Unpredictable Stress on Metabolic and Molecular Changes Associated with Memory Decline in Lipopolysaccharide-Challenged Rats

By

Khethelo Mayenziwe Sibiya

219013485

Supervisor: Dr. Mluleki Luvuno

Co-supervisor: Prof. Musa V Mabandla

Department of Human Physiology

School of Laboratory Medicine and Medical Sciences

College of Health Sciences

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Declaration

School of Laboratory Medicine and Medical Sciences, College of Health Sciences

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I, Khethelo Sibiya, declare that

The research reported in this dissertation, except where otherwise indicated, is my original work. This dissertation has not been submitted for any degree or examination at any other university. This dissertation does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. This dissertation does not contain text, graphics, or tables copied and pasted from the internet, unless specifically acknowledged, and the source is in the dissertation and the Reference sections.

Student: Miss K.M. Sibiya Signature: [redacted] Date: 19/08/2025

Supervisor: Dr M. Luvuno Signature: [redacted] Date: 19/08/2025

Co-supervisor: Prof M.V. Mabandla Signature: [redacted] Date: 19 August 2025

PLAGIARISM DECLARATION

School of Laboratory Medicine and Medical Sciences, College of Health Sciences [BACHELOR OF MEDICAL SCIENCE MASTERS IN HUMAN PHYSIOLOGY, 2025]

I, Khethelo Sibiya, Student Number: 219013485, declare that:

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Abbreviations

CUMS -Chronic unpredictable mild stress

LPS - Lipopolysaccharide

ACh -Acetylcholine

ROS -Reactive Oxygen Species

OFT -Open Field Test

CORT -Corticosterone

CNS -Central Nervous System

COX2 – Cyclooxygenase-2

IL-1β -Interleukin 1 beta

SOD1 -Superoxide Dismutase 1

4-HNE -4-Hydroxynonenal

APO-E -Apoliprotein E

TLR-4 -Toll-like receptor -4

NF-kB - Nuclear factor kB

PGE_2 - Prostaglandin E2

BBB -Blood-Brain Barrier

CNS - Central Nervous System

RNS - Reactive Nitrogen Species

BRU - Biomedical Research Unit

UKZN - University of KwaZulu-Natal

Study outline

Chapter 1: represents the introduction, aims, objectives, and methodology of the study. This chapter consists of a brief background, knowledge about the research, and the gaps. The studies aim to fill the gaps that were not answered in another research. Objectives show how the aims of the studies were answered. The methodology section offers insight into how the aims and objectives of the study were achieved.

Chapter 2: consists of the literature review. It gives us a brief knowledge of the research and the gaps. It represents other researchers' findings.

Chapter 3: represents the first research paper in a manuscript format that investigated the effects of unpredictable stress on memory decline, associated molecular changes in LPSchallenged Sprague-Dawley rats. This chapter contains the introduction of the study, aims and objectives, methodology section, results, discussion, and conclusion.

Chapter 4: consists of the second research study in a manuscript format. In this study, we evaluated the effects of chronic unpredictable mild stress on metabolic and associated changes in Sprague-Dawley rats. This chapter consists of the introduction of the study aims and objectives, the methodology section, results, discussion, and conclusion.

Chapter 5: The last chapter of the study gives a narrative of the discussion of the themes covered in the two studies. The disclosure focuses on the link between the content covered by the studies, given the broad study aims and objectives.

Chapter 6: consists of the conclusion of our study.

Main Abstract:

Chronic stress damages neurons and supporting cells in the brain and its periphery by inducing inflammation and increasing oxidative stress. It alters emotional regulation and impairs cognitive function; these changes significantly promote anxiety-like behaviours. Similarly, exposure to LPS interferes with weight regulations, increases inflammation and oxidative stress, affects cognitive function, and elevates anxiety-like behaviours. However, the effects of CUMS on memory decline, molecular changes, and metabolic alterations when combined with LPS are not clearly understood. Therefore, this study explores the combined effects of CUMS and a single dose of LPS on memory, molecular changes, and metabolic alterations in rats. 24 male Sprague-Dawley rats were divided into 4 groups: control, stressed, LPS, and stressed + LPS groups (n=6, per group). The concentrations of SOD1, 4-HNE, IL-1β, and Acetylcholine (ACh) in the plasma and the hippocampus were measured using ELISA, and APO-E expression was measured using PCR. The results were presented as a mean ± SEM and analysed using ANOVA where $p<0.05$ was considered significant. Rats exposed to both CUMS and LPS showed increased oxidative stress, inflammation, cholinergic dysfunction; marked by decreased SOD1 and ACh, and increased 4-HNE, IL-1β, and APO-E, suggesting that chronic stress and LPS might contribute to cognitive deficits. In a related study, metabolic and behavioural changes were assessed by monitoring body weight, food and water consumption, and anxiety-like behaviour using the Open Field Test (OFT), while plasma IL-1β, hippocampal acetylcholine, and plasma corticosterone were measured by ELISA. The stressed + LPS group exhibited decreased body weight without changes in feeding and spent more time near the walls in the OFT, indicating no increase in anxiety-like behaviour. These findings suggest that chronic stress and LPS interact to worsen cognitive and metabolic functions, likely through mechanisms involving oxidative stress, inflammation, and neurotransmitter imbalances, providing insight into potential pathways underlying neurodegenerative diseases.

Keywords: Lipopolysaccharide, chronic unpredictable mild stress, oxidative stress, acetylcholine, interleukin-1β

Chapter 1: Introduction

1.1.Background

The prevalence of neurodegenerative and neuropsychiatric diseases has significantly increased, contributing to the burden on society and healthcare systems (1, 2). According to the World Health Organisation (WHO), neurological diseases accounted for 63% of all disability-adjusted life years (DALYs) in 2006, making it one of the greatest public health issues (3). The number of disability-adjusted life-years (DALYs) worldwide attributable to neuropsychiatric disorders has grown from 80.8 million (95% UI 59.5–105.9) to 125.3 million (93.0–163.2) over the past three decades, while the percentage of DALYs worldwide attributable to mental disorders has increased from 3.1% (95% UI 2.4–3.9) to 4.9% (3.9–6.1) (1).

There is no solution for the debilitating disorders known as neurodegenerative diseases. In general, little is currently known about the mechanisms behind the start and progression of disease. Therefore, significant attempts are being made to better comprehend their pathophysiology. Among the various components that contribute to these conditions, immune activation is regarded as a key contributor. Neurodegenerative diseases are associated with cognitive impairment, motor dysfunction, neuronal death, and age-dependent accumulation of misfolded and aggregated proteins (4). Numerous experimental studies have shown that inflammation can trigger neuronal cell death, and inflammation can trigger cell death on its own (5). Exposure to chronic stress has a wide range of negative health impacts, from immunological response dysregulation to an elevated risk of neuropsychiatric diseases (6).

Activation of the hypothalamic-pituitary-adrenal (HPA) axis during stress leads to glucocorticoid (corticosterone) secretion from the adrenal cortex (7). One of the key pathological features associated with chronic stress is oxidative stress, which plays a significant role in the onset and progression of various neuropsychiatric and neurodegenerative disorders (7). Oxidative stress arises when there is an imbalance between the production of reactive oxygen species (ROS) and the cell's ability to neutralise these reactive molecules or repair the resulting cellular damage (27). Among the multiple downstream effects of stress, increased ROS generation is recognised as one of the most critical mechanisms contributing to neuronal dysfunction and disease progression (8). Comprehending the molecular and

cellular mechanisms triggered during the stress response is crucial for the creation of pharmaceutical strategies aimed at mitigating and treating illnesses brought on by physiological stress.

Neurons require mitochondria to function, and any oxidative damage that happens in the mitochondria may be related to the aetiology of AD (9). Mitochondria are highly dynamic, complex organelles that primarily provide energy but also play a crucial role in cell death, signalling pathways, apoptosis, reactive oxygen species (ROS) production, and calcium homeostasis processes that are all disturbed in neurodegeneration (10). The imbalance in mitochondrial function contributes to increased ROS formation and decreased ATP production, and both of those conditions have a direct and detrimental impact on the cells, leading to oxidative stress (11, 12). Numerous studies have demonstrated a strong correlation between various age-related disorders and impaired mitochondrial activity, making mitochondria an intriguing prospective target for the treatment and prevention of neurodegenerative diseases (13). By releasing cytochrome c, decreased ATP synthesis may result in neural dysfunctions and possibly "cellular energetic depression (CED)" that activates the apoptotic cell death program (14). Neuronal loss and dysfunction, the most recent neurobiological event to explain clinical dementia, are caused by interactions between mitochondrial malfunction and the basic pathology of Alzheimer's disease (AD) (amyloid beta plaque and tau deposition) (15). Before amyloid deposition, alterations in mitochondrial function are observed in animal models of AD (16).

Previous research has shown that the accumulation of amyloid-β within mitochondria is linked to a disruption of mitochondrial activity. It may also cause mitochondrial activity to be disrupted directly, which results in oxidative stress (17, 18). A study suggested a link between mitochondrial dysfunction and tau (18). Over-expression of tau has been linked to mitophagy deficits, alterations in mitochondrial shape, increased retrograde mitochondrial transport, perinuclear distribution of mitochondria, and decreased Complex I and ATP activity (19). Numerous studies have documented metabolic changes in the brain and plasma linked to the use of the CUMS model (20, 21).

1.2.Justification of the Study

In experimental research, LPS and stress models are frequently used. Although several studies have investigated the effects of stress and LPS on parameters like those examined in this study, little is known about the interaction between LPS and CUMS, particularly at the specific LPS dosage and stress duration used in this study. The study suggests that the combination of

chronic stress (CUMS) and LPS exposure may intensify the effects of both, resulting in increased oxidative stress, neuroinflammation, and decreased neuroplasticity. In particular, the impacts of the combined model on neurochemistry, metabolic changes, and systemic alterations have not been well explored, as are the synergistic effects of these two factors on brain function and behaviour, including anxiety-like behaviour. To provide insights into potential therapeutic approaches, our study attempts to close significant gaps in our current understanding of how these factors contribute to neurodegeneration, metabolic alterations, and mood disorders.

1.3.Aims

We examined the impact of CUMS on oxidative stress and inflammatory markers as well as APO-E expression, and their correlation with acetylcholine following LPS challenge in rats. Furthermore, we examined the impact of CUMS on body weight and feeding changes in association with anxiety-like changes in LPS-challenged rats.

1.4.Objectives

- To monitor metabolic alterations by tracking body weight, food and water intake.
- To assess anxiety-like behaviour by performing an Open Field Test on the control, stressed, LPS, and combined model of CUMS and LPS.
- To evaluate oxidative stress and neuroinflammatory response by quantifying 4-HNE, SOD1, and IL-1β concentrations using ELISA on a control, CUMS, LPS and combined model of CUMS and LPS.
- To assess mitochondrial dysfunction by measuring APO-E expression in isolated mitochondria using Mitochondrial Isolation Kit and measuring mtDNA expression using RT PCR on the control, stressed, LPS, and combined model of stress and LPS.
- To examine plasma CORT and ACh in the control, stressed, LPS, and combined model of stress and LPS.

1.5.Potential Benefits

Examining the impact of CUMS on memory, especially when combined with LPS (which can mimic inflammatory responses), can provide insights into how chronic stress speeds up

cognitive decline and may contribute to conditions like Alzheimer's and other types of dementia. It may also help uncover the mechanisms underlying cognitive impairments frequently observed in these mental health conditions. When combined with CUMS, this research can shed light on the relationships between immune responses and chronic stress, which can alter energy metabolism, including lipid and glucose metabolism, which are vital for brain function and result in molecular changes that lead to neurodegeneration. Examining these alterations concerning CUMS and LPS treatment can provide information about how metabolic changes brought on by stress may worsen memory loss and cognitive deterioration. Examining this connection in rats given LPS may reveal pathways where stress and the immune system interact, offering options for treatment. New therapeutic strategies targeted at lessening or reversing these effects may result from an understanding of how chronic stress speeds up inflammation and memory loss.

1.6.Methodology Overview

The animal experiments were permitted by the Animal Ethics Committee of the University of KwaZulu-Natal (UKZN) (AREC/00004514/2022). Twenty-four male Sprague-Dawley rats, 10 weeks old, weighing 200- 250 g, were housed and bred at UKZN Biomedical Research Unit (BRU) in cages with food and water availability *ad libitum*. They were maintained in a room on a 12-hour light/dark cycle and at constant temperature (22 ± 2 °C) with relative humidity (55 ± 10%). Animals were acclimatised for 5 days before the experiment. Animals were divided into 4 groups, each group containing 6 rats per group. Thereafter, baseline measurements of body weight as well as food and water intake using metabolic cages were taken from all the groups. Baseline measurements refer to measurements undertaken before animals are subjected to any interventions. They serve as a reference point to assess changes later. The control group was sham-treated with a single 0.9 % saline solution injection (5 mg/kg, i.p.) (Sigma-Aldrich, JHB, South Africa). The stressed groups underwent a chronic, unpredictable, mild stress protocol for 10 days. Animals that were subjected to stress using a chronic mild unpredictable stress procedure were exposed to 4 stressors for 10 days. The stress protocol consisted of daytime darkness for 12 hours, a 45° tilted cage for 5 hours with food and water on the inclined (23), and cold exposure for 5 minutes at 4°C (24) which consisted of the rats being kept in a cold room at the BRU with the temperature adjusted to 4°C and restraint for 1.5 hours (25). Rats received two random stressors per day over the 10 days. During the stress protocol, each stress was administered three times. Two of the stressors were applied

daily to animals. To maximise unpredictability, one of the stressors administered on the second day was randomly paired with one of the stressors from the first day. Inducing stress in rats has effects on metabolism, and stress induces changes in the hippocampal structure and function (26). The LPS group received a single dose of LPS (5 mg/kg, i.p), and the stressed + LPS group was exposed to both 10 10-day chronic unpredictable mild stressor protocol and LPS injection (5 mg/kg, i.p.). 5 mg/kg of LPS, which is a safe dosage in experimental rats (22), was administered intraperitoneally after 10 days of CUMS protocol in the appropriate groups. This was followed by an Open Field Test (OFT) 24 hours after LPS administration. The OFT was conducted at the BRU behavioural test room for one day. Final measurements of body weight, as well as food and water intake using metabolic cages, were taken a day after an OFT. Animals were decapitated a day after the final metabolic measurements to collect blood and hippocampal tissues. The blood was centrifuged immediately after decapitation to collect plasma. The plasma and hippocampal tissues collected were stored at -80°C in a Bio freezer (Snijders Scientific, Holland) until ready for biochemical analysis of SOD1, 4-HNE, APO-E expression, corticosterone, IL-1β, and ACh concentrations. The concentrations of SOD1, 4-HNE, IL-1β, corticosterone, and ACh were measured using ELISA Kits. The expression of APO-E was measured using PCR. All data analyses from ELISA were performed with GraphPad software version 8 and were analysed by one-way ANOVA followed by Tukey multiple comparisons test, and the results were presented as the mean ± SEM. Results with $p<0.05$ were considered statistically significant.

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Chapter 2: Literature Review

2.1. Stress

Stress is a general adaptive response brought on by social and environmental circumstances. Acute stress is a short-term response to an immediate threat, typically involving rapid activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. This results in the release of catecholamines and glucocorticoids, which prepare the body to cope with the stressor. Chronic stress is a prolonged stress that persists for weeks or months; it disturbs homeostasis and impacts almost all organs and tissues (76). Chronic psychological stress is increasingly recognised as a serious health concern in modern society, negatively impacting both physical and mental health (76). Chronic stress can also alter the immune system, produce heightened inflammatory responses (77), and impair antioxidant defenses, reducing their effectiveness (78). Chronic unpredictable mild stress (CUMS) refers to a set of minor psychosocial and physiological stressors that occur regularly over several weeks and is widely recognised as a reliable model for studying stress responses (10). Compared to acute stress, chronic stress more accurately mimics long-term stress exposure and cumulative brain damage, which can contribute to disease progression. Specifically, CUMS consistently induces behavioural and neurobiological changes in animals that reflect those seen in humans, including anxiety, HPA axis dysregulation, and hippocampal abnormalities.

Individual differences in stress response exist even when exposed to the same stressors because some people are more susceptible to stress, which can lead to changes in homeostasis and disease, while others are better equipped to handle similar stressful situations (1, 2). To have an effective stress tolerance, the HPA axis must be activated quickly and then effectively terminated when the stressor either goes away or persists for a longer period (3, 4). Both crucial functions rely on how the immunological, endocrine, and neurological systems regulate the brain (5, 6). Furthermore, the brain, in particular, is susceptible to stress, and when stress levels are beyond acceptable thresholds, mental and physical illnesses arise (7).

2.2.CUMS in neuronal processes and brain function

Stress can lead to an imbalance in the brain circuits supporting mood, anxiety, decision-making, and cognition, which can either increase or decrease the manifestation of such behavioural states and actions (8). This imbalance subsequently impacts systemic physiology

through neuroendocrine, autonomic, immunological, and metabolic mediators (9). Chronic stress has been shown in numerous studies to be a risk factor for the development of cognitive deficits

(10). Chronic psychological stress and the resulting physiological dysregulations are increasingly thought to be agitators of disease trajectories and accelerators of hastened ageing (11). Chronic stress is a risk factor for the development of depression, anxiety, and a decline in cognitive function because stress generates several neuropsychiatric disorders that show up as clinical pathological changes in specific brain regions (12, 13). In the CUMS model, prolonged stress exposure significantly affects how the parts of the rodent's brain involved in memory and learning work (10). Chronic stress alters the body's physiology in several ways. A primary factor is the activation of the HPA, which is linked to an excessive release of cortisol, sometimes known as the "stress hormone," into the bloodstream (10).

Chronic psychological stress is thought to promote inflammation by inducing the release of pro-inflammatory cytokines. It also exacerbates oxidative stress through the overproduction of ROS in the mitochondria (14). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) overproduction are a major cause of oxidative stress (14). However, excessive accumulation of what causes extensive cellular and molecular damage and is frequently brought on by environmental stressors, chronic inflammation, or mitochondrial dysfunction. Lipid peroxidation, protein oxidation, DNA strand breakage, and mitochondrial dysfunction are all included in ROS production (31). Furthermore, oxidative stress exacerbates tissue damage by inducing inflammatory reactions through activating redox-sensitive transcription factors like nuclear factor κB (NF-κB) (31). The pathophysiology and progression of neurodegenerative diseases are eventually influenced by these processes, which interfere with cellular signalling pathways and disturb the metabolism of essential macromolecules (14).

It is known that neuroinflammation is an innate immune response that is generally induced by different types of stresses imposed by the surroundings (16). In response to stress, the body detects and evaluates stressors, triggering an immunological response that initiates a cascade of local and systemic inflammatory processes. These responses disrupt normal homeostasis and contribute to psychological disturbances and functional impairments across various systems(16-18).

2.3.The central role of the brain in stress and neuroinflammation

Sensory inputs are first relayed via the thalamus to the amygdala and prefrontal cortex, where the amygdala rapidly appraises threat-related cues and the hippocampus provides contextual memory to distinguish between safe and dangerous situations. The prefrontal cortex integrates these inputs to guide responses and regulate amygdala activity. If a stimulus is deemed stressful, these circuits activate the hypothalamic–pituitary–adrenal (HPA) axis, where corticotropinreleasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus trigger the release of ACTH and downstream glucocorticoids and the sympathetic-adrenal-medullary (SAM) system, which releases cortisol, adrenaline and noradrenaline. Together, these specialised cells, structures, and neuroendocrine systems enable the brain to assess threats and initiate adaptive stress responses (75). It also controls the physiological and behavioural reactions, whether they are beneficial or detrimental to one's health (19). The brain is a biological organ that changes its structure, molecular makeup, and neurochemistry in response to both acute and chronic stress (19). It also controls numerous bodily systems, including the immune, cardiovascular, and metabolic systems, which contribute to the short- and long-term effects of being "stressed out" and the behaviours that harm one's health (8). The hippocampus, a unique brain component that supports mood and memory, was the first region outside of the hypothalamus to be identified as a stress hormone target (19). The hippocampus plays an essential role in regulating the immune processes. Deficits in memory and cognitive decline can occur in healthy older adults without any other indication of neurological impairment (20). Chronic stressful experiences have been shown in several animal models to remodel hippocampus neurons and alter the hippocampus's overall shape (19). In the hippocampus, a crucial area of the brain linked to mood and cognition, CUMS can directly activate M1-type microglia to produce pro-inflammatory mediators (21), which disrupts astrocyte and neuronal function and lowers neurotrophin levels, monoamine neurotransmitter concentration, and neurogenesis (22).

Microglia are the primary active immune defense mechanism in the central nervous system (23). Microglia continue to function and accelerate the inflammatory process in chronic neuroinflammation (24). In addition to PGE2, COX-2, mitogenic factors, chemoattractant protein (MCP-1), and macrophage colony-stimulating factor (M-CSF), activated microglia release pro-inflammatory cytokines like TNF-α, IL-1β, IL-12, and IFN-γ, which can be neurotoxic to neurons (25, 26). Dysregulation of immune-to-brain communication may result

in maladaptive alterations in the brain's reaction to inflammatory stimuli, which can exacerbate neuropsychiatric illnesses (19, 27).

Inflammation is a complicated adaptive and defensive process that can be local or universal; it’s a process that occurs in response to pathogenic stimulus or injury (28). Inflammatory systems play a role in preserving homeostasis in the face of numerous obstacles, including infections, tissue damage, stress, and toxic chemicals (28).

2.4.Oxidative stress in neurodegeneration

The brain is thought to be especially vulnerable to the harmful effects of ROS because of its high metabolic rate and comparatively lower potential for cellular regeneration when compared to other organs (29). To cope with oxidative stress and related oxidative damage, the cell has developed several defense and repair mechanisms (29). However, under these circumstances, the activities of different antioxidant defense molecules that ordinarily counteract the harmful effects of ROS are diminished. For instance, impacted brain regions exhibit decreased activity of the antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHPx), and glutathione reductase (GSHRd) (29). Because neurons are postmitotic, they may be especially vulnerable to oxidative stress, which is increasing in the brain with age. Cells' capacity to respond to oxidative protein damage also appears to diminish with age, which may be a factor in protein accumulation.

Oxidative stress is a redox condition brought on by an imbalance between the production and detoxification of reactive oxygen species (ROS) (30). ROS are physiological byproducts that are unavoidable yet have unfavourable effects on the biological system; they can oxidise all main macromolecules, including nucleic acids (DNA, RNA), proteins, and lipids. Therefore, when present in excess, they can harm the biological system (31, 32). They can serve important tasks like signalling molecules under well-controlled circumstances (33). By monitoring antioxidant concentrations or antioxidant enzyme activity, ROS could potentially be evaluated indirectly. Because of its comparatively low levels of antioxidants, high levels of polyunsaturated fatty acids, and higher need for oxygen, the brain is particularly susceptible to oxidative stress (30). Studies have shown that oxidative stress plays a major role in several nervous system damage processes, including memory loss, learning disabilities, neuroinflammation, and several neurodegenerative diseases (34, 35).

One of the main mechanisms involved in oxidative stress is lipid peroxidation and protein oxidation. Lipid peroxidation is a process that results in the production of lipid peroxidation

products when lipids are damaged by ROS via a free radical chain reaction mechanism (36). Protein oxidation is the covalent alteration of a protein caused by direct interactions with ROS or indirect interactions with secondary by-products of oxidative stress (37). Lipid peroxidation and protein oxidation are the hallmarks of oxidative stress; therefore, lipid and protein oxidation have been considered indicators of oxidative stress (37). It is significantly increased in AD, according to a considerable body of data. Along with the general increase in oxidative biomolecule products, a notable reduction in antioxidant levels or antioxidant enzyme activity has also been repeatedly recorded.

2.5.Lipopolysaccharide

Lipopolysaccharide (LPS) has been utilised to stimulate the brain's innate immunological response (38, 39). Endogenous LPS's pathogenic functions are receiving a lot of attention because it is known to be essential in fostering neuroinflammation in several neuropsychiatric conditions (such as depression, schizophrenia, anxiety, autism spectrum disorders, attentiondeficit hyperactivity disorder, etc.) and neurodegenerative conditions (such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS)) (40). Excessive cytokine production brought on by LPS disrupts immune homeostasis and increases oxidative/nitrative stress in the nervous system, gut, and peripheral regions through the gut-brain axis (GBA) (41). Accordingly, LPS is regarded as a neurotoxic and immunotoxin since it causes long-term inflammation in both the central and peripheral nervous systems (41). Molecular research has demonstrated that LPS-mediated inflammatory responses include impaired autophagy and elevated oxidative/nitrative stress, which manifests as increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), decreased activity of antioxidant enzymes (glutathione peroxidase and superoxide dismutase), dysfunctional mitochondria and endoplasmic reticulum, and decreased respiratory chain efficiency (41).

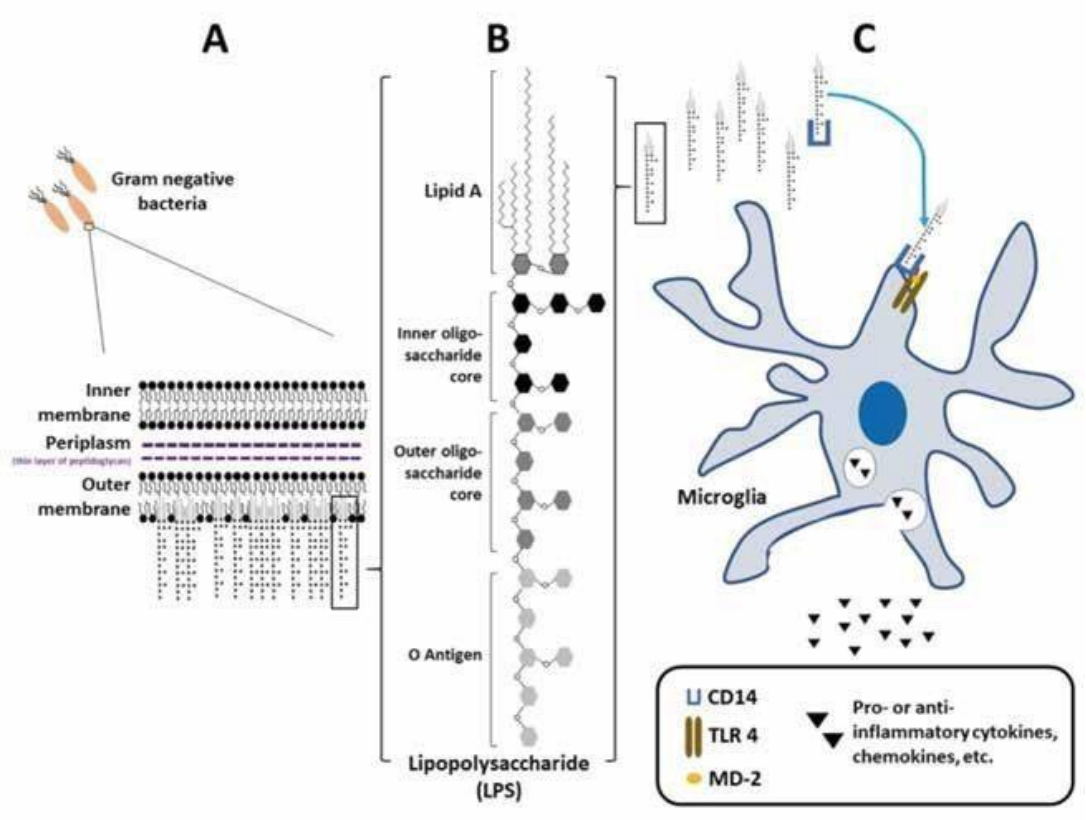

Figure 2.5.1.: Lipopolysaccharide (LPS) (A) location, (B) molecular structure, and (C) effects on immune cell signalling. [Image extracted from Ramirez et al., 2018 (64)]

LPS is the main inflammatory component of the endotoxin found in gram-negative bacterial cell walls (42). It is a potent endotoxin that causes inflammation for a long time. Even at low dosages, systemic administration of LPS causes the production of pro-inflammatory cytokines that trigger the neuroimmune and neuroendocrine systems, leading to symptoms of illness (43). In rodents, inflammatory drugs are frequently administered to cause neuroinflammation, which is linked to cognitive deficits. In rodents, LPS is frequently administered to cause neuroinflammation, which is linked to cognitive deficits (44). Systemic inflammation can lead to persistently high levels of circulating pro-inflammatory cytokines (45, 46) and has been associated with cognitive impairments in patients (47-49). Strong correlations between systemic inflammation and cognitive performance have been reported (50). Several potential mechanisms have been proposed for cognitive impairments following sepsis, including disruption of the blood-brain barrier, neuroinflammation, oxidative stress, microglial activation, and neuronal loss, particularly in the limbic system (45).

Numerous studies have demonstrated that LPS impairs cognitive function and induces neuronal cell death, particularly in brain regions critical for learning and memory, such as the

hippocampus and prefrontal cortex (51, 52). Following LPS administration, animals typically exhibit a constellation of behavioural and physiological symptoms, including anorexia, reduced locomotor activity, weight loss, diminished exploratory behaviour, and elevated anxiety-like behaviour, all of which reflect the onset of sickness behaviour and central nervous system dysfunction (53, 54). LPS exposure triggers the innate immune response at the molecular level, mainly via the Toll-like receptor 4 (TLR4) pathway, which in turn triggers the production of pro-inflammatory cytokines. Simultaneously, LPS induces the excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), further exacerbating oxidative stress and mitochondrial dysfunction (55) (56). Systemic LPS administration causes neuroinflammation, which is known to have detrimental effects on brain function (57-59). These effects include memory impairment and neuroinflammation through TLR-4/NF-κB signalling with increased expression of genes and/or molecules related to inflammation, such as different proinflammatory cytokines and chemokines (57). Acute LPS administration has been demonstrated to interfere with the consolidation of memory processes; for instance, acute LPS administration before training can disrupt cue-fear conditioning, while chronic LPS administration has been found to impair spatial memory and promote learning and memory deficits (60). A previous study demonstrated that a single administration of LPS in a dose of 1 mg/kg impaired cognitive performance in the Barnes Maze test and the inhibitory avoidance test (61).

Prolonged production of pro-inflammatory mediators by microglia activation increases oxidative and nitrosative stress, which can prolong neuroinflammation and potentially lead to neurodegenerative disorders (62). On microglia membranes, LPS attaches itself to CD14 to create the complex LPS-CD14, which then interacts with the Toll-like receptor (TLR) 4 (63). TLR-4 stimulates microglia by starting signal transduction cascades that result in the brain producing pro-inflammatory cytokines early on, including interleukin (IL)-1ß, IL-6, tumour necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (62).

Another study demonstrated that after 24 hours post-intraperitoneal injection, LPS (1 μg/kg to 250 μg/kg), significantly reduced body weight, increased corticotrophin hormone (ACTH) (doses ranging from 15 μg/kg to 250 μg/kg), and serum corticosterone levels (doses ranging from 5 μg/kg to 250 μg/kg) (64). In addition, LPS raised serum interleukins, specifically IL-1ß and IL-6 (doses ranging from 5 μg/kg to 250 μg/kg) and TNF-α (doses ranging from 1 μg/kg to 250 μg/kg). LPS can stimulate the immune system and cause behavioural alterations in rats at very low doses according to studies (64). LPS administration has been shown to stimulate

the immune system and cause inflammatory responses (56). Previous studies have reported that LPS injection impairs spatial memory in the Morris water maze (MWM), increases the levels of inflammatory cytokines, including TNF-α and IL-1β, in the hippocampus region, and reduces antioxidant defense (65, 66) through activating nuclear factor kβ (NFkβ). LPS injection leads to elevated insulin resistance, aberrant insulin signalling, oxidative stress response, increased advanced glycation end products, and altered blood-brain barrier (67).

2.6.CUMS and LPS administration

Both CUMS and LPS work as cytokine inducers to affect immune cell formation, distribution, and activation, as well as to elicit acute and chronic immunological activation (68). Chronic stress and inflammation can both impair metabolic functions (69). The LPS challenge normally generates a systemic inflammatory response, but CUMS could further worsen this by changing hormonal and metabolic processes (70). In rats given LPS, CUMS aggravates several brain changes linked to inflammation, neurotransmitter imbalances, and malfunction of the HPA axis (71, 72). Chronic stress and systemic inflammation work together to cause oxidative stress, neuroinflammation, and decreased neuroplasticity (73, 74). LPS administration can worsen the neurochemical, oxidative, and behavioural effects of stress (50), and chronic stress is known to enhance susceptibility to inflammatory stimuli (77). Because it reflects the intricate relationships between psychosocial stress and immunological dysregulation seen in people, this method is especially pertinent for investigating the processes underlying stress-related neuropsychiatric and neurodegenerative illnesses.

In addition to cognitive impairments, these effects are likely to have a role in the onset or exacerbation of mood disorders such as anxiety and depression. This combination aids in the understanding of how oxidative stress, neuroinflammatory processes, neurotransmitter imbalance, and cognitive or behavioural deficiencies may be exacerbated by stress-induced susceptibility. These models are useful for assessing possible treatment approaches and for examining the underlying mechanisms of neuropsychiatric and neurodegenerative illnesses linked to stress.

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Chapter 3: Manuscript 1

Prologue

CUMS exacerbates oxidative stress, triggers neuroinflammation, and impairs mitochondrial function, collectively contributing to cognitive deficits. Similarly, LPS, a component of a gramnegative bacterial cell wall, can also induce oxidative stress, inflammation, mitochondrial dysfunction, and memory impairment. Despite the well-established individual effects of CUMS and LPS, the combined impact of chronic stress and immune activation through a single LPS injection on these key neurobiological parameters remains largely unexplored. Therefore, this study aims to investigate how CUMS influences oxidative stress, inflammation, mitochondrial health, and cholinergic function both in the brain and in the body of rats exposed to LPS.

"Unpredictable Stress on Memory Decline Associated Molecular Changes in LPSchallenged Rats"

This manuscript is prepared according to the journal's guidelines to authors; this manuscript will be submitted for publication in the journal **Behavioural Brain Research.**

Unpredictable Stress on Memory Decline Associated Molecular Changes in LPSchallenged Rats

Khethelo Sibiya [1, a], Musa Mabandla[1], and Mluleki Luvuno[1]

[1]Discipline of Human Physiology, School of Laboratory Medicine & Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, South Africa, Private Bag X54001, Durban, South Africa

[a] Correspondence to: Email: 219013485@stu.ukzn.ac.za

Abstract

Chronic stress can disrupt homeostasis and lead to increased oxidative stress. Lipopolysaccharide (LPS) induces oxidative stress, neuroinflammation, mitochondrial dysfunction, and memory impairment. However, the impacts of LPS in chronically stressed rats on oxidative stress, mitochondrial function, and memory impairment have not been explored. Therefore, we investigated the impact of chronic unpredictable mild stress (CUMS) on SOD1, 4-HNE, IL-1β, and ACh concentrations as well as Apo-E expression in LPSchallenged rats. To achieve our aims, 24 male Sprague-Dawley rats were divided into 4 groups: control, stressed, LPS, and stressed + LPS groups (n=6, per group). The concentration of SOD1, 4-HNE, IL-1β, and ACh in the plasma and the hippocampus was measured using ELISA, and APO-E expression was measured using PCR. The results were presented as a mean

± SEM and analysed using one-way ANOVA, where $p<0.05$ was considered significant. Our findings demonstrated a decrease in plasma and hippocampal SOD1 concentration and an increased hippocampal 4-HNE concentration, accompanied by increased plasma and hippocampal IL-1β concentration, increased APO-E expression, and decreased ACh concentration in rats subjected to CUMS and later challenged with LPS. Our study implies that stress, if combined with LPS, exacerbates oxidative stress, neuroinflammation, mitochondrial dysfunction, and impairs cholinergic function, which are all factors that lead to cognitive deficits. However, LPS acts as an inflammatory trigger, aggravating the impacts of chronic mild stress. LPS helps by simulating the interaction of oxidative stress, inflammation, and cognitive failure; it sheds light on possible processes that could underlie neurodegenerative illnesses.

Keywords: Lipopolysaccharide, chronic unpredictable mild stress, oxidative stress, reactive oxygen species, acetylcholine

3.1.Introduction

Mitochondria are highly dynamic, complex organelles that primarily provide energy and ensure neuronal survival and function (1). Mitochondrial reactive oxygen species (ROS) trigger a variety of inflammatory cytokines and mitochondria-dependent apoptotic pathways, resulting in neuronal damage (2, 3). Under normal conditions, antioxidant compounds like glutathione and vitamin E, as well as intracellular antioxidant enzymes like superoxide dismutase, glutathione peroxidase, and catalase, could reduce ROS from the mitochondrial respiratory chain (4). However, in pathological conditions, due to their increased synthesis, these antioxidants might not be able to eliminate large amounts of ROS produced, resulting in oxidative stress and cellular damage (5).

Oxidative stress and mitochondrial dysfunction are the main pathological pathways underlying neurodegenerative diseases (6). In neurodegenerative disorders, neuroinflammation and mitochondrial dysfunction are linked to oxidative stress due to excessive generation and release of reactive oxygen (ROS) and nitrogen (RNS) species, which causes neuronal damage (7, 8). ROS are physiological byproducts that are unavoidable yet have unfavourable effects on the biological system. Oxidative stress is a redox condition brought on by an imbalance between the production and detoxification of ROS (9). Oxidative stress is known to contribute to neuronal degeneration in the central nervous system (CNS) in ageing as well as in neurodegenerative disorders such as amyotrophic lateral sclerosis, Alzheimer's dementia, and Parkinson's disease (10). Overproduction of ROS leads to oxidative damage, including lipid peroxidation, which leads to cell death (11). The brain is particularly susceptible to oxidative stress due to its comparatively low levels of antioxidants such as SOD1, high levels of polyunsaturated fatty acids such as 4-HNE and MDA, and a higher need for oxygen (9). SOD1 is a critical antioxidant enzyme involved in the detoxification of ROS generated during aerobic respiration. During oxidative stress, SOD1 plays a pivotal role in mitigating ROS accumulation, thereby preserving cellular homeostasis. In addition to its antioxidative function, SOD1 is implicated in the regulation of apoptotic signalling pathways and cell death mechanisms, highlighting its importance in cellular responses to oxidative insults. 4Hydroxynonenal (4-HNE) is a key byproduct of peroxidation and lipid oxidation. It is widely recognised as a key mediator of oxidative stress and is commonly used as a bioactive marker to assess the extent of oxidative damage in various biological systems. Studies have shown that oxidative stress plays a major role in several nervous system damage processes, including memory loss, learning disabilities, neuroinflammation, and several neurodegenerative diseases (12, 13).

With a range of acute and chronic insults to the central and peripheral nervous system, inflammation is believed to be a major factor in the development of pathological disorders. It has been suggested that the cholinergic system mediates the connection between the immune system and neurons (14). Acetylcholine (ACh) is a neurotransmitter that plays a role in cognitive processing, arousal, and attention in the brain (15). ACh is essential for controlling cognitive functions in the CNS, especially memory, attention, and learning (15). ACh is commonly evaluated to detect cognitive deficits, evaluate the integrity of the cholinergic system, and to the neurochemical effects of stress, neuroinflammation, and exposure to toxins (15). ACh inhibits the release of pro-inflammatory cytokines IL-1β, IL-6, and IL-18, but does

not alter IL-10 production in LPS-stimulated macrophages (16). IL-1β plays a crucial role in the host defense against pathogens, mediating short-term inflammatory responses and contributing to the development of adaptive immunity. Both chronic unpredictable mild stress (CUMS) and lipopolysaccharide (LPS) work as cytokine inducers to affect immune cell formation, distribution, and activation, as well as to elicit acute and chronic immunological activation (17).

Chronic unpredictable mild stress (CUMS) refers to a set of minor psychosocial and physiological stressors that occur regularly for several weeks in an unpredictable manner and is recognised as a reliable model for stress response (18). Stress has been linked to the progression of chronic neurodegenerative diseases such as AD, diabetes mellitus, cardiovascular disease, rheumatoid arthritis, and metabolic syndrome. It is well known that neuroinflammation is an innate immune response that is typically triggered by various environmental stressors (19). The duration of exposure affects how the immune system and stress response interact. Chronic stress can elevate pro-inflammatory cytokines, which are potential sources of ROS. Stressors activate pathways that generate ROS, such as NADPH oxidase enzymes and mitochondrial dysfunction (20). The brain is a vulnerable target organ for stress; if the stress intensity exceeds acceptable levels, it can lead to the onset of mental and physical diseases (21). A variety of studies have demonstrated that persistent stress increases the chance of developing cognitive impairment (22-24).

LPS is one of the pathogen-resistant components of gram-negative bacteria's cell walls and among pathogen-associated molecular in *vivo* immunological responses induced by patterns detected by the innate immune system, and it is said to induce memory impairment in rats (25). LPS has been demonstrated to induce hippocampal oxidative stress and enhance the generation of ROS in LPS-challenged animals (26), neuroinflammatory response, neuronal death via apoptosis, and memory impairment (27, 28). Memory impairment mediated by LPS is mostly due to the pathophysiology of oxidative stress brought on by abnormalities in the mitochondria (29). Inflammatory response triggered by mitochondria may include APO-E overexpression in response to mitochondrial malfunction (30); however, the impacts of combined chronic mild stress and immune activation on oxidative stress and mitochondrial function have not been fully studied. In this study, we investigated the impacts of LPS administration on oxidative stress, memory, and cognitive function in stressed rats. Therefore, this study aims to evaluate SOD1 and 4-HNE, IL-1β, APO-E expression, and acetylcholine (ACh) in stressed rats following LPS administration.

3.2.Materials And Methods

3.2.1. Chemicals

All chemicals and reagents used in this project were purchased from standard commercial suppliers and were of analytical grade. LPS was purchased from Sigma Chemical Co. (St. Louis, MO., USA). Pierce BCA Protein Assay Kit was purchased from Pierce in the USA, and ELISA assay kits were purchased from Elabscience Biotechnology Inc., USA.

3.2.2. Animals

Twenty-four male Sprague-Dawley rats aged 10 weeks and weighing 200- 250 g were bred and housed at the UKZN Biomedical Research Unit (BRU). The animals were kept in transparent cages (320 cm^2 x 14 cm^2) with access to food and water *ad libitum*. The animals were randomly divided into 4 groups, each group containing 6 rats per cage and allowed to acclimate for 5 days before the experiment. The animals were kept under standard laboratory conditions in a room on a 12-hour light/dark cycle and at constant temperature (22 ± 2 °C) with relative humidity (55 ± 10). The animal experiments were permitted by the Animal Ethics Committee of the UKZN (AREC/00004514/2022).

3.2.3. Sample Size

Animals were divided into four experimental groups, with six rats per group (n = 6/group), resulting in a total of 24 rats. A group size of six has been shown to provide adequate statistical power to detect significant differences in similar experimental designs involving chronic stress and LPS-induced models (67)

To further support the appropriateness of this sample size, the E-value was calculated using the formula:

E = Total number of animals – Total number of groups

E = (6 × 4) – 4 = 20

An E-value between 10 and 20 is considered optimal for ensuring a balance between ethical use of animals and statistical validity in biological studies, according to guidelines for

resource equation methods in animal research (67). The obtained E-value of 20 is at the upper end of this range, indicating a suitable sample size.

3.2.4. Experimental Design

All 24 male Sprague-Dawley rats were acclimatised for 5 days before any experiments; they were divided into 4 groups, each containing 6 rats. There was a control group that was shamcontrolled with saline solution; it received the saline solution of 5 mg/kg. The stressed group underwent chronic unpredictable mild stress procedures, the LPS group, which received a single dose of 5 mg/kg LPS, and the stressed + LPS group, which was exposed to both chronic unpredictable mild stressors and was administered 5 mg/kg of LPS. LPS was administered intraperitoneally after 10 days of CUMS protocol in the appropriate groups. Animals were decapitated a day after LPS administration to collect blood and hippocampal tissues. The blood and hippocampus collected were stored at -80°C in a Bio freezer (Snijders Scientific, Holland) until ready for biochemical analysis of SOD1, 4-HNE, IL-1β, APO-E expression and ACh concentrations.

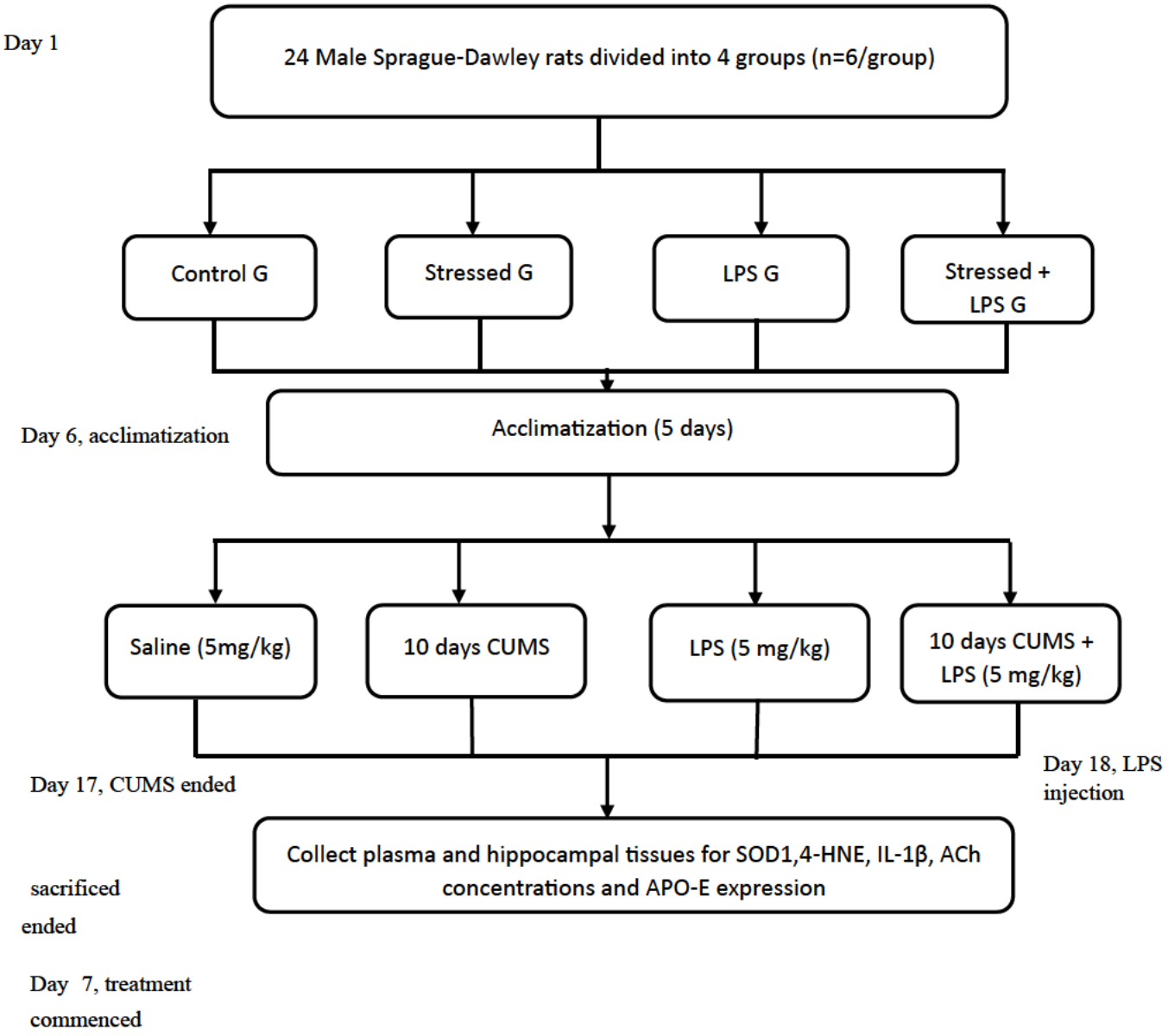

Figure 3.2.4.1.: A diagrammatic depiction of the experimental design

3.2.5. Stress protocol

Animals were subjected to stress using a chronic mild unpredictable stress (CUMS) procedure. These animals were exposed to 4 stressors for 10 days. A shorter timeline reduces cumulative animal distress and adheres to the 3Rs principle (Replacement, Reduction, and Refinement) in animal research ethics by minimising prolonged exposure to stressors, while still allowing for the collection of high-quality and biologically relevant data. However, CUMS procedures usually last 21–42 days to simulate depressive-like behaviours. New research suggests that shorter times (7–14 days) can still result in notable changes in the body's physiology, neuroendocrine system, and neurochemistry. The stress protocol consisted of daytime darkness for 12 hours, a 45° tilted cage for 5 hours with food and water on the inclined (31), cold exposure for 5 minutes at 4°C (32) which consisted of the rats being kept in a cold room at the BRu with the temperature adjusted to 4°C and restraint for 1.5 hours; for restraint stress, rats were placed in air-assessable cylinders (33), restraint alters the autonomic nervous system, increasing body temperature, and heart rate and behaviourally increasing anxiety-like behaviour (33). Rats received two random stressors per day over the 10 days. Each stressor was applied three times throughout the experimental period. Rats were exposed to two different stressors per day, and the same combination was not repeated on consecutive days. To reduce predictability, for instance, one of the stressors used on day one was alternated or combined with a different stressor on day two. Inducing stress in rats has effects on metabolism, and stress induces changes in the hippocampal structure and function (34).

3.2.6. LPS administration

The rats were administered LPS through intraperitoneal injection. Rats received a single dose of LPS (5 mg/kg). The single dose of LPS (5 mg/kg, i.p.), which is a safe dosage in experimental rats (35), was administered intraperitoneally to appropriate groups (LPS group and stressed + LPS group) a day after the stress procedure. While LPS was administered to the appropriate groups, a stressed group was injected with a vehicle.

LPS (Escherichia coli, serotype 0111:B4, Sigma-Aldrich Chemical Corp., St. Louis, MO., USA). To make 5 mg/ml of LPS, 5 mg of LPS powder was weighed carefully on a weighing boat using an analytical balance scale (Lasec® Group, South Africa). 5 mL of PBS was added to 5 mg of LPS powder. The mixture was vortexed until the solution was clear using an

analogue vortex mixer (Eins-Sci Laboratory Equipment, South Africa). The mixture of LPS and PBS was diluted to the working concentration of 0.2 µg/µL. The aliquots of 20 µL were prepared in small, sterile tubes to avoid repeated freeze-thaw cycles, which can degrade LPS.

LPS dosage used:

The LPS group had an average of 251.5 g. To obtain the volume of LPS, we multiplied the LPS dosage by the average body weight of the LPS group. The dosage of 5 mg/kg was multiplied by an average weight of rats, which was 0.2515 kg when converted to kilograms, resulting in 1.26 mg. The final volume of LPS used in each rat was 2 mL.

3.2.7. Tissue harvesting

The blood of the Sprague-Dawley rats was collected into an EDTA blood collection tube by cardiac puncture; rats were then decapitated using a sharp guillotine. Following decapitation, the external surface of the heads was disinfected with alcohol for brain section preparation. The skulls were immediately opened using straight scissors, surgical blades, a scalpel, and forceps after decapitation to remove the brain for hippocampi extraction. The hippocampi were carefully separated from the surrounding regions, including the cortex, corpus callosum, and thalamus, using the tools mentioned above. The hippocampi were carefully isolated using the probe. The hippocampi are C-shaped structures located between the cortex externally and the thalamus internally. The hippocampi were collected into Eppendorf tubes (Lasec® Group, South Africa), which were kept in ice to preserve tissues from degradation. The blood collected was immediately centrifuged to obtain the plasma. The tissues collected were stored at -80 °C in a Bio freezer (Snijders Scientific, Holland) until they were ready for analysis.

3.2.8. Biochemical Analysis

3.2.8.1. Enzyme-linked immunosorbent assay (ELISA)

Neurochemical changes and pro-inflammatory cytokines were determined by ELISA. Blood samples were collected via cardiac puncture, and the collected blood was centrifuged to obtain the plasma. The plasma was centrifuged for 15 minutes at 1000 x g at 8 °C to obtain a supernatant. The hippocampi were weighed and then homogenised in phosphate buffer saline (PBS) (0.01M, pH 7.4) in a ratio of 1:9 (tissue weight(g): PBS (ml) volume) using an ultrasonic homogeniser sonicator at 4°C. The homogenates were centrifuged for 10 minutes at 5000 x g at 8 °C to obtain a supernatant.

3.2.8.1.1. SOD1 (Superoxide dismutase 1) measurements.

The concentration of SOD1 was measured using ELISA Assay Kits according to the manufacturer's instructions. The plasma was centrifuged to collect supernatant, and the hippocampi collected were homogenised and centrifuged to collect supernatant according to instructions on **3.2.8.1.**

3.2.8.1.2. 4-HNE (4-Hydroxynonenal) measurements

The 4-HNE activity was measured using the 4-HNE ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The plasma was centrifuged to collect supernatant, and the hippocampi collected were homogenised and centrifuged to collect supernatant according to instructions on **3.2.8.1.**

3.2.8.1.3. IL-1β measurements

IL-1β was measured using the IL-1β ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The plasma was centrifuged to collect supernatant and the hippocampi collected were homogenised and centrifuged to collect supernatant according to instructions on **3.2.8.1**.

3.2.8.1.4. ACh measurements

ACh was measured using the ACh ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The hippocampi collected were homogenised and centrifuged to collect supernatant according to instructions on **3.2.8.1**.

3.2.8.2. Isolation of mitochondrial DNA

To isolate DNA from the mitochondria, the Quick-DNA Miniprep Kit was used. This kit has been optimised for maximal recovery of ultra-pure DNA without RNA contamination. In this study, we isolated mtDNA using plasma samples. The genomic lysis buffer (400µl) was added to a plasma sample (100µl), vortexed, and allowed to stand for 10 minutes. The mixture was transferred to a Zymo-Spin IICR Column according to the manufacturer's instructions. To further verify that mtDNA was not contaminated, PCR was performed with 10 µl mtDNA using primers for APOE, a nuclear-coded protein. PCR reaction mixtures of 50 µl contained GoTaq PCR Master Mix, Nuclease-Free Water, and 10 µl DNA. The primers used are shown in Table 1. These primers were supplied by Anatech Analytical Technology (Anatech Instruments (Pty) Ltd.). The following PCR conditions were used: 40 cycles at 95 °C for 15 seconds of gene denaturation, and 60 °C for 60 seconds of GAPDH primer annealing.

Table 3.2.8.2.1.: Sequences of primers used to determine mitochondrial DNA (mtDNA) using qPCR.

Primer	Gene Sequence
GAPDH forward	5′-GGCATTGCTCTCAATGACAA-3′
GAPDH reverse	5′-ATGTAGGCCATGAGGTCCAC-3′
APO-E forward	5’-GGCGCTCGCGGATGGCGCTGAG-3’
APO-E reverse	5’-GCACGGCTGTCCAAGGAGCTGCAGGC-3’

3.2.9. Statistical Analysis

All data analyses from ELISA were performed with GraphPad software version 8 and were analysed by one-way ANOVA followed by Tukey multiple comparisons test, and the results were presented as the mean ± SEM. Results with $p<0.05$ were considered statistically significant.

3.3. Results

3.3.1. SOD1 concentration

We measured plasma (a) and hippocampal (b) SOD1 concentration in the control, stressed, LPS, and stressed + LPS groups (n=6/group). The correlation between plasma and hippocampal SOD1 was also analysed (c). There was a decrease in the plasma and hippocampal SOD1 concentration across the groups, respectively ($F=3.57$, $p<0.05$, Fig 3.3.1a and $F=4.78$, $p<0.05$, Fig 3.3.1b).

In the plasma, we observed a decrease in SOD1 concentration in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, $p<0.05$, Fig 3.3.1a).

In the hippocampus, we observed a decrease in combined model on SOD1 concentration in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, $p<0.05$, Fig 3.3.1b). Also, hippocampal SOD1 showed an increase in the stressed + LPS group when compared to the LPS group, α (Stressed + LPS vs LPS group, $p<0.05$, Fig 3.3.1b).

There was no correlation between the plasma and hippocampal SOD1 (r=0.8946, Fig 3.3.1c).

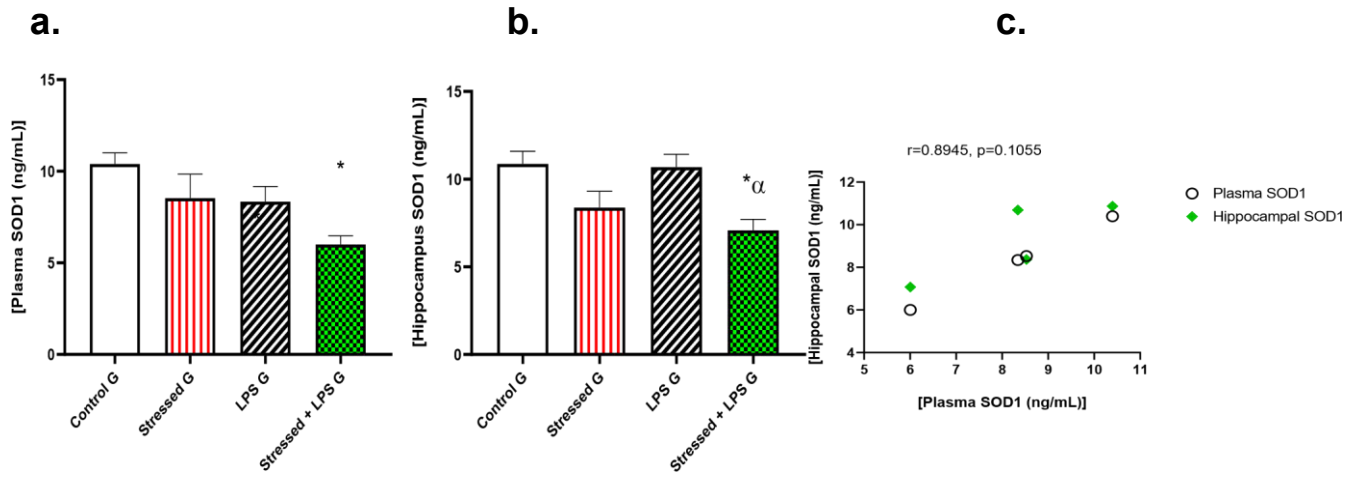

Figure 3.3.1: Plasma (a) and hippocampal (b) SOD1 concentration in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) as well as the correlation (c) between plasma and hippocampal SOD1. The results were presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group and α p<0.05 denotes a comparison to the LPS group.

3.3.2. 4-HNE concentration

Plasma (a) and hippocampal (b) 4-HNE concentration of the control, stressed, LPS, and stressed + LPS groups (n=6, per group) were measured during the experiment. The correlation between plasma and hippocampal 4-HNE (c) was also analyzed. There was no change in plasma 4-HNE concentration (F=0.28, Fig 3.3.2a). However, there was an increase in the concentration of 4-HNE (F=4.37, p<0.05, Fig 3.3.2b).

There was no effect of stress and/or LPS in the plasma 4-HNE concentration in the groups (Fig. 3.3.2a).

There was a significant increase on hippocampal 4-HNE concentration in the LPS group when compared to the control group, *(LPS vs control group, p<0,05, Fig 3.3.2b). Additionally, there was an increase on hippocampal 4-HNE concentration in the stressed + LPS group, *(Stressed + LPS vs control group, p<0,05, Fig 3.3.2b).

There was no correlation between plasma and hippocampal 4-HNE (r=0.1871, Fig 3.3.2c).

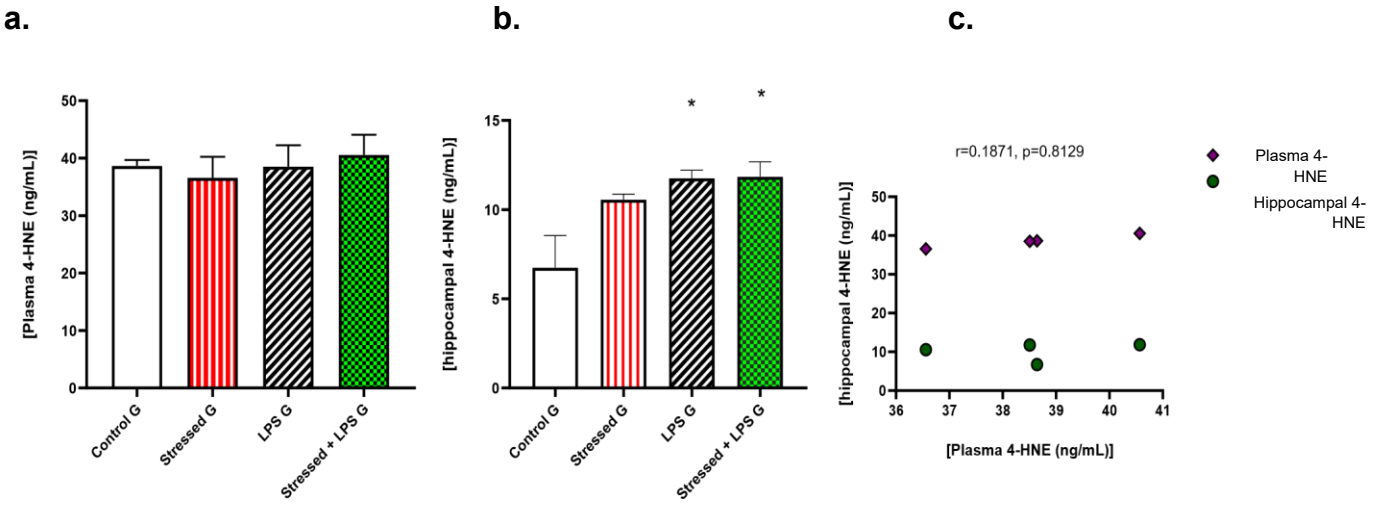

Figure 3.3.2: Plasma (a) and hippocampal (b) 4-HNE concentration in the control, stressed, LPS, and LPS + stressed group (n=6, per group) as well as the correlation (c) between plasma and hippocampal 4-HNE. The values are expressed as a mean ± SEM. *p<0.05 denotes a comparison to the control group.

3.3.3. IL-1β concentration

Plasma (a) and hippocampal (b) IL-1β concentration in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) were measured. The correlation (c) between plasma and hippocampal IL-1β was also analysed. There was an increase in the plasma and hippocampal concentration of IL-1β. In the plasma ($F=3.11$, $p<0.05$, Fig 3.3.3a) and in the hippocampus ($F=4.39$, $p<0.05$, Fig 3.3.3b).

In the plasma, there was an increase in IL-1β concentration in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, $p<0.05$, Fig 3.3.3a).

In the hippocampus, an increase in IL-1β concentration in the LPS-treated group when compared to the control group was observed, *(LPS vs control group, $p<0.05$, Fig 3.3.3b) and there was a stress + LPS effect in the stressed + LPS-treated group when compared to the control group, *(Stressed + LPS vs control group, $p<0.05$, Fig 3.3.3b).

There was no correlation between plasma 1L-1β and hippocampal 1L-1β ($r=0.8179$, Fig 3.3.3c).

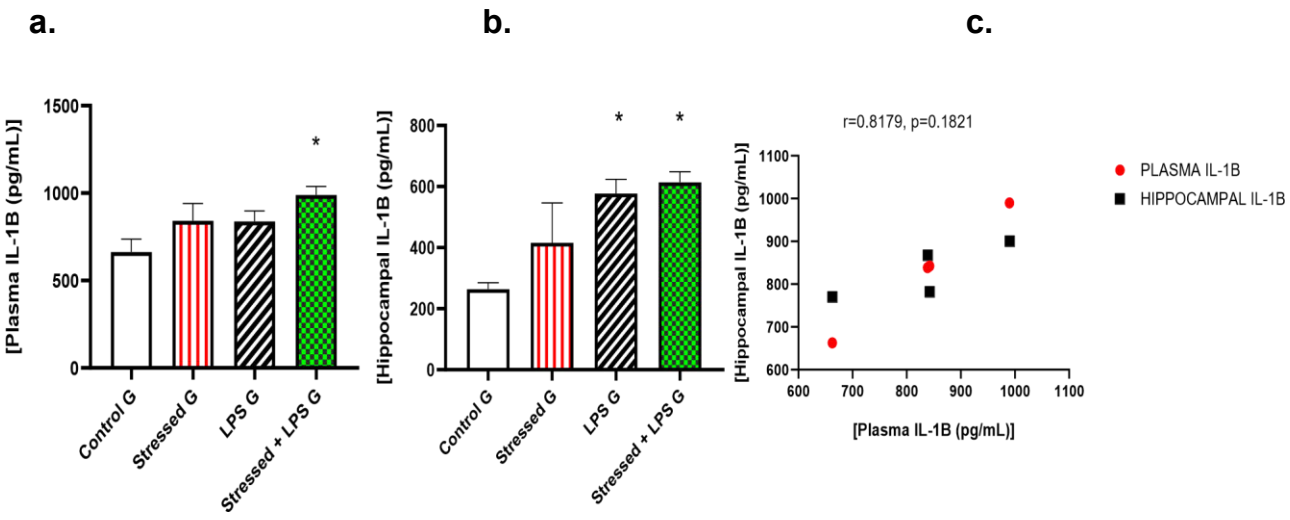

Figure 3.3.3: Inflammatory response results. Plasma (a) and hippocampal (b) IL-1β concentration in the control, stressed, LPS, and LPS + stressed group (n=6). Correlation between plasma and hippocampal IL-1β (c). The values are expressed as a mean ± SEM. *p<0.05 denotes a comparison to the control group.

3.3.4. APO-E expression

APO-E expression in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) was measured. There was a significant difference in the APO-E expression (F=37.79, p<0.05, Fig 3.3.4a).

There was an increase in APO-E expression in the stressed group when compared to the control group, *(Stressed vs control group, p<0.05, Fig 3.3.4a). There was an LPS effect, and in the LPS group when compared to the control group, *(LPS vs control group, p<0.05, Fig 3.3.4a). There was also an increase in APO-E expression in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, Fig 3.3.4a). Further analyses were conducted to explore the correlation (b) between APO-E expression and plasma IL-1β. There was no correlation between APO-E expression and plasma IL-1β (r=0.6748, Figure 3.3.4b).

a. b.

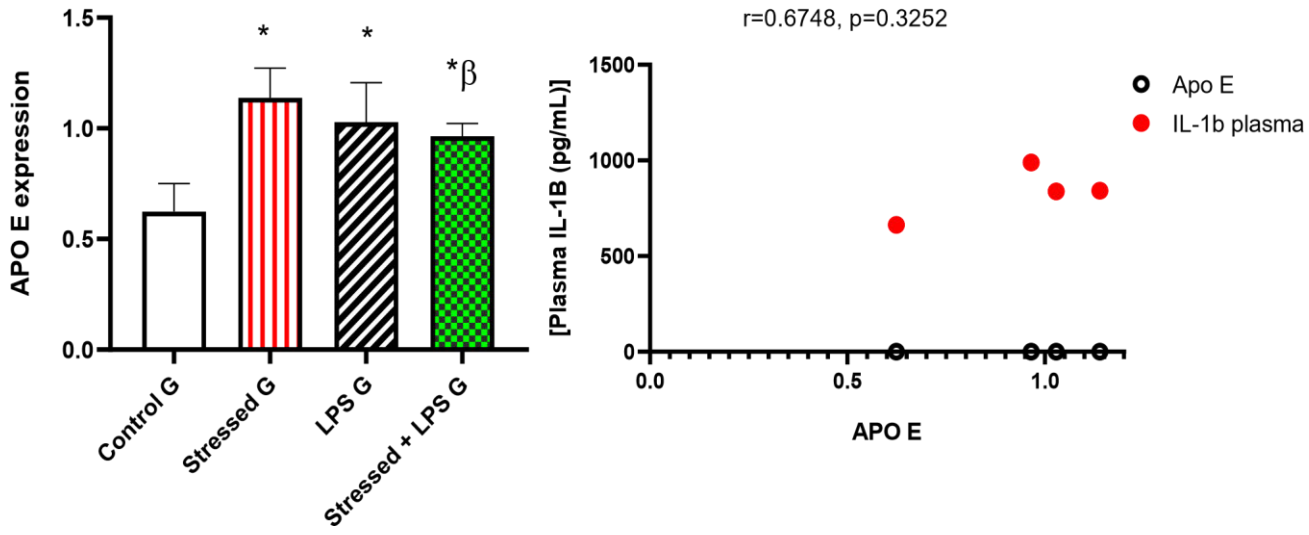

Figure 3.3.4: APO-E expression (a) in the control, stressed, LPS, and stressed + LPS groups (n=6, per group). The results were presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group. Correlation between APO-E expression and plasma IL-1β (b). The values are expressed as a mean ± SEM. *p<0.05 denotes a comparison to the control group, and β p<0.05 denotes a comparison to the stressed group.

3.3.5. ACh concentration

Hippocampal (a) ACh concentration in the control, stressed, LPS, and stressed + LPS groups (n=6, per group). There was a decrease in the concentration of ACh. (F=3.404, p<0.05, Fig 3.3.5a). Fig 3.3.5b was the correlation between ACh and plasma SOD1, Fig 3.3.5c was the correlation between ACh and plasma IL-1β, and Fig 3.3.5d was the correlation between ACh and APO-E.

There was a decrease in the concentration of ACh in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, p<0.05, Fig 3.3.5a) and a decrease in ACh concentration in the stressed + LPS group when compared to a stressed group, β (Stressed + LPS vs stressed group, p<0.05, Fig 3.3.5a) was also observed. There was no correlation in Fig 3.3.5b (r=0.9001), Fig 3.3.5c (r= -0.8282), and Fig 3.3.5d (r= -0.1458).

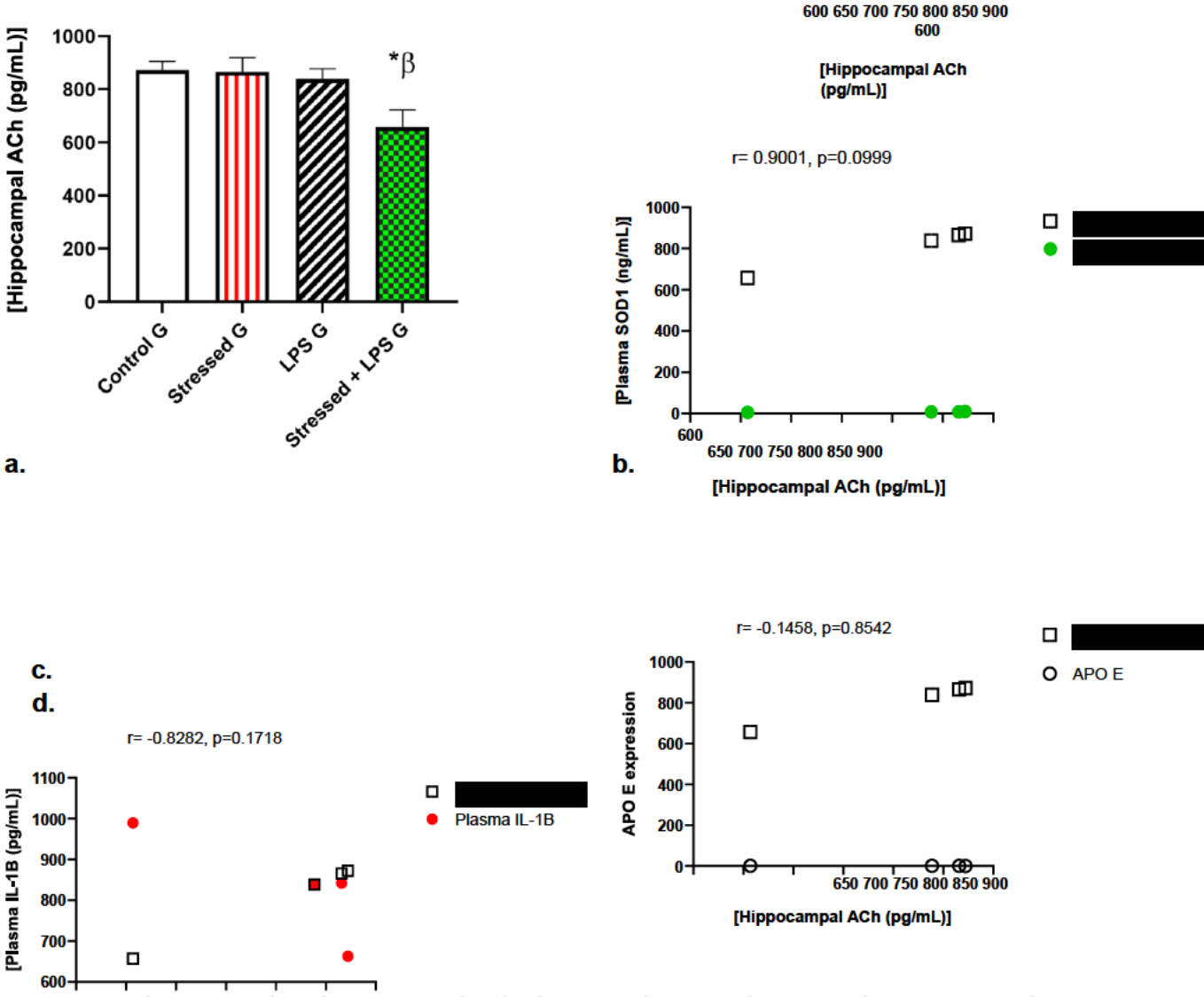

Figure 3.3.5: Hippocampal ACh concentration in the control, stressed, LPS, and LPS+ stressed groups (a), the correlation between ACh and plasma SOD1 (b), the correlation between ACh and plasma 1L-1β (c), the correlation between ACh and APO-E expression (d). The data are presented as a mean ± SEM (n=6, per group). *p<0.05 denotes a comparison to the control group and β p<0.05 denotes a comparison to the stressed group.

3.4. Discussion

In the current study, the effects of chronic unpredictable mild stress and LPS in oxidative stress, inflammatory response, mitochondrial function, and acetylcholine were evaluated. We demonstrated that chronic unpredictable mild stress in combination with LPS administration aggravates oxidative stress and inflammation, as evidenced by reduced SOD1 and elevated 4HNE, elevated IL-1β, increased APO-E expression, and reduced ACh concentration. These

results imply that stress in combination with LPS exacerbates systemic and brain oxidative stress and inflammation; the parameters measured in this study should be taken into consideration when treating neurodegenerative diseases. LPS also elicited a marked inflammatory response in rat brains, as evidenced by increased IL-1β. These findings are in line with several studies, which report that the production of IL-1β in the brain in general and in the hippocampus in particular is induced by LPS (36-38). These findings replicate the previous studies that demonstrated that activation of the immune system by LPS induces neuroinflammation (39, 40).

Chronic stress exposure is often linked to neurostructural changes and cognitive and affective problems. Long-term stress can lead to neurological disorders such as Alzheimer's disease (41). LPS is the major component of the outer membrane of Gram-negative bacteria (42). LPS may lead to memory and learning impairment as it causes oxidative stress in the brain and the overproduction of inflammatory cytokines (39). Pro-inflammatory cytokines can increase oxidative stress by stimulating the production of reactive species (ROS) (43).

Oxidative stress is a redox condition brought on by an imbalance between the production and detoxification of reactive oxygen species (ROS) (44). In this study, we performed a correlation between the plasma and the hippocampus. The correlation between plasma and hippocampus was conducted to investigate the interaction between the peripheral-brain relationship. Since the hippocampus is a key brain region involved in stress regulation, inflammation, and neuroplasticity, and plasma biomarkers often reflect systemic physiological changes, finding correlations between these compartments can provide insight into whether peripheral markers may serve as indirect indicators of brain alterations. Our findings showed no decrease in the plasma and hippocampal SOD1 concentration in the stressed animals; however, a tendency towards a decrease was observed. These results suggest a potential decrease in the plasma and hippocampal SOD1, highlighting that if the duration of the chronic mild stress was lengthened, we may have observed a significant change in systemic and brain antioxidant enzymes, as demonstrated in a previous study that showed a decrease in SOD1 concentration after 40-day chronic mild stress (45). In the LPS group, we observed no changes in the plasma and hippocampal SOD1 concentration; these results contradict the previous studies that looked at the effect of 1 mg/kg of LPS administration and observed a decrease in SOD1 concentration in the brain after LPS administration (36, 46). Our findings indicate that a single dose of LPS was not sufficient to induce systemic and brain oxidative stress. This might be due to the recovery of animals from LPS impact since the period before decapitation was longer. Animals were

induced with a single dose of LPS 3 days before decapitation. A single dose of LPS was measured after three days of LPS administration, whereas in the other study, LPS was administered for 5 consecutive days.

A decrease in SOD1 concentration when rats were exposed to chronic mild stress (45) and were administered with LPS (47) individually, positively influenced the combined stress + LPS effect. A decrease in plasma SOD1 concentration in stressed + LPS was also observed in the hippocampal SOD1 concentration; however, there was no correlation in the plasma and hippocampal SOD1, suggesting that systemic antioxidant defense mechanisms may operate independently of those in the brain. This finding implies that chronic mild stress, when combined with LPS, may exert separate and distinct effects on systemic and central oxidative stress responses. The lack of cross-compartmental influence may result in a disrupted balance between oxidative stress and antioxidant defense mechanisms in both the periphery and the brain. Such an imbalance could contribute to tissue damage and the progression of pathological processes associated with chronic stress and immune activation. Systemic and central antioxidants normally neutralise ROS and maintain redox balance. Peripheral antioxidants protect body organs, while central antioxidants safeguard the brain against oxidative damage that can lead to neurodegeneration, cognitive decline, and psychiatric disorders. However, the observations in this study deviate from this normal physiology, as increased oxidative stress was detected in both peripheral and central systems. A decrease in hippocampal SOD1 was also observed in the stressed + LPS group when compared to the LPS-treated. This might be due to other factors such as increased oxidative stress, inflammatory cytokine production, altered gene expression, mitochondrial dysfunction, and apoptotic cell death because oxidative stress, inflammation, and cell death all contribute to a multifactorial suppression of SOD1, which lowers the levels of this vital antioxidant enzyme.

Lipid peroxidation produces malondialdehyde (MDA) and 4-Hydroxy-2-nonenal (4-HNE), which are types of reactive aldehydes (48). 4-HNE has been implicated in the pathophysiology of neurological disorders. Previous research has consistently shown that plasma 4-HNE is a lipid peroxidation biomarker (49-51). When 4-HNE is overproduced in cells, it can form adducts with proteins, which can cause inflammation, apoptosis, and an antioxidant response (52, 53). Elevated 4-HNE levels indicate increased oxidative stress and lipid peroxidation. LPS induces oxidative stress according to a previous study (54), and chronic mild stress has been demonstrated to exacerbate oxidative stress by promoting the production of ROS (11). In this study, we observed no change in the plasma and hippocampal 4-HNE concentration of the

stressed group, implying that chronic unpredictable mild stress did not impact lipid peroxidation as in other studies that observed an increase in 4-HNE concentration in stressed rats (48, 55). Therefore, our results are not in agreement with the previously demonstrated result that lipid peroxidation is elevated after chronic mild stress induction in rats (45).

We found no change in the plasma 4-HNE concentration in the LPS group. Our results are not in agreement with the previous studies that observed an increase in plasma 4-HNE concentration after LPS administration, suggesting that plasma 4-HNE is not an indicator of lipid peroxidation (49, 56). However, an increase in hippocampal 4-HNE concentration has been demonstrated in the LPS-treated group; our result aligns with the studies that reported an elevated 4-HNE concentration (57, 58). Therefore, our findings are consistent with previous results suggesting that oxidative stress is involved in the pathogenesis of neurodegenerative diseases. In the stressed + LPS group, we demonstrated no effect of combined stress and LPS in the plasma 4-HNE. This is because the effects of oxidative stress may be more prominent in tissues (like the brain, liver, or heart) rather than in the plasma. An increase in the hippocampal 4-HNE concentration of the stressed + LPS group was observed which is in agreement with previous research (58). These findings suggest that LPS administration induced brain oxidative stress by increasing lipid peroxidation without influencing systemic oxidative stress, as also evidenced by no correlation found between plasma and hippocampal 4-HNE.

The findings of our study support the previously stated theory that an increase in oxidative processes, in the case of lipid peroxidation, ultimately causes the level of enzymatic antioxidant defense to decline. This study shows that rats, when exposed to stress + treated with LPS, exhibited oxidative stress by reducing antioxidant defense enzymes and increasing lipid peroxidation markers, as evidenced by decreased antioxidant defense enzyme in the plasma and hippocampal SOD1 concentration and increased hippocampal 4-HNE concentration. However, exposing rats only to chronic unpredictable mild stress for 10 days did not affect oxidative stress markers, and these results were not able to achieve what a 40-day study achieved with a 10-day study, which means that a shorter duration of stress may not lead to oxidative stress (45).

APO-E is essential for the transfer of lipids required for energy production in neurons, which are heavily reliant on mitochondrial function for ATP synthesis (59). In our study, we found an increased APO-E concentration in the stressed group; however, no previous study has been done to investigate the effect of chronic mild stress on APO-E expression. Our results suggest

that chronic mild stress induces mitochondrial dysfunction. An elevated APO-E expression was observed in the LPS group, and these results are consistent with reported results that observed an increase in APO-E expression (60). These results suggest that a single dose of LPS induced mitochondrial dysfunction. We found an increased APO-E expression in the stressed + LPS group. For this finding, no study has looked at the effect of stress in combination with LPS. The increase in APO-E expression in the stressed + LPS group suggests the involvement of chronic unpredictable mild stressors and LPS administration in exacerbating mitochondrial dysfunction. It was suggested that the increased oxidative damage was the cause of the increased mtDNA mutations (61). The findings of our current study suggest that chronic mild stressors and LPS administration impaired mitochondrial function by increasing the APO-E gene.

In this study, we found no changes in the plasma and hippocampal IL-1β concentration in the stressed group, implying that exposing rats to chronic mild stressors for 10 days was not enough to induce neuroinflammation. These results contradict the results of the previous research that demonstrated that chronic mild stress leads to increased pro-inflammatory cytokines (37). We observed an increase in the hippocampal IL-1β in the LPS group and these results are consistent with a previous study that looked at the effect of LPS (1 mg/kg, i.p) in the brain and demonstrated an increase in the concentration of IL-1β when compared to a control group suggesting that a single dose of LPS administration induced inflammatory response (36), however, there were no changes in the plasma IL-1β concentration. This suggests that the hippocampus is more susceptible to LPS challenge than the plasma. Our results are in line with the previously proposed theory that LPS induces neuroinflammation (36)

Both CUMS and LPS work as cytokine inducers to affect immune cell formation, distribution, and activation, as well as to elicit acute and chronic immunological activation (37). In a stressed + LPS group, an increase in the plasma and hippocampal IL-1β concentration was observed, suggesting that systemic inflammatory response is associated with brain inflammatory response. However, the systemic inflammatory response may not influence the brain inflammatory response since there was no correlation between the plasma and hippocampal IL-1β concentration. Furthermore, this result also suggests that stress, when combined with LPS, increases the production of pro-inflammatory cytokines (37).

The cholinergic system is essential for memory function (14). ACh plays a crucial role in cognitive processes, particularly in the hippocampus, which is important for memory formation

(63). In our study, chronic mild stress showed no effects on the ACh concentration. These results suggest that the absence of an effect might be due to the duration of the experimental schedule (10-day chronic mild stress). These results are not in agreement with the reported results that chronic mild stress reduces and impairs ACh release in the hippocampus (64). LPS has been shown to impair learning and memory (39, 62), and when an LPS (40 mg/kg, i.p) was used (65). However, we observed no effect of LPS on ACh concentration in the LPS-treated group in our study. Our findings are not in agreement with a previous study (65).

A significant reduction in hippocampal ACh concentration was observed in the stressed + LPS group. Given that decreased hippocampal ACh levels are closely associated with impairments in learning and memory, and reduced ACh release has been shown to negatively affect cognitive function (64) (65), this finding may suggest potential cognitive deficits. However, as no behavioural tests were conducted in the present study, it is not possible to directly conclude that reduced hippocampal ACh concentration following the combination of chronic stress and LPS exposure led to memory impairment. Chronic stress exacerbated the inflammatory response that is caused by LPS and resulted in a more noticeable decrease in ACh concentration (66). However, we found no correlation between ACh, inflammation, and oxidative stress.

Our current study demonstrates that chronic mild stress in combination with LPS induces systemic and brain oxidative stress in rats by impairing antioxidant enzymes and promoting the production of ROS by increasing lipid peroxidation. We also demonstrated that LPS induces immune activation in the hippocampus of the rats. Furthermore, the findings also indicate that the peripheral and central immune activation was exacerbated because of systemic and brain inflammation brought on by the combined effect of chronic mild stress and LPS. Stress, when combined with LPS, may induce memory impairment. Our findings shed light on how chronic mild stress, together with LPS administration, could induce oxidative stress, mitochondrial dysfunction, cholinergic dysfunction, and immune activation and contribute to the pathophysiology of neurodegenerative diseases such as AD and may open the door to exploring novel therapy strategies.

3.5. Conclusion

In conclusion, we demonstrated that chronic unpredictable mild stress in combination with LPS may induce systemic and brain oxidative stress, and neuroinflammation as evidenced by decreased SOD1 and increased 4-HNE, increased IL-1β, increased APO-E expression, and decreased ACh concentration. To the best of our knowledge, our data presents the impact of

the chronic mild stress paradigm in combination with LPS on oxidative stress, neuroinflammation, mitochondrial function, and cholinergic dysfunction, measured for the first time. The findings of the study may contribute to a greater comprehension of the intricate relationship that exists between stress and LPS; they can also aid in developing strategies to mitigate the impact of chronic mild stress in combination with LPS on oxidative stress, mitochondrial dysfunction and neuroinflammation.

Declarations:

Funding

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Declaration of Interest

The authors declare no competing interests.

Ethical Approval

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/00004514/2022) of the University of KwaZulu-Natal.

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Chapter 4: Manuscript 2

Prologue

In the first study, it has been demonstrated that the combined effects of CUMS and immune activation by LPS increased oxidative stress, neuroinflammation, systemic inflammation, mitochondrial dysfunction and cholinergic dysfunction. Beyond the brain, these disruptions may also contribute to behavioural changes and metabolic alterations. Examining metabolic alterations under these circumstances may offer important insights into the relationship between stress and LPS, as peripheral and central inflammation, and cholinergic dysfunction are intimately related to body weight management. These impacts might also lead to metabolic disruptions, all of which can impair behaviour and brain function. Therefore, studying metabolic changes, anxiety-like behaviours and neurochemical alterations following CUMS and LPS may provide a more comprehensive understanding of how stress and immune activation through LPS can interact to influence both metabolic processes and brain function.

"Unpredictable Stress on Metabolic, Anxiety-like Behaviour and Neurochemical Changes in LPS-challenged Rats"

This manuscript is prepared according to the journal's guidelines to authors, it will be submitted for publication in the journal **Brain, Behaviour, and Immunity-Health.**

Unpredictable Stress on Metabolic, Anxiety-like Behaviour and Neurochemical Changes in LPS-challenged Rats

Khethelo Sibiya [1, a], Musa Mabandla[1], and Mluleki Luvuno[1]

[1]Discipline of Human Physiology, School of Laboratory Medicine & Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, South Africa, Private Bag X54001, Durban, South Africa

[a] Correspondence to: Email: 219013485@stu.ukzn.ac.za

Abstract

Chronic stress and LPS exposure induce sickness behaviours that disrupt body weight regulation and feeding, although the underlying mechanisms remain controversial. The combined impacts of stress and immune activation are not fully understood; therefore, this study examined how CUMS influences metabolism, anxiety-like behaviour, and biochemical markers in LPS-challenged rats. Twenty-four male Sprague-Dawley rats were assigned to four groups (n=6): control, stressed, LPS, and stressed + LPS. Body weight, food, and water intake were monitored in metabolic cages; anxiety-like behaviour was assessed with the Open Field Test, and blood and hippocampal tissues were collected to analyse plasma IL-1β, corticosterone, and hippocampal acetylcholine. Data were expressed as mean ± SEM and analysed by one-way ANOVA, where $p<0.05$ was considered significant. The stressed + LPS group showed a significant reduction in final body weight compared to controls, suggesting a synergistic effect on weight regulation, while final food and final water intake remained unchanged. This suggests that stress + LPS impacts body weight without modifying feeding. In the OFT, the stressed + LPS group spent less time near the walls compared to the stressed group, indicating reduced anxiety-like behaviour. Biochemically, the stressed + LPS group showed elevated plasma IL-1β and reduced ACh when compared to the control group, suggesting inflammation and cholinergic dysfunction. Overall, these findings imply that combining CUMS with LPS may promote weight loss and sickness behaviour through inflammatory mechanisms without promoting anxiety-like behaviours.

Keywords: Lipopolysaccharide, chronic unpredictable mild stress, body weight, interleukin1β, acetylcholine.

4.1.Introduction

Chronic unpredictable mild stress (CUMS) refers to a set of minor psychosocial and physiological stressors that occur regularly over several weeks and is recognised as a reliable

model for stress response (1). Numerous studies have documented metabolic changes in the brain and plasma linked to the use of the CUMS model (2, 3). Animals exposed to stressful stimuli exhibit a wide range of metabolic and behavioural alterations, such as modifications in body weight gain, feeding, and exploratory and anxiety-like behaviours (4). The brain is a vulnerable target organ for stress; if the stress intensity exceeds acceptable levels, it will lead to the onset of mental and physical conditions (5). Stressful conditions activate the hypothalamic-pituitary-adrenal system, which further leads to the release of corticosteroid hormones (6). Exposure to a wide range of stressors greatly increases the likelihood of negative behavioural effects, such as anxiety and depression (7). A variety of studies have demonstrated that persistent stress increases the chance of developing cognitive impairments (8, 9).

LPS is one of the pathogen-resistant components of Gram-negative bacteria's cell walls (10). LPS triggers the innate immune response via toll-like receptor (TLR)-4 on host immune cells, including macrophages, and signals through nuclear factor kappa beta to induce proinflammatory gene expression (11). When LPS activates TLR4, it results in the production of several immune mediators, such as cytokines, and a sickness response that includes decreased feeding and weight loss (12). LPS administration also leads to elevated corticosterone, which may result in metabolic disturbances and immune response (13). This then causes the brain to produce more cytokines, including IL-1β, which causes symptoms of illness, like anorexia and decreased activity, as well as anxiety and depression-like behaviours (14). Blood cytokines send signals to the brain via humoral and neuronal pathways, which can alter behaviour and mood (15, 16).

When rats are exposed to LPS, their innate immune system is activated and pro-inflammatory cytokines such as TNF-α and IL-1β are released, resulting in a robust immunological response, according to other researchers (17, 18). These cytokines play a role in systemic inflammation, which can significantly impact metabolism, and they lead to body weight loss (19). This systemic inflammation can also transcend to the brain and result in neuroinflammation. It has been demonstrated that changes in metabolic hormones can have severe implications for the aetiology of neurodegenerative disorders (20).

Acetylcholine (ACh) is a neurotransmitter that plays a role in cognitive processing, arousal, and attention in the brain (21). ACh has been demonstrated to affect appetite regulation by interacting with neural pathways involved in feeding behaviour (22). ACh plays an important role in regulating immune response, brain activity, and behaviour (23). A variety of behavioural and physiological alterations, such as anxiety, social disengagement, and cognitive impairment,

are influenced by decreased ACh activity, which is a major factor in the presentation of sickness behaviour (23). ACh inhibits the release of pro-inflammatory cytokine IL-1β in LPS-treated animals (24).

However, whether metabolic changes and anxiety-like behaviours are etiologically linked to cognitive dysfunction or are a consequence of a disease process itself remains poorly understood. Therefore, this study investigated the effect of chronic unpredictable mild stress on metabolic and associated changes in LPS-challenged rats. This study aims to investigate body weight changes, food and water intake, anxiety-like behaviours, corticosterone, IL-1β, and ACh in LPS-administered rats following stress procedures.

4.2.Materials And Methods

4.2.1. Chemicals

All chemicals and reagents used in this project were purchased from standard commercial suppliers and were of analytical grade. LPS was purchased from Sigma Chemical Co. (St. Louis, MO., USA). Pierce BCA Protein Assay Kit was purchased from Pierce in the USA, and ELISA assay kits were purchased from Elabscience Biotechnology Inc., USA.

4.2.2. Animals

24 male Sprague-Dawley rats, aged 10 weeks and weighing 200- 250 g, were bred and housed at UKZN Biomedical Research Unit (BRU) in cages with food and water availability *ad libitum. They* were divided into 4 groups, each group containing 6 rats per cage. Animals were acclimatised for 5 days before the experiment. The animal experiments were permitted by the Animal Ethics Committee of the University of KwaZulu-Natal (AREC/00004514/2022). They were maintained in a room on a 12-hour light/dark cycle and at constant temperature (22 ± 2 °C) with relative humidity (55 ± 10%). They were maintained in a room on a 12-hour light/dark cycle and at constant temperature (22 ± 2 °C with relative humidity (55 ± 10).

4.2.3. Sample Size

Animals were divided into four experimental groups, with six rats per group (n = 6/group), resulting in a total of 24 rats. A group size of six has been shown to provide adequate statistical power to detect significant differences in similar experimental designs involving chronic stress and LPS-induced models (47).

To further support the appropriateness of this sample size, the E-value was calculated using the formula:

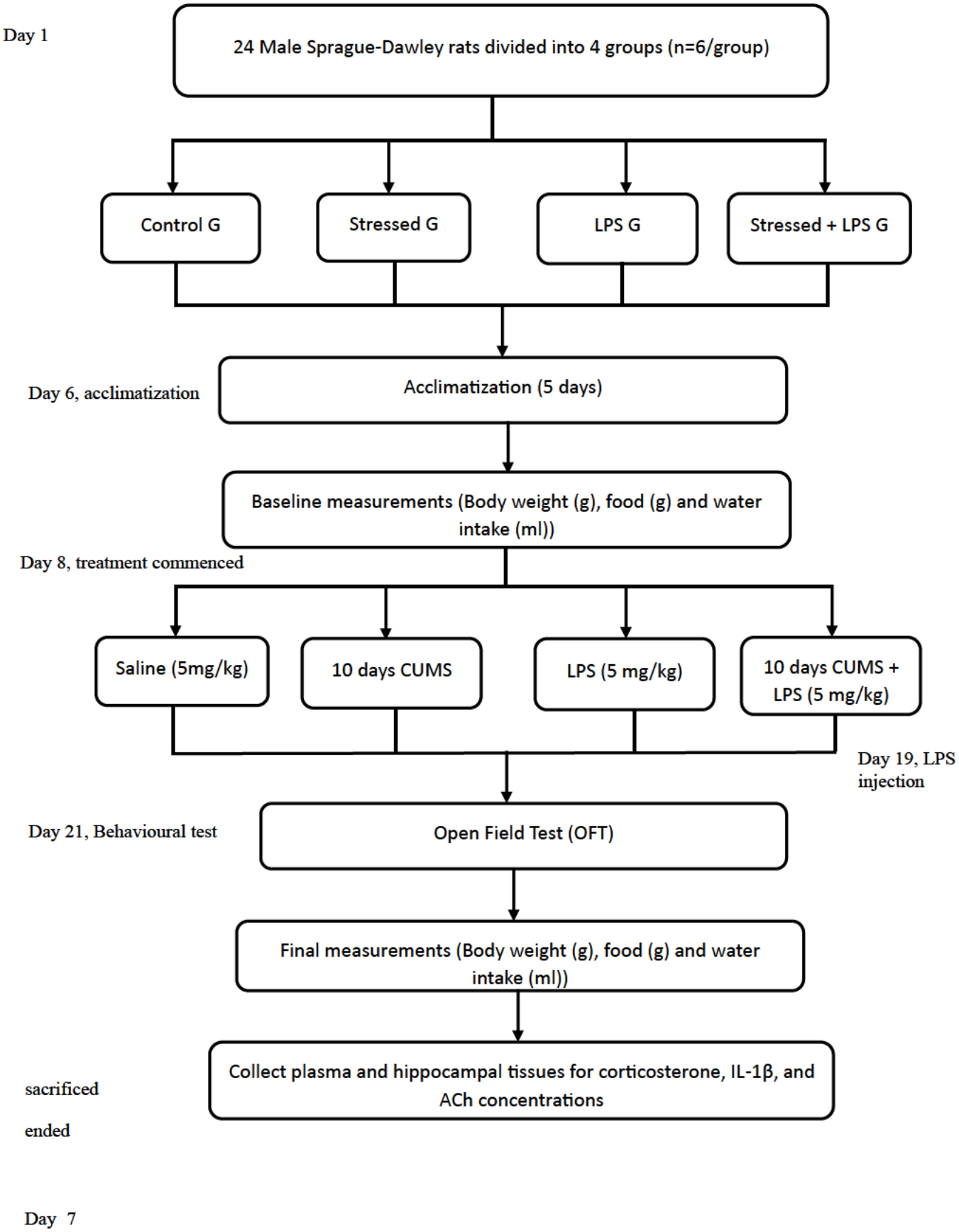
E = Total number of animals – Total number of groups

E = (6 × 4) – 4 = 20

An E-value between 10 and 20 is considered optimal for ensuring a balance between ethical use of animals and statistical validity in biological studies, according to guidelines for resource equation methods in animal research (47). The obtained E-value of 20 is at the upper end of this range, indicating a suitable sample size.

4.2.4. Experimental Design

All 24 male Sprague-Dawley rats were acclimatised for 5 days before any experiments; they were divided into 4 groups, each containing 6 rats. There was a control group that was shamcontrolled with saline solution (5 mg/kg, i.p.). Thereafter, baseline measurements of body weight as well as food and water intake using metabolic cages were taken. The stressed groups underwent chronic unpredictable mild stress (CUMS) protocol for 10 days, the LPS group received a single dose of LPS (5 mg/kg, i.p.), and the stressed + LPS group was exposed to both chronic unpredictable mild stressor protocol and LPS injection (5 mg/kg, i.p.). LPS was administered intraperitoneally after 10 days of CUMS protocol in the appropriate groups. A 3day delay was implemented to allow a sufficient washout period for acute LPS-induced sickness behaviours to subside and to accommodate 24-hour monitoring of metabolic parameters, ensuring that subsequent behavioural and biochemical assessments reflected more stable and sustained effects. This was followed by an Open Field Test (OFT) 24 hours after LPS administration. The OFT was conducted at the BRU behavioural test room for one day. Final measurements of body weight, as well as food and water intake using metabolic cages, were taken a day after an OFT. Animals were decapitated a day after the final metabolic measurements to collect blood and hippocampal tissues. The plasma and hippocampus collected were stored at -80°C in a Bio freezer (Snijders Scientific, Holland) until ready for biochemical analysis of corticosterone, IL-1β, and ACh concentrations.


Day 1
24 Male Sprague-Dawley rats divided into 4 groups (n=6/group)
Control G
Stressed G
LPS G
Stressed + LPS G
Day 6, acclimatization
Acclimatization (5 days)
Baseline measurements (Body weight (g), food (g) and water intake (ml))
Day 8, treatment commenced
Saline (5mg/kg)
10 days CUMS
LPS (5 mg/kg)
10 days CUMS + LPS (5 mg/kg)
Day 19, LPS injection
Day 21, Behavioural test
Open Field Test (OFT)
Final measurements (Body weight (g), food (g) and water intake (ml))
Collect plasma and hippocampal tissues for corticosterone, IL-1β, and ACh concentrations
sacrificed
ended
Day 7

Day 18, CUMS

ended

Day 22, final
measurements

Day 23, animals

Figure 4.2.4.1: A diagrammatic depiction of the experimental design

4.2.5. Stress protocol

Animals were subjected to stress using a chronic mild unpredictable stress (CUMS) protocol. These animals were exposed to 4 stressors for 10 days. A shorter timeline reduces cumulative animal distress and adheres to the 3Rs principle (Replacement, Reduction, and Refinement) in animal research ethics by minimising prolonged exposure to stressors, while still allowing for the collection of high-quality and biologically relevant data. However, CUMS procedures usually last 21–42 days to simulate depressive-like behaviours. New research suggests that shorter times (7–14 days) can still result in notable changes in the body's physiology, neuroendocrine system, and neurochemistry. The stress protocol consisted of daytime darkness for 12 hours, a 45° tilted cage for 5 hours with food and water on the incline (25), and cold exposure for 5 minutes at 4°C (26) which consisted of the rats being kept in a cold room at the BRU with the temperature adjusted to 4°C and restraint for 1.5 hours (27). Rats received two random stressors per day over the 10 days. Inducing stress in rats has effects on metabolism, and stress induces changes in the hippocampal structure and function (28). For restraint stress, rats were placed in air-assessable cylinders (27), restraint alters the autonomic nervous system, increasing body temperature, heart rate, and behaviourally increasing anxiety-like behaviour.

4.2.6. LPS administration

The rats were administered LPS through intraperitoneal injection. Rats received a single dose of LPS (5 mg/kg, i.p.). The single dose of LPS (5 mg/kg, i.p.), which is a safe dosage in experimental rats (29), was administered intraperitoneally to the animals a day after the stress procedure. The control animals were sham-treated with a single 0.9 % saline solution injection of the same dosage and route of administration as LPS.

LPS (Escherichia coli, serotype 0111:B4, Sigma Chemical Co., St. Louis, MO., USA). To make 5 mg/ml of LPS, 5 mg of LPS powder was weighed carefully on a weighing boat using an analytical balance scale (Lasec® Group, South Africa). 5 mL of PBS was added to 5 mg of LPS powder. The mixture was vortexed until the solution was clear using an analogue vortex mixer (Eins-Sci Laboratory Equipment, South Africa). The mixture of LPS and PBS was diluted to the working concentration of 0.2 µg/µL. The aliquots of f20 µL were prepared in small, sterile tubes to avoid repeated freeze-thaw cycles, which can degrade LPS.

LPS dosage used:
The LPS group had an average of 251.5 g. To obtain the volume of LPS, we multiplied the dosage by the average body weights of the LPS group. The dosage of 5 mg/kg was multiplied by an average weight of rats, which was 0.2515 kg when converted to kilograms, resulting in 1.26 mg. The final volume of LPS used in each rat was 2 mL.

4.2.7. Metabolic measurements

Following the acclimatisation period body weight of rats was measured on a digital weighing scale (Thermo Fisher Scientific Inc.) before stress procedures, LPS administration, and behavioural tests. Before measuring the baseline body weight, the container bowl used to measure the weights of rats was disinfected with alcohol, wiped using a paper towel and placed on a measuring scale. Each rat was measured in a container placed on a weighing scale; the same technique was applied to all rats. Baseline food and baseline water intake were monitored using metabolic cages in the experimental room: rats were moved from their home room for 24 hours before stress procedures, LPS administration, and behavioural tests. 40 g of food was weighed carefully on a digital weighing scale, and the food was placed in a food container provided in each metabolic cage with a rat; each rat was given 100 ml of water, which was poured into a water bottle of a metabolic cage. Food and water given to rats were measured carefully after 24 hours. Final body weights were measured using the same procedure, final

food and final water intake were also monitored for 24 hours in metabolic cages after being subjected to chronic stress, LPS administration and behavioural tests immediately after the behavioural test. Final body weight, final food and final water intake were measured a day after OFT on the last day of the experimental protocol.

4.2.8. Open Field Test (OFT)

The Open-field arena (90 cm length x 60 cm width x 35 cm height) was utilised. The animals were moved from their housing room to the testing room and acclimatised for at least 30 minutes before testing. The animals were placed in the central sector and their activity was recorded for 10 minutes by a video camera and taped for further analysis. The open field arena was thoroughly cleaned between each test. The time spent close to the walls and in the open arena was recorded and uploaded to the BORIS software (version 8.21.5).

The exploratory locomotor activity, which is quantified as the number of squares each rodent crossed in a testing chamber, was evaluated. Given that the test measures the rodent's ability and willingness to move about freely and participate in exploratory behaviour, exploratory locomotor activity is the ideal behavioural marker of acute illness behaviour (30). Exploratory locomotor activity is used to assess anxiety-like behaviour as well as depressive-like behaviour. This behaviour was scored by third-party observers blinded to the study protocol.

4.2.9. Tissue harvesting

The blood of the Sprague-Dawley rats was collected into an EDTA blood collection tube by cardiac puncture; rats were then decapitated using a sharp guillotine. Following decapitation, the external surface of the heads was disinfected with alcohol for brain section preparation. The skulls were immediately opened using straight scissors, surgical blades, a scalpel, and forceps after decapitation to remove the brain for hippocampus extraction. The hippocampi were carefully separated from the surrounding regions, including the cortex, corpus callosum, and thalamus, using the tools mentioned above. The hippocampi were carefully isolated using the probe. The hippocampi are C-shaped structures located between the cortex externally and the thalamus internally. The hippocampi were collected into Eppendorf tubes (Lasec® Group, South Africa), which were kept in ice to preserve tissues from degradation. The blood collected was immediately centrifuged to obtain the plasma. The tissues collected were stored at -80 °C in a Bio freezer (Snijders Scientific, Holland) until they were ready for analysis.

4.2.10. Biochemical Analysis

4.2.10.1. IL-1β, Corticosterone, and ACh measurements

IL-1β was measured using the IL-1β ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The plasma was centrifuged for 15 minutes at 1000 x g at 8 °C to obtain a supernatant. CORT was measured using the CORT ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The plasma was centrifuged for 15 minutes at 1000 x g at 8 °C to obtain a supernatant. ACh is a key neurotransmitter involved in several central and peripheral nervous system processes. ACh is essential for controlling cognitive functions in the CNS, especially memory, attention, and learning. ACh is commonly evaluated to detect cognitive deficits, evaluate the integrity of the cholinergic system, and to the neurochemical effects of stress, neuroinflammation, and exposure to toxins. ACh was measured using the ACh ELISA Kit (Elabscience Biotechnology Inc.) according to the manufacturer's instructions. The hippocampi were weighed and then homogenised in PBS (0.01M, pH 7.4) in a ratio of 1:9 (tissue weight(g): PBS (ml) volume) using an ultrasonic homogeniser Sonicator at 4°C. The homogenates were centrifuged for 10 minutes at 5000 x g at 8 °C.

4.2.11. Statistical Analysis

All data analyses from ELISA were performed with GraphPad software version 8 and were analysed by one-way ANOVA followed by Tukey multiple comparisons test, and the results were presented as the mean ± SEM. Results with $p<0.05$ were considered statistically significant.

4.3.Results

4.3.1. Body weight measurements

We measured baseline (a) and final (b) body weights in the control, stressed, LPS, and stressed + LPS groups (n=6, per group). There were no changes in the baseline body weights in all groups ($F=1.144$, $p>0.05$, Fig 4.3.1a). There was a significant decrease in final body weight ($F=5.070$, $p<0.05$, Fig 4.3.1b). A decrease in the final body weight in the stressed + LPS group was observed, *(Stressed + LPS group vs control group, $p<0.05$, Fig 4.3.1b).

a. **b.**

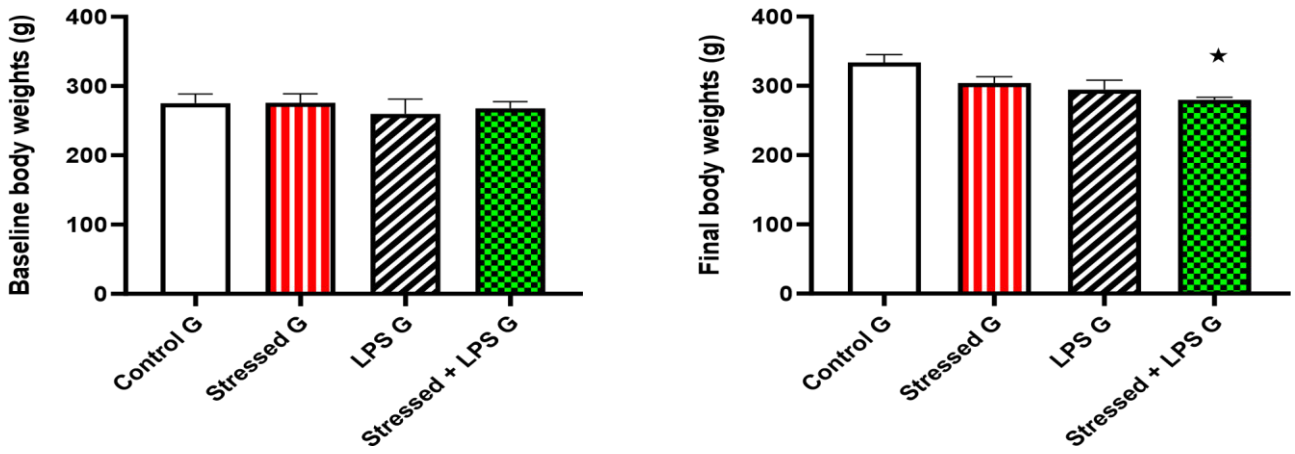


Figure 4.3.1: Baseline (a) and final (b) body weight changes in the control, stressed, LPS, and stressed + LPS group (n=6, per group). The results were presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group.

4.3.2. Food intake measurements

Baseline (a) and final (b) food intake in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) were monitored. There were no changes in the baseline food intake measurements (F= 1,586, p>0.05, Fig 4.3.2a). There was a significant decrease in final food intake measurements (F= 5.32, p<0.05, Fig 4.3.2b). There was a stress effect in the stressed group, *(Stressed vs control group, p<0.05, Fig 4.3.2b). There was an LPS effect in the LPS group, *(LPS vs control group, p<0.05, Fig 4.3.2b).

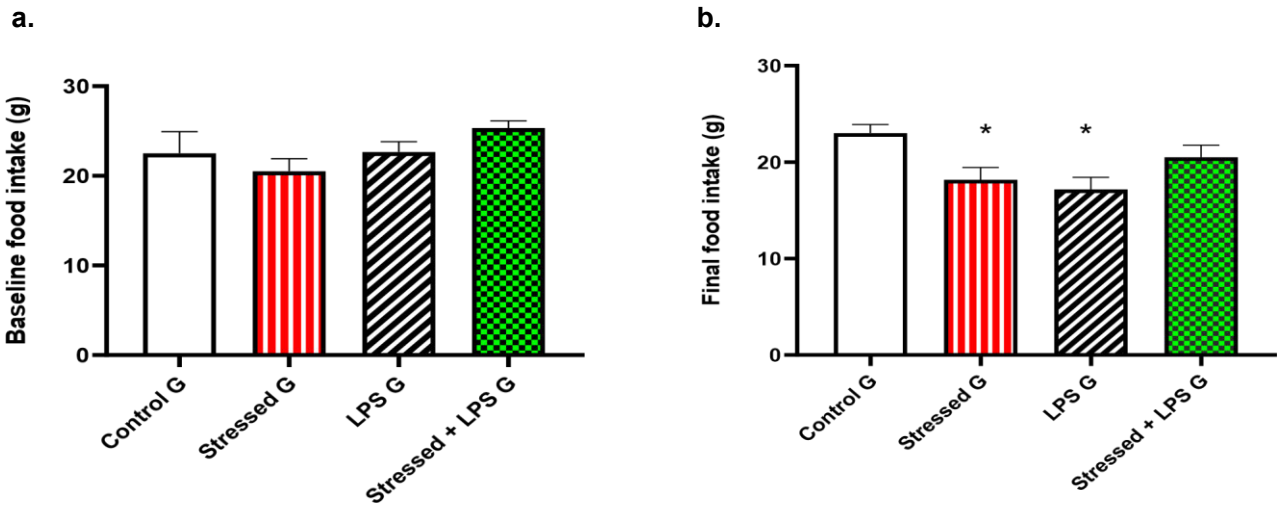


Figure 4.3.2: Baseline (a) and final (b) food intake measurements in the control, stressed, LPS, LPS + stressed group (n=6, per group). The results were presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group.

4.3.3. Water intake measurements

Baseline (a) and final (b) water intake in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) were monitored. There were no changes in baseline water intake measurements (F= 1.70, p>0.05, Fig 4.3.3a). There was a decrease in final water intake measurements (F=3.48, p<0.05, Fig 4.3.3b). There was an LPS effect in the LPS group, *(LPS vs control group, p<0.05, Fig 4.3.3b).

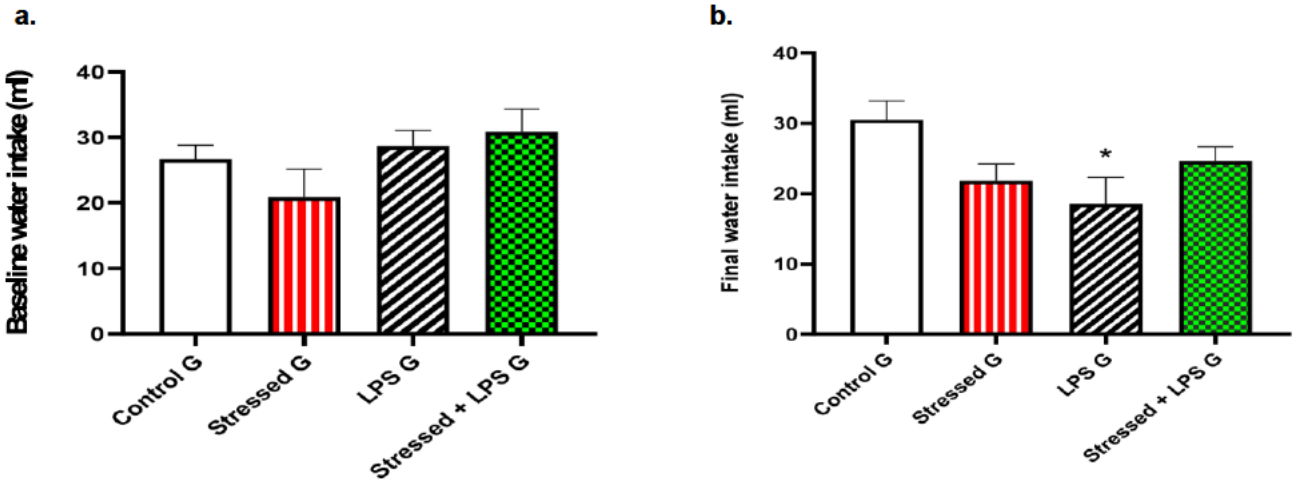


Figure 4.3.3: Baseline (a) and final (b) water intake measurements in the control, stressed, LPS, and stressed + LPS group (n=6, per group). The results were presented as a mean ± SEM. *p<0.05 denotes comparison to a control.

4.3.4. Open Field Test

Time spent close to the wall and time spent in the open arena of the OFT in the control, stressed, LPS, and stressed + LPS groups were evaluated. There was a decrease in time spent close to the wall (F=10.37, p<0.05, Table 4.3.4). There was an increase in the time spent in the open arena (F=3.518, p<0.05, Table 4.3.4).

Table 4.3.4.1: Time spent close to the wall and in the open arena of the OFT in the control, stressed, LPS, and stressed + LPS groups.

Animal groups (n=6)	Time spent Close to the Wall (s)	Time spent in an Open Arena (s)
Control	586.7 ± 3.33	2.33 ± 0.49
Stressed	381.7 ± 54.80*	22.5 ± 7.50*
LPS	565 ± 19.28$^{\beta}$	15 ± 6.46
Stressed + LPS	583.3 ± 12.82$^{\beta}$	17 ± 5.07

Values are presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group and β p<0.05 denotes a comparison to the stressed group.

4.3.5. Corticosterone concentration

Plasma corticosterone concentration in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) were measured. There was a significance difference in plasma corticosterone concentration. (F=3.937, p<0.05, Fig 4.3.5). There was a decrease in corticosterone concentration in the stressed group when compared to the control group, *(Stressed vs control group, p<0.05, Fig 4.3.5).

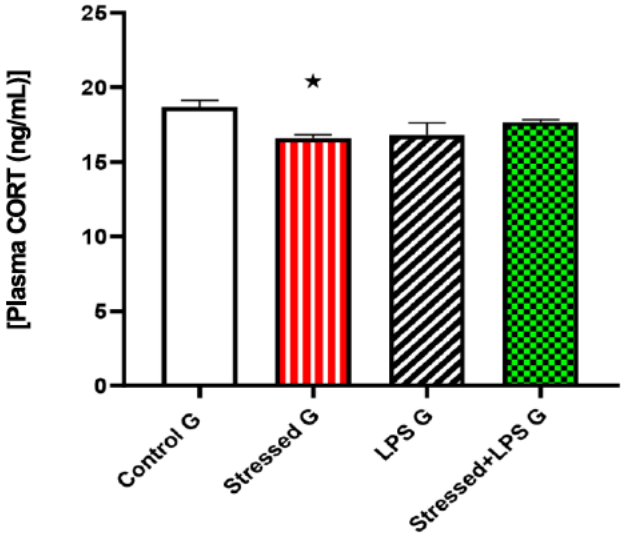


Figure 4.3.4: Plasma corticosterone concentration in the control, stressed, LPS, and LPS+ stressed groups. The data are presented as a mean ± SEM (n=6, per group). *p<0.05 denotes a comparison to the control group.

4.3.6. IL-1β concentration

Plasma IL-1β concentration was measured in the control, stressed, LPS, and stressed + LPS groups (n=6, per group). We observed a significant difference in the plasma concentration of IL-1β. ($F=3.11$, $p<0.05$, Table 4.3.6). There was a stress + LPS effect on IL-1β concentration in the stressed + LPS group, *(Stressed + LPS vs control group, $p<0.05$, Table 4.3.6).

Table 4.3.6.1.: Plasma IL-1β concentration in the control, stressed, LPS, and LPS + stressed group (n=6).

Animal groups (n=6, per group)	[Plasma IL-1β (pg/mL)]
Control	662.7 ± 74.7
Stressed	842.4 ± 97.68
LPS	838.4 ± 59.95
Stressed + LPS	989.8 ± 48.32*

Values are presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group.

4.3.7. ACh concentration

Hippocampal ACh concentration in the control, stressed, LPS, and stressed + LPS groups (n=6, per group) was measured. There was a significant in the concentration of ACh ($F(3.15) =3.404$, $p= 0.0377$, Table 4.3.7). There was a decrease combination effect in the stressed + LPS group when compared to the control group, *(Stressed + LPS vs control group, $p<0.05$, Table 3), and there was a decrease in the stressed + LPS group when compared to a stressed group, β (Stressed + LPS vs stressed group, $p<0.05$, Table 4.3.7).

Table 4.3.7.1: Hippocampal ACh concentration in the control, stressed, LPS, and LPS+ stressed groups.

Animal groups (n=6, per group)	[Hippocampal ACh (pg/mL)]
Control	872.1 ± 70.95
Stressed	865.8 ± 37.95
LPS	838.5 ± 53.10
Stressed + LPS	656.9 ± 32.20*β

Values are presented as a mean ± SEM. *p<0.05 denotes a comparison to the control group, and β p<0.05 denotes a comparison to the stressed group.

4.4.Discussion

In the present study, we evaluated the impacts of CUMS in LPS-challenged rats on metabolic changes, behavioural changes, stress response, inflammatory response, and memory impairment. Our results demonstrated that exposure to CUMS in combination with LPS led to decreased body weight gain. This was associated with sickness behaviour, inflammatory response, and cholinergic dysfunction as evidenced by elevated IL-1β and reduced ACh concentration. The combination of stress and LPS did not induce anxiety-like behaviour, as evidenced by increased time spent close to the wall in the OFT.

In contrast with other studies that demonstrated reduced body weight (31, 32), we observed no change in body weight in the stressed group, suggesting that exposing rats to CUMS for 10 days did not alter body weight. In the LPS-treated group, we found that LPS exposure did not induce body weight loss in rats; this may be due to a single low dose of LPS injection used in this study. These results are not in agreement with the previous study (33), suggesting that our rats may have recovered from a single dose of LPS since animals were assessed after 3 days of LPS administration. In the stressed + LPS group, we observed a decrease in the body weight of rats, suggesting that when stress and LPS are combined, they may amplify the impact on body weight. However, there is no study to support this hypothesis, since there is no previous study done to investigate this combined effect on body weight changes.

Consistent with other studies, which demonstrated that exposure to chronic stress affects feeding behaviour (12, 34), we observed decreased food intake in the stressed group. This indicates that CUMS may alter food intake. Stress-induced alterations in hypothalamic nuclei may disrupt homeostatic regulation of feeding behaviour (48). We found a decrease in food intake in the LPS group; these results suggest that LPS administration at a single low dose reduced food intake. These results align with the previous findings that showed lower food consumption in animals that were treated with LPS (12, 35). However, we observed no change in food intake in the stressed + LPS group. These results indicate that the combined exposure

to CUMS and LPS may induce metabolic or physiological alterations that reduce body weight independently of changes in feeding behaviour. Potential mechanisms underlying this effect may involve elevated energy expenditure or increased catabolic activity linked to hormonal changes brought on by stress or systemic inflammation (49). These findings also demonstrate the intricacy of the physiological response to body weight following simultaneous psychological and immunological stresses.

In the stressed group, we observed no effect of CUMS on water intake; these results align with the study that also demonstrated chronic stress did not affect water intake after exposing rats to 30-day chronic mild stressors (36). We demonstrated a decrease in water intake in the LPS group; however, our results are not in agreement with the previous study that reported that there were no differences in water consumption in the control group and the LPS group (37). Our findings suggest that a single dose of LPS influenced water intake. However, in the stressed + LPS group, we found no change in water intake, suggesting that the combination of stress and LPS did not influence water intake. Our results also indicate that body weight loss was not due to thirst regulation since we observed no effect of the combination of stress and LPS on water intake.

Our result demonstrates that CUMS did not induce anxiety-like behaviour as evidenced by a decrease in time spent close to the wall and an increase in time spent on the open arena in the OFT of the stressed group. We also observed no effect of LPS, indicating that a single dose of LPS did not induce anxiety-like symptoms, as evidenced by no change in exploratory activity. Our animals recovered from a single dose of LPS since we performed OFT after the LPS and CUMS protocol. Therefore, our results are not consistent with the previous studies that assessed animals after 20 hours of a single LPS injection and observed a decrease in locomotor and exploratory behaviour (12, 33). We observed no change in the stressed + LPS group in the time spent close to the wall as well as in the open arena; this finding suggests that chronic mild stress did not induce anxiety-like behaviour in LPS-challenged animals. This also suggests that there was no correlation between anxiety-like behaviour and body weight changes.

Decreased corticosterone concentration in the stressed group was demonstrated in our study, implying that CUMS modulated the body's stress response system. A previous study demonstrated that stress leads to increased corticosterone (38). However, our results do not agree with the previous study that observed an increase in corticosterone concentration after chronic mild stress (39); the decreased corticosterone in the stressed animals was one of the mechanisms to adapt to stressful situations, CUMS activated these mechanisms. Stressed

animals spent less time close to the wall zone and more time at the centre zone, suggesting that CUMS activated adaptation mechanisms. We observed no effect of LPS in stress responses; this means that LPS did not impair metabolic activity and immune response via mechanisms involving corticosterone (13). There is a possibility that stress, when combined with LPS, might not influence stress response, as evidenced by no change in the stressed + LPS group. These results are not in agreement with the previous study that observed an increase in corticosterone after exposure to stress and LPS (38). These results might be due to the shortened duration of CUMS. This suggests that adaptation mechanisms were still active even after acute immune activation. Furthermore, this confirms that stress hormones and associated behaviour were not involved in the low body weight observed in these animals, as we found no changes in the concentration of corticosterone in the stressed + LPS group, which is the group that had lower body weight.

We demonstrated no change in the plasma IL-1β concentration in the stressed group, implying that exposing rats to CUMS for 10 days was not enough to induce systemic inflammation. These results contradict the results of the previous research that demonstrated that chronic mild stress leads to increased pro-inflammatory cytokines (40). Following LPS injection, we observed no effect of LPS injection on plasma IL-1β; this may be due to the recovery of animals from a single dose of LPS, since animals were assessed after 3 days of LPS induction. The involvement of IL-1β, in particular, in sickness has been well documented (33). It has been demonstrated that elevated cytokine levels are associated with behavioural responses to sickness, which leads to body weight loss (14). Consistent with the previous study, we demonstrated an increase in IL-1β in the stressed + LPS group, suggesting that chronic mild stress in combination with LPS can amplify inflammation-associated responses culminating in impeded weight gain (40).

In our study, we observed that CUMS may not have induced memory impairment, as evidenced by no change in ACh in the stressed group. These results are not consistent with another study that demonstrated chronic mild stress reduces ACh release in the hippocampus (41). We observed no effect of LPS on ACh concentration compared to the control group, indicating that a single dose of LPS did not induce memory disturbance, which is a marker of sickness behaviour and has not disrupted cholinergic signalling, which may indicate risk of cognitive decline. These findings may be due to the single dose of LPS used in our study and the recovery period, because we measured ACh after 3 days of LPS administration. A single dose of LPS can significantly alter the acetylcholine (ACh) system (24, 42). A decrease in ACh

concentration was observed in the stressed + LPS group, implying that stress, when combined with LPS, may impair memory and learning. ACh impacts appetite regulation via its interaction with specific receptors in the brain, leading to body weight loss. Low ACh concentration led to body weight loss through disruption in metabolic and physiological processes.

In this study, body weight loss in stressed + LPS animals was brought on by non-behavioural factors such as changes in energy expenditure or metabolism. This can be the result of the systemic inflammation induced by LPS, which can cause body weight loss through enhanced catabolism, modified energy storage, or modifications in nutrient utilisation. These findings suggest that metabolic and inflammatory processes, rather than changes in feeding behaviour, underlie the observed weight loss (44). Both immune activation and chronic stress are known to disturb energy homeostasis by elevating pro-inflammatory cytokines, increasing muscle catabolism, altering nutrient metabolism, and raising overall energy expenditure (45). Inflammatory mediators such as IL-1β and TNF-α have been shown to contribute to weight loss by stimulating catabolic pathways and inhibiting anabolic processes (46).

Reduced hippocampus ACh levels and elevated systemic inflammation (IL-1β) suggest that cognitive deficits are a major consequence of stress and LPS exposure. The findings demonstrate the intricate relationships that exist between stress, inflammation, immunological activation, and memory function, and they imply that these relationships could be a factor in metabolic disorders and cognitive deterioration. Given that both stress and inflammation are believed to be significant factors in the development of neurodegenerative illnesses, our model may shed light on these conditions.

4.5.Conclusion

From the results of the current study, it can be concluded that chronic unpredictable mild stress in combination with LPS leads to reduced body weight. This may be due to the pronounced impact of stress and LPS on inflammatory response, evidenced by elevated IL-1β, which was accompanied by reduced ACh concentration. These changes in body weight are associated with sickness behaviour. Our study indicates that when developing strategies to mitigate neurodegenerative diseases, metabolic and associated changes should also be considered, as they are involved in the progression of neurodegenerative diseases.

Declarations:

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Declaration of Interest

The authors declare no competing interests.

Ethical Approval

Ethical approval was obtained from the Animal Research Ethics Committee (AREC/00004514/2022) of the University of KwaZulu-Natal.

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Chapter 5: Synopsis

Chronic unpredictable mild stress refers to the set of minor psychological stress that occurs randomly in an unpredictable manner, affecting the brain and the body. CUMS disrupts homeostasis, which results in behavioural, physiological, neurological, biochemical, and somatic disturbances. CUMS induces cognitive deficit and anxiety-like symptoms. Lipopolysaccharide (LPS), the primary chemical on the surface of Gram-negative bacteria, activates the host immune system during infection by identifying the bioactive lipid A domain. LPS leads to alterations in the brain as well as in the body.

The CUMS and LPS both tend to affect the brain and the body; however, no studies have been conducted to investigate their combined impact on metabolic, systemic, and neurological alterations. Therefore, this study investigated the impact of CUMS and LPS in rats on the parameters that represent the brain and the body. The mechanism linking stress and LPS administration involves the activation of stress response pathways, immune system activation as well as neuroinflammation and oxidative stress within the brain.

The study looked at how stress (CUMS) and an immune system trigger (LPS) affect the brain, specifically the hippocampus, which is important for memory, learning, and thinking. We measured inflammation, oxidative stress, and chemicals involved in brain function. We also looked at changes in the blood to see how stress and immune activation spread throughout the body. When the immune system is activated, it can make inflammation worse. LPS made this even worse by increasing a protein called IL-1β, which is a sign that the immune system is not working properly.

This study looked at how a combination of unpredictable stress (CUMS) and an immune system trigger (LPS) affects brain health in rats. The results showed that when stress and immune activation are combined, they make damage to the brain worse. This combination increases harmful effects like oxidative stress, inflammation, and memory problems. The rats had lower levels of protective enzymes (SOD1), higher levels of brain cell damage (4-HNE), more inflammation (IL-1β), and reduced levels of acetylcholine (ACh), a brain chemical important for memory. While stress on its own did not have much impact, adding LPS made things worse, suggesting that stress and immune activation together can speed up brain damage.

There were no signs of anxiety-like behaviour in the OFT, nor were there major changes in stress hormone (corticosterone) levels, suggesting that the usual stress response was not completely affected. These results imply that the classical stress response might not have been fully triggered or that the experimental setup may have hindered its manifestation. A minor or temporary reaction to the stressors or a limitation in the sensitivity of this specific behavioural test to identify subtle anxiety-like states could be the cause of the absence of behavioural alterations in the OFT. However, the rats did lose body weight, indicating that stress was still influencing their physical health. The results showed that when stress and LPS were combined, rats experienced body weight loss, sickness behaviour, inflammation, and memory problems. Specifically, inflammation markers like IL-1β were higher, and acetylcholine (ACh), a key chemical for memory, was lower. Interestingly, even though stress and LPS together caused body weight loss, they did not lead to changes in food or water intake, suggesting that the weight loss was not due to eating or drinking less, but rather due to changes in metabolism or energy use.

Neither stress nor LPS alone caused anxiety-like behaviour or changes in the rats' exploratory activities, indicating that the rats did not feel more anxious. Stress reduced corticosterone levels (a stress hormone), which suggests the rats' bodies were adapting to the stress. However, when

stress and LPS were combined, there was no significant change in corticosterone levels, indicating that the combination did not further disrupt the body's stress response.

In terms of inflammation, while stress alone did not cause major inflammation in the body, the combination of stress and LPS increased IL-1β, a marker of inflammation, suggesting that stress and immune activation together can heighten inflammation. Cholinergic dysfunctions were seen in the rats that experienced both stress and LPS, as shown by decreased ACh levels in the brain. This suggests that stress and immune activation together may induce oxidative damage and sickness behaviours in rats.

In conclusion, this study highlights the negative impacts of unpredictable stress and LPS on the body and the brain. Understanding how stress and immunological activation interact to cause neurological diseases and metabolic dysfunctions is crucial, according to the study. The findings of the study aid in developing therapeutic interventions for neurological diseases.

Chapter 6: Conclusion

The first study highlights that exposure to stress and immune activation can induce neurotoxicity, primarily mediated through enhanced oxidative stress and neuroinflammatory pathways. This is characterised by increased markers of oxidative damage and inflammatory cytokines, which may contribute to neural dysfunction and brain tissue damage. Such mechanistic insights provide a foundation for developing targeted therapeutic interventions aimed at mitigating the detrimental effects of combined stress and immune activation on brain health. The second study elucidates that the observed reduction in body weight in animals subjected to both stress and LPS is more likely attributable to metabolic alterations rather than behavioural changes alone. This indicates that the interplay between stress and immune factors disrupts metabolic homeostasis, potentially contributing to systemic sickness behaviours. Furthermore, the combination of stress and immune activation appears to impair cognitive function, likely through cholinergic dysfunction and inflammatory mechanisms, linking

metabolic disturbances with neurobehavioral deficits. These findings provide insight into how stress and inflammation could contribute to disorders like neurodegenerative diseases.

Limitations of the study

No behavioural tests were conducted to assess learning and memory, limiting the study's ability to evaluate cognitive outcomes associated with stress and immune activation. Additionally, both behavioural and hormonal assessments, particularly the Open Field Test (OFT) and corticosterone measurements, were performed at a single time point. This may have failed to capture the peak or temporal fluctuations in stress and immune responses, potentially underestimating their full impact. Furthermore, only one behavioural assay (OFT) was used to assess anxiety-like behaviour, which restricts the behavioural interpretation to a narrow set of parameters.

Recommendations

Future studies should examine the impact of repeated LPS injections in combination with repeated exposure to chronic unpredictable mild stress (CUMS). Along with monitoring body weight and dietary habits during the stress and immune activation period, behavioural tests that evaluate learning and memory should also be included.

APPENDICES

Appendix 1: Ethical clearance letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

08 December 2023

Renewal	
Protocol Reference number:	AREC/00004514/2022 (00024333)
Project title:	**The effects of LPS-induced memory impairment on selected metabolic changes in pre-stressed animals**
Main Applicant and School:	Ms Khethelo Sibiya (219013485) School of Lab Med & Medical Sciences

Dear Ms Sibiya,

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

About your approved protocol, please note:

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

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

Yours faithfully

[redacted]

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

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

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

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

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Appendix 2: Summary of guidelines to authors-Elsevier: Behavioural Brain Research
Essential title page information

- **Title.** Concise and informative. Titles are often used in information retrieval systems. Avoid abbreviations and formulae where possible. "New" and "novel" are not allowed within the title and abstract.

- **Author names and affiliations.** Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.

- **Corresponding author.** Indicate (marked by an asterisk) who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address

- **Present/permanent address.** If an author has moved since the work described in the article was done or was visiting at the time, a "Present address" (or "Permanent address") may be indicated as a footnote to that author's name. The address at which the author did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

The abstract should provide a concise and factual abstract that does not exceed 250 words. The abstract should briefly state the purpose of your research, principal results, and major conclusions.

Keywords

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

Article structure

Subdivision – numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1. (1.1.1., 1.1.2., …), 1.2., etc (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Methodology and Experimental Procedures

The methodology section should provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarised and indicated by a reference. If quoting directly from a previously published way, use quotation marks and cite the source. Any modifications to existing methods should also be described.

Subsections on the Experimental Procedures should be inserted as part of the first line of the text to which they apply. The experimental design should begin with a subsection entitled Experimental Design. This subsection will typically contain brief details of instruments used, and identification of sources of specialized chemicals, biochemicals, and molecular biology kits. The next subsection describes the source(s) and documentation of biological materials used. The Experimental Procedures employed should be concise but sufficiently detailed so that a qualified researcher will be able to repeat the studies undertaken, and these should emphasize either truly new procedures or essential modifications of existing procedures.

Theory/calculation

A theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise. For describing statistical results, please provide details of the statistical test used and full statistical reporting. Full statistical reporting should include the

statistical value and the p-value. For example, an ANOVA should be reported as F=3.11, $p<0.05$.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Acknowledgements

Include any individuals who helped you during your research, such as help with language, writing, or proof reading, in the acknowledgements section. Acknowledgements should be placed before the reference list. Do not include acknowledgements on your title page.

Appendices

Give separate numbering to formulae and equations within appendices using formats such as Eq. (A.1), Eq. (A.2), etc., and in subsequent appendices, Eq. (B.1), Eq. (B. 2), etc. Similarly, give separate numbering to tables and figures using formats such as Table A.1.; Fig. A.1, etc.

Appendix 3: Summary of guidelines to authors-Elsevier: Brain, Behaviour, and Immunity-Health

Essential title page information

- **Title.** Concise and informative. Titles are often used in information retrieval systems. Avoid abbreviations and formulae where possible. "New" and "novel" are not allowed within the title and abstract.

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- **Corresponding author.** Indicate (marked by an asterisk) who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address

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Theory/calculation

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Results

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Discussion

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Conclusions

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Acknowledgements

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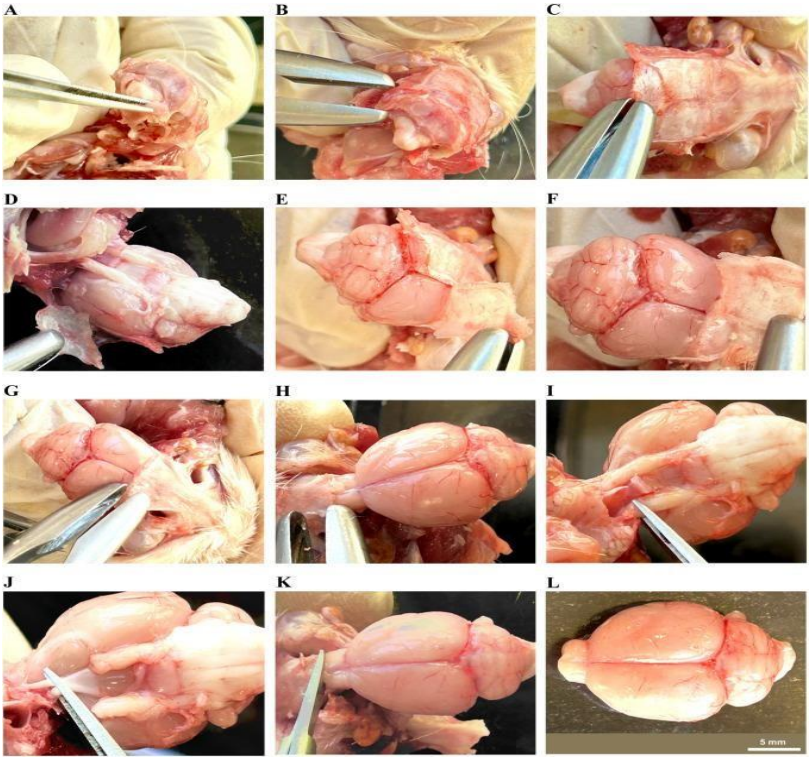
Appendices

Give separate numbering to formulae and equations within appendices using formats such as Eq. (A.1), Eq. (A.2), etc., and in subsequent appendices, Eq. (B.1), Eq. (B. 2), etc. Similarly, give separate numbering to tables and figures using formats such as Table A.1.; Fig. A.1, etc.

Appendix 4: Removal of the brain from the skull of rats (43)



Appendix 5: Dissection of the hippocampus. (43)