevated concentrations of inflammatory factors such as interleukins and TNF- α , which are crucial in the development of cognitive disorders (58, 61). Research conducted on animals indicates that neurodegenerative processes, neuroendocrine and neuraminic variables, and stress contribute to cognitive problems (58). But it's important to remember that depression might not necessarily result from an overreaction to stress triggered by the body (62).

2.6 Lipopolysaccharide Effect on Memory and Cognitive Function

LPS, also known as endotoxin, is produced by a gram-negative bacterium. LPS plays a crucial role in inducing immunological and neurological symptoms during bacterial infections. LPS works through cytokines, and administering it peripherally or to the brain causes the brain to produce more cytokines (63). IL-1 β and tumour necrosis factor- α (TNF- α) are two pro-inflammatory cytokines that are involved in LPS's mechanism of action. Additionally, the effects of (central) LPS and IL-1 β on the CNS are prevented or attenuated by the exogenous administration of a competitive endogenous inhibitor called IL-1 receptor antagonist (IL-1Ra). Over the past two decades, research using animal models has shown that inflammation brought on by LPS can mimic several aspects of Parkinson's disease (PD), such as significant microglial activation and a targeted loss of dopaminergic neurons in the nigrostriatal pathway (64). Due to its tiny size, LPS can cross the blood-brain barrier and accumulate in the central nervous system (65). Brain injury can be prevented by low-dose LPS by causing the body to develop cross-immune tolerance (66). According to another study, LPS can protect against subarachnoid haemorrhage, greatly increase tolerance against cerebral I/R injury, and potentially improve the prognosis for neurological function. Pretreatment with 0.2 mg/kg LPS dramatically decreased the cerebral ischemia inflammatory response (67). Low-dose LPS pretreatment has been shown by Ding and Li (68) to decrease glial cell inflammatory damage and the generation of pro-inflammatory cytokines, such as TNF- α . In another study, a rat model of cerebral I/R was developed. The pretreatment with low-dose LPS resulted in less damage to the brain tissue, indicating that low-dose LPS can suppress the inflammatory response in ischemic brain injury (69). LPS has also been used as a systemic immune activation and it has been shown to impair hippocampus-dependent memories, such as contextual fear and spatial memory (70). A single LPS injection leads to a sustained pro-inflammatory response

characterised by microglial activation, moderate astrocytic reaction, and increased interleukin-6 levels, correlating with decreased neurogenesis. In contrast, repeated intermittent LPS injections do not elicit a prolonged pro-inflammatory response, and the associated neurogenic decrease is not observed (71). Pro-inflammatory cytokines, elevated during immune activation, negatively impact brain-derived neurotrophic factor and nerve growth factor levels. Activation of the HPA axis during immune response further inhibits BDNF expression in the brain. These neurotrophins are essential for maintaining neuronal health, and their reduction compromises neuroprotection, leading to cognitive deficits and structural brain changes (72). Systemic administration of LPS, a potent immune activator, induces glial activation and promotes the expression of proinflammatory cytokines within the central nervous system (73). This neuroimmune response manifests behaviourally as a transient sickness state, characterised by reduced locomotion, anorexia, social withdrawal, and hyperalgesia. Importantly, this acute phase is often followed by a more persistent depressive-like phenotype, which can be reversed with antidepressant intervention (74). IL-1 β has emerged as a key cytokine mediating this neuroinflammatory cascade and is strongly implicated in stress-related depressive behaviours (74). Its maturation and release are regulated by the Nod-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, a cytosolic sensor activated by diverse physiological stressors (75). NLRP3 activation leads to caspase-1-mediated cleavage of pro-IL-1 β into its active form, thereby amplifying the inflammatory response (75). Additionally, neuroinflammation is often accompanied by oxidative stress, to which the brain is particularly susceptible due to its high oxygen consumption and lipid-rich composition. Oxidative damage, including lipid peroxidation and nucleic acid oxidation, is increasingly recognized as a contributing factor in the pathophysiology of mood disorders and other neuropsychiatric conditions (76). Oxidative stress occurs when there's an imbalance between free radicals (reactive oxygen species) and antioxidants in the body (77). Prolonged oxidative stress disrupts cellular redox balance, leading to tissue damage and the activation of proinflammatory pathways (8). This happens because the excess free radicals activate certain transcription factors (like NF- κ B and AP-1), which then increase the production of pro-inflammatory cytokines. These cytokines are signalling molecules that promote inflammation as part of the body's immune response. Oxidative stress triggers inflammation, which in turn produces more free radicals that can lead to further oxidative stress (78). Oxidative stress is implicated in the pathogenesis of numerous metabolic disorders, chronic diseases, and cancers (79). Although the free radical theory originated over half a century ago, only in recent decades have

researchers elucidated its role in disease development and the therapeutic potential of antioxidants (80).

At physiological levels, reactive oxygen species (ROS) such as superoxide and hydroxyl radicals serve essential roles in immune defence and in redox signalling critical for cellular homeostasis (81). However, excessive ROS production induces protein, lipid, and DNA damage through membrane lipid peroxidation, protein oxidation/fragmentation, and DNA strand breaks or base modifications (82) which disrupts cellular function and may trigger apoptosis (82). Uncontrolled ROS accumulation is linked to various pathologies, including cancer, cardiovascular events, diabetes, and neurodegeneration (83). In cancer, oxidative DNA damage can result in mutations that drive malignant transformation (84).

To counterbalance ROS, organisms rely on a network of enzymatic (e.g., SOD-1 & CAT) and non-enzymatic antioxidants that neutralise radicals and repair macromolecular damage (85). When ROS production exceeds antioxidant capacity, often due to stress, pollution, or metabolic dysregulation, oxidative stress ensues, compromising biomolecules and impairing redox-dependent signalling (86).

In autoimmune and allergic diseases, oxidative stress can modify self-antigens and amplify immune responses, while antioxidant supplementation can have mixed effects (87). Additionally, environmental factors such as pollutants and heavy metals contribute to ROS generation and chronic disease burden (88). The mitochondrial free radical theory of ageing posits that ROS-induced mitochondrial DNA damage diminishes cellular regenerative capacity and accelerates tissue ageing (89). This underscores the dual role of oxidative stress both in disease progression and the ageing process.

2.7 Genes Activated by Inflammation

The Forkhead/winged helix box gene, group O (FOXO) proteins are a group of transcription factors that have been conserved throughout evolution and serve as a key hub for the integration of numerous significant cellular inputs. Mammals possess four FOXOs, namely FOXO1, 2, 3, 4, and 6. Tissue-specific and temporal variances in expression are observed, even though most are expressed universally. At least in skeletal muscle, FOXO3 may decrease more with age (90). Different genes can be regulated by each FOXO based on the kind of cell (91). Numerous tissues, including the CNS, have high expression levels of FOXOs (92). In animals, the brains

express all four FOXO proteins. Studies have suggested that FOXOs play a role in the nervous system's development and homeostatic control. The requirement for FOXOs for neuronal development was shown by the knockdown of FOXO proteins in a study on neuronal polarity (93). According to research, Foxo1 regulates downstream targets to reduce necroptosis and neuroinflammation after traumatic brain damage, hence serving a neuroprotective function (94). By preventing neuronal autophagy in rats, small interfering RNA (siRNA) knockdown of FOXO3 showed a neuroprotective function (95).

FOXO3 plays a role in immune system regulation (96). The immune system weakens with age, making infections more likely. Therefore, among the elderly, infections are a common cause of death. FOXO3 induces *Puma* and *Bim* upregulation in response to cytokine or growth factor withdrawal, which results in T cell death (97). In the human kidney, lung, and gut, FOXO3 stimulates the synthesis of antimicrobial peptides (98). They act as effector molecules of innate immunity and combat microbial infections in a variety of animals.

FOXO3 regulates the generation of cytokines, inhibits NF- κ B activation, suppresses T cell activation and proliferation, decreases lymphatic cell proliferation, and lowers inflammation, according to evidence from knockdown studies in mice (99). Inhibiting the synthesis of inflammatory cytokines like interleukin-2 (100) and interleukin-6 (100) is one of its anti-inflammatory activities. The pleiotropic cytokine interleukin-6 is activated in response to infection (101), and this affects the immune system in a number of ways, including antibody formation, T cell activity, hemopoiesis, and inflammation. Elevated interleukin-6 in the elderly is linked to a higher risk of death from cancer and coronary heart disease, as well as all causes (102). Previous studies have shown that oxidative stress triggers the activation of FOXO3, which then enters the cell nucleus and stimulates the production of genes related to antioxidant defence, including CAT and SOD-1. This activation aids in preventing oxidative damage to neurons in the brain, which is a major contributing cause to numerous neurodegenerative illnesses like Alzheimer's, Parkinson's, and stroke. FOXO3 supports the maintenance of neuronal function and guards against cognitive decline by increasing the brain's capacity for antioxidants (103).

FOXO3 is known to directly regulate a network of autophagy genes, which are crucial for cellular maintenance and stress responses. This regulatory role of FOXO3 can intersect with various cellular pathways, potentially including pathways involving Cyclin-Dependent Kinase 10 (CDK10) (104). Autophagy is a natural process where your body cleans out damaged cells

and regenerates newer, healthier ones. Therefore, we measured the concentration of CDK10 in the cerebral cortex in the stressed group compared to the control group.

Recent research aimed to find and assess additional genes that are expressed in brain tissues and have molecular interactions with genes that are already known to cause neurodegenerative diseases. These genes could aid in the future discovery of diagnostic and prognostic indicators. The genes that also stand out as promising research targets include CDK10, CDK11, FOXO1 & FOXO3; these genes are involved in cell physiology, which are relevant to other disorders of the CNS and may influence the onset of neurodegenerative conditions. However, it is unknown how these genes are expressed in encephalic tissues (105). Through significant manipulation of the Raf-Merk-Erk1/2 signalling pathway, which is crucial for cell proliferation, differentiation, and even cell death, CDK10 may have a significant impact on the expression of brain progenitor cells (106). As a result, understanding CDK10's gene expression in the context of Alzheimer's disease, a neurodegenerative condition, can be useful for understanding this gene's role in neurodegeneration/neurogenesis. The differentiation of neural progenitor cells has been linked to CDK10. CDK10 is a serine/threonine kinase involved in cell cycle regulation, transcription, and cellular differentiation, and has emerging roles in neurological and inflammatory responses (11). While CDK10 has been studied primarily in the context of cancer and developmental disorders, recent evidence suggests it may also respond to cellular stress and inflammatory stimuli (11). Some studies have shown that CDK10 expression can be modulated by external stressors, including oxidative and endoplasmic reticulum stress, and may contribute to cell survival pathways (11). However, the specific effects of psychological stress and immune activation through LPS on CDK10 expression, particularly in the brain, remain poorly understood. There is currently a lack of direct evidence linking LPS-induced neuroinflammation or chronic stress exposure to changes in CDK10 levels in neuronal or glial cells. As such, the role of CDK10 in stress-related cognitive impairment or recovery remains an underexplored but promising area for investigation. Neurodegenerative diseases or developmental abnormalities could result from this gene's dysregulation (107). Both genes have been mainly investigated in aged individuals; hence, our study will contribute to the little to non-existent literature on FOXO3 and CDK10 neuroprotective effects in the brains of young adults.

2.8. Conclusion

Although substantial evidence links chronic stress and immune activation to neuroinflammation and oxidative damage, most studies focus on aged populations, leaving a critical gap in understanding how these mechanisms operate in young adults. Moreover, the roles of neuroprotective genes such as FOXO3 and CDK10 in stress adaptation and recovery remain underexplored. FOXO3 and CDK10 are popularly known as molecular targets due to their established roles in cellular stress regulation and neuroprotection. There is limited understanding of how these markers respond during post-stress recovery and immune activation. Therefore, this study investigates their role in animals exposed to chronic stress and inflammation. With the intention of providing insight into molecular mechanisms underlying resilience and inflammation in the brain. Given the growing relevance of stress-related cognitive decline in younger populations, it is essential to investigate whether recovery periods can mitigate these effects. This study is therefore justified in examining the impact of post-stress recovery on cognitive function, oxidative stress, inflammation, and neuroprotective gene expression in a young animal model, to better inform strategies for early intervention and resilience-building in humans. Most studies focus on the damaging effects of chronic stress, but this research takes a different approach by exploring what happens during recovery. By looking at how the brain responds after stress or inflammation. This study offers a new insight into resilience and the biological processes that support it.

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Chapter 3: Manuscript 1

Prologue

Literature evidence has shown that chronic stress and immune activation are known to disrupt neuroendocrine regulation, yet the capacity for recovery to restore HPA axis function, particularly under subsequent inflammatory challenge, remains poorly understood. This study investigates the effects of a post-stress recovery period following CUMS on HPA axis dynamics, memory performance, oxidative stress, neuroinflammation, and neuroprotective markers (FOXO3 and CDK10) in LPS-challenged rats.

Post-stress recovery on the HPA Axis activity and associated neurochemistry changes in LPS-challenged rats.

This manuscript was prepared according to the journal's guidelines for authors and was submitted for publication in the *Journal of Brain, Behaviour and Immunity (Experimental Neurology)*.

POST-STRESS RECOVERY ON THE HPA AXIS ACTIVITY AND ASSOCIATED NEUROCHEMISTRY CHANGES IN LPS-CHALLENGED RATS

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ABSTRACT

Chronic stress and immune activation are recognized as major disruptors of HPA axis function; however, the extent to which recovery following chronic stress can restore HPA axis regulation, particularly in the context of subsequent immune challenges, remains insufficiently understood. This study aimed to investigate the impact of recovery following exposure to CUMS on HPA axis function in LPS-challenged rats. 12-week-old Male Sprague-Dawley rats were randomly assigned to five experimental groups: Control, CUMS, Post-Stress Recovery (PSR), LPS, and Post-Stress Recovery + LPS (PSR+LPS). The CUMS protocol was applied for 10 days, which is a duration commonly used in rodent models to induce reliable behavioural and physiological stress responses. A 7-day recovery period was selected to allow sufficient time for potential normalization of stress-related biomarkers and to assess the brain's capacity for resilience. These time frames were chosen to align with previous studies and to explore under-investigated aspects of post-stress recovery. The animals also received a single intraperitoneal injection of LPS (5 mg/kg). Subsequently, concentrations of CRH, ACTH, CORT, and GR were assessed in the hippocampus, plasma, and cerebral cortex. The CUMS group exhibited significantly elevated CRH, ACTH, and CORT concentrations, indicating sustained HPA axis activation and impaired negative feedback regulation. LPS exposure alone led to increased CORT and GR concentration, likely reflecting a compensatory mechanism in response to immune-induced stress. Importantly, the PSR+LPS group demonstrated a more adaptive neuroendocrine profile, characterized by elevated GR concentrations and reductions in ACTH and CORT in specific brain regions, indicative of partial restoration of HPA axis function. Collectively, these findings suggest that post-stress recovery facilitates normalization of HPA axis dynamics and enhances resilience to subsequent inflammatory challenges, highlighting the potential therapeutic relevance of recovery-oriented interventions in the context of stress- and inflammation-related neuroendocrine dysfunction.

Keywords: Chronic stress, Stress Recovery, Lipopolysaccharide, HPA activity & Glucocorticoid Receptor.

3.1 INTRODUCTION

Stress activates the release of two primary types of hormones: glucocorticoids (corticosterone in animals and cortisol in humans) and catecholamines (adrenaline and noradrenaline) (14). The rapid release of these hormones in response to a stressor initiates the hormonal cascade of the stress response. Together, they stimulate the fight-or-flight reaction, leading to changes like elevated heart rate and blood pressure (18). This fight or flight response then activates the HPA axis, the HPA axis is critical for life and is a major part of our homeostatic regulatory system (19). When a stressor activates the hypothalamus, it triggers the release of CRH (20). These hormones stimulate the production of ACTH from the posterior pituitary, and ACTH stimulates the adrenal cortex to produce cortisol. Normally, CRH and ACTH concentrations fluctuate in a regular circadian rhythm, with high cortisol concentrations providing negative feedback to inhibit their production. This predictable rhythm and response of the HPA axis make it useful for both experimental and clinical studies (20). Cortisol concentrations rise during stress and affect the cortex, particularly areas involved in higher cognitive functions, like decision-making, working memory, and emotional regulation (21). The hippocampus plays a crucial role in the negative feedback loops that ultimately terminate the physiological stress response and is therefore particularly important in the regulation of glucocorticoid concentrations (22), because of its high density of mineralocorticoids receptors(MR) and glucocorticoid receptors(GR), the hippocampus is the region of the brain that is most susceptible to alterations in glucocorticoids concentrations (23). Animal studies have demonstrated that prolonged severe stress causes damage to hippocampal CA3 pyramidal neurons in the CA1 region (24), and that neurogenesis in the dentate gyrus is reduced by stress & enhanced by adrenalectomy (25). Prolonged exposure to elevated concentrations of corticosterone has also been associated with hippocampal neuronal cell death, changes to cell morphology, including atrophy of dendritic processes, and reduced neurogenesis in animals (26) and stress-induced memory impairment has been reported in rats (27). The HPA axis helps the body adapt to stress by triggering hormonal responses that mobilize energy to manage challenges and maintain balance (28). To prevent overactivation, glucocorticoids provide feedback inhibition at all stages of the axis (29). These hormones bind to receptors, including low-affinity GRs and high-affinity MR, which are essential for cellular function and brain processes like learning and memory (30). Glucocorticoid release follows a circadian rhythm regulated by MR, but during stress, glucocorticoid concentrations rise, activating GR significantly (31). The hippocampus and cortex are essential in regulating the HPA axis through their interactions with glucocorticoids. In the hippocampus, both GRs and MRs are highly concentrated, especially in areas such as

the ventral hippocampus and subiculum (32). These receptors play a critical role in modulating stress responses; for instance, damage to the ventral hippocampus or its major output pathways can impair the ability of corticosteroids to suppress HPA activation, leading to altered stress responses (32). In the cortex, GRs are primarily found in pyramidal cells, and the genetic deletion of GRs in these cells results in increased circulating glucocorticoid concentrations, emphasizing the key role of GRs in maintaining balance within the HPA axis (32). Glucocorticoids, such as cortisol and corticosterone, play a key role in regulating cognitive functions and stress response through their actions in the hippocampus and prefrontal cortex (PFC) (33). Chronic stress and elevated glucocorticoid concentrations in the hippocampus can lead to dendritic retraction, impairing memory and learning processes, a phenomenon known as the “glucocorticoid vulnerability hypothesis”. In the PFC, glucocorticoids enhance memory consolidation but can also impair working memory, with these effects being mediated by noradrenergic activity and the activation of membrane-bound steroid receptors (45). Prolonged chronic stress exposure can cause dendritic atrophy in hippocampal neurons, impairing memory formation and spatial navigation. However, studies have shown that these stress-induced changes in the hippocampus can be reversible within 10 days after the termination of chronic stress, suggesting a degree of plasticity and recovery potential in this region (46). Chronic stress and systemic immune activation have profound effects on brain function and structure, leading to cognitive impairments and emotional disturbances. The hippocampus and cortex were selected for analysis due to their sensitivity to stress-induced molecular changes, particularly in relation to BDNF expression. As outlined in previous studies, chronic stress downregulates hippocampal BDNF and disrupts neurogenesis, dendritic branching, and neuronal survival (108). These regions are central to cognitive and emotional processing, making them critical targets for understanding the impact of stress and recovery (109). LPS, a component of Gram-negative bacteria, is commonly used to model systemic immune activation and has been shown to impair hippocampus-dependent memories, such as contextual fear and spatial memory (70). A single LPS injection leads to a sustained pro-inflammatory response characterized by microglial activation, moderate astrocytic reaction, and increased interleukin-6 concentrations, correlating with decreased neurogenesis. In contrast, repeated intermittent LPS injections do not elicit a prolonged pro-inflammatory response, and the associated neurogenic decrease is not observed (71). To investigate the biological mechanisms underlying recovery from chronic stress, this study incorporates an LPS challenge during the post-stress phase. LPS is widely used to model systemic inflammation and immune activation, allowing us to assess how prior exposure to chronic stress influences

the brain's response to a secondary immune insult. By introducing LPS after the CUMS protocol, we hope to explore whether stress recovery alters neuroimmune sensitivity, thereby providing insight into resilience and vulnerability mechanisms in the hippocampus and cortex. Pro-inflammatory cytokines, elevated during immune activation, negatively impact brain-derived neurotrophic factor and nerve growth factor concentrations. Activation of the HPA axis during immune response further inhibits BDNF and nerve growth factor expression in the brain. These neurotrophins are essential for maintaining neuronal health, and their reduction compromises neuroprotection, leading to cognitive deficits and structural brain changes (72). While chronic stress and immune activation are well-established disruptors of HPA axis function, the mechanisms by which the HPA axis recovers following prolonged stress exposure remain incompletely understood. Most existing research has focused either on acute stress models or on the effects of chronic stress in adult or aged animals, leaving a critical gap in knowledge regarding how the developing brain of young adult animals adapts or recovers. Therefore, this study aims to investigate the impact of recovery following exposure to CUMS on the HPA axis function in LPS-challenged rats. It remains unclear whether a recovery period following chronic stress can normalize HPA axis regulation in the presence of an immune challenge.

3.2 METHODS & MATERIALS

3.2.1 Reagents

All chemicals and reagents were purchased from commercial suppliers and were of analytical grade.

Normal Saline (0.9% Sodium Chloride) S8776-100ML and LPS 437627 were procured from Sigma Aldrich, Boksburg, South Africa. The guillotine used in this study was obtained from the UKZN Biomedical Research Unit (BRU) in Durban, South Africa.

3.2.2 Experimental Animals

Twenty-five Male Sprague-Dawley rats that were 12 weeks of age, weighing 245g were randomly used in this study. The rats were obtained from the University of KwaZulu-Natal Biomedical Research Unit (BRU). The animals were housed under standard laboratory

conditions of temperature (22 ± 2 , CO₂ content (<5000 P.M), relative humidity ($55 \pm 5\%$) and illumination (12h light/dark cycle, lights on at 07H00). The noise levels were less than 65 decibels. All animals were provided with environmental enrichment and were given *ad libitum* access to standard rat chow and tap water. All animal experimentations were approved by the animal research ethics committee of the University of KwaZulu-Natal (AREC/00004699/2022).

3.2.3 Experimental Protocols

Animals were randomly group-housed ($n=5/\text{group}$) according to other studies (110) with the same mates throughout the acclimation and testing periods. The animals were acclimatized for 8 days and exposed to environmental enrichment, which included hiding structures and tunnels. These objects were used to support different natural behaviors such as burrowing & gnawing. The first group included the rats that were treated with saline as a vehicle, and this group was used to control for all contextual conditions in the study. The second group was the stressed group; in this group, the animals were exposed to chronic unpredictable mild stress (CUMS) for 10 consecutive days. This group was referred to as the CUMS group. The third group was the post-stress recovery group (PSR G), which was also exposed to CUMS for 10 consecutive days. This was followed by a week of non-exposure to stress for one week to allow the animals to recover from the CUMS effect. The fourth group was the LPS administration group (LPS G), to induce microglial activation, where a single dose of 5mg/kg was injected intraperitoneally into the animals. The last group of rats was exposed to CUMS for 10 consecutive days, allowed to recover for a week, and injected with a single dose of LPS (5mg/kg, i.p.) (PSR+LPS).

On day 8, environmental enrichment material was removed from CUMS, PSR, and PSR+LPS groups to allow the introduction of the CUMS protocol. It was initiated on day 8 and lasted for 10 days. We incorporated it for 10 days because it is widely recognized for inducing behavioural and physiological changes that resemble human depression (111). It involves exposing animals to a variety of mild stressors delivered daily and in a random order, which prevents habituation and mimics the unpredictability of real-life stress. It included the following stressors: cold exposure, cage tilt at an angle of 45° , daytime darkness, and animal restraint. The animals received two of the stressors per day. On day 19, the animals in the CUMS group were euthanized, and the blood was collected during the decapitation procedure

from the blood flow and then transferred into labelled tubes, which were then centrifuged at $10000 \times g$ for 10 min. Post centrifuge, plasma was pipetted into newly cooled Eppendorf tubes and stored at -80°C in a bio ultra-freezer until further analysis. The hippocampus & cerebral cortex were harvested and stored at -80°C in a Bio-freezer (Snijders Scientific, Holland) until ready for analysis. They were harvested to measure the CRH, ACTH, CORT, and GR concentrations. The PSR and PSR+LPS groups were given 7 days of recovery from the stress protocol. A 7-day recovery period was selected to allow sufficient time for the normalization of stress-related physiological and molecular changes following the 10-day CUMS protocol (112). During the recovery period, the environmental enrichment material was returned to the PSR groups, and the animals returned to their normal housing conditions. On day 26, the animals from the LPS group received an intraperitoneal injection of a 5mg/kg single dose of LPS, and the control group received a single dose of 5mg/kg of saline. Also, on day 26, the animals from all remaining groups were euthanized to collect tissues and store them for analysis. The hippocampus & cortex was harvested to measure HPA axis hormones. Blood was collected during the decapitation procedure from the blood flow and then transferred into labelled heparin tubes, which were then centrifuged. Post centrifuge, plasma was pipetted into newly cooled Eppendorf tubes and stored at -80°C in a bio ultra-freezer until further analysis.

3.2.4 Stress (CUMS) Protocol

The protocol involved using a CUMS procedure with minor modifications(113). The stress protocol consisted of daytime darkness (2 hours), 45° angle tilted cage (5 hours), cold exposure (5 minutes) at 4°C , and restraint (rat cylinder container) for 90 minutes. For the cold room exposure, animals were singly placed in new cages, and all animals from each group were placed in the cold room for 10 minutes simultaneously. There was always a 5-minute break from the previous group to the next group. This procedure was repeated 3 times per day. The animals received two random types of stressors per day over 10 days. The animals were carried to different rooms, where they were stressed according to the stress protocol. Food and water were available *ad libitum* during the stress procedure (114). The other group of animals, which didn't undergo the stress procedure, were still in their respective cages receiving environmental enrichment until the day of euthanization.

3.2.5 LPS Administration

LPS purchased from Sigma Aldrich, South Africa was used to induce memory impairment-like symptoms. A single dose of LPS(5mg/kg) was injected intraperitoneally on the LPS group in the morning between 9-10 am to minimize variability due to circadian fluctuations in stress hormone levels, which are known to influence immune and neuroendocrine responses. The calculation of the dosage was determined with the animal weight according to (115). The average body weight of the animals in the LPS-treated group was calculated to be 251.5 g (0.2515 kg). Based on the standard dosage of 5 mg/kg, the corresponding LPS dose was determined to be approximately 1.25 mg per animal. This dosage was then diluted to a final volume of 2 mL for intraperitoneal injection.

3.2.6 Tissue Collection

At the end of the experimental period, animals were sacrificed by a sharpened guillotine to collect blood. Brain tissue was removed and placed in a frozen 0.9% saline slush to preserve tissue quality. The tissues were collected at a fixed time between 09:00 and 10:00 am to minimize variability due to circadian fluctuations in stress hormone levels and ensure consistency across experimental groups. The tissues were removed from the skull with a surgical scissor and a surgical blade. The hippocampus and cortex were stored in differently labelled Eppendorf tubes, then snap-frozen in liquid nitrogen and stored at -80°C in a Bio Ultra freezer (Snijders Scientific, Holland), until the day of biochemical analysis. Blood was collected during the decapitation procedure from the blood flow and then transferred into labelled heparin tubes, which were then centrifuged at $10000 \times g$ for 10 min. Post centrifuge, plasma was pipetted into newly cooled Eppendorf tubes and stored at -80°C in a bio ultra-freezer until further analysis.

3.2.7 ELISA (Enzyme-linked Immuno-sorbent Assay)

Brain samples were thawed in ice, and then homogenized in PBS (0.01M, pH 7.4) with an ultrasonic cell disrupter (sonicator, Bio-Equip, South Africa) in a ratio of 1:9 (tissue weight(g); PBS (ml) volume). The homogenate was then centrifuged for 10 minutes at $5000 \times g$ at 2°C to get the supernatant. Plasma was used obtained from the centrifuged blood collected from the Male Sprague-Dawley rats. The ELISA from Elabscience 12(Gauteng ,South Africa) was conducted according to the manufacturer's instructions to quantify the concentrations of CRH (RTDL00249), ACTH (RTFI01483), Corticosterone (A79228) and GR (MBS752565).

3.2.8 Statistical analysis

Data was analysed using GraphPad PRISM version 8 software, it was also examined for outliers using boxplots and tested for normality of variance using the Shapiro-Wilk test. Data were expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) was used, followed by a Tukey's post hoc test. A p-value < 0.05 was considered significant. Each column & each point is the mean $\pm$ SEM of 5 rats per group.

3.3 Results

3.3.1 Corticotropin-releasing hormone concentration

CRH concentrations in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group (n=5), were measured during the experiment (Figure 3.3.1). There was a significant difference in CRH concentration between the groups ($F = 4.44$, $p < 0.05$; Figure 3.3.1). The concentration was higher in the CUMS group compared to the control group ($p = 0.0001$), with a mean difference of 30.83 & SE diff. of 0.6577. The concentration was higher in the CUMS group compared to the PSR group ($p < 0.0001$), with a mean diff. of 31.77 & SE diff. of 0.6577. The concentration was also higher in the CUMS group compared to the PSR+LPS group ($p < 0.0001$), with a mean diff. of 29.78 & SE diff. of 0.6577.

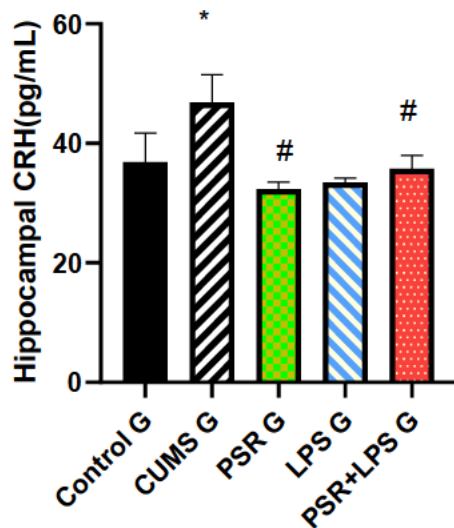


Figure 3.3.1: CRH concentration in the Control, CUMS, PSR, LPS and PSR+LPS (n=5/group). Values are presented as mean $\pm$ SEM. $p < 0.05$ was considered significant.

3.3.2 Adrenocorticotrophic hormone concentration

Plasma ACTH (a) & Hippocampal ACTH(b) concentration in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group (n=5/group), were measured during the experiment (Figure 2). There was a significant difference in the plasma ACTH concentration between the groups ($F=9.77$, $p < 0.05$, Figure 3.3.2(a)). There was a decrease in concentration in the control group compared to the CUMS group ($p=0.0286$), with a mean diff. of -29.34 & SE diff of 0.6788. There was a higher concentration in the control group compared to the PSR group ($p=0.0034$), with a mean diff. of 31.87 & SE diff. of 0.6788. There was also a higher concentration in the CUMS group compared to the PSR group ($p < 0.0001$), with a mean diff of 34.82 & SE diff of 0.6788.

There was a significant difference in the Hippocampal ACTH concentration between the groups ($F=17.50$, $p < 0.05$, Figure 3.3.2(b)). We found a CUMS effect in the CUMS group compared to the control group *(control vs CUMS group, $p < 0.005$, Figure 3.3.2(b)). There was a decrease in the concentration of the control group compared to CUMS group ($p=0.0127$), with a mean diff of -27.65 & SE diff. of 0.766. There was an increase in the concentration of the

CUMS group compared to the PSR group ($p < 0.0001$), with a mean diff. of 30.89 & SE diff. of 0.766.

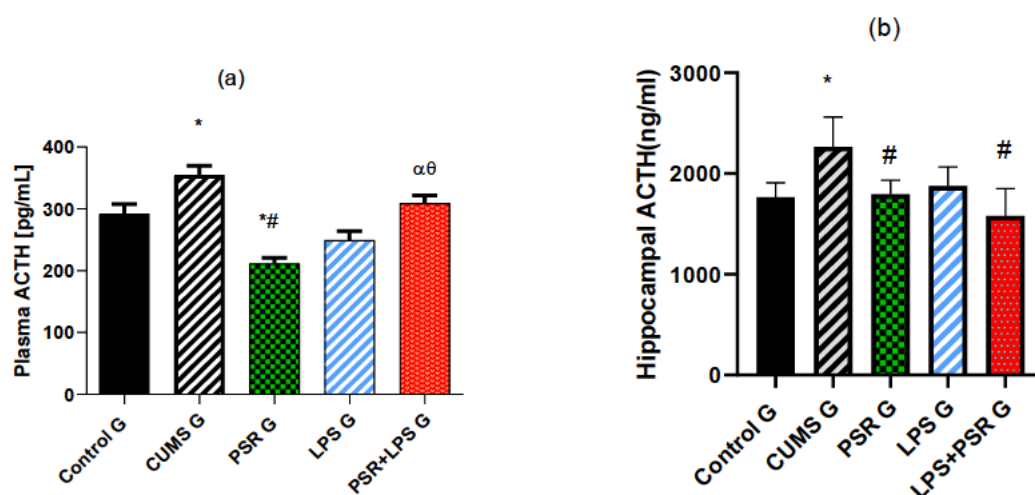


Figure 3.3.2: Plasma ACTH(a) & Hippocampal ACTH(b) concentration in the Control, CUMS, PSR, LPS and PSR+LPS ($n=5/\text{group}$). Values are presented as mean $\pm$ SEM. $p < 0.05$ was considered significant.

3.3.3 Corticosterone Concentration

Plasma CORT(a), Hippocampal CORT(b), and Cortical Cort (c) concentrations in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group ($n=5/\text{group}$), were measured during the experiment (Figure 3.3.3(a)). In the Corticosterone plasma, we found no significant differences between the groups. There was a decrease in the concentration of the control group compared to the CUMS group ($p=0.0019$), with a mean diff of -27.86 & SE diff. of 0.576. There was an increase in the concentration of the CUMS group compared to the PSR group ($p < 0.001$), with a mean diff of 30.78 & SE diff. of 0.576.

There was a significant difference in hippocampal corticosterone concentration between the groups ($F=4.44$, $p < 0.05$, Figure 3.3.3(b)). There was also a significant difference in the cortex's corticosterone concentration between the groups ($F=34.02$, $p < 0.05$, Figure 3.3.3(c)). There was a decrease in the concentration of the control group compared to the CUMS group ($p=0.0007$), with a mean diff. of -29.78 & SE diff. of 0.455. There was an increase in the

concentration of the CUMS group compared to the PSR group ($p<0.001$), with a mean diff. of 29.91 & SE diff. of 0.455.

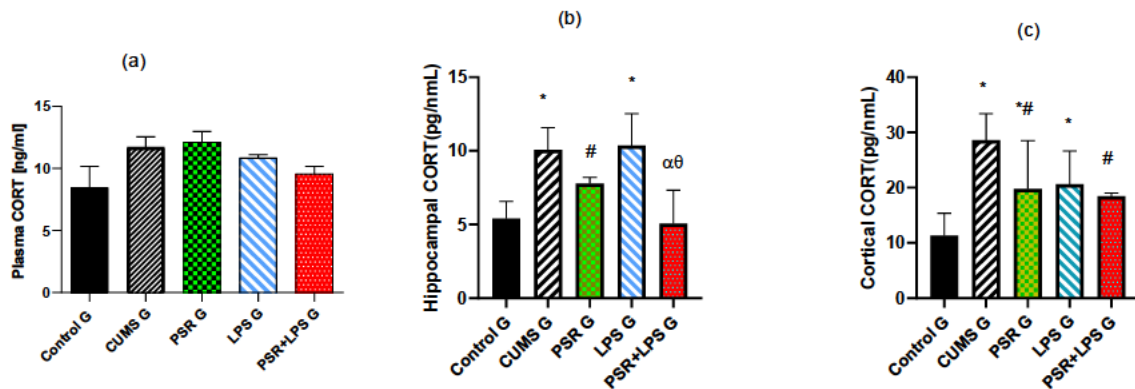


Figure 3.3.3: Plasma CORT, Hippocampal CORT & Cortical CORT concentration in the Control, PSR, LPS and PSR+LPS ($n=5/\text{group}$). Values are presented as mean $\pm$ SEM. $P < 0.05$ was considered significant.

3.3.4 Glucocorticoid receptor concentration

Hippocampal GR (a) & Cortical GR (b) concentration in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group ($n=5/\text{group}$), were measured (Figure 3.3.4(a)). There was a significant difference in the Hippocampal GR concentration between the groups ($F=11.08$, $p < 0.05$, Figure 3.3.4(a)). There was a decrease in the concentration of the CUMS group when compared to the LPS group ($p=0.003$), with a mean diff. of -27.56 & SE diff. of 0.675. There was another decrease in the concentration of the control group compared to the PSR+LPS group ($p<0.0001$), with a mean diff. of -30 & SE diff. of 0.675.

There was also a significant difference in the Cortical GRH concentration between the groups ($F=6.02$, $p<0.05$, Figure 3.3.4(b)). There was an increase in the concentration of the control group compared to the CUMS group ($p<0.001$), with a mean diff. of 30.78 & SE diff. of 0.675.

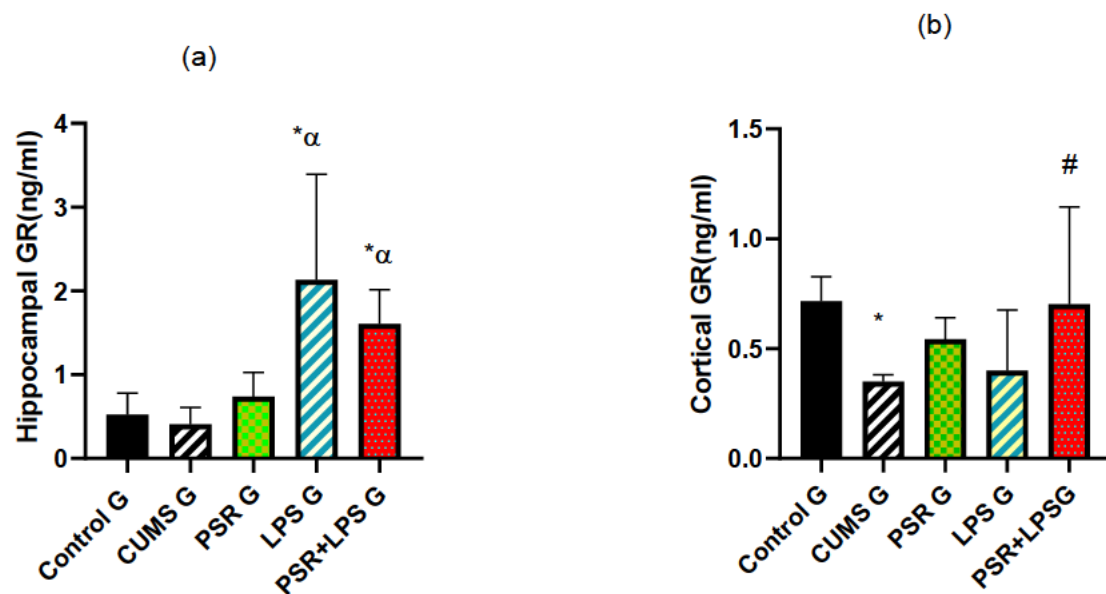


Figure 3.3.4: Hippocampal GR(a) & Cortical GR (b) concentration in the Control, PSR, LPS and PSR+LPS (n=5/group). Values are presented as mean±SEM. p<0.05 was considered significant.

3.4. Discussion

The extent to which recovery following chronic stress restores HPA axis function under subsequent inflammatory challenge remains poorly understood, particularly in the developing brain of young adult rats. The study sought to investigate the impact of recovery after CUMS in LPS-challenged rats on the HPA axis activity, as reflected by plasma ACTH and corticosterone concentrations. Furthermore, we sought to examine the associated changes in HPA axis-related hormones such as CRH, ACTH, and corticosterone within the cortex and hippocampus, with a focus on their potential effects on cognitive and stress response functions. Reductions in CRH, ACTH, and corticosterone concentrations, as well as high GR concentration in the PSR group despite acute immune challenge, suggest that post-stress recovery mitigates the long-term impact of chronic stress on HPA axis function and allows for a more adaptive response to subsequent immune challenges.

CUMS exposure led to elevated hippocampal CRH, ACTH, and corticosterone levels, consistent with prolonged HPA axis activation and impaired feedback regulation. These changes were attenuated in the post-stress recovery (PSR) group, suggesting partial normalisation of HPA axis function (116). Notably, CRH concentrations in the hippocampus decreased in both PSR+LPS groups compared to CUMS, indicating that recovery may restore negative feedback control even under immune challenge. This finding aligns with previous research showing that hippocampal CRH modulates excitability and memory-related circuits, and its dysregulation under chronic stress can impair cognitive function (117), (118).

Plasma ACTH levels mirrored this trend, increasing in the CUMS group and returning to or below baseline in the PSR group. ACTH levels in the PSR+LPS group remained comparable to controls, suggesting that recovery preserved the HPA axis's ability to respond adaptively to immune stress. The ability to mount an ACTH response to LPS reflects intact hypothalamic-pituitary sensitivity and highlights neuroendocrine plasticity following chronic stress.

Interestingly, plasma corticosterone levels remained unchanged across all groups, contrasting with significant increases in hippocampal and cortical corticosterone in both CUMS and LPS groups. This dissociation underscores the importance of measuring both peripheral and central hormone levels, as brain-specific regulation may be more sensitive to stress and inflammation. The elevated brain corticosterone in CUMS and LPS groups may reflect impaired local feedback mechanisms, while its reduction in the PSR group suggests recovery-driven recalibration of glucocorticoid signalling.

GR expression patterns further support this interpretation. In the hippocampus, GR levels were reduced in the CUMS group and restored in the PSR and PSR+LPS groups, indicating that recovery may enhance feedback sensitivity and buffer against subsequent immune activation. In the cortex, GR expression also improved in the PSR+LPS group, suggesting region-specific modulation of HPA axis recovery. These findings align with the role of GR in maintaining glucocorticoid homeostasis and regulating stress-responsive gene transcription.

Although previous studies have linked GR signalling to synaptic plasticity, memory, and emotional regulation, our data do not directly assess these outcomes. Therefore, interpretations regarding cognitive resilience remain speculative. Future studies incorporating behavioural assessments and synaptic markers are needed to establish functional links between hormonal recovery and neurocognitive outcomes.

In summary, our results demonstrate that post-stress recovery may exert a restorative effect on HPA axis regulation, particularly within the brain. Despite an acute immune challenge, recovered animals maintained hormonal balance and GR expression, reflecting a regained capacity for neuroendocrine adaptation. These findings highlight the potential of recovery periods to enhance stress resilience and mitigate long-term neuroendocrine dysfunction following chronic stress exposure.

3.5. Conclusion

Taken together, these results suggest that post-stress recovery mitigates the long-term impact of chronic stress on HPA axis function and allows for a more adaptive response to subsequent immune challenges. This was evidenced by reduced CRH, ACTH, and corticosterone concentrations as well as high GR concentration in the PSR group despite acute immune challenge. This highlights the therapeutic potential of recovery periods or interventions that mimic recovery in stress-related and inflammation-linked conditions.

3.6. Declarations:

None

Conflict of interest:

None

Author's Contribution :

This research project was conceptualized by Miss Siku, who also designed the methodology, conducted the experiments, and carried out data collection and analysis. She independently interpreted the findings and composed the dissertation. Dr. Luvuno and Prof. Mabandla provided critical guidance throughout the study, helping to shape the research direction and refine the research question. They reviewed multiple drafts, offered constructive feedback, and supported the development and structure of the dissertation. Their contributions also included insightful input during data analysis and interpretation of results. As Supervisors, they provided expert oversight and support across all stages of the research process.

Funding :

The National Research Foundation, South Africa supported this work.

Ethical Approval :

Ethical approval was obtained from the Animal Research Ethics Committee((AREC/00004699/2022) of the University of KwaZulu-Natal.

Acknowledgements :

The authors are grateful to the National Research Foundation for providing funding for this study and to the Biomedical Research Unit for supplying us with rats.

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Chapter 4: Manuscript 2

Prologue

Dysregulation of the HPA axis is a central feature of chronic stress, contributing to downstream effects such as oxidative stress and neuroinflammation. Chronic stress and immune activation are known to impair brain function through mechanisms involving oxidative stress and neuroinflammation. However, the extent to which a recovery period following stress can reverse these effects, particularly in the presence of subsequent immune challenges, remains unclear. This study investigates the impact of post-stress recovery on memory performance, oxidative and inflammatory markers, and the expression of FOXO3 and CDK10, two genes implicated in neuroprotection.

Recovery after stress on memory performance and associated molecular changes in LPS-challenged rats.

This manuscript was prepared according to the journal's guidelines for authors and will be submitted for publication in the Journal of Brain, Behaviour and Immunity (Experimental Neurology).

Recovery after stress on memory performance and associated molecular changes in LPS-Challenged rats.

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ABSTRACT

The long-term effects of chronic stress and immune activation on neuroinflammation and oxidative stress, as well as the roles of FOXO3 and CDK10 in young adults, remain poorly understood. We investigated the impact of a recovery period following exposure to chronic unpredictable mild stress (CUMS) on memory performance, oxidative stress, inflammation, the expression of FOXO3 and CDK10 in LPS-challenged rats.

Animals were assigned to five groups: Control, CUMS, Post-stress Recovery (PSR), LPS, and Post-Stress Recovery + Lipopolysaccharide (PSR+LPS). After 10 days of CUMS and a 7-day recovery period, animals received a single intraperitoneal injection of LPS (5mg/kg) followed by the assessment of spatial learning and memory following sacrifice, the cerebral cortex to measure 4-Hydroxynonenal (4-HNE), Superoxide Dismutase 1 (SOD-1), and Interleukin-1 β (IL-1 β) concentrations, as well as FOXO3 and CDK10 expression was collected.

Recovery after stress partially improved memory performance, as indicated by reduced escape latency and increased goal quadrant time in the PSR group compared to the CUMS group. However, the recovery period did not attenuate LPS-induced oxidative stress. Recovery reduced IL-1 β concentration compared to CUMS, indicating attenuated neuroinflammation; however, it remained elevated with LPS co-administration. Recovery partially restored FOXO3 and CDK10 expression, indicating incomplete molecular recovery after chronic stress.

These results suggest a partial restoration of cognitive and molecular function, but persistent oxidative stress, inflammation, and disrupted FOXO3/CDK10 signalling hinder full neurobiological recovery.

Keywords: Stress Recovery, Lipopolysaccharide, Oxidative stress, Inflammation, Cognitive function & Neuroprotection

4.1 INTRODUCTION

Stress has an impact on both physical and mental health across the lifespan of an individual (14). The body's response to stress is shaped by several variables, including the duration and nature of the stressor, as well as individual differences such as age and gender (15). Recent data from the American Institute of Stress reports that 33% of Americans experience high stress levels, with notable effects on physical (77%) and mental (73%) health, and sleep disturbances in 48% of individuals (16). Globally, stress affects around 35.1% of people, with a higher prevalence in men(64%) and women(36%). Notably, stress levels have increased over time in low- and middle-income countries. According to the WHO, stress-related issues contribute to the loss of 12 billion workdays and cost the global economy about \$1 trillion annually (17). Chronic exposure to stress impairs cognitive function through mechanisms such as allostatic load, HPA axis dysregulation, and sympathetic activation (34). These processes lead to sustained elevations in glucocorticoids and catecholamines, which disrupt neural homeostasis. In particular, the hippocampus is a key region for learning and memory. It is highly sensitive to stress hormones. Prolonged exposure results in dendritic retraction, reduced neurogenesis, and synaptic loss, all of which contribute to cognitive deficits and emotional dysregulation(35, 36). The gap lies in understanding the effect of recovery after chronic mild stress in young adults on cognitive function and performance.

Research has increasingly shown that the immune system influences CNS function, including cognitive abilities (119). In aged adults, microglia exhibit a primed or sensitised state, making them more prone to adopting a pro-inflammatory phenotype in response to various stimuli, which contributes to low-grade chronic neuroinflammation (120). This neuroinflammation is thought to contribute to cognitive deficits and age-related disorders. Understanding the mechanisms behind microglial activation and developing interventions to reduce inflammation while preserving their neuroprotective roles could improve cognitive function and neural plasticity. Therefore, identifying factors that induce neuroinflammation and potential preventative treatments is crucial for maintaining normal microglial activity in the ageing brain (121). LPS, an endotoxin, stimulates the activation of microglia and astrocytes within the brain, which in turn leads to elevated levels of proinflammatory cytokines, including IL-1 β and TNF- α (122). This inflammatory activation triggers acute inflammatory responses and is associated with the manifestation of depression-like behaviours (123).

Oxidative stress is closely linked to inflammation, as activated inflammatory cells release reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), cytokines, proteases, and other mediators, all of which contribute to oxidative damage (124). This oxidative stress, in turn, activates transcription factors like Nrf2 and NF- κ B, as well as proinflammatory cytokines such as TNF- α , which lead to the altered expression of inflammatory cytokines, chemokines, and anti-inflammatory molecules (125). LPS exposure reduces antioxidant concentrations, including SOD, CAT, and GSH-PX, resulting in an imbalance between oxidative and antioxidant activities (126). Similarly, psychological stress activates the HPA axis and leads to elevated concentrations of stress hormones, increased ROS production, and inflammatory signalling mechanisms implicated in reduced synaptic plasticity and cognitive decline (127).

Several genes involved in stress regulation and neuroprotection have garnered interest due to their potential role in mitigating these effects. Among them, FOXO3 and CDK10 have been identified as key regulators of antioxidant responses, cellular repair, and neuroinflammation (128). FOXO3 is a multifunctional transcription factor that suppresses inflammation and promotes oxidative defence mechanisms (69). While its role in peripheral immune response is well established, less is known about its function in the brain under conditions of chronic stress and LPS-induced inflammation (129). FOXO3 is believed to protect neurons from damage by reducing neuroinflammation and regulating autophagy, a process critical for cellular homeostasis (104).

CDK10 is a serine/threonine kinase involved in cell cycle regulation, transcription, and cellular differentiation, and has emerging roles in neurological and inflammatory responses (11). It has been suggested that CDK10 may also respond to cellular stress and inflammatory stimuli (11). Another study has shown that CDK10 expression can be modulated by external stressors, including oxidative and endoplasmic reticulum stress, and may contribute to cell survival pathways (11). However, the specific effects of psychological stress and immune activation through LPS on CDK10 expression, particularly in the brain, remain poorly understood. There is currently a lack of direct evidence linking LPS-induced neuroinflammation or chronic stress exposure to changes in CDK10 expression in neuronal or glial cells. As such, the role of CDK10 in stress-related cognitive impairment or recovery remains an underexplored but promising area for investigation. Neurodegenerative diseases or developmental abnormalities could result from this gene's dysregulation (107). Both FOXO3 and CDK10 have complementary roles that may reveal coordinated regulatory patterns under stress conditions.

Both genes have been mainly investigated in aged individuals; hence, our study will contribute to the little to non-existent literature on FOXO3 and CDK10 neuroprotective effects in the brains of young adults.

Despite growing evidence linking chronic stress to neuroinflammation and oxidative stress, most studies have focused on aged populations or individuals already experiencing neurodegenerative decline. However, young adults who often face prolonged psychological stress represent an understudied group in this context, despite being at a critical stage of brain maturation and plasticity. The long-term neurobiological effects of chronic stress combined with peripheral immune activation (e.g., via LPS exposure) in young brains remain poorly understood. Moreover, the role of key neuroprotective regulators like FOXO3 and CDK10, which are known to influence oxidative defence, inflammation, and neural function, has not been well characterised in young, stressed individuals. Understanding how these molecular pathways respond to compounded stressors in early adulthood is essential for identifying early biomarkers and therapeutic targets to prevent long-term cognitive and emotional dysfunction. Therefore, this study aimed to investigate the impact of recovery following exposure to chronic unpredictable mild stress on memory performance, oxidative stress, inflammation, and the expression of FOXO3 and CDK10 in LPS-challenged rats.

4.2. METHODS & MATERIALS

4.2.1 Reagents

All chemicals and reagents were purchased from commercial suppliers and were of analytical grade.

Normal Saline (0.9% Sodium Chloride) and LPS were procured from Sigma Aldrich, South Africa.

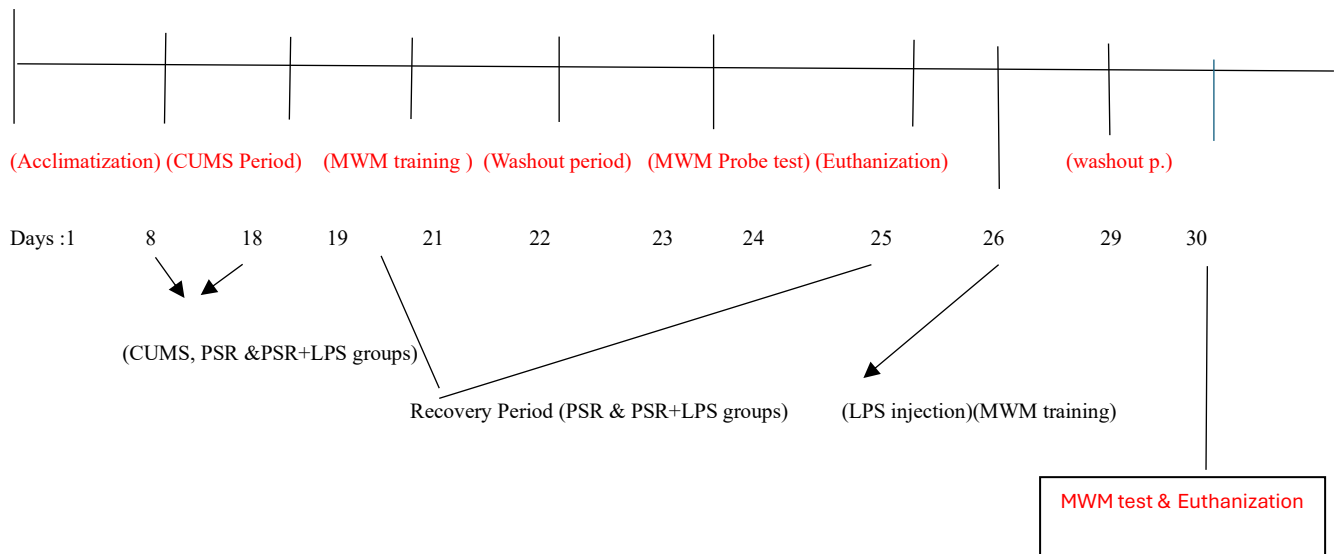
4.2.2 Experimental Animals

Twenty-five Male Sprague-Dawley rats that were 12 weeks of age, weighing 245 g, were randomly used in this study. The rats were obtained from the University of KwaZulu-Natal Biomedical Research Unit (BRU). The animals were housed under standard laboratory conditions of temperature (22 ± 2 °C), CO₂ content (<5000 ppm), relative humidity (55±5%) and illumination (12h light/dark cycle, lights on at 07H00). The noise levels were less than 65

decibels. All animals were provided with environmental enrichment and were given *ad libitum* access to standard rat chow and tap water. All animal experimentations were approved by the animal research ethics committee of the University of KwaZulu-Natal (AREC/00004699/2022).

4.2.3 Experimental Protocol

Timeline



Animals were randomly group-housed (n=5/group) with the same mates throughout the acclimation and testing periods. The animals were acclimatised for 8 days and exposed to environmental enrichment, which included hiding structures and tunnels. These objects were used to support different natural behaviours such as burrowing & gnawing. The first group included the rats that were treated with saline as a vehicle, and this group was used to control for all contextual conditions in the study. The second group was the stressed group; in this group, the animals were exposed to unpredictable mild chronic stress for 10 consecutive days. This group was referred to as the CUMS group. The third group was the post-stress recovery group (PSR G), which was also exposed to CUMS for 10 consecutive days. This was followed by a week of non-exposure to stress for one week to allow the animals to recover from the CUMS effect. The fourth group was the LPS administration group (LPS G) to induce microglial activation. A single dose of 5mg/kg was injected intraperitoneally into the animals in this group. The last group of rats was exposed to CUMS for 10 consecutive days, allowed to recover for a week, and then injected with a single dose of LPS (5mg/kg, i.p.) (PSR+LPS).

On day 8, environmental enrichment material was removed from CUMS, PSR, and PSR+LPS groups to allow the introduction of the CUMS protocol. The CUMS protocol was initiated on day 8 and lasted for 10 days. CUMS included the following stressors: cold exposure, the cage is set at a 45° tilt, daytime darkness, and animal restraint. The animals received 2 of the stressors per day. On day 19, the CUMS group was exposed to the Morris Water Maze test (MWM) training for 3 consecutive days. A 24-hour washout period was allowed before the MWM PROBE test was performed on day 23. On day 24, the animals were euthanised, and the cerebral cortex was harvested and stored at -80°C in a Bio-freezer until ready for analysis. The cortex was harvested to measure 4-HNE, SOD-1, IL-1 β , FOXO3, and CDK10. The PSR and PSR+LPS groups were given 7 days of recovery from the stress protocol. During the recovery period, the environmental enrichment material was returned, and the animals returned to their normal acclimatisation conditions. On day 26, the animals from the LPS group received an intraperitoneal injection of a 5mg/kg single dose of LPS, and the control group received a single dose of 5mg/kg of saline. Also, on day 26, the animals from all remaining groups were subjected to the MWM training for 3 consecutive days. LPS was administered on Day 26, immediately before the start of MWM training. Therefore, all behavioural data for the LPS and PSR+LPS groups reflect performance following LPS exposure. Day 29 was a rest day. On Day 30, the MWM test was performed, after which the animals were euthanised using a guillotine, and the cerebral cortex was collected and stored in the bio freezer.

4.2.4. Stress (CUMS) Protocol

The protocol involved using a chronic unpredictable mild stress procedure with minor modifications (113). The stress protocol consisted of daytime darkness (2 hours), the cage was set at 45° tilt (5 hours), cold exposure (5 minutes) at 4°C, and restraint (rat cylinder container) for 90 minutes. For the cold room exposure, animals were singly placed in new cages, and all animals from each group were placed in the cold room for 10 minutes simultaneously. There was always a 5-minute break from the previous group to the next group. This procedure was repeated 3 times per day. The animals received two random stressors per day over 10 days. The animals were carried to different rooms, where they were stressed according to the stress protocol. Food and water were available *ad libitum* during the stress procedure (114). The other group of animals, which didn't go under the stress procedure, were still in their respective cages receiving environmental enrichment until the day of MWM training.

4.2.5. LPS Administration

LPS purchased from Sigma Aldrich, South Africa, was used to induce memory impairment-like symptoms. A single dose of LPS(5mg/kg) was injected intraperitoneally in the LPS group. The calculation of the dosage was determined with the animal's weight according to (115)The average body weight of the animals in the LPS-treated group was calculated to be 251.5 g (0.2515 kg). Based on the standard dosage of 5 mg/kg, the corresponding LPS dose was determined to be approximately 1.25 mg per animal. This dosage was then diluted to a final volume of 2 mL for intraperitoneal injection.

4.2.6. Behavioural test

The MWM test was used to assess spatial learning and memory. The maze was a circular tank of 100 cm in diameter and 60 cm high, which was filled with water at an ambient temperature range between 22-23°C up to the 24 cm mark. The tank was divided into 4 equal quadrants that had visual cues of different colours and shapes, placed equidistant from each other. The platform was submerged in the middle of the 4th quadrant during the entire training phase. This quadrant was referred to as the goal quadrant. Each rat was placed in a quadrant other than where the hidden platform was located, facing the wall of the pool. The animals were given 120s to find the platform and 15s to stay on it. Learning was assessed as the time taken for the animal to locate the hidden platform, referred to as the latency time; time spent in the quadrant of the hidden platform was a measure of long-term memory assessment. Each animal underwent 4 trials per day, for 3 days. The trials were 20 minutes apart. Then the animals underwent a probe test for 1 day. The rats were trained on all 4 quadrants. Each time, the animal was released near the wall of the quadrant, the trajectory/path of the rat to the hidden platform was video recorded. After the trials, the animals were dried with a warm towel warmed with an electric heater, and returned to the cage (130). There was a 24-hour washout period before the probe test.

The probe trial of the MWM was conducted to assess spatial memory retention following the training phase. In this trial, the hidden platform was removed, and each animal was allowed to swim freely for 60 seconds. Key parameters that were measured included the time spent in the goal quadrant and the time spent trying to find the location of the hidden platform. Greater time spent in the goal quadrant and more frequent crossings of the platform site were interpreted as indicators of stronger spatial memory, while reduced engagement with the goal area suggested memory impairment. During the probe trial, spatial memory retention was assessed by

measuring the time spent in the goal quadrant where the platform was previously located. Time spent in the target is quantified by measuring the total duration or percentage of the trial during which the subject remains within the defined target area, usually via video tracking software. Statistical analysis was used to analyse the results from the video recordings (69).

4.2.7. Tissue collection

At the end of the experimental period, animals were sacrificed by a sharpened guillotine at the Biomedical Research Unit, University of KwaZulu-Natal, Durban, South Africa. The cortex was removed from the skull with a surgical scissor and a surgical blade. It was then stored in the tubes, 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.

4.2.8. Biochemical Analysis

4.2.8.1. ELISA (Enzyme-linked Immunosorbent Assay)

Cortex samples were thawed on ice and then homogenised in PBS (0.01M, pH 7.4) with an ultrasonic cell disrupter(sonicator) in a ratio of 1:9(tissue weight(g); PBS volume (mL). The homogenate was then centrifuged for 10 minutes at 5000xg at 2 degrees Celsius to get the supernatant. The ELISA was conducted according to the manufacturer's (Elabsciences) instructions to quantify the concentrations of IL-1beta (EL-R0012),4-HNE(E-EL-0128), and SOD-1(E-EL-R1424).

4.2.8.2. Real-time quantitative polymerase chain reaction (Rt-qPCR)

Cortex samples were thawed on ice, and then fresh samples of cortex were sliced and weighed into Eppendorf tubes of 25mg each. Total RNA was extracted from the brain tissue with RNA mini prep kit (Inqaba Biotech, Pretoria, South Africa). Reverse transcription reactions were performed with the First Strand cDNA Synthesis Protocols(E6300) (Inqaba Biotech, Pretoria, Pretoria, South Africa) according to the manufacturer's instructions. Real-time PCR was performed with One-step RT-PCR Kit (Inqaba Biotech, Pretoria, South Africa). The specific real-time PCR primers for FOXO3, CDK10, SYBR Green & GAPDH were purchased from Inqaba Biotech (Pretoria, South Africa). The GAPDH served as an internal control. The cycle threshold (Ct value) was acquired using the Light Cycler 96 analysis software. The data were analysed using $2^{-\Delta\Delta Ct}$ Method and the values were represented by a relative quantity.

4.2.9. Statistical analysis

Data were analysed using GraphPad PRISM version 8 software. Data were expressed as mean $\pm$ SEM. There was a strong positive correlation ($r = 0.9764$, $p = 0.0206$) between the MWM probe test and FOXO3 concentration. A one-way analysis of variance (ANOVA) was used to compare the five independent groups, namely, Control, CUMS, PSR, LPS, and PSR+LPS, followed by a Tukey's post hoc test. A p -value < 0.05 was considered significant. Data were expressed as $\pm$ SEM.

4.3 RESULTS

4.3.1 MWM training & probe test (Cortex)

MWM Training (a.) and MWM Probe Test (b.) of the Cortex in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group ($n=5$), were measured during the experiment (Figure 4.3.1(a)). LPS was administered on Day 26, immediately before the start of MWM training. Therefore, all behavioural data for the LPS and PSR+LPS groups reflect performance following LPS exposure. In the Day 1, Day 2, and Day 3 training phase (the time to find the platform). There was a significant difference between the days but not between the groups. There was a significant difference between Day 1 vs Day 2 of the Control (Saline) Group. All experimental groups (PSR, LPS, and PSR+LPS) showed a significant reduction in escape latency from Day 1 to Day 2 and 3, indicating improved learning across trials.

There was a significant difference in the MWM Probe test (time spent in the goal quadrant) between the groups ($F=1.98$, $p<0,05$, Figure 4.3.1(b)). There was an increase in the concentration of the control group compared to the LPS group ($p=0.0183$) mean difference between the groups is 11.80 & SE difference is 6.652.

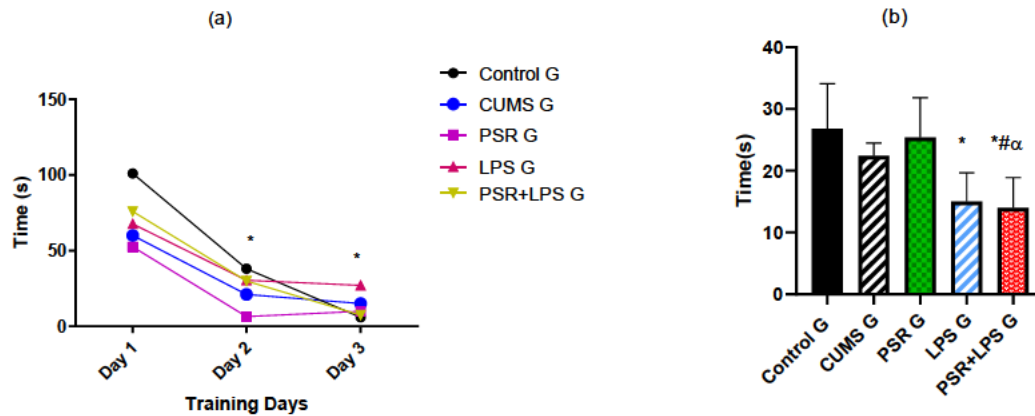


Figure 4.3.1: MWM training phase (a.) and MWM probe test (b.) concentrations of the Cortex in the Control, CUMS, PSR, LPS, and PSR+LPS (n=5/group).

4.3.2 Cortical SOD-1 and 4-HNE concentration

Cortical 4-HNE (a.) and SOD-1 (b.) concentration in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group (n=5), were measured from the cortex during the experiment (Figure 4.3.2(a)). There was a significant difference in the 4-HNE concentration between the groups ($F=117.43$, $p < 0.05$, Figure 2a). There was a decrease in the concentration of the control group compared to the CUMS group ($p=0.0001$), mean difference is -38 & SE of difference is 6.652. There was another decrease in the concentration of the control group compared to the LPS group ($p=0.0004$), mean difference is -34.60 and SE difference is 6.652. There was another decrease in the concentration of the Control group compared to the PSR+LPS group ($p=0.0001$), mean difference is -38.00 & SE of diff. is 6.652.

There was also a significant difference in the SOD-1 concentration between the groups ($F=34.02$, $p < 0.05$, Figure 4.3.2(b)). We found a CUMS effect in the SOD-1 concentration of the CUMS group compared to the control group *(control group vs CUMS group, $p < 0.05$, Figure 2b). There was an increase in the concentration of the control group when compared to the CUMS group ($p=0.0009$), mean diff. is 29.95 & SE of diff. is 5.771. There was a decrease in the control group compared to the LPS group ($p=0.0139$), mean diff. is -21.68 & SE of diff. is 5.775.

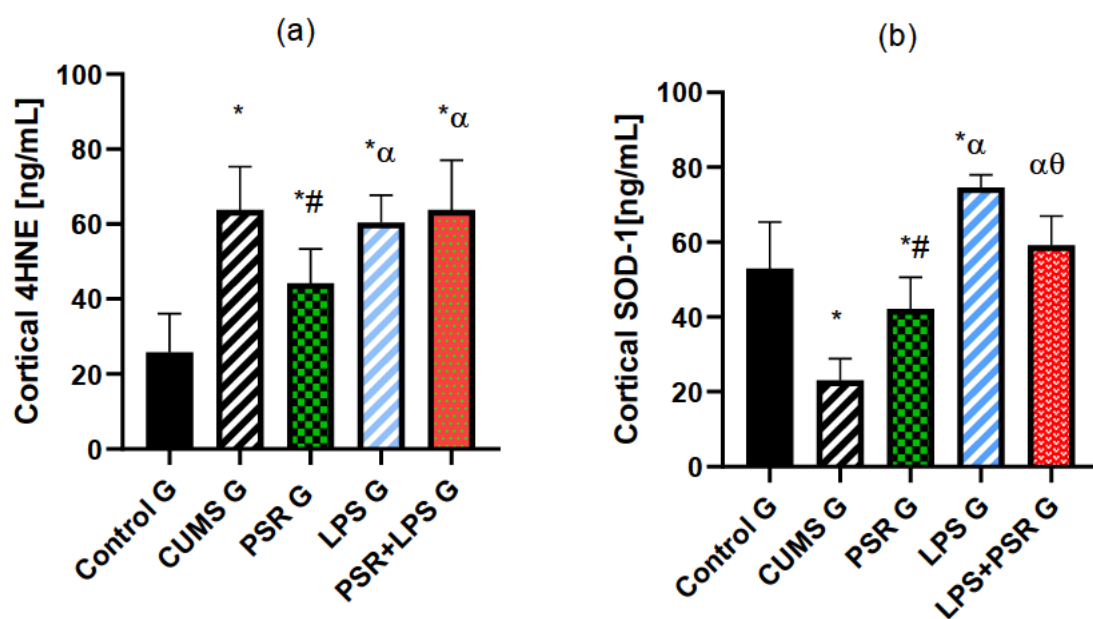


Figure 4.3.2.: Cortical 4-HNE (a.) and cortical SOD-1 (b.) concentrations in the Control, CUMS, PSR, LPS, and PSR+LPS (n=5/group).

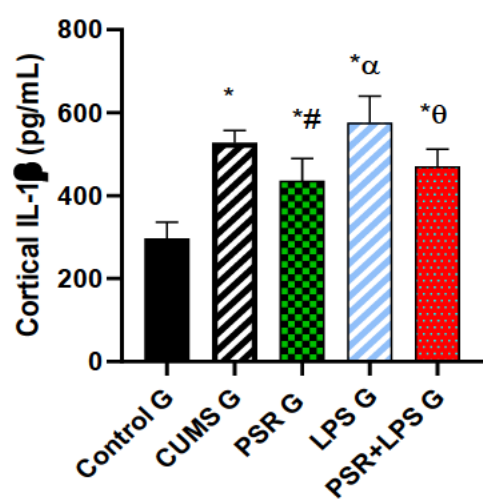


Figure 4.3.4: Cortical IL-1 β concentration in the Control, CUMS, PSR, LPS and PSR+LPS(n=5/group).

4.3.5 Cortical FOXO3 and CDK10 concentrations

Cortical FOXO3 (a.) and CDK10 (b.) concentrations in the Control group, CUMS group, PSR group, LPS group, and PSR+LPS group (n=5/group), were measured during the experiment (Figure 4.3.5(a)). There was an effect in the FOXO3 expression between the groups ($F=25.40$, $p < 0.05$, Figure 4.3.5(a)). There was an increase in the concentration of the control group compared to the CUMS group ($p<0.0001$), mean diff. is 1.595 & SE of diff. is 0.2899. There was another increase in the control group compared to the PSR group ($p=0.0281$), mean diff 0.9267 & SE diff. is 0.2899. There was another increase in the control group compared to the LPS group ($p=0.0210$), mean diff is 0.9633 & SE diff. is 0.2899. There was another increase in the control group compared to the PSR+LPS group($p=0.0320$), mean diff is 0.9100 & SE diff. is 0.2899.

There was an effect in the CDK10 expression between the groups ($F=22.45$, $p<0.05$, Figure 4.3.5(b)). There was an increase in the control group compared to the CUMS group($p<0.0001$), mean diff. is 1.595 & SE diff. is 0.2899. There was another increase in the control group compared to the PSR group ($p=0.0281$), mean diff. is 0.9267 & SE diff. is 0.2899. There was another increase in the control group compared to the LPS group($p=0.0210$), mean diff. is 0.9633 & SE diff. is 0.2899.

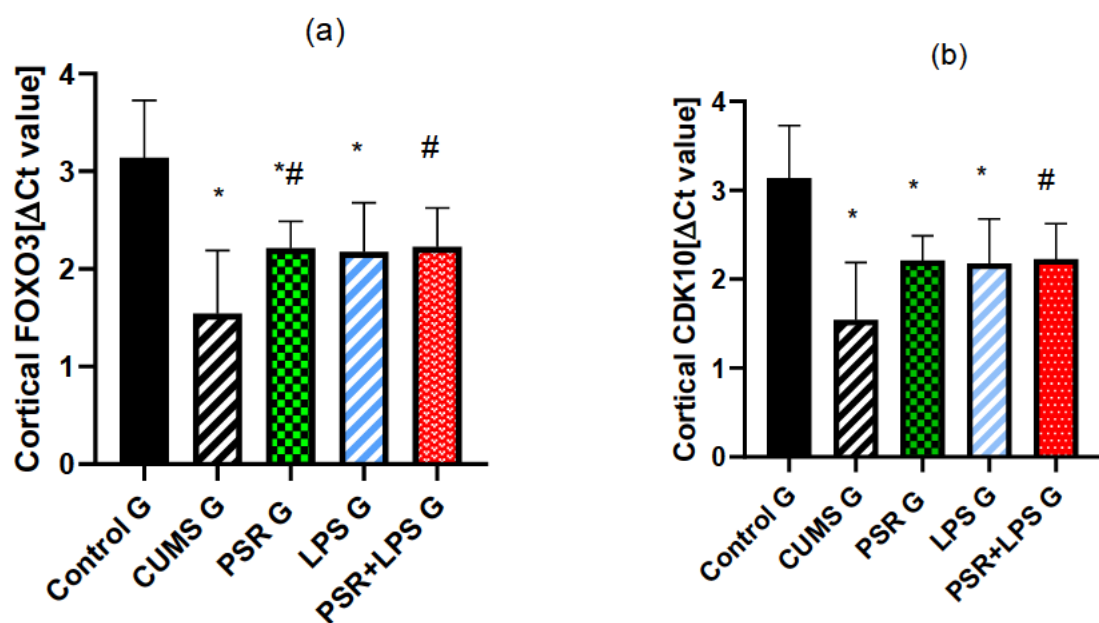


Figure 4.3.5: Cortical FOXO3(a) and CDK10(b) concentration in the Control, CUMS, PSR, LPS, and PSR+LPS (n=5/group).

4.3.6 Correlation analysis (Cortex)

A strong positive correlation ($r = 0.9764$, $p=0.0206$, Figure 4.3.6) was observed between MWM probe test performance and FOXO3 concentration, suggesting that reduced FOXO3 concentration is associated with poorer memory performance. This correlation was statistically

significant ($p < 0.05$), suggesting a robust relationship between the two variables.

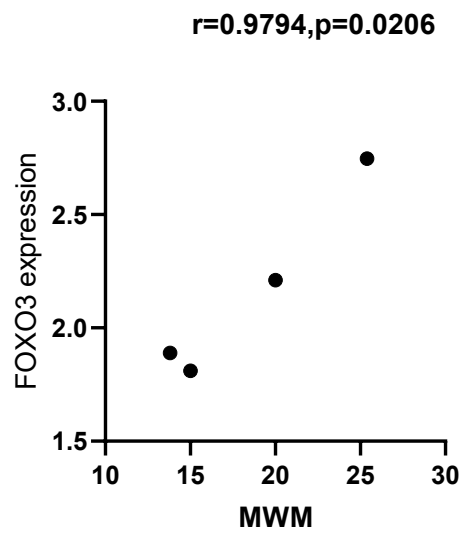


Figure 4.3.6: MWM probe test correlation with FOXO3 concentration.

4.4 DISCUSSION

Chronic stress disrupts cognitive function and induces inflammation and oxidative damage in the brain (131), yet the mechanisms underlying recovery from these effects remain unclear. This study aimed to address how the physiological recovery process following stress influenced brain function, particularly focusing on cognitive abilities, inflammatory responses, and oxidative damage. By examining the role of FOXO3, a regulator of stress resistance, and CDK10, a cell cycle regulator, this study aimed to provide insight into the molecular mechanisms underlying the recovery process and the potential implications for therapeutic strategies to mitigate against the detrimental effects of stress on memory performance and overall health. MWM was used to assess spatial learning and memory, providing insights into cognitive dysfunction linked to stress and immune activation. A progressive decrease in latency over the three-day training period was observed, indicating that the animals were successfully acquiring spatial learning and locating the hidden platform in the MWM.

We observed a decrease in the time spent in the goal quadrant in the LPS group when compared to the control group. Our findings corroborate with existing literature where LPS impairs spatial memory, as evidenced by a reduction in time spent in the goal quadrant during the probe test (132). A decrease in time spent in the goal quadrant could also suggest that the animals failed to properly learn the task or that the learning process was impaired due to LPS-induced stress or inflammation (133). Therefore, these MWM findings suggest that combined exposure to inflammation and psychological stress exerts a detrimental effect on spatial cognition, likely through converging impacts on hippocampal function. Notably, the lack of an additive effect beyond LPS alone may reflect shared underlying mechanisms or a threshold beyond which additional stress does not further impair cognitive function. We also found a decrease in the PSR+LPS group when compared to the PSR group. This difference suggests that the recovery period following CUMS may allow for partial cognitive restoration, potentially through normalisation of neuroendocrine or neuroimmune pathways. However, the co-administration of LPS with the recovery group appears to attenuate or negate these cognitive benefits. This finding underscores the critical sensitivity of the post-stress recovery window and suggests that inflammatory insults during this period may disrupt adaptive neuroplastic changes or prolong stress-induced neurobiological alterations. Therefore, while recovery alone may promote cognitive resilience, concurrent immune activation may undermine these protective mechanisms and hinder full functional restoration.

Oxidative stress markers play a critical role in the body's response to stress, as they indicate the imbalance between reactive oxygen species (ROS) and antioxidant defences, which can lead to cellular damage and contribute to various diseases, including neurodegenerative conditions (134). Our findings show that there was a higher 4-HNE concentration and a lower SOD-1 concentration in the CUMS group when compared to the control group, which is in line with published research. Five days after CUMS exposure, oxidative stress markers such as 4-HNE may remain elevated due to sustained oxidative damage, while SOD-1 levels may remain reduced or return to baseline gradually during the recovery period (131). Since SOD-1 concentrations are low and 4-HNE concentrations are high, this means that there was an elevated accumulation of free radicals, which resulted in oxidative stress and potential cellular damage to the brain (135). A decrease in antioxidant enzymes is an indication of these enzymes failing to suppress lipid peroxidation, which is a marker of oxidative stress (136). Recovery after stress improved oxidative stress found in the CUMS group, as evidenced by lower 4-HNE concentration and higher SOD-1 concentration in the PSR group compared to the CUMS group. However, this improvement did not restore oxidative stress balance to normal, as there was still a higher 4-HNE concentration and lower SOD-1 concentration in the PSR group compared to the control group. These findings show that recovery after stress led to a partial improvement in oxidative stress, as shown by decreased 4-HNE concentrations and increased SOD-1 concentrations in the PSR group compared to the CUMS group. However, these markers did not return to baseline, with the PSR group still showing elevated oxidative damage and reduced antioxidant levels compared to controls.

Our findings show a higher 4-HNE concentration in the LPS group despite higher SOD-1 concentrations in these animals when compared to the control group, suggesting that antioxidant enzymes were trying to suppress lipid peroxidation and restore balance between pro-oxidants and antioxidants. Our results corroborate findings by Skrzypczak-Wiercioch A et al., who suggested that LPS administration induces oxidative stress, leading to lipid peroxidation as indicated by elevated 4-HNE levels (137).

We found an increase in both 4-HNE and SOD-1 levels in the PSR+LPS group compared to the PSR group. These findings suggest a dual response to the introduction of an acute inflammatory stimulus (LPS) after a period of stress recovery. The elevation in 4-HNE indicates renewed oxidative damage, likely triggered by LPS-induced systemic inflammation. Simultaneously, the increase in SOD-1 suggests that the brain initiated a compensatory antioxidant response to counteract this oxidative effect; hence, in this acute phase, we do not

see the impact of oxidative damage. We also observed a negative correlation between 4HNE and MWM. The observed negative correlation between 4-HNE levels and MWM performance underscores the behavioural impact of oxidative stress on spatial memory. Elevated 4-HNE may reflect lipid peroxidation-induced neurotoxicity, which compromises hippocampal integrity and impairs cognitive function. These findings suggest that oxidative stress is a key mediator of stress-related memory deficits and highlight 4-HNE as a potential biomarker for neurocognitive vulnerability

An increase in oxidative stress may cause a neuroinflammatory response; hence, we also looked at IL-1 β concentration as a measure of inflammatory cytokine production. IL-1 β serves as a key indicator of inflammation, reflecting immune system activation and neuroinflammation (138). Elevated IL-1 β concentrations provide insight into the inflammatory responses triggered by chronic stress and LPS administration, which are linked to behavioural changes, cognitive decline, and the development of various stress-related diseases (139). Furthermore, we observed a decrease in IL-1 β concentrations in the PSR group compared to the CUMS group, indicating a reduction in neuroinflammatory response during the recovery phase. Therefore, the downregulation of IL-1 β in the PSR group suggests that the physiological stress recovery process may promote anti-inflammatory effects that support cognitive restoration. Our study supports previous research indicating that post-stress recovery mechanisms can downregulate inflammatory markers such as IL-1 β , even following prolonged stress exposure (140). Furthermore, we observed a decrease in the IL-1 β concentration in the PSR+LPS group compared to the LPS group; these findings may suggest that the recovery phase was effective in attenuating the acute neuroinflammatory response induced by LPS. These findings also suggest that the recovery period effectively dampened the inflammatory response, supporting cognitive restoration. This aligns with our study's aim by demonstrating that physiological recovery mechanisms can suppress proinflammatory cytokine production, thereby potentially facilitating the restoration of cognitive function. These findings emphasise the importance of recovery dynamics in mitigating the negative impact of both chronic stress and acute immune activation on brain health. These contrasting results underscore the complex and dynamic interplay between stress recovery and immune challenges, reinforcing the importance of recovery periods in mitigating inflammatory responses within the brain. These findings also suggest that in the context of chronic stress and immune activation, recovery is not simply delayed but may become pathologically dysregulated.

FOXO3 plays a critical role in regulating the cellular response to oxidative stress, inflammation, and cell survival, making it a key parameter in studies involving LPS and chronic stress (141). By modulating inflammatory pathways, antioxidant defences, and apoptosis, FOXO3 helps maintain cellular homeostasis under stress-induced conditions (141). Alterations in FOXO3 activity can contribute to the development and progression of neuroinflammatory and neurodegenerative diseases, highlighting its potential as a therapeutic target for managing chronic stress-related disorders and inflammatory diseases. We found an increase in the PSR group when compared to the CUMS group; these findings suggest that recovery after stress may help restore or enhance the expression of protective regulatory proteins involved in stress resistance and cellular repair. These results imply that the recovery phase may activate adaptive mechanisms that counteract stress-induced damage, possibly by promoting antioxidant defences and reducing long-term cellular dysfunction. Other studies also indicate that the neuroprotective action of FOXO3 could occur through FOXO3 increasing the expression of glutamine synthetase, which could promote an increase in the response to DNA damage (11). Therefore, a decrease in SOD-1 as well as an increase in 4HNE and FOXO3 expression can lead to impaired cellular defence against oxidative stress, which can result in higher susceptibility to oxidative damage and potentially contribute to neurodegenerative diseases and can be exacerbated with age (142). We also found a decrease in the FOXO3 gene expression in the LPS Group compared to the control in the presence of oxidative stress, suggesting the neurons were left vulnerable to oxidative damage in the acute stress induced by LPS. This may lead to neurodegeneration and cognitive decline. Our results contradict another study that revealed LPS-induced cytotoxicity in hippocampal neurons was associated with the overexpression of FOXO3 (143). As discussed earlier, a decrease in FOXO3 concentration can compromise cellular defences against oxidative stress, reducing the brain's capacity to recover from neuroinflammatory challenges. However, we found a significant positive correlation between FOXO3 concentration and escape latency in the MWM, indicating that higher FOXO3 concentrations are associated with poorer spatial memory performance. This suggests that elevated FOXO3 may reflect an incomplete cognitive recovery following stress. While FOXO3 is known for its role in stress response and neuroprotection, its sustained expression after stress may interfere with memory restoration. These findings highlight FOXO3 as a potential molecular marker or modulator of impaired recovery in stress-related memory dysfunction.

CDK10 plays a pivotal role in cell cycle regulation, cell survival, and stress response pathways. By controlling cell proliferation and apoptosis, CDK10 helps to maintain cellular homeostasis

in the face of oxidative stress and inflammation (144). Its dysregulation could contribute to excessive inflammation, cell damage, and the development of neurodegenerative diseases (145). We found an increase in the PSR+LPS groups relative to the PSR group, indicating that inflammatory stimuli may trigger a compensatory molecular response. We also found a decrease in all the groups when compared to the control group. CDK10 expression was reduced across all experimental groups compared to the control, with a modest increase in the PSR+LPS group relative to the PSR group. While these changes may reflect altered neuronal regulatory dynamics, the interpretation remains correlative. CDK10 downregulation may indicate disrupted recovery processes or stress-related transcriptional changes, but further investigation is needed to determine its functional role in neuroplasticity or resilience

4.5 Conclusion

This study shows that recovery from chronic stress only partially reactivates key molecular pathways needed for cognitive and neural restoration. Persistent oxidative stress (elevated 4-HNE, reduced SOD-1) and incomplete resolution of inflammation (IL-1 β) indicate ongoing neurobiological disruption. While FOXO3 was upregulated during recovery, likely as a compensatory response, its association with impaired memory suggests a maladaptive role when sustained. Downregulation of CDK10 across stress and LPS groups points to compromised cell cycle regulation and repair. These findings suggest that effective recovery requires timely reactivation of protective FOXO3 and CDK10 signalling, which is disrupted by additional inflammatory insults.

4.6 Declaration of interest:

None

Author contributions:

This research project was conceptualised by Miss Siku, who also designed the methodology, conducted the experiments, and carried out data collection and analysis. She independently interpreted the findings and composed the dissertation. Dr Luvuno and Prof. Mabandla provided critical guidance throughout the study, helping to shape the research direction and refine the research question. They reviewed multiple drafts, offered constructive feedback, and supported the development and structure of the dissertation. Their contributions also included

insightful input during data analysis and interpretation of results. As Supervisors, they provided expert oversight and support across all stages of the research process.

Funding:

The National Research Foundation provided funding for this study.

Ethical approval:

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/00004699/2022) of the University of KwaZulu-Natal.

Acknowledgements:

The authors are grateful to the National Research Foundation for providing funding for this study and to the Biomedical Research Unit for supplying us with rats.

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CHAPTER 5: Synopsis

Chronic stress negatively affects both physical and mental health, with long-term exposure linked to dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and structural damage to stress-sensitive brain regions such as the hippocampus. This dysregulation impairs negative feedback control, leading to sustained elevations in stress hormones like corticosterone, which can compromise cognitive functions, including learning and memory. When combined with immune activation such as lipopolysaccharide (LPS) exposure, stress exacerbates neuroinflammation and oxidative damage, further impairing brain function. LPS is a bacterial endotoxin known to induce systemic inflammation and oxidative stress and has been shown to impair hippocampus-dependent memory tasks. To assess the impact of chronic stress and immune challenge on spatial memory, we employed the Morris Water Maze (MWM), a well-established test of hippocampal function. We also measured markers of oxidative stress (4-HNE, SOD-1), inflammation (IL-1 β), and stress-related gene expression (FOXO3, CDK10) to evaluate molecular changes associated with recovery and immune activation.

Our study aimed to investigate how a recovery period following chronic unpredictable mild stress (CUMS) influences HPA axis activity, cognitive performance, and neurobiological resilience in young adult male Sprague-Dawley rats. Animals were assigned to five groups: Control, CUMS, Post-Stress Recovery (PSR), LPS, and PSR+LPS. After habituation, rats underwent 10 days of CUMS, followed by 7 days of recovery. LPS (5 mg/kg) was administered on day 26, immediately before MWM training. Behavioural testing was followed by biochemical analysis of plasma and brain tissue (hippocampus and cortex).

Results show that recovery may have contributed to improvements in spatial memory and is associated with partial normalisation of HPA axis activity. Plasma corticosterone remained stable across groups, while brain corticosterone increased in response to stress and LPS, indicating region-specific sensitivity. GR expression, which was reduced by CUMS, was restored in the PSR and PSR+LPS groups, suggesting improved feedback regulation and neuroendocrine resilience.

MWM performance suggested that LPS impaired memory, as indicated by reduced time in the goal quadrant. Although recovery was associated with reduced IL-1 β levels, indicating lower neuroinflammation, the observed elevations in 4-HNE suggest that oxidative damage may persist despite partial recovery. The increase in SOD-1 likely reflects a compensatory antioxidant response rather than a direct marker of damage. Thus, while recovery may initiate protective mechanisms, the continued presence of 4-HNE implies that oxidative stress is not fully resolved.

FOXO3 and CDK10, both involved in cellular stress regulation, were differentially expressed across groups. FOXO3 increased in LPS and PSR+LPS groups, potentially reflecting ongoing stress signalling or neuroprotective activation. CDK10 was downregulated across stress conditions, with a modest increase in the PSR+LPS group. While these changes may indicate disrupted recovery or compensatory mechanisms, interpretations remain correlative and require further investigation.

In summary, our findings demonstrate that post-stress recovery can partially restore HPA axis function and cognitive performance, even under acute immune challenge. Recovery mitigates neuroinflammatory and oxidative damage and modulates stress-responsive gene expression. These results highlight the importance of recovery periods in promoting neurobiological resilience following chronic stress.

Conclusion

This study shows that taking time to recover after stress can help the brain start to heal and return to normal, especially in important areas like the hippocampus and cortex. Recovery also helped improve how the body's stress system (the HPA axis) works and improved memory in some ways. However, because we used a small amount of LPS to cause inflammation, we can't say for sure if recovery can fully protect the brain from immune system effects. Some stress hormones also did not return to normal with recovery alone; it seemed that the immune system

also played a role. More research with stronger doses of LPS is needed to better understand how recovery helps reduce brain inflammation.

Appendices

Ethical clearance



Renewal	
Protocol Reference number:	AREC/00004699/2022(00024308)
Project title:	LIPOPOLYSACCHARIDE INDUCED MEMORY IMPAIRMENT: EFFECTS ON DEPRESSIVE LIKE BEHAVIOUR IN PRE-STRESSED ANIMALS
Main Applicant and School:	Ms Esethu Siku (219002885) School of Lab Med & Medical Sciences

Dear Ms Siku,

Regarding your revised 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



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

cc Supervisor: Dr Mluleki Luvuno, Dr Musa Vuyisile 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)

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