NEUROPROTECTIVE EFFECTS OF SIMVASTATIN PRE-TREATMENT AGAINST ALCOHOL-INDUCED BRAIN DAMAGE IN ADOLESCENT MICE

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DECLARATION

I, Robin du Preez, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the Department of Anatomical Sciences, School of Biomedical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work in this thesis has not been submitted for any degree or examination in this or any other University.

Robin du Preez

13th day of October 2025 in Roodepoort

DEDICATION

To God be all the glory, honour and praise.

This work is dedicated to my Lord and Savior, whose grace, wisdom, and strength have sustained me throughout this journey. In moments of doubt, You provided clarity; in times of struggle, You granted endurance. I am nothing without You, and this achievement is a testament to Your faithfulness.

"Commit to the Lord whatever you do, and He will establish your plans."
- Proverbs 16:3
May this work serve a greater purpose beyond myself ♥

AND

To the "DuDe's"

I also dedicate this work to my beloved family, whose unwavering love, prayers and support have been my anchor. To my husband, Mom, and Dad, your sacrifices, encouragement, and faith in me have been invaluable. I could not have done this without you.
I love you!

PREFACE

This thesis is the result of a rewarding journey in the field of neuroscience, one that has been both challenging and inspiring. With a background in physiology (neurophysiology) and molecular biology, I was eager to apply my knowledge in a new realm of research, one that would push the boundaries of my prior experience. The decision to explore the effects of alcohol on brain development marked the beginning of a profound learning experience, transitioning from molecular techniques to an animal-based study. This shift significantly expanded my skill set and deepened my understanding of the complex mechanisms governing brain development. Additionally, my research explores the potential of drug repurposing as a strategy to protect the brain from the damaging effects of alcohol, which could offer new therapeutic avenues for safeguarding brain health.

The journey to completing this work was not without challenges, particularly the unprecedented disruptions caused by the COVID-19 pandemic. The global health crisis disrupted research timelines and forced me to adapt to new working conditions, which at times felt overwhelming. However, these obstacles provided valuable lessons in resilience and adaptability, and ultimately, I was able to continue my work with renewed focus and determination.

Furthermore, the lack of a functional cryotome in our department posed another challenge, necessitating travel to the University of Johannesburg's Doornfontein campus to use their cryotome in the Department of Anatomy and Physiology. The repair process for our cryotome was significantly delayed due to COVID-19-related disruptions, further complicating histological preparations. This additional logistical hurdle required careful planning and extended the time required for tissue processing, but ultimately ensured the success of the histological analyses.

Another significant hurdle was navigating the revised importation and control processes for biological products in South Africa. The introduction of the Section 20 application under the Medicines Control Council (MCC), later transitioning to SAHPRA, required extensive paperwork and adherence to new regulations. These changes affected the importation of critical research reagents, such as rabbit polyclonal antibodies, causing delays and added complexity to the research process.

Additionally, the time-consuming process of manually analyzing each section of brain tissue, either through Stereology or using QuPath software, added another layer of complexity to the research. These methods required meticulous attention to detail and significant time investment to ensure accurate and reliable data, ultimately deepening my understanding of the neurobiological processes involved.

Despite these challenges, each obstacle provided an opportunity for growth and strengthened my passion for research. By dedicating long hours, including weekends and public holidays, to ensure continuous progress, I demonstrated persistence and adaptability, which allowed me to stay focused and maintain steady momentum.

I am deeply grateful to those who supported me throughout this journey: my supervisor, colleagues, friends, and family. Their encouragement, guidance, and patience have been invaluable in shaping this work.

The work presented in this thesis reflects years of academic exploration, experimentation, and collaboration. I hope that these findings contribute meaningfully to our understanding of the effects of alcohol on the adolescent brain and inspire future research in this critical area. While this thesis is the product of my efforts, it is also a tribute to those who have shaped my academic and personal growth. I am honoured to share the insights and discoveries of this research with the broader scientific community, and I look forward to future advancements in the field.

ABSTRACT

Adolescence is a critical period of brain development characterised by increased vulnerability to alcohol-induced neurotoxicity, particularly in the hippocampus and prefrontal cortex, regions essential for memory, executive function, and emotional regulation. In South Africa, adolescent alcohol misuse is widespread and contributes significantly to long-term neurocognitive and structural impairments. Chronic alcohol exposure during adolescence disrupts neurogenesis and induces oxidative stress and neuroinflammation, with sex-specific differences in susceptibility driven by hormonal and developmental factors. Drug repurposing offers a promising intervention strategy, and simvastatin, a lipid-lowering agent with known antioxidant and anti-inflammatory properties, has shown potential for neuroprotection.

This study investigated the neuroprotective potential of simvastatin against alcohol-induced brain damage in adolescent C57BL/6J mice, with a focus on oxidative stress, neuroinflammation, and neurogenesis in the hippocampus and prefrontal cortex. Four-week-old male and female mice were grouped separately and assigned to treatment conditions. Mice received daily intraperitoneal alcohol (20% v/v) for 28 days, alone or in combination with oral simvastatin (5 or 15 mg). Control groups received simvastatin only or remained untreated. Biochemical assays measured oxidative stress markers (MDA, GSH-Px, SOD) in the hippocampus and prefrontal cortex. Immunohistochemistry assessed neurogenesis (PcNA, DCX), neuronal and glial populations (NeuN, GFAP), and cortical laminar structure (Nissl stain). RT-PCR was used to examine hippocampal expression of IL-6, IL-10, CREB1, and BDNF.

Alcohol exposure increased hippocampal GSH-Px activity, a response attenuated by simvastatin in a dose- and sex-dependent manner, particularly in females. Neurogenesis in the dentate gyrus was suppressed by alcohol and partially restored by simvastatin, with high-dose treatment more effective in females and low-dose treatment in males. Although NeuN-positive neuronal populations and dentate gyrus volume remained stable, GFAP expression revealed marked sex-specific astrocytic responses: alcohol-induced astrocyte reactivity in females was reduced by simvastatin, whereas in males, simvastatin exacerbated alcohol-induced GFAP loss. In the prefrontal cortex, simvastatin enhanced alcohol-induced increases in Nissl- and NeuN-positive cell densities in females but not in males. Molecular analyses revealed divergent IL-6, IL-10, and CREB expression patterns across sexes, while BDNF levels remained unchanged.

These findings highlight simvastatin's capacity to mitigate alcohol-related neurotoxicity in adolescence through antioxidant, neurogenic, and anti-inflammatory mechanisms that are both sex- and region-specific. The study highlights the importance of glial modulation in early alcohol-induced neuropathology and positions simvastatin as a candidate for sex-specific neuroprotective intervention. By integrating biochemical, molecular, and stereological analyses, this research offers a comprehensive framework for understanding adolescent alcohol neurotoxicity and informs future translational efforts aimed at protecting neurodevelopmental health in at-risk youth.

Keywords: Adolescent, Brain, Neurodevelopment, Alcohol, Simvastatin, Neurogenesis, Oxidative stress, Neuroinflammation, Sex differences, Hippocampus, Prefrontal Cortex, Neuroprotection

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I would like to extend my heartfelt thanks to my supervisor, Dr. Oladiran Olateju, for his unwavering support, thoughtful guidance, and meticulous attention to detail throughout this research journey. His mentorship has been both encouraging and intellectually challenging, consistently pushing me to refine my thinking and elevate the quality of my work. I am especially grateful for the balance he maintained between support and high expectations, which fostered both personal and academic growth. It has been a privilege to work under his supervision, and I sincerely appreciate the time, insight, and dedication he invested in this project.

I would also like to express my sincere thanks to Ms. Hasiena Ali for her guidance and support throughout my research. Her expertise, mentorship, and thoughtful advice were instrumental in helping me navigate the challenges of the lab and refine my work. I am truly grateful for her encouragement and the positive environment she cultivated, which made my research experience both productive and rewarding.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

5-HT3	Serotonin Type 3 Receptor
ACh	Acetylcholine
AD	Alzheimer's Disease
ADH	Antidiuretic hormone
AMPA Receptors	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ARBD	Alcohol-Related Brain Damage
BAC	Blood Alcohol Concentration
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CA	Cornu Ammonis
cAMP	Cyclic Adenosine Monophosphate
CAT	Catalase
CNS	Central Nervous System
COVID-19	Coronavirus Disease of 2019
CPM	Central Pontine Myelinolysis
CREB1	CAMP Responsive Element Binding Protein 1
CRP	C-Reactive Protein Levels
CVD	Cardiovascular Disease
CYP450	Cytochrome P450
DCX	Doublecortin
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
EC	Entorhinal Cortex
eNOS	Endothelial Nitric Oxide Synthase
FDA	Food and Drug Administration
fMRI	Functional Magnetic Resonance Imaging
GABA	Gamma-aminobutyric Acid
GABA-A	GABA (gamma-aminobutyric acid) Type A Receptor
GCL	Granule Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GSH	Glutathione

GSH-Px	Glutathione Peroxidase
H_20	Water
H_2O_2	Hydrogen Peroxide
hA	Horizontal Astrocytes
HIV	Human Immunodeficiency Virus
HMG-CoA	Hydroxymethylglutaryl-CoA (3-hydroxy-3-methylglutaryl) Coenzyme A
IL-10	Interleukin-10
IL-6	Interleukin-6
KS	Korsakoff Syndrome
LDL	Low-density Lipoprotein
LDL-C	Low-density Lipoprotein Cholesterol
LDLR	Low Density Receptor
LTP	Longterm Potentiation
MBD	Marchiafava-Bignami Disease
MDA	Malondialdehyde
MMPs	Matrix Metalloproteinases
MOR	μ-opioid receptor
MVA	Mevalonate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NeuN	Neuronal Nuclear Protein
NMDA	N-Methyl-D-Aspartate
NO	Nitric Oxide
NSc	Neural Stem cells
O_2	Oxygen
PCNA	Proliferating Cell Nuclear Antigen
PD	Parkinson's Disease
PFC	Prefrontal Cortex
QNPs	Quiescent Neural Progenitors
rA	Radial Astrocytes
RMS	Rostral Migratory Stream
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

SES	Socioeconomic Status
SGZ	Subgranular Zone
SOD	Superoxide Dismutase
SOX2	Sex Determining Region Y-Box 2
SREBP's	Sterol Regulatory Element Binding Proteins
SVZ	Subventricular Zone
TBI	Traumatic Brain Injury
TNF-α	Tumour Necrosis Factor Alpha
TrkB	Tropomyosin-Related Tyrosine Kinase B
VTA	Ventral Tegmental Area
WE	Wernicke's Encephalopathy
WHO	World Health Organization
δ	Delta

CHAPTER ONE

INTRODUCTION AND RESEARCH PROBLEM

1.1. Introduction

Ethanol, commonly referred to as alcohol, is the most common and oldest recreational substance worldwide (Marshall, 2014; Dey and Meenu Singh, 2015; Jones, 2022). In South Africa, the use of alcohol has a long history and is deeply embedded in many people's lives, regardless of their socio-economic background. Although the method of measurement varies, reports estimate South Africa's per capita alcohol consumption at 11 liters per person, placing the country among the top 20 worldwide and making it the highest in Africa (Rashied, 2023). The high levels of alcohol consumption are primarily driven by a combination of cultural, social, and economic factors, including the acceptance of heavy drinking in some communities and the widespread availability of alcohol, often due to regulatory lapses (Charman, Petersen and Piper, 2013; Trangenstein et al., 2018). Alcohol abuse remains a significant health concern, with extensive social, economic, and health consequences (Sudhinaraset, Wigglesworth and Takeuchi, 2016).

1.1.1. Alcohol Use Among South African Adolescents

The World Health Organization (WHO) describes adolescence as the developmental stage between childhood and adulthood, typically spanning the ages of 10 to 19 (Poznyak and Rekve, 2014). During this period, significant alterations in behavioural patterns, endocrine activity, and neurochemical processes contribute to the maturation required for independent living (Carvajal and Lerma-Cabrera, 2015; Claborn, 2015; Dick and Ferguson, 2015). Other characteristics include pubertal development, cognitive maturation, identity formation, and increased independence (Sawyer et al., 2018). In addition, this stage is also accompanied by heightened exploration and risk-taking behaviours, including experimentation with drugs and alcohol, smoking, and sexual activities (Steinberg and Morris, 2001; Carvajal and Lerma-Cabrera, 2015; Sanci, Webb and Hocking, 2018). Adolescents may engage in binge drinking, typically defined as consuming four or more standard alcoholic beverages for women and five or more for men within a two-hour period, which can cause blood alcohol levels to spike to 0.08 g/dL (Naimi and Brewer, 2005). This threshold is met when women have four or more standard alcoholic beverages, and men have five or more, within two hours (Bethesda, 2007).

More specifically, from a public health perspective, the WHO describes binge drinking as heavy episodic drinking where a person consumes 60 grams or more of pure alcohol (equivalent to six or more standard drinks) on a single occasion at least once in 30 days (WHO, 2019). While adolescents' frequency of consumption may be lower than that of adults, the volume of alcohol ingested per occasion tends to be significantly greater (MacPherson et al., 2010; Chung et al., 2018; Ojeda et al., 2022), resulting in a higher incidence of alcohol-related illness (e.g., alcohol poisoning, increased risk of injuries) and neurocognitive impairments (short and long term) (Courtney and Polich, 2009; Hermens and Lagopoulos, 2018).

Rising levels of alcohol use among adolescents represent a significant public health concern. Research indicates that consumption within this age group remains high, due to a complex interplay of socio-cultural, economic, and environmental influences, including peer dynamics, family structure, and socioeconomic conditions (Mothibi, 2014). An estimated 32% of South African youth aged 11 to 20, with males more likely to consume alcohol than females, have reported drinking in the past month (Reddy et al., 2013; Morojele and Ramsoomar, 2016; Morojele et al., 2018; Letsela et al., 2019). This is further supported by data from a nationwide survey on youth risk behaviors conducted among Grade 8 to 11 pupils in public schools, where half of the students reported having consumed alcohol at some point, a quarter had engaged in binge drinking, and 12% had begun drinking before the age of 12 (Morojele et al., 2018). Additionally, many students admitted to driving or walking under the influence, engaging in physical altercations (including gender-based violence), and participating in sexual experimentation (Burton and Leoschut, 2013; Shisana et al., 2014; Russell et al., 2014). These patterns of risky behaviour reflect the harmful impact of alcohol on young people, often leading to negative economic (e.g., poverty, unemployment), social (e.g., lack of education, violence) and health consequences (e.g., HIV transmission, unplanned teenage pregnancies), placing substantial strain on South Africa’s public resources and healthcare system (Harrison et al., 2010; Scott-Sheldon et al., 2013; Khuzwayo, Taylor and Connolly, 2020; Matzopoulos, Corrigall and Bowman, 2014).

The South African context presents unique challenges in regulating alcohol use among the adolescent population, specifically due to the country’s socio-economic disparities, high levels of unemployment, and the normalising of alcohol use in social settings. Studies indicate that adolescents from lower-income or disadvantaged backgrounds are more prone to start consuming alcohol at a younger age, influenced by peer pressure and a lack of access to

education about the risks of alcohol abuse (Donovan, 2004; Leung, Toumbourou, and Hemphill, 2011). Additionally, familial patterns of alcohol use greatly influence adolescent drinking behaviour, with parental drinking habits often serving as a model for their children (McMorris et al., 2011; Stephenson et al., 2023). Moreover, the media's portrayal of alcohol use, coupled with the aggressive marketing strategies employed by alcohol companies, substantially contributes to the normalisation of drinking, framing it as a central feature of social interaction among young South Africans (Osuafor, Okoli, and Chibuzor, 2023).

1.1.2. Preventative Measures

Many preventative and intervention strategies have been proposed and implemented to address adolescent alcohol consumption in South Africa. The National Department of Health aims to raise awareness of the dangers linked to alcohol abuse and promote responsible drinking by implementing various public health campaigns (Parry, 2005). The Department of Basic Education also incorporates substance abuse prevention into the school curriculum through its "Alcohol and Drug Use Prevention and Management Programme." This is achieved via subjects like Life Orientation and Life Skills, along with co-curricular activities such as Peer Education programs and initiatives promoting healthy lifestyle choices (Department of Basic Education, 2025). Furthermore, the Liquor Act was introduced in 2003 to regulate the sale and consumption of alcohol and aims to restrict access to minors (Davies, 2016). Unfortunately, despite all these efforts, the enforcement of these policies remains inconsistent, and alcohol continues to be widely accessible to adolescents in both rural and urban areas, indicating a need for more comprehensive and culturally sensitive interventions (Morojele and Ramsoomar, 2016; Letsela et al., 2019; Mathibe, Cele and Modjadji, 2022; Mmereki et al., 2022).

Research indicates that brief interventions and motivational interviewing, commonly conducted by nurses, can effectively reduce harmful and risky alcohol consumption in low- and middle-income countries like South Africa (Ghosh et al., 2022). These interventions have demonstrated encouraging results, highlighting the need to incorporate them into more comprehensive prevention strategies. Another proposed preventive measure is the restriction or ban of alcohol advertising to reduce exposure to alcohol-related risks at both the individual and community levels. In South Africa, the "Control of Marketing of Alcoholic Beverages Bill" was introduced to enforce such restrictions, but it has not been passed into law due to opposition from the alcohol industry and economic concerns (Morojele et al., 2018). While some

regulations, such as warning labels on advertisements and sponsorship limitations, are in place, a comprehensive ban has yet to be implemented (Bertscher, London, and Orgill, 2018; Morojele et al., 2018). Nevertheless, this policy reformation could be key to diminishing the normalization of alcohol use among the adolescent population. While long-term regulatory measures, such as restrictions on alcohol advertising, remain a subject of debate, temporary interventions implemented during the COVID-19 pandemic offered valuable insight into the potential effectiveness of stricter alcohol control policies. The pandemic introduced a range of factors that influenced alcohol consumption behaviours in South Africa. Initially, the ban on alcohol sales led to a temporary reduction in alcohol-related harm; however, a sharp increase in consumption was observed once the restrictions were lifted (Mapanga et al., 2023).

Effectively addressing alcohol use in this demographic requires a multi-dimensional approach that includes public health campaigns, policy reforms, education, and targeted health and community-based interventions tailored to the needs of South African youth. Given that alcohol-related diseases are largely preventable, prioritizing prevention over treatment is essential to reducing long-term health and social burdens (Walmisley et al., 2024).

1.2. Brain Maturation during Adolescence

Undoubtedly, numerous factors influence the rise in alcohol use during adolescence. One particularly important aspect is the ongoing development of the brain at this stage, during which critical neural connections are being formed and reinforced (Alert, 2006; Lees et al., 2019). This dynamic process involves structural, functional, and neurochemical changes that shape cognitive abilities, emotional regulation, and behaviour. During mid-adolescence (between the ages of 14 and 17), the subcortical limbic regions mature, causing an imbalance in brain region development. More specifically, “The Dual Systems Model of Adolescent Brain Development” explains this functional maturation of brain networks (Steinberg, 2010; Lambert et al., 2014). This model proposes that the increased tendency for risk-taking seen during adolescence arises from an imbalance in the maturation of two key brain systems: the socioemotional network, primarily involving limbic structures, and the cognitive control network, largely governed by the prefrontal cortex. The socioemotional system, encompassing regions such as the nucleus accumbens (associated with reward), amygdala (involved in processing emotions), and hippocampus (linked to learning and memory), develops more rapidly than the prefrontal cortex. This developmental gap results in heightened emotional sensitivity and an increased

motivation for novelty and pleasure-driven behaviour (Casey, Jones and Hare, 2008; Shulman et al., 2016). In addition, adolescents are highly sensitive to dopamine surges, which make the reward feel more intense and reinforce risk-taking behaviours (Steinberg, 2004; Ernst and Mueller, 2008; Somerville, Jones and Casey, 2010). In contrast, the cognitive control system is mainly centered in the PFC and governs executive functions such as planning, impulse regulation, reasoning, and problem-solving. The PFC is one of the final brain regions to fully mature, with its development continuing into mid-to-late 20s (Arain et al., 2013; Friedman and Robbins, 2022). Adolescents often exhibit immature decision-making and poor impulse control because the PFC is not yet fully connected to other brain regions, particularly the limbic system. Functional MRI (fMRI) studies show that adolescents rely more on the amygdala (emotion-driven processing) than on the PFC in response to stimuli, contributing to heightened emotional reactivity (Scherf, Smyth, and Delgado, 2013). This temporal disparity results in a period where heightened reward sensitivity is not yet properly regulated by fully developed cognitive control. As a consequence, adolescents develop a reward bias that increases their attraction to new, high-risk, and impulsive behaviors, including alcohol use (Steinberg and Morris, 2001; Sylwester, 2007; Yurgelun-Todd, 2007; Murty, Calabro, and Luna, 2016; Shulman et al., 2016).

Along with structural modifications, significant changes occur in neurotransmitter activity and hormonal regulation, influencing behaviour, emotional stability and social interactions. A key change occurs in the dopaminergic system, which is crucial for processing pleasure, rewards and motivation. (Ernst and Luciana, 2015). During adolescence, dopamine release increases in response to novel and rewarding stimuli, which leads to heightened sensation-seeking behaviors, a stronger drive for social rewards and a preference for immediate gratification over long-term benefits (Wahlstrom et al., 2010; Telzer, 2016). This neurochemical change partly explains why adolescents are more prone to risk-taking and peer influence. Additionally, serotonin levels, which regulate mood and anxiety, fluctuate during this developmental period, contributing to greater emotional variability, increased susceptibility to anxiety and depression, and challenges in emotional regulation (Kepser and Homberg, 2015; Garcia-Garcia et al., 2017). Alongside these neurotransmitter changes, pubertal hormones like testosterone and estrogen significantly impact adolescent behaviour by amplifying emotional intensity, shaping social dynamics, and increasing aggression (Sisk and Zehr, 2005; Blakemore, Burnett and Dahl, 2010; Herting and Sowell, 2017). Furthermore, an increase in oxytocin, a hormone associated with social bonding, heightens sensitivity to peer relationships and social

acceptance, reinforcing the importance of social interactions during adolescence (Doom, Doyle, and Gunnar, 2017; Sannino, Chini, and Grinevich, 2017; Knoop et al., 2022). Collectively, these neurochemical changes contribute to the unique emotional, cognitive, and social patterns observed in adolescents.

Environmental and cultural factors are also crucial in shaping the development of the adolescent brain, influencing cognitive, emotional, and social outcomes. Socioeconomic status (SES) significantly impacts brain maturation, with lower SES being associated with increased exposure to stress, limited access to educational resources, and higher risks of mental health challenges, all of which can affect cognitive function and emotional well-being (Hackman and Farah, 2009; Farah, 2017). These stressors, in particular, may heighten emotional regulation difficulties, increasing the likelihood of maladaptive coping strategies, such as alcohol misuse (Tartaglia and Bergagna, 2020; Wang, Anderson-White and Jin, 2022). Similarly, education and cognitive stimulation promote brain development, particularly in areas related to executive function and decision-making, which are essential in moderating risky behaviors like substance use (Petrosini et al., 2009; Rakesh et al., 2024). Lack of cognitive stimulation, however, may contribute to poorer decision-making abilities and increased susceptibility to peer pressure, both of which are linked to higher rates of alcohol use among adolescents (Stautz, 2013). Furthermore, the increasing use of technology and social media among adolescents impacts brain development, especially in areas linked to attention control, reward processing, and social cognition (Crone and Konijn, 2018; Firth et al., 2019; Marciano, Camerini and Morese, 2021). While digital engagement can provide opportunities for learning and social interaction, excessive use may contribute to altered attention spans and increased sensitivity to social validation, which can encourage risky behaviors, including alcohol consumption, as adolescents seek social acceptance (Shanmugasundaram and Tamilarasu, 2023). Together, these environmental factors illustrate how external influences on adolescent brain development can create vulnerabilities, increasing the likelihood of alcohol misuse and other risky behaviors.

1.3. Alcohol

1.3.1. Mechanism of Action

Recent advancements have deepened scientific understanding of the pharmacodynamic properties of alcohol (Narahashi et al., 2001; Santhakumar, Wallner and Otis, 2007), particularly concerning its effects on the brain and body, including interactions with

neurotransmitter systems and receptors involved in behaviour, cognition, and motor control. Due to its hydrophilic properties, alcohol readily passes through biological membranes and crosses the blood-brain barrier. Alcohol metabolism primarily occurs in the liver and brain after systemic absorption, due to the presence of enzymes including alcohol dehydrogenase (ADH), catalase, and P450 (CYP2E1) (Dharavath et al., 2023). These metabolic processes generate three main by-products: acetaldehyde, salsolinol (a neurotoxic substance formed from dopamine and acetaldehyde), and acetate, which often cause the toxic effects of alcohol (Burcham, 2013; Gil-Mohapel et al., 2019). In the brain, alcohol and its metabolites function as a CNS depressant, leading to several disruptions such as impaired glucose uptake, increased monocarboxylate uptake, and changes in neurotransmitter activity (Peana et al., 2017). Voltage-gated calcium channels are essential for neurotransmitter release, hormone secretion, gene expression, and cellular differentiation. Acute exposure to ethanol has been found to inhibit these channels at pharmacologically significant concentrations, potentially disrupting their physiological functions (Wang et al., 1994; Widmer, Lemos, and Treistman, 1998; Lobo and Harris, 2008; Möykkynen and Korpi, 2012). In addition, alcohol elevates the concentration of inhibitory neurotransmitters such as gamma-aminobutyric acid (GABA) (Santhakumar, Wallner and Otis, 2007; Lobo and Harris, 2008) and negatively affect other neurotransmitter systems such as acetylcholine (nicotinic acetylcholine receptor), serotonin (5-HT3 receptor), dopamine, glycine, and ionotropic glutamate (Narahashi et al., 2001; Lobo and Harris, 2008; Möykkynen and Korpi, 2012).

Research indicates that alcohol functions as a GABA_A receptor agonist (positive allosteric modulator), increasing the activity of GABA, the primary inhibitory neurotransmitter in the brain, resulting in increased feelings of sedation and relaxation, decreased anxiety, and impaired motor coordination (Santhakumar, Wallner and Otis, 2007; Edwards and Preuss, 2025). Concurrently, alcohol inhibits N-methyl-d-aspartate (NMDA) receptors, disrupting glutamate-mediated excitatory signalling, leading to cognitive impairments, memory deficits and difficulties in learning (Nagy, 2008; Chandrasekar, 2013; Mira et al., 2019). Additionally, alcohol stimulates the mesolimbic reward pathway by increasing dopamine release in the nucleus accumbens, reinforcing reward-seeking behaviour, increasing the risk of addiction, and creating a feeling of euphoria (Boileau et al., 2003; Gonzales, Job and Doyon, 2004; Pierce and Kumaresan, 2006). Acute alcohol intake temporarily elevates serotonin levels, affecting mood regulation, sociability and impulsivity, whereas chronic alcohol use disrupts serotonin function, leading to anxiety, depression and emotional instability (Heinz et al., 2001; Chastain, 2006;

Ketcherside, Matthews and Filbey, 2013). Alcohol also stimulates the release of endorphins, which bind to mu-opioid receptors (MORs), producing analgesic (pain-relieving) and euphoric effects, reinforcing alcohol's rewarding effects and increasing the risk of addiction (Herz, 1997; Gianoulakis, 2001).

1.3.2. The Impact of Alcohol on the Adolescent Brain

Heavy drinking during brain maturation adversely affects brain development, structure, and neuropsychological performance in areas responsible for memory, decision-making, impulse control, and emotional regulation (Bechara, 2005; Crews and Boettiger, 2009). In addition, alcohol also affects brain blood flow, electrical brain activities, and compromises brain plasticity, leading to impairment of neurocognitive function, slowed cognition, and unconsciousness (Tapert, Caldwell and Burke, 2004; American Psychiatric Association, 2013; Spear, 2013; Feldstein, Sakhardande and Blakemore, 2014). More serious cases include Alcohol-related brain damage (ARBD), liver cirrhosis, cardiovascular disease, malnutrition, and even death (Victor, 1989; Hillbom, Juvela and Karttunen, 2007; Guerri and Pascual, 2010; Bruha, Dvorak and Petrtyl, 2012; Erdozain et al., 2014).

Neurons, glial cells, and brain regions including the PFC, hippocampus, and cerebellum demonstrate heightened sensitivity to the effects of alcohol (Markwiese et al., 1998; Crews et al., 2000; Alfonso-Loeches and Guerri, 2011; Spear, 2014; Squeglia, Jacobus and Tapert, 2014). The PFC, which regulates working memory, judgment, reasoning, and self-control, remains immature during this period, and alcohol exposure can hinder its development, leading to impaired executive functioning and increased impulsivity (Abernathy, Chandler and Woodward, 2010). Similarly, the hippocampus, which is responsible for learning and memory, is highly vulnerable to alcohol-related neurotoxicity, often resulting in memory deficits and difficulty retaining new information (Meda et al., 2018; Wooden et al., 2021). Research indicates that adolescents who regularly consume alcohol tend to have smaller hippocampal and prefrontal lobe volumes compared to their healthy peers (De Bellis et al., 2005; Nagel et al., 2005). Additionally, alcohol disrupts the integrity of white matter, which is essential for neural communication between different brain regions, effectively leading to reduced cognitive processing abilities, attention deficits, and poor emotional regulation (Crews and Nixon, 2009; Monnig et al., 2014). Adolescent alcohol consumption is connected to an elevated risk of mental health disorders, such as depression, anxiety, and mood disturbances, through its

disruption of neurotransmitter balance, particularly serotonin and dopamine (Ward, Lallemand and De Witte, 2009; Sinclair et al., 2014). Considering alcohol's effects on brain maturation, cognitive abilities, and mental health, adolescent alcohol use presents considerable risks, highlighting the importance of implementing preventive measures to promote healthy neurodevelopment.

1.3.3. Sex Differences in Alcohol-Induced Changes in the Adolescent Brain

Alcohol affects male and female adolescents differently due to variations in brain development, hormone levels, and metabolism. Studies suggest that females are typically more vulnerable to alcohol's neurotoxic effects because their brains mature at an earlier rate than males (De Bellis, 2001). Additionally, differences in body fat composition and metabolic rate between males and females can influence alcohol distribution and clearance, resulting in higher blood alcohol concentrations in females for the same intake (Baraona et al., 2001; De Bellis, 2001). Females are also more sensitive to hormonal influences. Fluctuations in estradiol levels throughout the menstrual cycle have been linked to various mental health challenges in adult women, including alcohol consumption (Handy et al., 2022). Similarly, adolescent girls have shown a comparable pattern, with elevated estrogen levels being associated with greater alcohol use (de Water et al., 2013; Erol et al., 2019). This relationship (between estradiol and alcohol) is particularly well-documented in the animal literature (see a review by Finn, 2020). Additionally, women metabolize alcohol less efficiently because of lower levels of the enzyme alcohol dehydrogenase, resulting in higher blood alcohol concentrations (BAC) compared to men who consume the same amount of alcohol (Mumenthaler et al., 1999; Baraona et al., 2001). Conversely, male adolescents, influenced by testosterone, tend to engage in riskier behaviours and consume alcohol in larger amounts, resulting in increased exposure to its long-term effects (Hicks, Iacono and McGue, 2010). Studies indicate that alcohol consumption in males frequently correlates with impairments in spatial memory and decision-making, while female adolescents are more prone to deficits in memory and heightened emotional responses (Caldwell et al., 2005; Noorbakhsh et al., 2020). Additionally, although males face a higher risk of developing alcohol use disorders, females may experience more significant cognitive effects, such as greater reductions in brain volume over time (Medina et al., 2008; Grace et al., 2021). These variations highlight the importance of accounting for biological and developmental factors when evaluating alcohol's effects on adolescent brain health.

1.3.4. Alcohol-Related Brain Damage (ARBD)

ARBD refers to the damage imposed on the brain's structure and function caused by the direct neurotoxic effects of chronic or excessive consumption of alcohol (Zahr, Kaufman and Harper, 2011). The damage manifests as an increased density of microglia and heightened expression of pro-inflammatory cytokines in the brain, reflecting neuroinflammatory responses that may contribute to subsequent neurodegeneration (Crews and Boettiger, 2009). Depending on the location of the damage, these neurological consequences can range from mild cognitive impairments to more severe impairments. ARBD affects impacts brain regions including the frontal lobe (Oscar-Berman and Marinkovic, 2003), hippocampus, limbic system, and cerebellum (Jaatinen and Rintala, 2008; Arathi et al., 2019) and contributes to various neurological conditions such as acute Wernicke's encephalopathy (WE), Korsakoff's syndrome (KS), Wernicke-Korsakoff's syndrome (thiamine deficiency), alcohol-related dementia, frontal lobe syndrome, cerebral and cerebellar degeneration, hepatic encephalopathy, Marchiafava–Bignami disease (MBD) and central pontine myelinolysis (CPM) (White, 2003; Crews, He and Hodge, 2007; Marshall, Guerrini and Thomson, 2009; Zuccoli et al., 2010; Zahr, Kaufman and Harper, 2011). At the cellular level, alcohol induces oxidative stress, neuroinflammation, and apoptosis, further accelerating neuronal damage. It disrupts neurotransmitter systems, including GABA, glutamate, dopamine, and serotonin, leading to deficits in cognition, emotional regulation, and reward processing (Nunes et al., 2019). While some alcohol-induced brain damage may be reversible with abstinence, nutritional support, and cognitive rehabilitation, prolonged and excessive consumption can cause permanent cognitive and structural impairments, increasing the risk of dementia and neurodegenerative diseases (Bartsch et al., 2007).

1.4. The Hippocampus

1.4.1. The Anatomical Structure of the Hippocampus and Associated Regions

The hippocampus is a brain structure found in humans and other vertebrates, located deep within the temporal lobe of each cerebral cortex in mammals (Eberhard et. al., 2006). The hippocampus, situated next to the entorhinal cortex (EC), along with the cingulate gyrus, parahippocampal gyrus, amygdala, septal area, and hypothalamus, forms the limbic system, a network essential for regulating emotion, behaviour, and memory (Kaushal et al., 2024). The larger hippocampal formation within the limbic system consists of four key areas: the dentate gyrus (DG), the Cornu Ammonis subregions CA3, CA2, and CA1 (collectively called the

hippocampus proper), the subicular complex, and the EC (Rajmohan and Mohandas, 2007). The word "hippocampus" generally refers to both the DG and the Cornu Ammonis (CA) subfields, which are further divided into distinct layers based on cell types (Mark et al., 1993; Sadock, Sadock and Ruiz, 2017). As a key component of the limbic system, the hippocampus plays a vital role in transforming short-term memories into long-term ones, as well as in regulating emotions and facilitating learning (Anand and Dhikav, 2012). It is among the most extensively studied regions of the mammalian CNS (Johnston and Amaral, 2004), due to its frequent involvement in various neuropsychiatric disorders.

1.4.2. The Flow of Information in the Hippocampus

The hippocampus operates through a highly structured trisynaptic circuit that processes and encodes information that is essential for learning, memory, and spatial navigation (Vakilna et al., 2021). This process is influenced by a dynamic interaction between internal factors, such as growth factors and neurotransmitters, and external factors, including environmental conditions and drug exposure (Åberg et al., 2005; Cameron and Glover, 2015; Opendak, Briones and Gould, 2016). Memory formation and learning in the hippocampus are fundamentally supported by long-term potentiation (LTP). This process involves the sustained strengthening of synaptic connections after high-frequency stimulation, leading to more efficient neural signal transmission (Lynch, 2004; Whitlock et al., 2006).

Sensory and cognitive information from the neocortex is conveyed to the EC, where it then projects to the DG via the perforant pathway (Canals et al., 2008). This process is considered to be the first synapse in the trisynaptic circuit and is driven by glutamate release and NMDA receptor activation, allowing calcium influx into the postsynaptic neuron, which triggers intracellular signalling pathways that enhance synaptic strength. Thereafter, the granule cells in the DG process incoming information from where signals are relayed to CA3 pyramidal neurons via the mossy fibre pathway, enabling associative memory retrieval and pattern completion. GABA receptors help control pattern separation and memory precision, ensuring that similar inputs do not overlap excessively in memory encoding (i.e., similar memories are distinguished, preventing confusion between overlapping experiences) (Ginsberg, 2005). Interneurons in the hippocampus (e.g., basket cells, chandelier cells) release GABA to inhibit pyramidal neurons in CA1 and CA3 that help maintain oscillatory rhythms (theta waves, gamma waves), which are essential for encoding memory (Iwakiri et al., 2006; Kia, Shojaei

and Ghafouri, 2023). CA3 neurons then send signals to CA1 pyramidal neurons via the Schaffer collateral pathway, facilitating synaptic plasticity. This connection is essential for pattern completion, allowing the reconstruction of complete memories from partial information (i.e., it aids in recalling full memories from incomplete cues) (Remondes and Schuman, 2002; Piskorowski and Chevaleyre, 2012).

Acetylcholine (ACh) contributes to memory modulation by regulating attention, learning, and synaptic plasticity within the hippocampus. It enhances the formation of new memories while inhibiting the recall of older ones (Hasselmo, 2006). CA3 then forms recurrent collaterals within itself, facilitating the retrieval of associative memories. Finally, the processed information is transmitted to the subiculum via CA1 projections and back to the entorhinal cortex, from where the information is redistributed to the prefrontal cortex, amygdala, and other neocortical areas for long-term memory storage and retrieval (White, Matthews and Best, 2000; White and Swartzwelder, 2004; Eberhard et. al., 2006). Dopamine influences hippocampal plasticity, motivation, and the detection of novel stimuli. Neurons from the ventral tegmental area (VTA) release dopamine as they project to the hippocampus through the mesolimbic pathway, where it supports the consolidation of long-term memories in reward-based learning (Lisman and Grace, 2005; Shohamy and Adcock, 2010). Lastly, LTP leads to an upregulation of AMPA receptors, increasing postsynaptic sensitivity to neurotransmitters and promoting structural modifications such as dendritic spine growth and synaptic remodelling, facilitating long-term memory storage (Malenka, 2003; Santos et al., 2009). Given its role in synaptic efficiency, LTP is widely regarded as a fundamental mechanism underlying learning and memory.

This intricate flow of information is tightly regulated by excitatory and inhibitory neurotransmitters that shape hippocampal activity and cognitive functions. Imbalances in these neurotransmitter systems are linked to cognitive deficits, psychiatric disorders, and neurodegenerative diseases. An excess of glutamate causes excitotoxicity, which contributes to conditions like Alzheimer's disease, epilepsy, and stroke damage. Conversely, reduced glutamate availability has been connected to learning and memory deficits, as well as cognitive deterioration (Volk et al., 2015; Giorgi et al., 2020). Reduced GABA leads to hyperexcitability, seen in epilepsy, schizophrenia, and anxiety disorders, while an excess can impair memory formation and cognitive flexibility (Möhler, 2006; Prévot and Sibille, 2021). In addition, reduced levels of ACh are linked to Alzheimer's disease (due to the loss of cholinergic

neurons), while an excess of ACh can cause confusion by interfering with retrieval processes (Garcia-Alloza et al., 2005; Foong et al., 2018). Low dopamine levels have been linked to motivational and memory impairments, contributing to Parkinson's disease and depression, while excessive dopamine has been linked to schizophrenia and cognitive inflexibility (Seeman, 2013; Nobili et al., 2017).

In addition, the hippocampus also helps with spatial navigation through specific cells in the CA1 and CA3 regions, known as "place cells," which encode spatial locations and help with navigation (Chersi and Burgess, 2015). Furthermore, the hippocampus modulates stress regulation and emotional responses through its neural pathways involving the amygdala and hypothalamus (McEwen, Nasca and Gray, 2016). Therefore, the precise coordination of hippocampal information flow and neurotransmitter activity is fundamental for optimal cognitive function and memory processing.

1.4.3. Neurogenesis

In the adult mammalian brain, new neurons are primarily generated in two regions: the hippocampus and the olfactory system. Within the olfactory system, progenitor cells are located in the anterior subventricular zone (SVZ), which lines the lateral ventricles. These progenitors travel via a mechanism known as "chain migration" along the rostral migratory stream (RMS) toward the olfactory bulb, where they differentiate into granule cells or periglomerular inhibitory interneurons (Doetsch et al., 1999; Balu and Lucki, 2009).

Hippocampal neurogenesis describes the formation of new neurons and glial cells within the subgranular zone (SGZ) of the DG (Lazarov and Hollands, 2016). Located between the hilus and the granule cell layer, the subgranular zone (SGZ) provides a nurturing niche where neural stem cells (NSCs) produce new neurons. Unlike many other brain areas, the hippocampus retains neurogenic capacity throughout adulthood. This process is influenced by both internal factors, such as gene expression, growth factors (like BDNF) and neurotransmitters (glutamate, GABA, serotonin, and dopamine), as well as external factors, including physical activity, stress, environmental enrichment and pharmacological agents (Bond, Ming and Song, 2015). Therefore, neurogenesis is a promising target for therapeutic strategies aimed at enhancing cognitive function and treating mood disorders. In addition, hippocampal neurogenesis is essential for learning and memory, especially for being able to distinguish between similar

experiences or environments (Deng, Aimone and Gage, 2010). Furthermore, neurogenesis allows for cognitive flexibility, allowing an individual to adapt to new information and maintain hippocampal plasticity for overall cognitive health, especially during ageing (Klempin and Kempermann, 2007). The generation of new neurons contributes significantly to stress resilience and emotional regulation. Decreased or impaired neurogenesis has been associated with neurodegenerative diseases such as Alzheimer's, as well as psychiatric conditions including depression and anxiety, along with cognitive decline (Hill, Sahay and Hen, 2015; Babcock et al., 2021).

The hippocampal neurogenesis pathway involves multiple distinct yet connected steps, from the initial proliferation of neural stem cells (NSCs) to the final integration of mature neurons into the existing network. Quiescent neural progenitors (QNPs) are multipotent cells with a unique morphology resembling radial glial cells, featuring a triangular-shaped cell body and a prominent apical process that extends into the granule cell layer (GCL), where it forms extensive branches (Seri et al., 2001). Typically, QNPs are dormant and do not divide, yet they may resume cell division when prompted by certain signals. It is suggested that QNPs undergo asymmetric division, yielding one daughter cell that preserves stem cell characteristics and another that is committed to becoming a neuron (Kempermann et al., 2004). Progenitor cells in the SGZ are classified into two types: radial astrocytes (rA) and horizontal astrocytes (hA), based on their orientation (Seri et al., 2004). The presence of GFAP, an astrocyte-specific protein, together with Nestin and Sox2, identifies these QNPs and highlights their multipotent stem cell characteristics (Seri et al., 2004). When QNPs become activated, they enter a phase of proliferation, actively dividing to generate a new population of intermediate neural progenitor cells (Balu and Lucki, 2009). This stage is characterized by the expression of several proliferation-associated proteins, notably Proliferating Cell Nuclear Antigen (PcNA). During the S-phase of the cell cycle, PCNA, an important cofactor for DNA polymerase δ, is highly expressed to assist in DNA replication and repair (Strzalka and Ziemienowicz, 2011). PcNA expression serves as a reliable indicator of proliferative activity, distinguishing actively dividing neural progenitor cells from quiescent NSCs and post-mitotic neurons (Zerjatke et al., 2017).

After leaving the cell cycle, progenitor cells proceed to differentiate, predominantly becoming neurons, while some give rise to glial cells, including astrocytes and oligodendrocytes (Purves et al., 2001). Neurons in their immature stage express doublecortin (DCX), a microtubule-

associated protein crucial for neuronal migration and the initial development of dendrites (Couillard-Despres et al., 2005; Kempermann, Song and Gage, 2015). Following differentiation, the newly generated immature neurons migrate a short distance from the SGZ into the granule cell layer of the DG, within which they undergo maturation. During this phase, they extend dendrites and axons, establish synaptic connections, and progressively express markers characteristic of mature neurons (Gonçalves, Schafer and Gage, 2016). Ultimately, the mature neurons become incorporated into the existing hippocampal network, particularly within the trisynaptic pathway, where they support hippocampus-dependent cognitive functions like learning and memory. At the final integration stage, these neurons express neuronal nuclei (NeuN), a marker of mature post-mitotic neurons, signifying their functional incorporation into the hippocampal circuitry (Gusel'nikova and Korzhevskiy, 2015). Each stage of this process is meticulously regulated by both internal genetic mechanisms and external environmental factors, ensuring the accurate development and incorporation of newly generated neurons into the hippocampal circuitry (Deng, Aimone and Gage, 2010).

1.4.4. Alcohol-Induced Damage in the Hippocampus

Alcohol serves as a potent amnestic agent (White and Swartzwelder, 2004) that significantly impairs memory formation by disrupting hippocampal activity (Aggleton, 2014; Jauhar, Marshall and Smith, 2014; Aggleton and Morris, 2018), leading to memory impairments that are often seen in alcohol-related brain disabilities, especially during adolescence (Hanson et al., 2011; Jacobus and Tapert, 2013). The hippocampus is widely recognised for its increased sensitivity to alcohol-induced neurotoxicity, given its ongoing refinement and maturation during adolescence and into early adulthood. This region is crucial for integrating emotion, reward processing, homeostasis, and memory (Kutlu and Gould, 2016; Mira et al., 2019; Walker, Kuhn and Risher, 2021).

Moreover, alcohol significantly disrupts the formation of LTP (Roberto et al., 2002; Hicklin et al., 2011). At sufficiently high concentrations, alcohol disrupts normal synaptic signalling, potentially preventing the induction of LTP altogether, thereby hindering learning and memory processes, an effect particularly pronounced during adolescence (Titterness and Christie, 2012). Additionally, alcohol suppresses the proliferation and survival of neural progenitor cells (Nixon and Crews, 2002), resulting in impaired hippocampal neurogenesis. This disruption contributes to alcohol-induced neurodegeneration and is associated with subsequent cognitive

impairments (Crews et al., 2004, 2006; Zeigler et al., 2005; Broadwater et al., 2014; Mira et al., 2020; Doremus-Fitzwater and Deak, 2021; Anand et al., 2023).

1.5. Neurodegeneration

Neurodegeneration refers to the gradual and irreversible degeneration of neurons and synapses in specific regions of the CNS (Przedborski, Vila and Jackson-Lewis, 2003). This degenerative process results in deficits across cognitive, motor, and sensory domains, depending on the specific brain regions affected. Neuronal loss in Alzheimer's disease (AD) primarily affects the hippocampus and cortical regions, resulting in gradual memory decline and cognitive impairment (Crews and Masliah, 2010). In Parkinson's disease (PD), selective loss of dopaminergic neurons in the substantia nigra leads mainly to motor dysfunction (Dawson and Dawson, 2003). Huntington's disease, a genetic disorder, causes widespread neuronal degeneration, particularly in the striatum, contributing to a combination of motor abnormalities, cognitive decline, and psychiatric symptoms (Gil and Rego, 2008). Neurodegeneration is considered a multifactorial process involving complex interactions between genetic factors, environmental influences, and age-related changes. However, the precise molecular mechanisms driving disease progression are still not fully understood (Skovronsky, Lee and Trojanowski, 2006; Jellinger, 2009). Key mechanisms implicated include protein aggregation, inflammation, oxidative stress, mitochondrial dysfunction, and genetic mutations (Golde and Miller, 2009; Glass et al., 2010; Gandhi and Abramov, 2012; Johri and Beal, 2012).

Neuroinflammation, a critical aspect of neurodegenerative processes, involves the activation of microglia (the brain's immune cells), cytokines (small signalling proteins involved in immune responses, inflammation, and neuroimmune modulation), and neurotrophic signalling molecules (Allan and Rothwell, 2001; Levy et al., 2005). Chronic neuroinflammation can cause neuronal damage and trigger apoptosis (Kempuraj et al., 2016; Kwon and Koh, 2020). Furthermore, when reactive oxygen species (ROS) exceed the brain's antioxidant capacity, oxidative stress occurs, leading to neuronal damage (Shukla, Mishra and Pant, 2011).

1.5.1. Molecular Regulators of Neuroplasticity and Neuroinflammation

In the hippocampus, key molecular mediators, including interleukin-6 (IL-6), interleukin-10 (IL-10), cAMP response element-binding protein (CREB), and brain-derived neurotrophic

factor (BDNF), play essential roles in modulating neuronal plasticity, neurogenesis, and inflammatory responses, thereby affecting cognitive functions such as learning and memory.

Among these, IL-6 stands out due to its multifaceted role as a pro-inflammatory cytokine and regulator of neuronal development, regeneration, and degeneration. As a significant pro-inflammatory cytokine, IL-6 is involved in various processes, including neuronal development, differentiation, regeneration, and degeneration (Kummer et al., 2021). Within the CNS, IL-6 is primarily produced by neurons, astrocytes, microglia, and endothelial cells. Under normal conditions, IL-6 expression is tightly regulated; however, various stimuli, including infections, injuries, and environmental stressors, can trigger increased IL-6 release (Erta, Quintana and Hidalgo, 2012). As a central mediator of neuroinflammation, IL-6 expression coordinates immune responses within the CNS (Koper et al., 2018; Kölliker-Frers et al., 2021). Its effects are dualistic: acute IL-6 expression promotes neuronal survival and neurogenesis, aiding recovery after injury and supporting memory processes such as acquisition, consolidation, and retrieval (Gadient and Otten, 1997; Elderkin-Thompson et al., 2012; Chesnokova, Pechnick and Wawrowsky, 2016). In contrast, sustained high levels of IL-6 are associated with chronic inflammation and neurodegeneration, facilitating neuronal apoptosis and playing a role in the development of disorders like multiple sclerosis and Alzheimer's disease (Fisman and Tenenbaum, 2010; Mammana et al., 2018). Furthermore, prolonged high IL-6 levels impair neurogenesis, disrupt LTP, and are linked to reductions in hippocampal gray matter volume, potentially leading to cognitive decline (Marsland et al., 2008; Harrell et al., 2022).

Adolescence represents a critical period for IL-6 regulation, as hormonal fluctuations, brain maturation, and environmental factors influence its expression (Allen et al., 2018; Sciarra et al., 2023). Studies indicate heightened basal IL-6 levels during this developmental stage, particularly in the hippocampus and PFC, where it modulates neurogenesis and synaptic refinement (Stumper et al., 2020; Kummer et al., 2021). However, excessive IL-6 during adolescence can impair synaptic plasticity and LTP, increasing vulnerability to cognitive deficits. Chronic stress exposure during adolescence has also been linked to prolonged IL-6 upregulation, elevating the risk of anxiety, depression, and cognitive impairments later in life (Pandey et al., 2012). Furthermore, abnormal IL-6 expression during this critical developmental period has been linked to the pathophysiology of neurodevelopmental disorders such as schizophrenia and autism spectrum disorders (Meyer, Feldon and Dammann, 2011; Goines and Ashwood, 2013; Velloso et al., 2022).

IL-10 is an anti-inflammatory cytokine mainly produced by microglia and astrocytes. It is well known for its neuroprotective effects, as it inhibits pro-inflammatory cytokines such as IL-6 and TNF-α, thereby helping to maintain immune balance within the CNS (Porro, Cianciulli and Panaro, 2020; Barabási et al., 2023). The neuroprotective function of IL-10 has been confirmed in several experimental models, including ischaemic stroke (Frenkel et al., 2005; Sharma et al., 2011), spinal cord injuries (Zhou et al., 2009a), and acute brain injuries (Garcia et al., 2017). IL-10 provides direct neuronal support (Zhou et al., 2009b), preserves synaptic integrity (thereby facilitating learning and memory), and promotes neurogenesis by protecting hippocampal neurons from inflammation-induced damage (Perez-Asensio et al., 2013; Pereira et al., 2015). These effects on immune regulation suggest that IL-10 may contribute to slowing neurodegeneration in diseases like Alzheimer's and Parkinson's, where ongoing inflammation is a fundamental aspect of disease progression (Porro, Cianciulli and Panaro, 2020). During adolescence, fluctuations in puberty-related hormones (e.g., increased sex hormones such as estrogen and testosterone) can influence cytokine production, including IL-10. Some studies suggest that IL-10 levels may vary during adolescence compared to other developmental stages, particularly in response to stress or neuroinflammation, where it may be upregulated as a compensatory anti-inflammatory response (Grassi-Oliveira et al., 2016; Nelson and Lenz, 2017).

As a key transcription factor, CREB1 regulates genes involved in maintaining neuronal survival, facilitating synaptic plasticity, supporting memory consolidation, and enhancing resistance to apoptosis under stressful or injurious conditions (Herold et al., 2011; Sakamoto, Karelina and Obrietan, 2011). Activation of CREB1 occurs through multiple intracellular signalling pathways, notably those linked to brain-derived neurotrophic factor (BDNF) and cyclic AMP (cAMP) (Wen, Sakamoto and Miller, 2010). In neurodegenerative diseases, CREB1 activation can be impaired, leading to deficits in neuronal plasticity and cognitive decline. Decreased CREB1 activity has been linked to neurodegenerative disorders like Alzheimer's disease, potentially contributing to synaptic dysfunction, cognitive decline, and memory loss (Mantamadiotis et al., 2002; Bhardwaj et al., 2024). Within the hippocampus, CREB1 is predominantly localized in regions such as CA1, CA3, and the dentate gyrus, where it is essential for facilitating long-term potentiation, supporting neurogenesis through regulation of downstream genes, and controlling the expression of genes like BDNF that are

critical for synaptic enhancement and neuronal development (Herold et al., 2011; Kandel, 2012).

As a neurotrophin, BDNF supports neuronal growth, sustenance, and maintenance (Cohen-Cory et al., 2010) and plays a crucial role in neuroplasticity, learning, and memory (Gómez-Pinilla et al., 2002; Kapczinski et al., 2008). By interacting with the TrkB receptor, BDNF initiates signalling cascades that sustain neuronal survival and function (Nagappan and Lu, 2005). In neurodegenerative diseases, BDNF levels are often reduced, which can contribute to neuronal loss, synaptic dysfunction, and cognitive decline. Neurodegenerative disorders like Alzheimer's, Parkinson's, and Huntington's have been associated with diminished BDNF expression (Jellinger, 2009; Azman and Zakaria, 2022). Enhancing BDNF expression or its signalling pathways has demonstrated neuroprotective effects by reducing neuroinflammation and oxidative stress, which are hallmarks of these conditions. BDNF is highly expressed in the hippocampus, especially within the CA1, CA3, and DG regions, where it supports neuronal survival, dendritic growth, and differentiation (Schaaf, De Kloet and Vreugdenhil, 2000). BDNF also supports LTP and synaptic plasticity, key mechanisms underlying learning and memory (Hall, Thomas and Everitt, 2000). Moreover, BDNF is crucial for adult neurogenesis, facilitating the proliferation, maturation, and synaptic integration of new neurons in the DG (Rossi et al., 2006). The expression of BDNF is modulated by neuronal activity and enhanced by activation of CREB1 (Esvald et al., 2020).

During adolescence, the expression levels of CREB1 and BDNF undergo significant changes, influenced by the neurodevelopmental and hormonal shifts characteristic of this period. CREB1 activity is heightened during adolescence, reflecting increased neuronal activity and ongoing brain maturation. The increase in CREB1 activity is essential for promoting the expression of BDNF, which peaks during adolescence, especially in areas like the hippocampus. This supports key processes such as synaptogenesis, neuronal growth, and synaptic pruning. However, exposure to stress during this sensitive period may dysregulate BDNF expression, potentially increasing vulnerability to psychiatric disorders (Juhasz et al., 2011; Li et al., 2022).

1.5.2. Alcohol-Induced Dysregulation of IL-6, IL-10, CREB1 and BDNF

Several external (environmental or lifestyle) factors are known to contribute to neurodegeneration and neuroinflammation. These factors can either trigger harmful processes in the brain or worsen existing vulnerabilities, ultimately leading to neuronal damage, cognitive decline, or neurodegenerative diseases (Jellinger, 2009). Chronic alcohol consumption and drug abuse result in oxidative damage, excitotoxicity, and increased microglial activation, accelerating neuronal loss (Olney et al., 2002; Crews et al., 2004).

Adolescent alcohol consumption exerts significant modulatory effects on key molecular markers involved in neurodevelopment, immune regulation, and cognitive function. Elevated IL-6 levels in response to alcohol consumption have been observed mainly in the hippocampus, PFC, amygdala, and hypothalamus (Doremus-Fitzwater et al., 2015; Gano, Doremus-Fitzwater and Deak, 2017), promoting neuroinflammatory responses that are especially harmful during adolescence, a period characterized by heightened vulnerability to immune-mediated neuronal damage (Zago and Pedrotti Moreira, 2016). Alcohol exposure simultaneously lowers levels of IL-10, impairing the balance between pro-inflammatory and anti-inflammatory responses and thereby amplifying inflammation in the developing brain (Marshall et al., 2017). Additionally, alcohol impairs the activation of CREB1, which is critical for synaptic plasticity, LTP, and memory formation. This inhibition negatively affects learning and memory processes, which are still maturing during adolescence (Soares-Simi et al., 2013). Moreover, alcohol consumption is consistently linked to decreased expression of BDNF, a neurotrophin critical for neurogenesis, synaptic connectivity, and neuronal survival. This reduction in BDNF during adolescence disrupts synaptogenesis and cognitive development, potentially heightening the risk of mood disorders and neuropsychiatric conditions in later life (Miguez et al., 2020; Cutuli and Sampedro-Piquero, 2022). Collectively, these alterations suggest that alcohol-induced dysregulation of IL-6, IL-10, CREB1, and BDNF may have profound and lasting consequences on adolescent brain maturation and function.

1.5.3. Alcohol-Induced Oxidative Stress

Chronic alcohol consumption causes oxidative stress in the brain, resulting from an imbalance between ROS generation and antioxidant protection. Due to the presence of unpaired electrons, reactive ROS exhibit high reactivity and can harm cellular components (Bergamini et al., 2004). During alcohol metabolism, acetaldehyde is produced as the primary metabolite. The

oxidation process produces superoxide free radicals that can interact with hydrogen peroxide, leading to the formation of additional reactive molecules, including ROS and reactive nitrogen species (RNS). These reactive agents cause damage to vital cellular structures like lipids, proteins, and DNA, which contributes to neuronal death, neuroinflammation, disrupted synaptic activity, cognitive impairments, memory loss, and neurodegenerative changes (Betteridge, 2000; Smith et al., 2005; Teleanu et al., 2022). Alcohol additionally promotes the generation of nitric oxide (NO), a cytotoxic free radical that harms neurons and glial cells by triggering lipid peroxidation, which involves the degradation of phospholipids in cell membranes (Lancaster, 1995; Baraona et al., 2002; Osakada et al., 2003). A strong correlation exists between oxidative stress and the pathogenesis of several neurodegenerative conditions, including epilepsy, Alzheimer's, Huntington's, and Parkinson's diseases (Aguiar et al., 2012; Kim et al., 2015; Singh et al., 2019).

The brain's elevated oxygen consumption combined with its abundance of polyunsaturated fatty acids, especially within neuronal membranes, renders these membranes particularly vulnerable to ROS and oxidative damage (Singh et al., 2010). One major consequence of ROS exposure is lipid peroxidation, an oxidative degradation process that damages the integrity and function of membrane phospholipids (Negre-Salvayre et al., 2008). Lipids are essential structural components of neuronal membranes and function as bioactive signalling molecules (Tracey et al., 2021). Because polyunsaturated fatty acids are especially prone to oxidation, these membranes represent key targets for oxidative stress (Lushchak and Bagnyukova, 2006; Monaghan, Metcalfe and Torres, 2009).

A key byproduct of lipid peroxidation is malondialdehyde (MDA), a highly reactive compound generated when ROS degrade polyunsaturated fatty acids in cell membranes (Ayala, Muñoz and Argüelles, 2014). MDA is broadly acknowledged as a marker of oxidative stress and cellular injury, frequently utilized in both clinical and research contexts to evaluate oxidative damage in neurological and systemic diseases (Sidorova and Domanskyi, 2020). Elevated MDA levels indicate increased lipid peroxidation, which contributes to neurodegeneration, inflammation, and various pathological conditions (Lushchak and Bagnyukova, 2006; Monaghan, Metcalfe and Torres, 2009). In the brain, excessive MDA accumulation is linked to neuronal apoptosis, cognitive decline, and impaired synaptic function, particularly in conditions such as alcohol-related brain damage, neurodegenerative diseases, and metabolic disorders (Itoh et al., 2013; Plascencia-Villa and Perry, 2023). Although the body has

antioxidant defence mechanisms, prolonged exposure to oxidative stressors, such as alcohol, environmental toxins, and metabolic imbalances, can overwhelm these systems, resulting in sustained MDA levels and exacerbating tissue damage and dysfunction.

The body relies on antioxidant defense systems to counteract the harmful effects of oxidative stress and protect cells from damage caused by ROS. Among the most vital antioxidants are glutathione (GSH) and superoxide dismutase (SOD), which are crucial for neutralizing free radicals and preserving cellular balance (Huang et al., 2009; Sharma, 2014; He et al., 2017). GSH, a tripeptide made up of glutamate, cysteine, and glycine, functions as a major intracellular antioxidant. It neutralizes ROS through enzymatic reactions catalyzed by glutathione peroxidase (Marí et al., 2009; Averill-Bates, 2023). GSH plays a role in cell signalling, immune response, and maintaining redox balance. However, its depletion is linked to heightened oxidative damage, neurodegeneration, and compromised detoxification, particularly in conditions like alcohol-related brain damage, neurodegenerative diseases, and liver disorders (Averill-Bates, 2023).

Superoxide dismutase (SOD) is a crucial enzymatic antioxidant that serves as the primary defense against RNS, ROS and other harmful molecules. It catalyzes the conversion of the superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), which is subsequently broken down into water (H_2O) and oxygen (O_2) by the enzymes catalase (CAT) and glutathione peroxidase (GPx) (Younus, 2018; Chidambaram et al., 2024). SOD is widely expressed throughout the CNS, both within and outside neurons and glial cells. In adults, SOD1 (Cu/Zn-SOD) is predominantly found in neurons of the substantia nigra, hippocampal pyramidal neurons and dentate gyrus granule neurons. In contrast, during early central nervous system development, SOD1 expression is mainly found in cortical layers II, III, and V, along with neurons in the pyriform cortex, hippocampus, and hypothalamus (Chidambaram et al., 2024). Following excitotoxic injury, neuronal SOD1 expression declines prior to neurodegeneration, while astrocytic expression increases (Peluffo et al., 2005). SOD2 (Mn-SOD), primarily found in neurons of the brain and spinal cord, is additionally upregulated in astrocytes following injury (Maier and Chan, 2002). Importantly, research by Massaad et al. (2009) demonstrated that SOD2 overexpression reduced superoxide concentrations in the hippocampus and protected against memory deficits in a mouse model of Alzheimer's disease (AD). Likewise, SOD supplementation was reported to enhance outcomes in a different AD mouse model (Persichilli et al., 2015). Extracellular SOD (EC-SOD or SOD3), which is located in the extracellular

matrix, plays a crucial role in managing oxidative stress within the hippocampal microenvironment and supports all phases of neurogenesis (Zou et al., 2012).

Adolescence is a critical developmental phase characterized by intense neurodevelopment, synaptic remodelling, myelination, and neuroplasticity in response to genetic and environmental influences (Yahfoufi, Matar and Ismail, 2020). This period also leads to increased oxidative stress due to higher energy demands (Patel et al., 2021; Ene et al., 2023). Due to significant neuronal reorganization and remodelling, the adolescent brain is particularly susceptible to stress and immune challenges (Paus, Keshavan and Giedd, 2008). Alcohol-induced oxidative stress particularly affects regions such as the PFC, hippocampus, and cerebellum (Enache et al., 2008; Fowler et al., 2014; Rajput et al., 2017; Kamal et al., 2020; Tsermpini, Plemenitaš Ilješ and Dolžan, 2022). This initiates a series of processes that result in mitochondrial dysfunction, disrupted neuronal signalling, neuronal cell death, and suppression of neurogenesis (Givens, Williams and Gill, 2000; Hovatta, Juhila and Donner, 2010; Brocardo, Gil-Mohapel and Christie, 2011). Alcohol-induced oxidative stress in the PFC has been associated with deficits in executive functions, including decision-making, impulse regulation, and working memory (Abernathy, Chandler and Woodward, 2010; Fowler et al., 2014). Oxidative stress further exacerbates brain injury linked to thiamine deficiency, increasing the risk of Wernicke-Korsakoff syndrome and other cognitive disorders related to alcohol use. Chronic oxidative damage can accelerate neurodegenerative processes, playing a role in alcohol-related dementia and increasing the likelihood of neurodegenerative disease onset (Bhat et al., 2015).

GSH and SOD together constitute a vital defense mechanism that safeguards neurons and other cells against oxidative damage, inflammation, and programmed cell death. However, chronic exposure to oxidative stressors such as alcohol, environmental toxins, and metabolic disorders can overwhelm these antioxidant systems, leading to cellular dysfunction and disease progression. Enhancing antioxidant capacity through dietary antioxidants, lifestyle modifications, and potential therapeutic interventions is a key strategy for reducing oxidative damage and promoting overall health.

1.6. Drug Repurposing

Drug repurposing, sometimes called drug repositioning, refers to identifying new medical applications for drugs that have previously received approval or are nearing the final phases of development. This approach is emerging as a vital approach for both industrial investigators and the academic community (Oprea et al., 2011; Chen et al., 2018; Pushpakom et al., 2019; Ardakani et al., 2023). This strategy provides a quicker and more economical option compared to conventional drug discovery, largely by leveraging pre-existing pharmacokinetic, safety, and toxicity information, which shortens development timelines and lowers the risk of failure (Oprea et al., 2011). Drug repurposing has become particularly valuable for addressing unmet medical needs, including rare diseases, neglected conditions, and emerging health crises, where conventional drug development may not be economically viable or fast enough (Pushpakom et al., 2019). For instance, drugs like sildenafil, originally developed for erectile dysfunction, were later approved for pulmonary hypertension (Cruz-Burgos et al., 2021; Eid, 2021), and thalidomide, once used as a sedative, was repurposed to treat leprosy and multiple myeloma (Rahmat, Sklavenitis and Ghobrial, 2019; Amare, Meharie and Belayneh, 2021).

Several prominent examples demonstrate the effectiveness of drug repurposing in neurological and psychiatric conditions. For example, amantadine, initially created as an antiviral medication for influenza, has been effectively redirected for use in treating Parkinson's disease due to its effects on dopamine pathways (Danysz et al., 2021; Fletcher et al., 2021), while valproic acid, first approved for epilepsy, is now widely used in bipolar disorder and migraine management (Fava, 2018; Aroosa et al., 2023). Similarly, minocycline, an antibiotic, has demonstrated promise in managing neurodegenerative disorders such as Alzheimer's and Huntington's disease because of its neuroprotective effects (Sanshita et al., 2023), while ketamine, traditionally an anaesthetic, has been repurposed as a rapid-acting antidepressant (Das, 2020; Carboni et al., 2021). Additionally, statins, originally prescribed to lower cholesterol and prevent cardiovascular disease, have been explored for their anti-inflammatory and neuroprotective effects, showing promise in conditions such as Alzheimer's disease and multiple sclerosis (Ferrarelli, 2015; Bhat et al., 2021).

The process of drug repurposing typically involves computational techniques, data mining, or machine learning to identify promising candidates, followed by preclinical and clinical evaluation to confirm efficacy and safety for the new indication (Peyvandipour et al., 2018;

Parvathaneni et al., 2019). A major benefit of this approach is that repurposed drugs have already undergone human testing, with comprehensive data on pharmacology, dosing, toxicity, and formulation, making it a quicker, safer, and more cost-efficient alternative to developing new medications (Badria, 2020). Since these drugs have already demonstrated safety, they do not require Phase 1 clinical trials, allowing them to enter pivotal studies much quicker (Cha et al., 2018). For example, repurposing efforts have played a critical role in the response to COVID-19, with drugs from AbbVie being evaluated for their potential to treat the disease (Kulkarni et al., 2023). Repurposing also offers potential benefits even for drugs that have failed in some indications, as they may still offer therapeutic promise in different therapeutic areas (Begley et al., 2021). This makes drug repurposing an increasingly attractive strategy, particularly in the context of rising drug prices and slow drug discovery. Additionally, repurposing can be a key component of a company's lifecycle management strategy, helping to address unmet clinical needs or develop age-appropriate dosage forms (Cha et al., 2018; Hering et al., 2018).

While drug repurposing has many advantages, challenges such as regulatory approval complexities, intellectual property issues, and difficulties in securing funding, specifically for generic drugs, remain significant barriers to widespread implementation (Pushpakom et al., 2019; Begley et al., 2021). Nonetheless, the strategy remains a powerful tool for expanding therapeutic options efficiently and effectively, offering the potential to bring effective treatments to market more quickly and at a fraction of the cost associated with novel drug development.

1.7. Statins

Statins belong to a group of drugs that lower lipid levels, mainly targeting low-density lipoprotein cholesterol, which helps reduce the risk of cardiovascular diseases such as heart attacks and strokes (Byrne et al., 2019). Simvastatin, atorvastatin, and rosuvastatin are widely used lipid-lowering medications that have received approval from the Food and Drug Administration (FDA) (Adeli, Zahmatkesh and Dezfouli, 2019; Schade, Shey and Eaton, 2019).

1.7.1. Mechanism of Action

Statins, also referred to as reductase inhibitors, function primarily by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Schachter, 2005; Adeli, Zahmatkesh and Dezfouli, 2019). Under normal physiological conditions, this enzyme facilitates the conversion of HMG-CoA to mevalonate, an essential step in the biosynthesis of cholesterol and isoprenoids (Friesen and Rodwell, 2004). By disrupting the NADPH-dependent transformation of HMG-CoA into mevalonic acid, statins effectively block the mevalonate pathway, resulting in reduced cholesterol production and decreased intracellular cholesterol content in liver cells (Holstein and Hohl, 2004; Schachter, 2005). A decrease in intracellular cholesterol levels triggers the activation of Sterol Regulatory Element-Binding Proteins (SREBPs), which in turn promote the transcription of the gene responsible for encoding the Low-Density Lipoprotein Receptor (LDLR) (Horton, Goldstein and Brown, 2002). This leads to an increased number of LDL receptors on the surface of hepatocytes, enhancing the clearance of circulating low-density lipoprotein cholesterol (LDL-C) from the bloodstream and thereby lowering plasma LDL-C concentrations (Duan et al., 2022). In addition to lowering cholesterol, statin-induced inhibition of the mevalonate pathway also reduces the production of downstream molecules like ubiquinone (Coenzyme Q10), vital for mitochondrial energy metabolism, and prenylated proteins that play key roles in cellular signalling and proliferation (Friesen and Rodwell, 2004). These additional biochemical effects contribute to the broader therapeutic benefits, referred to as pleiotropic effects, of statin therapy.

1.7.2. Pleiotropic Effects of Statins

The pleiotropic effects of statins involve a variety of cholesterol-independent benefits that extend beyond lipid reduction, supporting improved cardiovascular health (Davignon, 2004). These effects include reduced cardiovascular risk, enhanced overall vascular health, and anti-inflammatory and anti-thrombotic benefits (Oesterle, Laufs and Liao, 2017).

One key pleiotropic effect is the enhancement of endothelial function. Statins increase NO production, which promotes vasodilation, improves blood flow, and reduces vascular resistance, lowering the risk of hypertension and atherosclerosis (Liu et al., 2023). Statins also possess potent anti-inflammatory properties, notably lowering C-reactive protein (CRP) levels, which contributes to the stabilization of atherosclerotic plaques. By reinforcing plaque integrity, statins help prevent rupture and the ensuing risk of thrombosis, thereby playing a vital

role in reducing the likelihood of acute cardiovascular events such as myocardial infarctions and strokes (Blum and Shamburek, 2009; Nesti, Mengozzi and Natali, 2020). Statins also exhibit antithrombotic properties by inhibiting platelet aggregation and clot formation, further decreasing cardiovascular risk (Nesti, Mengozzi and Natali, 2020). Moreover, their ability to reduce oxidative stress helps protect blood vessels from damage, contributing to long-term vascular health (Kadhim et al., 2020). Statins also influence immune function by dampening immune cell activation and vascular inflammation, processes that are critical to slowing the advancement of atherosclerosis (Palaniswamy et al., 2010; Bahrami et al., 2018). From a metabolic perspective, statins have been reported to enhance insulin sensitivity and lower blood glucose levels, offering potential benefits for individuals with metabolic syndrome or diabetes; however, these effects warrant careful monitoring (Muscogiuri et al., 2014). Finally, statins offer cardiac benefits beyond cholesterol lowering, improving heart muscle function and reducing myocardial inflammation, which can help prevent heart failure and improve outcomes in coronary artery disease (Rohilla et al., 2016; Nchodu et al., 2024a).

Emerging research highlights additional neuroprotective pleiotropic effects of statins that may benefit brain health. These include anti-inflammatory and antioxidant effects that help attenuate neuroinflammation and oxidative stress, key pathological processes underlying neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Tramontina et al., 2011; Abdanipour et al., 2018; Carroll et al., 2019; Kosowski et al., 2021). Statins may mitigate neuronal damage by reducing C-reactive protein (CRP) and other pro-inflammatory markers, thereby attenuating microglial activation. Additionally, they improve endothelial function, enhance cerebral blood flow, and protect against ischemic damage in conditions such as stroke and vascular dementia (Asahi et al., 2005; Van Der Most et al., 2009). Statins also influence cholesterol metabolism in the brain, potentially reducing the accumulation of amyloid beta plaques, a hallmark of AD (Li, Lin and Huang, 2018). Furthermore, statins may enhance cognitive function and memory by upregulating brain-derived neurotrophic factor (BDNF), thereby supporting neurogenesis and synaptic plasticity (Wu et al., 2008). Statins have also demonstrated neuroprotective benefits in promoting recovery after spinal cord injury (Nazli et al., 2015; Mirzaie et al., 2022), minimizing brain damage following intracerebral haemorrhage (Gomis et al., 2010; Chen et al., 2019) and alleviating neuronal injury associated with epileptic seizures(Vitturi and Gagliardi, 2020; Amanlou et al., 2023). Statins have also demonstrated promise in alleviating symptoms of mental health conditions, including depression and anxiety (Kim et al., 2014; Yan et al., 2020; Avan et al., 2021).

1.7.3. Simvastatin

Simvastatin is derived from lovastatin, a naturally occurring compound produced by the fungus *Aspergillus terreus*. Lovastatin, the earliest statin identified, serves as the foundational compound for the synthesis of simvastatin (Alberts, 1988). As a lipophilic (fat-soluble) drug, simvastatin is readily absorbed through the intestinal tract and distributed to lipid-rich tissues, with the liver being its primary site of action (Sirtori, 2014). Similar to atorvastatin and lovastatin, simvastatin is distinct from hydrophilic statins like pravastatin and rosuvastatin, which primarily remain in the bloodstream (Hamelin and Turgeon, 1998; Climent, Benaiges and Pedro-Botet, 2021). Its lipophilicity (i.e., ability to readily cross cell membranes through passive diffusion) enhances simvastatin's capacity to penetrate tissues and effectively reduce cholesterol production (Bhandary et al., 2009). Classified as a moderate-intensity statin, simvastatin is among one of the older-generation statins. While it is less potent than newer statins like atorvastatin and rosuvastatin, it remains effective in reducing LDL cholesterol levels (Jones et al., 2003; Pedersen, 2005).

1.7.4. Neuroprotective Properties of Simvastatin

1.7.4.1. *Antioxidant and Neurovascular Effects of Simvastatin*

Simvastatin demonstrates significant antioxidant properties by modulating key markers of oxidative stress including MDA, GSH-Px and SOD (Mansouri et al., 2022). MDA is a well-established marker of lipid peroxidation and oxidative stress affecting cellular membranes. Increased MDA concentrations have been linked to numerous pathological conditions, including neurodegenerative diseases, cardiovascular disorders, and metabolic syndromes. Studies have shown that simvastatin markedly reduces MDA levels, indicating diminished lipid peroxidation and a decrease in overall oxidative stress (Lovrić et al., 2008; Su et al., 2010). This effect is likely attributed to simvastatin's ability to inhibit ROS production by targeting the mevalonate pathway, thereby downregulating NADPH oxidase activity, a key source of ROS (Shang et al., 2006).

GSH-Px, an essential component of the antioxidant defense system, catalyzes the reduction of hydrogen peroxide and lipid peroxides into water and harmless alcohols by utilizing glutathione as a reducing substrate. Simvastatin has been shown to enhance GSH-Px activity, thereby improving the detoxification of peroxides and reinforcing cellular protection against oxidative stress (Zinellu and Mangoni, 2021). By increasing GSH-Px activity, simvastatin

helps maintain redox balance within cells, particularly in tissues susceptible to oxidative stress, such as the brain, liver, and vascular endothelium (Tong et al., 2018).

SOD is another essential antioxidant enzyme that catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, serving as a first-line defence against oxidative stress. Evidence suggests that simvastatin enhances SOD activity, facilitating more efficient clearance of superoxide radicals. This improvement in antioxidant defence helps mitigate oxidative stress-induced cellular damage and offers protection against inflammation and apoptosis (Zinellu and Mangoni, 2021).

1.7.4.2. *Immunomodulatory and Neurotrophic Actions of Simvastatin*

Simvastatin's neuroprotective effects are largely attributed to its ability to diminish neuroinflammation by blocking microglial activation (Li et al., 2009a) and decreasing the secretion of pro-inflammatory cytokines, including TNF-α and IL-6, which play key roles in the development of neurodegenerative diseases such as Alzheimer's and Parkinson's (Fassbender et al., 2001; Huang et al., 2017; McFarland, Davey and Anoopkumar-Dukie, 2017). Simvastatin further supports an anti-inflammatory environment by upregulating the production of IL-10, a cytokine known for its anti-inflammatory properties (Balduini et al., 2003). Besides its role in immune regulation, simvastatin influences key intracellular signalling pathways that govern neuronal survival and synaptic plasticity. It enhances the expression of the anti-apoptotic gene Bcl-2, which is essential for protecting neurons against programmed cell death (Ko et al., 2011; Spampanato et al., 2012; Fu et al., 2014). Furthermore, simvastatin stimulates the activation of CREB1, a transcription factor crucial for synaptic plasticity and memory consolidation, thereby enhancing the production of BDNF (Crespo et al., 2005; Wu et al., 2008; Carloni et al., 2009; Han et al., 2011). Increased BDNF availability supports neurogenesis, synaptic restructuring, and overall cognitive performance (Wang et al., 2015).

1.7.4.3. *Simvastatin's Role in Enhancing Hippocampal Neurogenesis*

Simvastatin has been shown to enhance hippocampal neurogenesis, a key process for learning, memory, and overall cognitive function. Research shows that simvastatin promotes the proliferation, differentiation, and survival of neural progenitor cells in the hippocampus (Robin et al., 2014). This neurogenic effect is primarily attributed to simvastatin's modulation of molecular pathways governing cellular growth and survival. A key mechanism involves the

upregulation of BDNF, an essential neurotrophin that facilitates neuronal differentiation and synaptic plasticity (Wu et al., 2008). By increasing BDNF expression, simvastatin fosters an environment conducive to neuronal development. Additionally, its inhibition of the mevalonate pathway decreases the production of isoprenoid intermediates such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, compounds implicated in cell cycle regulation and apoptosis (Mans, McMahon and Li, 2012). Reducing their availability may lift inhibitory signals on progenitor cell proliferation (Li et al., 2012). Complementing these actions, simvastatin's antioxidant and anti-inflammatory properties counteract oxidative stress and neuroinflammation, both of which negatively impact neurogenesis. Furthermore, by enhancing nitric oxide (NO) production, simvastatin improves cerebral blood flow and endothelial function, thereby contributing to a microenvironment that supports hippocampal neurogenesiss (McGirt et al., 2002; Tan et al., 2016).

Additionally, apart from its involvement in neurogenesis, simvastatin enhances synaptic plasticity and glutamatergic signalling in the CA1 area of the hippocampus, thereby supporting improved cognitive function (Chen et al., 2016). It has also been shown to improve memory in patients with cerebrovascular damage, as well as enhance learning, memory, and LTP (Li et al., 2006; Tong, Lecrux and Hamel, 2012). Animal studies further support simvastatin's neurogenic potential. For instance, research on traumatic brain injury (TBI) has shown that simvastatin, along with atorvastatin, increases neurogenesis in the DG, reduces neuronal loss in the hippocampus by preventing oxidative stress damage, and improves spatial learning (Lu et al., 2007; Wang et al., 2007).

1.7.4.4. *Simvastatin Improves Endothelial Function*

Simvastatin has been reported to support endothelial function, especially within the central nervous system. The blood-brain barrier (BBB), an essential structure that protects the brain by controlling the movement of substances between the blood and neural tissue, is frequently disrupted in neurological conditions like stroke, Alzheimer's disease, and multiple sclerosis (Ortiz et al., 2014; Kurz et al., 2022). Simvastatin contributes to the maintenance of BBB integrity by upregulating tight junction proteins, including claudins and occludins, that are essential for preserving the cohesion of endothelial cells lining cerebral vessels (Jiang et al., 2012; Yang et al., 2013). Furthermore, simvastatin reduces the expression of matrix

metalloproteinases (MMPs), enzymes that break down the extracellular matrix and compromise the integrity of the BBB (Izidoro-Toledo et al., 2011).

Apart from these effects, simvastatin promotes endothelial cell migration and proliferation, aiding in vascular repair, particularly after ischemic events or brain injury (Pan et al., 2014). This process is crucial for neurovascular health and brain recovery. Additionally, simvastatin increases the production of NO by activating endothelial nitric oxide synthase (eNOS) (McGirt et al., 2002). NO plays a crucial role in vasodilation, thereby increasing cerebral blood flow and facilitating the supply of oxygen and nutrients to brain cells, processes that are especially important in conditions such as ischemia and neurodegenerative diseases (Wang et al., 2011; Saeedi et al., 2017). By acting through these pathways, simvastatin supports endothelial health, preserves vascular integrity, and offers protection to the brain's circulatory system. Its capacity to cross the BBB and safeguard its structure is especially advantageous in mitigating neuronal injury associated with ischemic events, such as stroke, and in the context of neurodegenerative disorders (Van Der Most et al., 2009; Sierra et al., 2011; Chang et al., 2014; Fong, 2014; Griffin et al., 2016).

These neuroprotective properties suggest that simvastatin's therapeutic potential extends well beyond its cardiovascular benefits, offering promise in the treatment of neurodegenerative diseases and cerebrovascular conditions. According to findings by Sierra et al. (2011) simvastatin exhibited the most favourable profile among statins for preventing neurodegenerative disorders, making it a strong candidate for managing various brain pathologies (Bedi et al., 2016).

1.7.5. Simvastatin and Alcohol Consumption

The potential of simvastatin to mitigate alcohol-induced brain damage is an emerging area of investigation, with early evidence indicating neuroprotective effects mediated by its anti-inflammatory, antioxidant, and cerebrovascular actions (Jafari et al., 2021a; Nasef et al., 2021a). Chronic alcohol consumption is known to lead to cognitive impairments, brain atrophy, and neuroinflammation, driven by mechanisms such as oxidative stress, glutamate toxicity, and disrupted neuronal signalling. Research indicates that simvastatin, similar to other statins, may alleviate neuroinflammation and oxidative stress, key contributors to brain damage caused by alcohol (Bell, O'Connell and Lohoff, 2022). Furthermore, statins have demonstrated

neuroprotective properties by enhancing cerebral blood flow and decreasing oxidative damage, which may help lessen the cognitive impairments and neurodegenerative effects associated with long-term alcohol consumption (Simakova et al., 2017). While animal studies have demonstrated simvastatin's protective effects against alcohol-related damage in the heart (Nchodu et al., 2024a) and kidneys (Nchodu et al., 2024b), its impact on alcohol-induced brain damage remains less explored. Therefore, additional research is needed to explore the potential of repurposing simvastatin for the treatment and management of alcohol-related brain damage.

1.8. Summary

Alcohol consumption in South Africa has a longstanding and multifaceted history, with elevated usage rates closely linked to socio-economic and cultural influences, especially among adolescents. Early alcohol use is a significant concern, as adolescence represents a critical period for brain maturation marked by extensive neurodevelopmental changes. Alcohol primarily affects the brain by modulating neurotransmitter systems, such as enhancing GABAergic activity while inhibiting glutamatergic signalling, disrupting normal neural function. In adolescents, chronic alcohol consumption is linked to both structural and functional brain impairments, especially in the hippocampus and PFC, regions crucial for learning, memory, and executive functions. Gender differences further influence susceptibility to alcohol-related brain damage, with hormonal and metabolic variations playing key roles. On a molecular level, prolonged alcohol use during adolescence contributes to neuroinflammation, oxidative stress, and impaired neurogenesis. These effects are driven by inflammatory cytokines, including IL-6 and IL-10, coupled with decreased levels of neuroprotective factors like CREB1 and BDNF. Together, these processes contribute to neurodegeneration and the deterioration of cognitive function.

Although public health initiatives and regulatory policies strive to reduce underage drinking, new therapeutic strategies are being investigated to prevent, mitigate, or reverse the harmful impacts of alcohol. Drug repurposing strategies have identified statins, particularly simvastatin, as promising candidates due to their pleiotropic effects beyond lipid-lowering. Simvastatin has shown anti-inflammatory, antioxidant, and neuroprotective properties, including enhancement of endothelial function, reduction of oxidative stress, modulation of inflammatory cytokines, and upregulation of BDNF and CREB1. Through these mechanisms, simvastatin may support hippocampal neurogenesis and protect against alcohol-induced neurodegeneration,

highlighting its potential as a therapeutic strategy for mitigating the harmful effects of adolescent alcohol exposure.

1.9. From Background to Purpose

Due to the broad lipid-lowering effects and diverse pleiotropic benefits, statins are among the most widely prescribed medications worldwide (McFarland et al., 2014; Bedi et al., 2016; Kosowski et al., 2021). Additionally, statins are generally well-tolerated and considered remarkably safe (Schachter, 2005; Hu, Cheung and Tomlinson, 2012; Maji et al., 2013). Although extensive research has investigated the neuroprotective potential of statins (Whyte et al., 2019; Kosowski et al., 2021; Nasef et al., 2021a), their impact on alcohol-induced brain damage has yet to be thoroughly examined. Consequently, this study investigated whether simvastatin could mitigate alcohol-induced brain damage by reducing oxidative stress, regulating neuroinflammatory responses, and promoting neuronal survival and neurogenesis in the hippocampus and prefrontal cortex of adolescent mice. The findings of this study will offer important insights into the potential of simvastatin to protect the brain against alcohol-related damage, with significant implications for the treatment of alcohol-induced neurodegenerative conditions.

1.10. Aims and Objectives

1.10.1. Aim

This project aimed to assess the neuroprotective effects of simvastatin against alcohol-induced brain damage by targeting oxidative stress, neuroinflammation, and capacity to promoting neurogenesis in adolescent C57BL/6J mice.

1.10.2. Specific Objectives

The specific objectives of this study were:

1.10.2.1. To investigate the effect of simvastatin on oxidative stress and antioxidant defence mechanisms in the hippocampus of adolescent mice following alcohol exposure using biochemical assays for MDA, GSH-Px and SOD.

1.10.2.2. To investigate the effect of simvastatin on oxidative stress and antioxidant defence mechanisms in the prefrontal cortex of adolescent mice following alcohol exposure using biochemical assays for MDA, GSH-Px and SOD.

1.10.2.3. To investigate the neurogenic capacity of simvastatin against alcohol-induced brain damage by determining the distribution of proliferative cells and immature neurons in the suprapyramidal blade of the dentate gyrus of the hippocampus of adolescent mice using immunohistochemistry.

1.10.2.4. To investigate the effect of simvastatin on the population of neuronal cells, the distribution of astrocytic cells, and the hippocampal volume of the dentate gyrus following alcohol exposure in adolescent mice using immunohistochemistry and stereology.

1.10.2.5. To investigate the effect of simvastatin on neuronal density in the prefrontal cortex following alcohol exposure in adolescent mice, using Nissl staining and NeuN immunolabelling in combination with stereological analysis

1.10.2.6. To investigate the anti-inflammatory and neuroprotective effects of simvastatin against the effects of alcohol by determining the expression levels of some neurotrophic markers (IL-6, IL-10, CREB1, and BDNF) in the hippocampus of adolescent mice using RT-PCR (Reverse Transcription Polymerase Chain Reaction).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Animal Model

2.1.1. Experimental Animals

The animal study received ethical approval from the Animal Research Ethics Committee (AREC) of the University of the Witwatersrand, Johannesburg, South Africa (Ethics Clearance Number: 2019/11/63/C). C57BL/6J mice aged between 4 and 8 weeks, representing the adolescent period (Lach et al., 2020) were obtained from the National Health Laboratory Services (NHLS), Johannesburg. Both female and male mice were included, given their differential responses to drugs under various disease conditions (Arnold and Chen, 2009; Miller et al., 2017). Mice of the same sex and experimental group were housed together in polycarbonate cages (200 × 200 × 300 mm) within the Wits Research Animal Facility (WRAF) at the Faculty of Health Sciences, University of the Witwatersrand. The animal room was maintained on a reversed 12-hour light/dark cycle, with lights off from 06:00 to 18:00. Environmental enrichment was provided according to WRAF's standard operating procedures (SOP) for mice including PVC pipes, suspended toys, small balls, and tissue paper for nest building. No more than four animals of the same sex and treatment group were housed per cage to minimise stress and maintain consistency across conditions.

2.1.2. Experimental Model

Similar to previous studies (Nchodu et al., 2024a; Nchodu et al., 2024b), three-week-old female and male C57BL/6J mice were allocated to five treatment groups: control (no treatment), simvastatin-only (5 mg, oral gavage), alcohol-only (intraperitoneal injection), and two combined alcohol + simvastatin groups (5 mg and 15 mg simvastatin via oral gavage followed by alcohol via intraperitoneal injection, administered 30 minutes later). Treatment details are presented in Table 2.1. In total, 100 mice (50 males and 50 females) were used in this study. Each experimental group included both sexes, with 25 males and 25 females distributed across the five groups.

The mice were acclimatized for one week before the commencement of treatment, during which they were handled daily and weighed to habituate them to human interaction and experimental procedures, thereby minimizing stress during the subsequent treatment period. Simvastatin (Cat. no: 1612700, Merck, South Africa; prepared as described by McKay et al., 2004) and pharmacological-grade absolute alcohol (99.9%; Sigma-Aldrich, South Africa; Cat. no: 24105, diluted with 0.9% NaCl to a 20% solution) were freshly prepared each day and filter-sterilized prior to administration. Oral gavage and intraperitoneal (i.p.) injections were carefully carried out by trained personnel at the Wits Research Animal Facility (WRAF) to minimise stress to the animals. Oral gavage is routinely used in developing models of different disorders, especially in alcohol-related studies (Hoggatt et al., 2010). It allows for a more precise dosage to be administered and for faster peak absorption of unpalatable compounds (e.g., simvastatin, which has a bitter taste) as compared to other methods such as voluntary feeding (Nebendahl, 2000). Treatment was administered daily for a duration of 28 consecutive days.

Mice were marked using an ear-notching system to facilitate individual identification throughout the study (Kasanen et al., 2011). Unique combinations of notches were applied to the left and/or right ears to distinguish each mouse. The identification scheme included NT (No Tag), LT (Left Tag), RT (Right Tag), and BT (Bi-Tag), corresponding to the specific placement of notches. This method allows for accurate monitoring, treatment allocation, and data collection while minimizing handling stress.

All mice were allowed unrestricted access to food and water for the duration of the study. However, to partially mimic the reduced intake associated with alcohol intoxication, food and water were withheld for approximately two hours from mice not receiving alcohol. The amount of food and water consumed per experimental group was monitored throughout the treatment period, and each mouse was weighed daily to monitor growth patterns.

Table 2.1 Experimental Design - Group Descriptions and Treatments

Experimental Group	Abbreviation	Dose	Duration
Control	C	No treatment. This group is used as a control for procedures and treatments	Nurtured for 28 days
Simvastatin only	SIM	5 mg/Kg/day Simvastatin via oral gavage	28 days
Alcohol only	ALC	2.5 g/Kg/day of 20% alcohol (via intraperitoneal injection	28 days
Alcohol + Simvastatin (5 mg)	ALC+SIM5	5 mg/Kg/day of Simvastatin via oral gavage, followed by 2.5 g/Kg/day of 20% alcohol via intraperitoneal injection	28 days
Alcohol + Simvastatin (15 mg)	ALC+SIM15	15 mg/Kg/day Simvastatin via oral gavage, followed by 2.5 g/Kg/day of 20% alcohol via intraperitoneal injection	28 days

2.1.3. Determination of Blood Alcohol Concentration (BAC)

On the last day of treatment, blood samples were collected 30 minutes after the intraperitoneal alcohol injection from all mice that received alcohol. This time point was selected based on evidence that blood alcohol concentration (BAC) typically peaks around 30 minutes post-administration (Donovan, 2009; Mitchell, Teigen and Ramchandani, 2014). A small incision was made at the saphenous vein on the hind leg, and approximately 50 µL of blood was collected from each animal into heparinized capillary tubes. Samples were stored at 4 °C overnight before centrifugation using Vivaspin500© 100 µm membrane tubes (Biotech, South Africa) at 5000 rpm for 15 minutes to isolate serum. BAC levels were then determined using the EnzyChrom™ Ethanol Assay Kit (Cat no: MAK076-1KT, BioVision, South Africa). The average BAC measured ranged between 178.85 and 384.36 mg/dL.

2.2. Animal Sacrifice and Brain Tissue Collection

2.2.1. Immunohistochemical Processes

On postnatal day 56 (PND 56), following the final treatment, mice allocated for immunohistochemical analysis were weighed and euthanized with an intraperitoneal injection of Euthanaze (sodium pentobarbital, 80 mg/kg). This was followed by transcardial perfusion with cold 0.9% saline, immediately succeeded by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were extracted, weighed, and post-fixed in 4% PFA (0.1 M PB) for 48 hours. Tissues were then cryoprotected in a 30% sucrose solution in PB for an additional 48 hours and subsequently stored in antifreeze solution at 4 °C until further processing. For downstream analyses, 50 brains (left hemispheres) were used for neurogenesis (PcNA and DCX), and the corresponding 50 right hemispheres were processed for NeuN, GFAP, and Nissl staining. The prefrontal cortex was dissected from the same animals for parallel studies, ensuring efficient use of tissue across immunohistochemical endpoints.

Histological and stereological assessments were conducted using standardized protocols and consistent anatomical landmarks across all groups. Analyses were not performed blinded to treatment allocation. While this approach allowed for efficient tissue utilization and consistency of data acquisition, the absence of blinding is acknowledged as a limitation. To minimize potential observer bias, all staining procedures were carried out under uniform conditions, multiple technical replicates were used, and standardized counting criteria were

applied. Future studies will incorporate blinded analyses to further reduce the risk of observer bias

2.2.2. Biochemical Assays and Molecular Analyses

At the end of the treatment period, on postnatal day 56 (PND 56), mice designated for biochemical and molecular analyses were decapitated following the standard operating procedures (SOP) of the Wits Research Animal Facility (WRAF). Brains were promptly extracted, weighed, and documented. Each brain was divided into left and right cerebral hemispheres in an ice-cold 0.1 M phosphate-buffered (PB) solution under a dissecting microscope. To isolate the prefrontal cortex (PFC), the olfactory bulb at its anterior margin was carefully removed, followed by a cut anterior to the corpus callosum. The prefrontal cortex was immediately removed and snap-frozen. For hippocampal isolation, the cerebellum was first removed, after which the midbrain and thalamus were carefully dissected to expose the hippocampus. The hippocampus was quickly snap-frozen in liquid nitrogen and then stored at −80 °C until further analysis. Of these brains, the left hemispheres from 50 animals were analysed for biochemical assays, while the corresponding right hemispheres were used for RT-PCR. This strategy optimised tissue utilisation and enabled parallel biochemical and molecular profiling from the same animals

2.3. Hippocampal Brain Region Selection

For all analyses, the left hemisphere of the hippocampus was used for neurogenesis assessment and biochemical assays (MDA, GSH-Px, and SOD), while the right hemisphere was used for neuroinflammation analyses and RT-PCR targeting IL-6, IL-10, CREB1, and BDNF. This division was implemented to optimize tissue utilization and minimize variability within each assay. Biochemical assays were paired with neurogenesis studies due to the important role of oxidative stress in neuronal survival and plasticity. In parallel, the analysis of inflammatory markers and gene expression profiling provided insight into immune responses and neuroplasticity. This approach ensured efficient use of tissue and supported a comprehensive investigation of alcohol and simvastatin effects on hippocampal plasticity and degeneration.

2.4. Biochemical Analyses of Oxidative Stress in the Hippocampus and Prefrontal Cortex

2.4.1. Tissue Processing and Protein Quantification

Snap-frozen hippocampus or PFC samples from the left cerebral hemisphere were retrieved from storage, accurately weighed, and gently thawed on ice. Once thawed, each tissue was homogenized in cold 0.01 M PBS (pH 7.4) at a 1:9 (weight/volume) ratio using a handheld tissue homogenizer (Tissue Grinder, Model: G50, Cat no: 2002000301, Coyote Bioscience Co., LTD., China). The homogenized tissue-PBS solution was then centrifuged at 10,000 rpm for 10 minutes in Eppendorf tubes. Following centrifugation, the clear supernatant was carefully collected, and total protein content was determined using the Pierce™ BCA Protein Colorimetric Assay Kit (Cat. no. 23227, ThermoFisher Scientific) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the standard to generate the standard curve. Protein concentration was measured using a microplate reader (A51119500C, Multiskan SkyHigh Microplate Spectrophotometer, ThermoFisher Scientific) at a wavelength of 562 nm. These protein concentrations were subsequently used for the biochemical analyses of oxidative stress.

2.4.2. Malondialdehyde (MDA) Levels

MDA levels, serving as a marker of oxidative stress, were quantified in adolescent hippocampal and PFC tissues using a commercially available colorimetric assay kit (Elabscience®, Cat no: E-BC-K025-M), following the manufacturer's protocol. Briefly, 0.02 mL aliquots of standards, samples, and controls were placed into labelled 1.5 mL Eppendorf tubes. Subsequently, 0.02 mL of clarificant reagent was added to each tube, followed by 0.6 mL of acid application solution. Chromogenic application solution (0.2 mL) was added to the standard and sample tubes, while control tubes received 0.2 mL of 50% acetic acid instead. After sealing the tubes with Parafilm and mixing thoroughly, a small vent was created in each lid to release pressure. The tubes were then incubated in a 100 °C water bath for 40 minutes. Once incubation was complete, samples were cooled to room temperature and centrifuged at 10,000 rpm for 10 minutes. An aliquot of 0.25 mL of the resulting supernatant, free from precipitate, was carefully transferred to a microplate for absorbance measurement at 532 nm using a microplate reader. Quantification of MDA concentrations was performed based on a generated standard curve. The following formula was applied to calculate the MDA content in each sample:

$$\text{MDA (μmol/gprot)} = (\Delta A - b) \div a \times f \div \text{Cpr}$$

Where:

- ΔA = absolute optical density (OD), ($OD_{sample} - OD_{blank}$)
- a = slope of the standard curve
- b = y-intercept of the standard curve
- f = dilution factor of the sample before testing
- Cpr = concentration of the protein sample (g protein/L)
- MDA levels were expressed in micromoles per gram of protein (μmol/g protein)

2.4.3. Glutathione Peroxidase (GSH-Px) Activity

GSH-Px activity was determined using a commercially available assay kit (Cat. no: E-BC-K096-M; Elabscience®) following the manufacturer's protocol. Tissue homogenate aliquots were utilized for the measurement. This assay is based on the enzymatic function of GSH-Px, which catalyzes the reduction of hydrogen peroxide (H_2O_2) by oxidizing reduced glutathione (GSH) to glutathione disulfide (GSSG). For the reaction setup, 20 μL of a 1 mmol/L GSH standard was added to labelled 1.5 mL Eppendorf tubes. In the enzyme reaction tubes, 20 μL of the GSH standard and 20 μL of the sample were combined and mixed thoroughly. Both the tubes and the stock reagent were pre-incubated at 37 °C for 5 minutes. Following this, 10 μL of the stock reagent was added to the enzyme tubes, and the reaction proceeded for an additional 5 minutes at 37 °C. For the control tubes (non-enzyme), 200 μL of acid reagent and 20 μL of sample were added; the enzyme tubes contained 200 μL of acid reagent. The tubes were then mixed and centrifuged at 3100 rpm for 10 minutes. A 100 μL volume of the clear supernatant was carefully collected for the chromogenic assay. Subsequently, 100 μL aliquots from both enzyme and non-enzyme tubes were transferred to separate wells of a microplate. Wells containing GSH standard solutions at different concentrations served as standards. To each well, 100 μL of phosphate buffer and 50 μL of DTNB reagent were added, followed by incubation at room temperature for 5 minutes. Absorbance readings were taken at 412 nm using a microplate reader. GSH-Px activity in the hippocampus or prefrontal cortex tissue was calculated by reference to a standard curve and quantified using the equation below:

$$\text{GSH-Px activity (U/mgprot)} = (\Delta A412 - b) \div a \times [0.23+V / 0.03+V] \div (V \times Cpr) \times f$$

Where:

- ΔA_{412} = The absolute OD value of the sample ($OD_{Non\text{-}enzyme\ tube}$-$OD_{Enzyme\ tube}$)
- (0.23+V)/(0.03+V) = Dilution factor of sample in enzymatic reaction
- V = The volume of sample added to the reaction system
- f = Dilution factor of the sample before testing
- Cpr = Concentration of protein in the sample (mg protein/mL)
- GSH-Px activity was expressed in units per milligram of protein (U/mg protein)

2.4.4. Superoxide Dismutase (SOD) Activity

SOD activity was assessed using a commercial SOD Determination Kit (Cat no: 19160, Sigma-Aldrich), which employs the water-soluble tetrazolium salt WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt]. This salt is reduced by the superoxide anion to form a water-soluble formazan dye. The reduction rate in the presence of oxygen (O_2) reflects xanthine oxidase (XO) activity and is inhibited by SOD. For the assay, aliquots of tissue homogenates were used. Briefly, 20 µL of each sample was pipetted into the Sample and Blank 2 wells, while 20 µL of ultrapure water was added to Blank 1 and Blank 3 wells. Subsequently, 200 µL of WST Working Solution was added to all wells and mixed carefully. Next, 20 µL of Dilution Buffer was added to the Blank 2 and Blank 3 wells. To initiate the enzymatic reaction, 20 µL of Enzyme Working Solution was added to the Sample and Blank 1 wells, followed by thorough mixing. The microplate was then incubated at 37 °C for 20 minutes. Absorbance was read at 450 nm using a microplate reader after incubation. The percentage inhibition representing SOD activity in hippocampal and prefrontal cortex tissue was calculated according to the following equation:

$$\text{SOD activity (inhibition rate \%)} =$$

$$[(A_{Blank\ 1} - A_{Blank\ 3}) - (A_{Sample} - A_{Blank\ 2})] / (A_{Blank\ 1} - A_{Blank\ 3}) \times 100\%.$$

SOD activity was expressed as units per millilitre (U/mL).

2.5. Immunohistochemical Analyses to Determine the Distribution of Proliferative and Immature Neurons in the Suprapyramidal Blade of the Dentate Gyrus of the Hippocampus

2.5.1. Tissue Processing

Brain samples were cryoprotected in a cold 30% buffered sucrose solution before further processing. Ten animals per group (n = 50; balanced 50% male/female ratio) were included in the analysis. The left cerebral hemisphere was cut into 50 µm sagittal sections using a cryostat. One to three serial free-floating sections were collected and placed into 24-well plates containing 0.01 M phosphate buffer (PB). Endogenous peroxidase activity was inhibited by incubating the sections in a solution composed of 49.2% methanol, 49.2% 0.1 M PB, and 1.6% 30% hydrogen peroxide for 30 minutes at room temperature (~37 °C), followed by three washes of 10 minutes each in 0.01 M PB. To block nonspecific binding sites, the sections were pre-incubated for two hours at room temperature with gentle agitation in blocking buffer consisting of 0.25% Triton X-100 in 0.1 M PB, 3% normal goat serum, and 2% bovine serum albumin (BSA). Every third section was immunolabeled with either anti-proliferating cell nuclear antigen (PcNA) antibody (1:200 mouse anti-PcNA; Cat no: MAB424R, Merck), which targets proliferating cells in the G1/S phase, or anti-doublecortin (DCX) antibody (1:2000 rabbit anti-DCX; Cat no: ab18723, Abcam), a marker for immature neurons indicating newly generated cells. Immunolabelling was performed over 48 hours at 4 °C under gentle agitation. Following primary antibody incubation, sections were exposed to the corresponding biotinylated secondary antibodies for two hours at room temperature with gentle shaking: goat anti-mouse IgG (1:1000; Cat no: BA-9200, Vector Labs) for PcNA or goat anti-rabbit IgG (1:1000; Cat no: BA-1000, Vector Labs) for DCX. Subsequently, sections were treated with avidin-biotin complex solution (1:125; Vectastain® Elite® ABC-HRP Kit, Cat no: PK-6100) for one hour. Visualization of immunoreactivity was achieved by immersing sections in 0.05% diaminobenzidine (DAB) in 0.1 M PB for 15 minutes, followed by the addition of 3.3 µL of 30% hydrogen peroxide per mL of DAB solution to develop the chromogen. The reaction was stopped by adding 2 mL of 0.1 M PB at room temperature. Finally, sections were mounted on gelatin-coated (0.5%) glass slides, air-dried, dehydrated through graded alcohols, cleared in xylene, and coverslipped using DPX mounting medium.

Sections designated for Nissl staining were initially mounted onto slides and allowed to dry. They were subsequently subjected to a defatting procedure by immersion in a 1:1 mixture of chloroform and ethanol (DEFAT solution) overnight to remove residual lipids and enhance stain penetration. Following defatting, sections were rehydrated through a graded ethanol series, beginning with two 5-minute incubations in absolute ethanol (100%), followed by sequential 2-minute incubations in descending ethanol concentrations (95%, 70%, and 50%) to achieve gradual rehydration of the tissue. The rehydrated sections were then stained with cresyl violet for 2 minutes, selectively binding to the Nissl substance (comprising rough endoplasmic reticulum and ribosomes) within neuronal somata to facilitate cellular visualization. Excess stain was removed by rinsing in distilled water for 2 minutes. Subsequent dehydration was performed by reversing the ethanol gradient with 2-minute washes in 50%, 70%, and 95% ethanol, followed by two 5-minute washes in 100% ethanol. The sections were cleared in xylene with two 5-minute incubations to render the tissue transparent and compatible with mounting media. Finally, sections were coverslipped for histological examination under light microscopy.

2.5.2. Distribution of PcNA- and DCX-Positive Cells in the Suprapyramidal Blade of the Dentate Gyrus

The quantification of PcNA- and DCX-positive cells within the suprapyramidal blade of the DG was performed for all experimental groups, stratified by sex (Fig. 2.1). To evaluate the spatial distribution of immunopositive cells within each section, tissue sections were digitized using a VS200 Slide Scanner (Evident, USA). The resulting high-resolution images were analysed with QuPath software (Version 0.5.0; USA). Adopting the methodology described by Olateju et al. (2021), PcNA- and DCX-immunopositive cells were manually counted along the entire rostro-caudal axis of the suprapyramidal blade utilizing the counting point annotation tool. Cell counts were restricted to those located within or in direct contact with the subgranular zone (SGZ) (Fig. 2.1A). Concurrently, the length of the suprapyramidal blade (expressed in micrometers; µm) was measured using the polyline annotation tool in QuPath (Fig. 2.1B). The density of positively stained cells in each section was then calculated by dividing the total number of PcNA- or DCX-positive cells by the measured blade length.

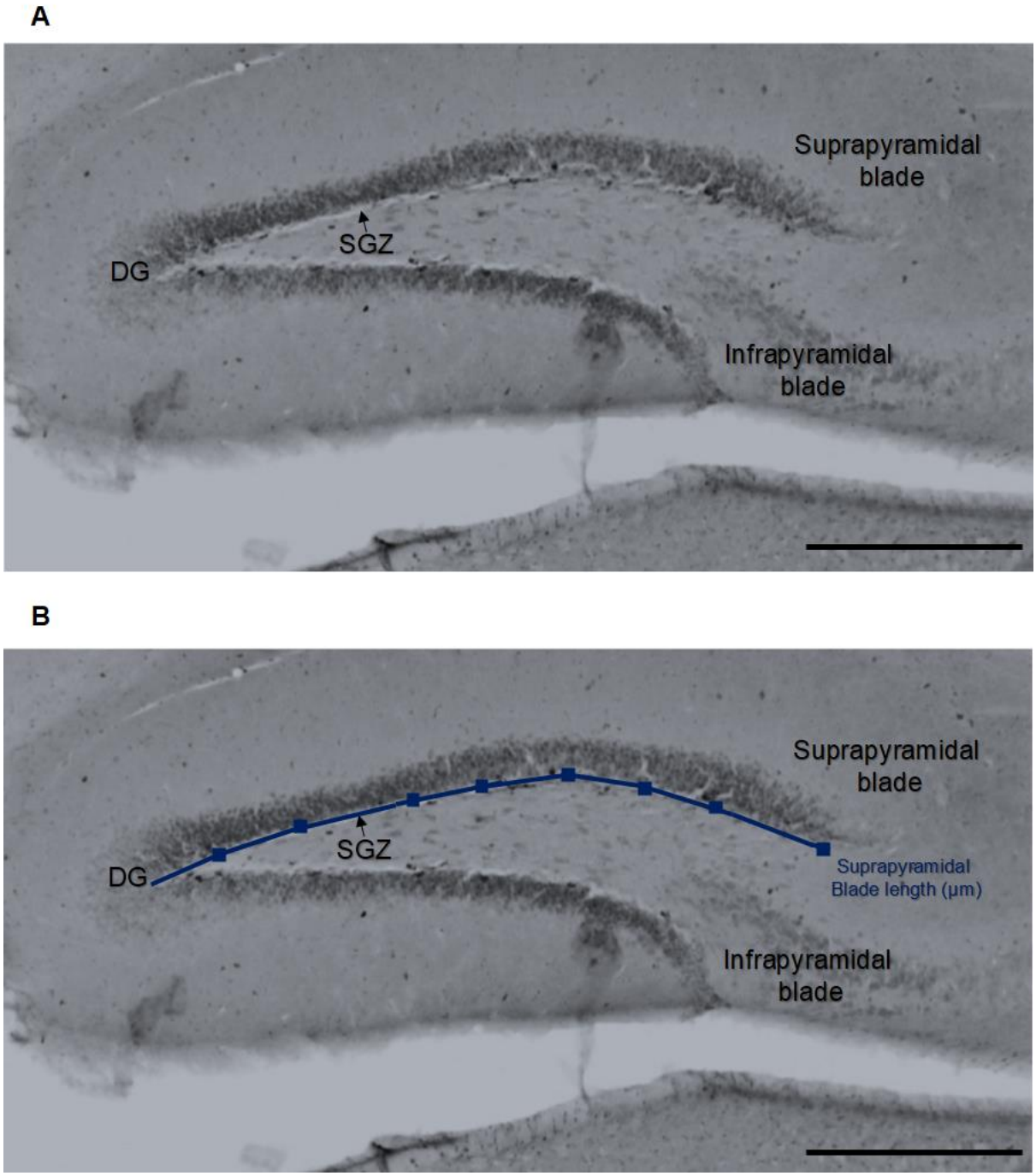


Figure 2.1: PcNA-Immunolabelled Hippocampal Sections Demonstrating Qupath Software Analysis. A) Representative photomicrograph of a hippocampal section from a control animal, illustrating the dentate gyrus (DG), including the suprapyramidal and infrapyramidal blades, as well as the subgranular zone (SGZ). B) Illustration of suprapyramidal blade length measurement (µm) performed using the polyline annotation tool in QuPath, as denoted by the blue line. Scale bar = 200 µm.

2.6. Immunohistochemical and Histological Analyses to Estimate the Population of Neuronal Cells, the Distribution of Astrocytic Cells, and the Hippocampal Volume

2.6.1. Tissue Processing

As detailed in Section 2.5.1, brain samples were cryoprotected by immersion in a 30% buffered sucrose solution prior to further processing. A total of ten animals per group (n = 50; 50% male/female ratio) were included in the analysis. The right cerebral hemispheres were coronally sectioned at a thickness of 50 µm using a cryostat. One to three serial free-floating sections were collected and transferred to 24-well plates containing 0.01 M phosphate buffer (PB). To block endogenous peroxidase activity, sections were incubated in a solution containing 49.2% methanol, 49.2% 0.1 M PB, and 1.6% 30% hydrogen peroxide for 30 minutes at room temperature, followed by three 10-minute washes in 0.01 M PB. Non-specific antibody binding was minimized by pre-incubation in a blocking solution comprising 0.25% Triton X-100 in 0.1 M PB, 3% normal goat serum, and 2% bovine serum albumin for two hours at room temperature under gentle agitation. Every third section was immunolabeled for either Glial Fibrillary Acidic Protein (GFAP), a marker for astrocytes (1:1000 rabbit anti-GFAP; Cat. no: AB5804, Merck Millipore), or NeuN, a marker of mature neurons (1:500 rabbit anti-NeuN, Cy3 conjugated; Cat. no: ABN78C3, Sigma), for 48 hours at 4 °C under gentle shaking. Subsequently, sections were incubated in a biotinylated secondary antibody solution (1:1000 goat anti-rabbit IgG; Cat. no: BA-1000, Vector Labs) for two hours at room temperature. Sections were then treated with an avidin–biotin complex (1:125; Vectastain® Elite® ABC-HRP Kit, Peroxidase; Cat. no: PK-6100) for one hour. Chromogenic detection was achieved by incubation in 0.05% 3,3'-diaminobenzidine (DAB) in 0.1 M PB for 15 minutes, followed by the addition of hydrogen peroxide to initiate the reaction. The reaction was terminated with the addition of 2 mL of 0.1 M PB. Sections were mounted on 0.5% gelatin-coated glass slides, air-dried at room temperature, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped using DPX mounting medium.

Serial sections designated for Nissl staining were first mounted onto 0.5% gelatin-coated slides and air-dried. Before staining, the sections were defatted overnight in a 1:1 mixture of chloroform and ethanol to remove lipids and enhance staining quality. Thereafter, sections were rehydrated through a descending alcohol series, stained with cresyl violet for two minutes, and rinsed in distilled water. Following staining, sections were dehydrated in an ascending alcohol series, cleared in xylene, and coverslipped for microscopic evaluation.

2.6.2. Distribution of GFAP-Positive Cells in the Suprapyramidal Blade of the Dentate Gyrus

As described in Section 2.5.2, the number of GFAP-immunopositive cells in the right suprapyramidal blade of the DG was quantified across experimental groups in both sexes. To assess the distribution of GFAP-positive cells, tissue sections were scanned using a VS200 Slide Scanner and analysed with QuPath software (Version 0.5.0). GFAP-positive cells within the granule cell layer (GCL) and in contact with the subgranular zone (SGZ) were counted along the entire rostro-caudal extent of the suprapyramidal blade using the software's counting point annotation tool (Fig. 2.2A). Blade length (in μm) was measured using the polyline annotation tool (Fig. 2.2B), following the method described by Olateju et al. (2021). The distribution of GFAP-positive cells was expressed as the number of immunopositive cells per unit length of the suprapyramidal blade.

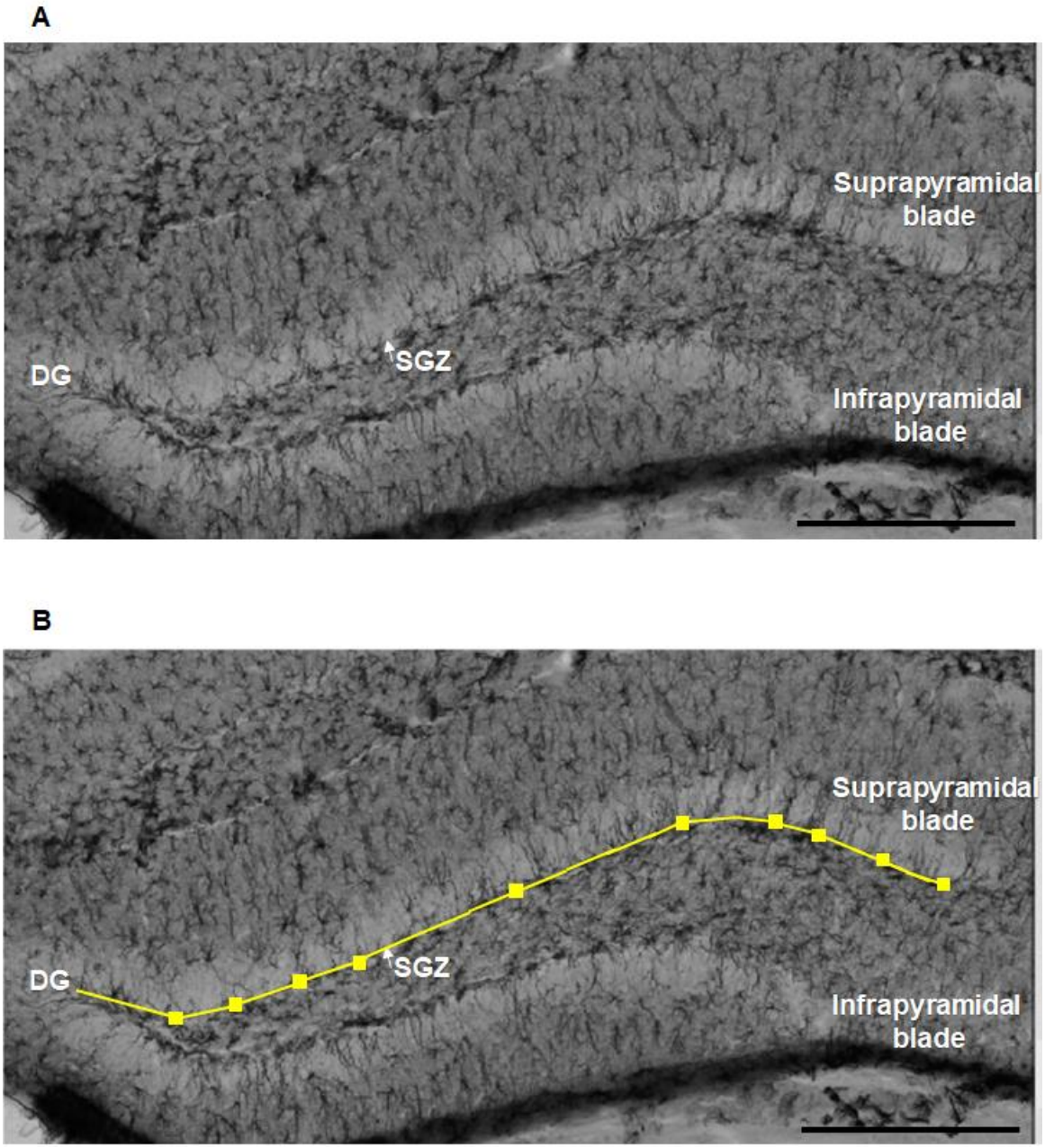


Figure 2.2: GFAP-Immunolabelled Hippocampal Sections Demonstrating QuPath Software Analysis. A) Representative photomicrograph of a control hippocampal section illustrating the dentate gyrus (DG), including the suprapyramidal and infrapyramidal blades and the subgranular zone (SGZ). B) Length of the suprapyramidal blade (in µm) measured using the polyline annotation tool in QuPath, as indicated by the yellow line. Scale bar = 200 µm.

2.6.3. Estimation of the Population of NeuN-Immunolabelled Neurons in the Suprapyramidal Blade of the Dentate Gyrus

An unbiased, design-based stereological method incorporating systematic random sampling was employed to estimate the total number of NeuN-immunolabeled neurons in the suprapyramidal blade of the DG across experimental groups and both sexes (Fig. 2.3). NeuN immunostaining was used to specifically label mature neurons within hippocampal subregions. Figure 2.3A presents a representative hippocampal section with the DG, including the suprapyramidal and infrapyramidal blades, delineated. Figure 2.3B highlights the traced suprapyramidal blade, outlined in blue. Figure 2.3C illustrates the application of systematic random sampling using the optical fractionator method. Stereological analyses were conducted using an MBF Bioscience system (LLC, Williston, VT, USA), which included a Zeiss Z2 Axio Imager Vario microscope equipped with a three-axis motorized stage and controlled by Stereo Investigator software (version 2018.1.1.1; 64-bit, MBF). The optical fractionator probe was employed to obtain unbiased estimates of neuron number.

Preliminary pilot studies were conducted to optimise the stereological parameters, including the counting frame dimensions (45 µm × 45 µm), sampling grid size (100 µm × 100 µm), and section thickness (50 µm), to ensure that the coefficient of error (CE) remained within acceptable thresholds (Gundersen, 1988). The dissector height (Z) was established at 14 µm, and estimation precision was assessed using the Gundersen CE with a smoothness factor of m = 1, assuming a uniform distribution of neurons. A CE value of ≤ 0.10 was considered acceptable, ensuring the reliability and reproducibility of the NeuN-positive cell estimates (Gundersen, 1988). All stereological analyses were performed using a 20× objective lens. A summary of the number of animals per group, total number of sections per group, and average number of sampling sites per group used for NeuN-positive cell quantification is shown in Table 2.2.

For stereological analyses, the average defined mounted thickness (40 µm per group) was applied rather than measuring the actual section thickness before counting the cells. This approach was selected to minimize variability introduced by tissue compression, mounting medium effects, and sectioning artifacts, which can cause inconsistencies when measuring individual section thicknesses. Using a standardized thickness value across all samples ensured consistent application of the optical dissector method for unbiased cell counting. Measuring

the actual thickness for each section before analysis is time-consuming and may introduce additional variability due to fluctuations in section integrity. The use of average defined mounted thickness aligns with established stereological protocols (Theofilas et al., 2014; Mehrabi et al., 2018), allowing for reliable and reproducible NeuN-positive cell quantification in the suprapyramidal blade of the DG.

Following the quantification of NeuN-positive cells, neuronal density was calculated to enable standardized comparisons of neuronal abundance across sexes and experimental groups. The density of NeuN-positive neurons, expressed in cells per cubic millimeter (cells/mm^3), was calculated by dividing the stereologically estimated cell count by the sampled volume (μm^3) of the respective brain region. Sampled volume was calculated using the optical fractionator principle, which incorporates the counting frame, sampling grid, dissector height, and the average mounted section thickness. This method ensured accurate and reproducible estimates while minimizing variability introduced by tissue processing. It was applied consistently in both the hippocampus and PFC to assess neuronal populations in response to alcohol exposure and simvastatin treatment.

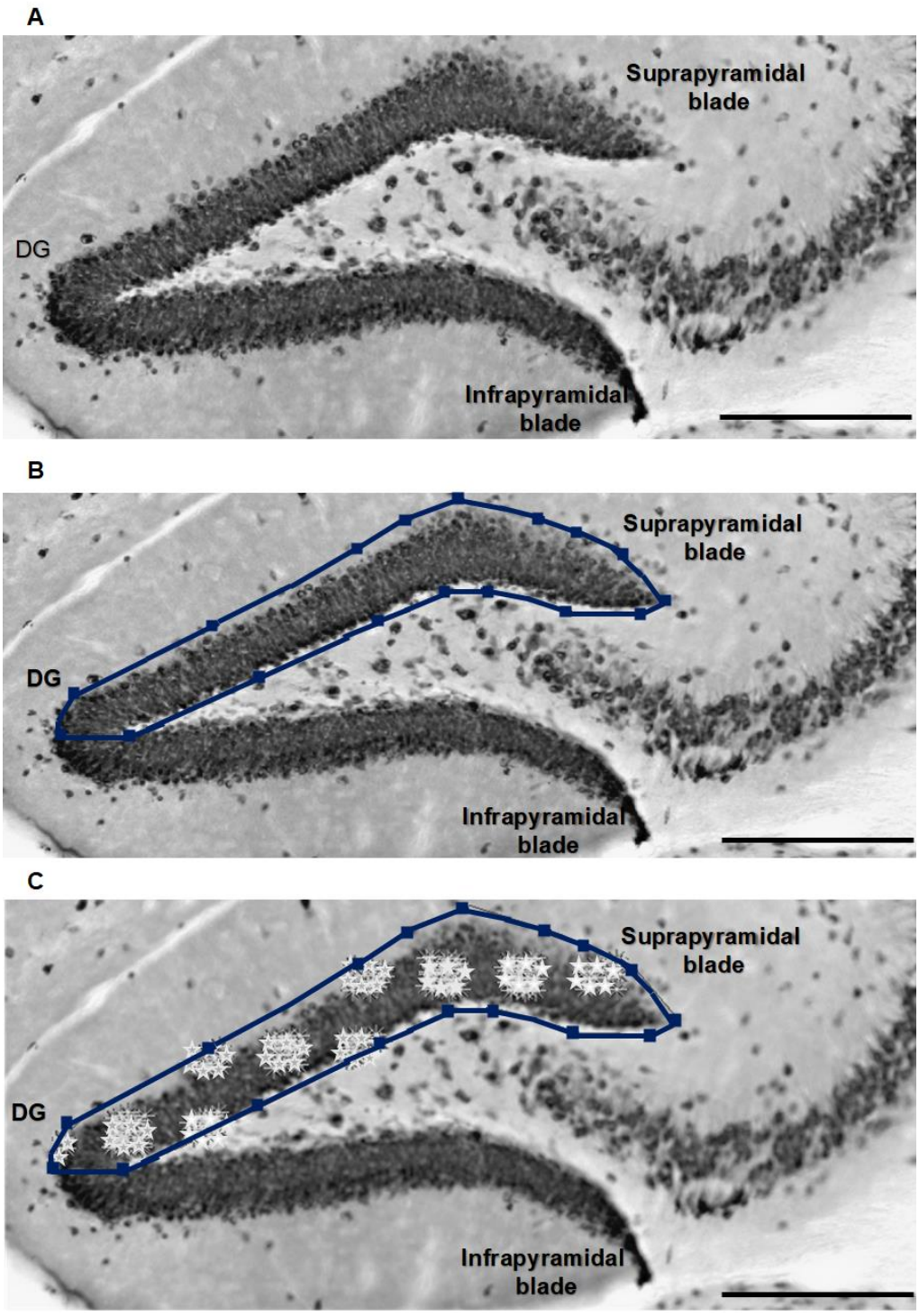


Figure 2.3: NeuN-Immunolabelled Hippocampal Sections Demonstrating Stereological Analysis. A) Representative photomicrograph of a control hippocampal section showing the delineated dentate gyrus (DG) including the suprapyramidal and infrapyramidal blades. B) Representative photomicrograph highlighting the traced suprapyramidal blade (outlined in blue) used for analysis. C) Example section illustrating systematic random sampling with the optical fractionator method. Stars (*) indicate positively immunolabelled neurons counted within the counting frames. Magnification: 20× objective. Scale bar: 200 µm.

Table 2.2 Animals, Sections per Animal, and Total Sections Used for NeuN-Positive Cell Quantification in the Suprapyramidal Blade of the Dentate Gyrus by Treatment Group and Sex

Treatment Group	Number of Animals per Group	Total Number of Sections Per Group	Average Number of Sampling Sites per Group
Female			
Control	5	30	141
SIM	4	27	119
ALC	5	30	131
ALC+SIM5	5	30	142
ALC+SIM15	5	29	143
Male			
Control	5	30	111
SIM	5	30	116
ALC	5	30	107
ALC+SIM5	5	30	121
ALC+SIM15	5	30	108

2.6.4. Volume Estimation in the Dentate Gyrus (Suprapyramidal Blade) Using the Cavalieri Method

Volume estimation of the DG was carried out using the Cavalieri method, a well-established and commonly applied stereological approach for quantifying tissue volumes in biological samples (Golub et al., 2015). Every one in three serial coronal brain sections (50 μm) was collected throughout the hippocampus, ensuring a representative sample of the entire structure. Each section was stained with Cresyl violet to enhance contrast and allow for accurate identification of the hippocampal regions. Identification of hippocampal subregions, including the suprapyramidal blade, was guided by anatomical references from the Allen Mouse Brain Atlas (Allan Institute for Brain Science, 2004) as shown in a representative Nissl-stained hippocampal section (Fig. 2.4A).

Systematic, random sampling was performed on a series of sections obtained from the entire hippocampus. The sampling interval was optimized to balance statistical efficiency while accounting for the practical limitations of tissue preparation. The areas of hippocampal regions, including the suprapyramidal blade (outlined in red) and the entire dentate gyrus (outlined in yellow), were measured using a point grid overlay on each section. Grid points were systematically measured to estimate the area of each section (Fig. 2.4B). To determine the total volume, the measured cross-sectional areas from each sampled section were added together and multiplied by the interval between sections (t = 50 μm), following this formula:

$$V = \Sigma A \times t$$

Where:

- V = total volume
- ΣA = sum of the measured cross-sectional areas
- t = distance between sections

Volume estimation for Nissl-stained sections in the hippocampus followed the same approach as described for NeuN-immunolabelled sections in Section 2.6.3. Briefly, the analysis was conducted using the MBF Bioscience system and Stereo Investigator software, as detailed in Section 2.6.3. To correct for potential over-projection errors caused by section thickness and tissue shrinkage during processing, a correction factor was applied to the calculated volume.

This adjustment accounted for tissue loss during sectioning and processing, providing a more accurate volume estimate. To assess the reliability of the volume estimates, the Gundersen coefficient of error (CE) was employed as a measure of their variability. A Gundersen CE value of ≤ 0.10 was considered acceptable, ensuring the reliability and reproducibility of the volume measurements (Gundersen, 1988). The corrected volume of the entire DG was then used for subsequent analyses. The average number of sections per animal and the total number of sections per experimental group are summarised in Table 2.3. Pilot studies were conducted to refine and optimise stereological parameters for improved reliability. Tissue sections were prepared at a thickness of 50 µm, and a sampling grid measuring 50 µm by 50 µm was applied.

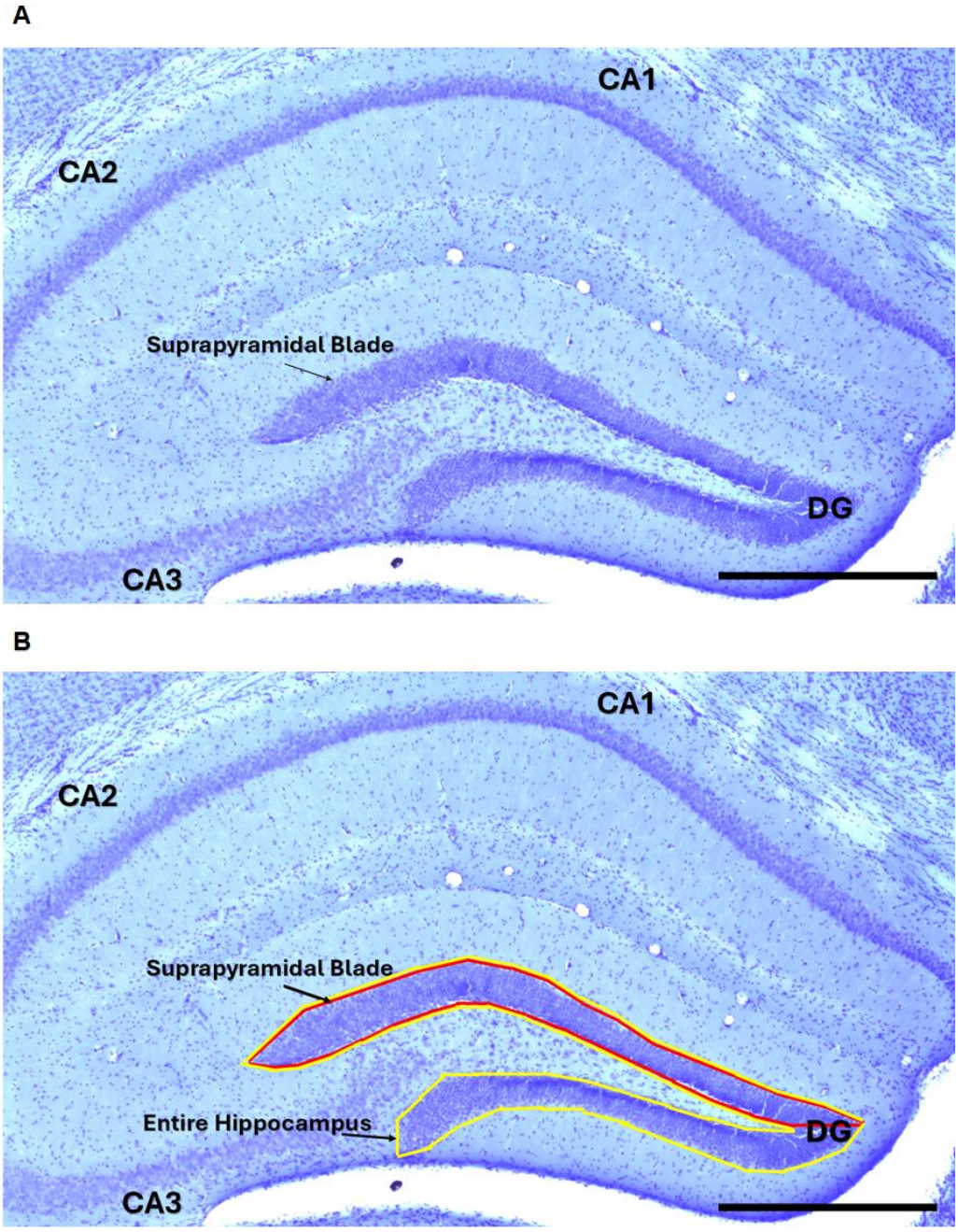


Figure 2.4: Nissl-Stained Hippocampal Sections Demonstrating Stereological Volume Estimation Using The Cavalieri Principle.

A) Representative photomicrograph of a Nissl-stained hippocampus from the control experimental group showing the delineated regions for volume analysis, including the dentate gyrus (DG), cornu ammonis 1 (CA1), cornu ammonis 2 (CA2), cornu ammonis 3 (CA3), and the suprapyramidal blade. B) The traced areas for volume analyses using the Cavalieri principle, where the suprapyramidal blade is outlined in red and the entire DG in yellow. Scale bar = 200 µm.

Table 2.3 Summary of the number of sections analysed per animal and the total number of sections per experimental group for the suprapyramidal blade and entire dentate gyrus in both sexes

Treatment Group	Sex	Suprapyramidal blade		Entire Dentate Gyrus	
		Average Number of Sections per Animal	Total number of sections per group	Average Number of Sections per Animal	Total number of sections per group
Control	Female	9.17	55	9.17	55
	Male	10.17	61	10.17	61
SIM	Female	10.03	60	10.20	51
	Male	9.67	58	9.67	58
ALC	Female	10.50	63	10.60	53
	Male	10.44	63	10.60	53
ALC+SIM5	Female	9.58	58	9.40	47
	Male	9.07	54	8.80	44
ALC+SIM15	Female	9.10	55	9.00	45
	Male	8.85	53	8.80	44

2.7. Immunohistochemical and Histological Analyses to Estimate the Population of Neuronal Cells in the Prefrontal Cortex

2.7.1. Tissue Processing

The immunohistochemical staining protocol for NeuN immunolabelling in the PFC followed the same procedure as described in Section 2.6.1 for the hippocampus. Briefly, brain samples were cryoprotected in a 30% buffered sucrose solution before sectioning. Coronal brain slices (50 μm thick) from the right hemisphere were sectioned using a cryostat and maintained as free-floating samples in 0.01 M phosphate buffer. Immunohistochemistry was carried out, involving steps such as peroxidase activity suppression, antigen retrieval, and incubation with the primary antibody (rabbit anti-NeuN, Cy3-conjugated, 1:500 dilution; Cat. no. ABN78C3, Sigma) followed by a secondary biotinylated goat anti-rabbit IgG antibody (1:1000 dilution; Cat. no. BA-1000, Vector Laboratories). Chromogen development, tissue mounting, dehydration, and coverslipping followed the same procedures. Nissl staining with Cresyl violet was performed on designated serial sections to assess general brain morphology.

2.7.2. Stereological Estimation of Neuronal Populations in Cortical Layers I–IV of the Prefrontal Cortex Following Alcohol Exposure and/or Simvastatin Treatment Using Nissl Staining and NeuN Immunolabelling

A design-based stereological method, employing systematic random sampling, was applied to estimate the total population of Nissl-stained and NeuN-immunopositive cells in the PFC. This unbiased approach allows for accurate and representative quantification, independent of cellular size, form, or spatial arrangement. Anatomical boundaries of the PFC were defined using the criteria outlined by Conte et al. (2019) and the "Allen Mouse Brain Atlas" (Allan Institute for Brain Science, 2004). The delineation of the PFC is illustrated in Fig. 2.5 and was applied to both NeuN-positive (Fig. 2.5A) and Nissl-stained (Fig. 2.5B) sections.

The stereological assessment of Nissl-stained and NeuN-immunolabelled neuronal populations in the PFC was performed using the same methodology outlined for the hippocampus in Section 2.6.3. This analysis utilized the MBF Bioscience system alongside Stereo Investigator software, as previously described in Section 2.6.3.. The optical fractionator probe was used to obtain neuron counts, with pilot studies conducted to optimize sampling parameters, including counting frame dimensions, grid spacing, and dissector height. A counting frame of 50 × 50

µm, a sampling grid of 75 × 75 µm, a section cut thickness of 50 µm, and a dissector height of 10 µm were applied to ensure efficient and unbiased sampling. These parameters were applied to both Nissl-stained and NeuN-immunolabelled sections of the PFC. The Gundersen coefficient of error (CE) with a smoothness factor of m = 1 (Gundersen, 1988) was used to assess the precision of the estimates, with a CE value of ≤ 0.10 considered acceptable. All stereological analyses were performed using a 20× objective lens. Table 2.4 provides a summary of the stereological parameters, including the group sample sizes, the mean number of sampling sites per group, and the average measured mounted section thickness.

Neuronal density was calculated to enable standardized comparisons of neuronal abundance across experimental groups and sexes. After quantifying NeuN-positive cells in the PFC, cell density (cells/mm³) was calculated by dividing the stereologically estimated neuronal count by the volume estimated for the corresponding brain area. A similar approach was applied to Nissl-stained sections to assess overall neuronal populations, where neurons were identified based on soma size, morphology, and the presence of a prominent nucleolus to distinguish them from glial cells. The volume sampled (µm³) for analyses based on both NeuN immunolabelling and Nissl staining was calculated using the optical fractionator method outlined in Section 2.6.3, which included factors such as counting frame size, sampling grid dimensions, dissector height, and mean mounted tissue thickness. This method ensured that density values were standardised and minimally influenced by tissue processing variability, facilitating meaningful cross-group comparisons.

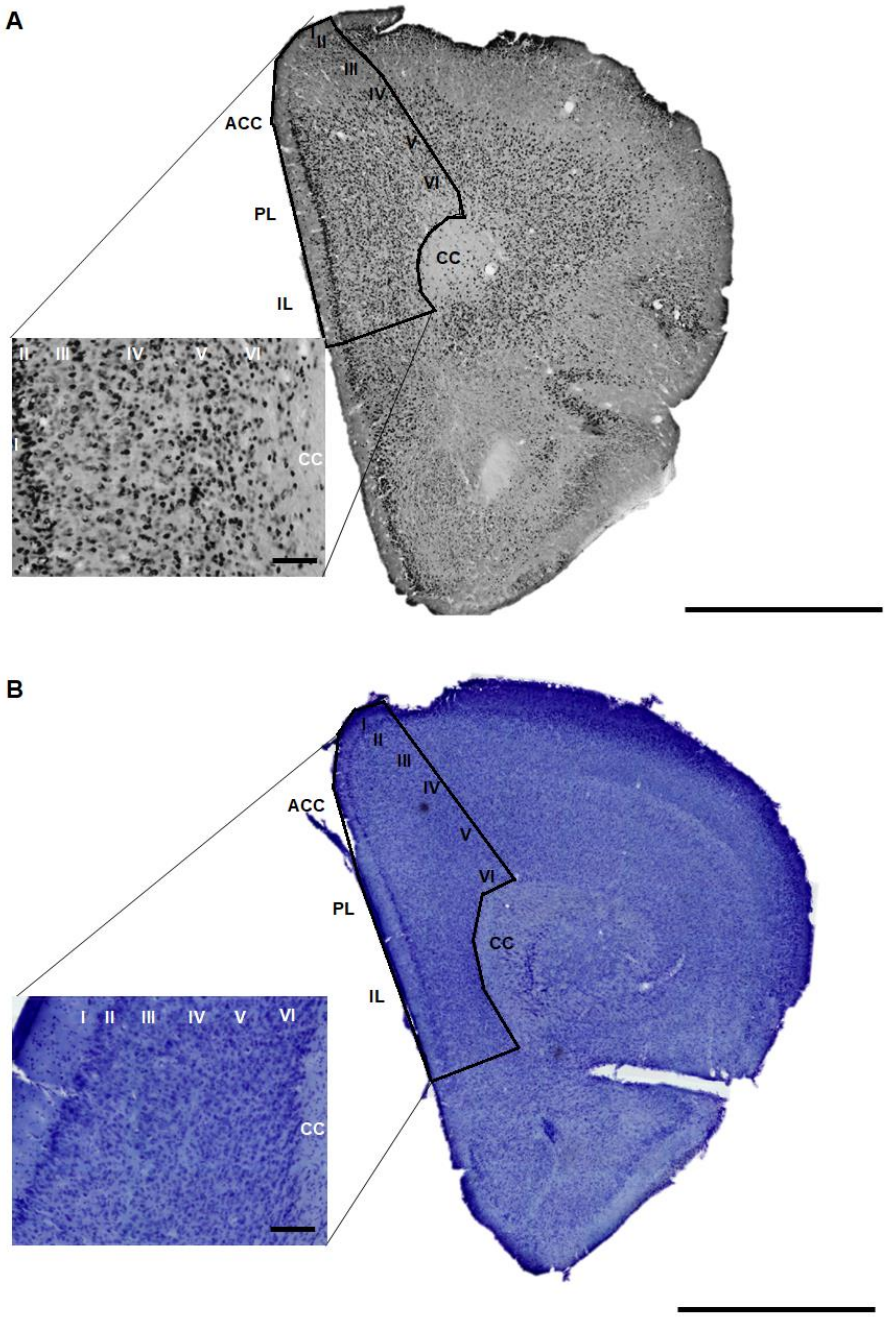


Figure 2.5: Delineation of Prefrontal Cortex Subregions for Neuronal Quantification.

Representative coronal sections of the prefrontal cortex from a control mouse brain showing A) NeuN-immunolabelled and B) Nissl-stained tissue used for neuronal analysis. Both panels illustrate the laminar organisation (layers I–VI) and anatomical subdivisions of the prefrontal cortex, including the anterior cingulate cortex (ACC), prelimbic cortex (PL), and infralimbic cortex (IL), as well as the corpus callosum (CC). Scale bars: 200 µm (low magnification), 20 µm (high magnification).

Table 2.4 Quantitative Stereological Assessment of Nissl-Stained and NeuN-Immunolabelled Neuronal Populations in Cortical Layers I–IV of the Prefrontal Cortex

Experimental groups	Average Defined Measured Mounted Thickness per Group	Number of Animals per Group	Total Number of Sections per Group	Average Number of Sampling Sites per Group
Nissl Stain				
Female				
Control	32	5	15	159
SIM	33	5	14	279
ALC	34	5	15	217
ALC+SIM5	35	5	15	293
ALC+SIM15	33	5	15	293
Male				
Control	33	5	14	166
SIM	34	5	15	275
ALC	33	5	15	229
ALC+SIM5	33	5	15	238
ALC+SIM15	29	4	12	243
NeuN Immunolabelling				
Female				
Control	32	5	15	170
SIM	33	5	14	250
ALC	34	5	15	286
ALC+SIM5	35	5	15	307
ALC+SIM15	33	5	15	293
Male				
Control	33	5	13	181
SIM	34	5	15	184
ALC	33	5	15	232
ALC+SIM5	40	5	15	273
ALC+SIM15	36	5	15	250

2.8. Assessment of Neurotrophic Marker Expression (IL-6, IL-10, CREB1, and BDNF) in the Hippocampus via RT-PCR Analysis

2.8.1. cDNA Synthesis for Relative Gene Expression Analysis via RT-PCR

Right hippocampal tissues, snap-frozen from 50 mice (10 per group, with an equal male-to-female ratio), were carefully thawed on ice before homogenization using a Misonix Ultrasound Liquid Processor (XL-2000 series, Misonix Inc., New York, USA). Total RNA was isolated employing the ReliaPrep™ RNA Tissue Miniprep System following the manufacturer's protocol (Catalog No: Z6111, Promega, Madison, WI, USA). For cDNA synthesis, 2 µL of the extracted RNA was reverse transcribed using 4 µL of SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Waltham, USA). The reaction mixture, loaded into a 96-well plate, was incubated at 25 °C for 10 minutes, followed by a 60-minute step at 42 °C, and enzyme inactivation at 85 °C for 5 minutes, using a T100™ thermal cycler (Bio-Rad, California, USA). The resulting cDNA was quantified and evaluated for quality using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Life Technologies, Waltham, USA).

2.8.2. Evaluation of mRNA Expression Levels Using RT-PCR

Quantitative gene expression analysis via RT-PCR was carried out using 1 µL of synthesized cDNA per reaction as the template. Each 10 µL reaction mixture comprised a preformulated TaqMan™ probe specific to the reference gene TATA-box binding protein (Tbp) (0.25 µL, Thermo Fisher Scientific, Life Technologies, Waltham, USA), 0.5 µL of a gene-specific TaqMan™ expression assay for the target gene, 5 µL of TaqMan™ Fast Advanced PCR master mix, and 3.25 µL of RNase-free water (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The amplification was executed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) under the following thermal cycling parameters: 2 minutes at 50 °C for initial hold, 10 minutes at 95 °C for enzyme activation, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute. Samples from individual mice that exhibited Ct values beyond the assay's quantification range across duplicate plates were excluded from subsequent analyses. The relative gene expression changes were determined using the 2^-ΔΔCt method as described by Livak and Schmittgen (2001).

Expression analysis was conducted for four genes of interest, IL-6, IL-10, CREB1, and BDNF, associated with neuroimmune signalling, neurotrophic function, and neuroinflammation, as

outlined in Table 2.5. TaqMan® probe-based assays were employed for quantification. cDNA samples from all experimental conditions, including both male and female subjects (n = 5 per sex per group), were included in the analysis. TATA-box binding protein (Tbp) was used as the internal reference in duplex reactions with a total volume of 10 μL. Each RT-PCR assay was run in duplicate using pre-validated TaqMan® primer/probe sets (Applied Biosystems) and the TaqMan® Advanced PCR Master Mix. Amplification was carried out on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). Negative control reactions included all reagents except cDNA to confirm the absence of contamination.

Table 2.5 Summarised Descriptions of the Selected Genes Used for the Comparative Gene Expression RT-PCR Analyses in Hippocampal Tissue of Adolescent Mice

Gene code	Full Gene Name	TaqMan™ Assay ID	Gene Function	RefSeq ID (mRNA)	References
Tbp	TATA-box binding protein	Mm01277042_m1	A key transcriptional component that binds the TATA box, aiding RNA polymerase II initiation. In the brain, it regulates genes important for neuronal development, synaptic function, and cognition	NM_013684.3	(Luna-Arias and Castro-Muñozledo, 2024)
IL-6	Interleukin-6	Mm00446190_m1	A cytokine crucial for immune regulation and inflammation. In the brain, it influences neuroinflammation, neurogenesis, and synaptic plasticity, and is linked to neurodegenerative and psychiatric disorders	NM_001314054.1	(Arnaud et al., 2005; Gruol et al., 2023)
IL-10	Interleukin-10	Mm01288386_m1	This cytokine plays an anti-inflammatory role within the CNS by regulating immune activity, mitigating neuroinflammation, minimizing neuronal damage, and promoting tissue recovery. It contributes to neuroprotection in conditions like Alzheimer's disease and traumatic brain injury.	NM_010548.2	(Marshall et al., 2017; Bobermin et al., 2024)
CREB1	cAMP Response Element-Binding protein	Mm01342452_m1	This transcription factor responds to intracellular signals such as cAMP and calcium, controlling the expression of genes involved in neuronal survival, synaptic adaptability, memory formation, learning processes, neurogenesis, and the maintenance of long-term potentiation.	NM_001037726.1	(Josselyn and Nguyen, 2005; Kandel, 2012)

BDNF	Brain-derived neurotrophic factor	Mm04230607_s1	A neurotrophic factor vital for neuron survival, growth, synaptic plasticity, learning, memory, and neurogenesis, particularly in the hippocampus.	NM_001048139.1	(Zuccato and Cattaneo, 2009)

2.9. Statistical Analyses

Descriptive and summary statistics were calculated for each variable, with results presented as either mean ± standard deviation (SD) or median with interquartile range (IQR), depending on the data distribution. Statistical evaluations were conducted using Paleontological Statistics Software (PAST, version 4.03) (Hammer and Harper, 2001). Data normality was examined using the Shapiro-Wilk test. Depending on the normality outcomes, group comparisons were made using One-Way ANOVA for normally distributed datasets or the Kruskal-Wallis test for non-normally distributed data. For post-hoc analysis, Tukey's test was applied to parametric data, while Dunn's test was used for non-parametric data to identify statistically significant group differences.

The following datasets were analysed:

- Body and brain weight indices
- Oxidative stress markers (MDA levels, GSH-Px and SOD activity) and the distribution of PcNA/DCX-positive cells
- Stereological estimates of NeuN-immunolabeled and Nissl-stained neuronal counts in both the PFC and the hippocampus, as well as the volume estimations
- Distribution of GFAP-immunopositive cells in the hippocampus
- Expression levels of neurotrophic and inflammatory genes (IL-6, IL-10, BDNF, and CREB1)

All analyses employed two-tailed statistical tests, with a threshold for significance established at $p < 0.05$. Data visualization, such as histograms and boxplots, was carried out using Microsoft Excel (Microsoft Corporation, 2018).

CHAPTER THREE

RESULTS

3.1. Overall Assessment of Brain and Body Weight Among Experimental Groups

Table 3.1 presents the effects of control, SIM, ALC, ALC+SIM5, and ALC+SIM15 treatments on body weight, brain weight, and brain-to-body mass ratio in female and male mice.

In females, Day 1 body weight differed significantly among groups ($p = 0.042$), with ALC mice weighing less than SIM ($p = 0.039$) and ALC+SIM5 ($p = 0.004$), and ALC+SIM5 weighing more than ALC+SIM15 ($p = 0.037$). By Day 28, however, no significant differences were observed in body or brain mass ($p > 0.05$). For the brain-to-body mass ratio, the SIM group displayed the highest values, significantly exceeding those of the control ($p = 0.035$) and ALC ($p = 0.001$) groups, while ALC was lower than both ALC+SIM5 ($p = 0.043$) and ALC+SIM15 ($p = 0.037$). These findings suggest simvastatin exerts a modulatory effect on growth and brain development in females, even under alcohol exposure.

In males, no significant group differences were found for body weight (Day 1 or Day 28), brain mass, or brain-to-body ratio (all $p > 0.05$), suggesting a limited effect of treatment in males.

Table 3.1 Variation in Body Mass, Brain Mass, and Brain-to-Body Mass Ratio by Treatment Group and Sex

		Control	SIM	ALC	ALC+SIM5	ALC+SIM15	*p*
Female							
Day 1	Body mass (mean ± SD) (g)	12.70 ± 0.57	12.40 ± 0.22[a]	13.20 ± 0.57[a,b]	12.10 ± 0.55[b,c]	13.00 ± 0.87[c]	**0.042**
	Brain mass (mean ± SD) (g)	0.42 ± 0.01	0.42 ± 0.02	0.41 ± 0.01	0.40 ± 0.02	0.40 ± 0.02	0.453
Day 28	Body mass (mean ± SD) (g)	15.40 ± 0.65	14.30 ± 1.04	15.70 ± 0.84	14.20 ± 1.04	14.20 ± 0.98	0.069
	Brain/Body ratio (mean ± SD)	0.027 ± 0.002[a]	0.030 ± 0.002[a,b]	0.026 ± 0.002[b,c,d]	0.028 ± 0.001[c]	0.028 ± 0.001[d]	**0.023**
Male							
Day 1	Body mass (mean ± SD) (g)	15.20 ± 0.76	14.50 ± 0.35	14.50 ± 2.06	14.20 ± 0.57	14.10 ± 2.01	0.739
	Brain mass (mean ± SD) (g)	0.42 ± 0.04	0.43 ± 0.01	0.41 ± 0.02	0.42 ± 0.01	0.40 ± 0.01	0.145
Day 28	Body mass (mean ± SD) (g)	18.30 ± 1.48	18.30 ± 1.64	16.80 ± 1.35	17.20 ± 1.44	16.20 ± 2.95	0.329
	Brain/Body ratio (mean ± SD)	0.023 ± 0.001	0.023 ± 0.002	0.025 ± 0.002	0.024 ± 0.002	0.025 ± 0.005	0.690

SD – Standard deviation

[a,b,c,d] - Superscript letters indicate significant differences between treatment groups within the same row ($p < 0.05$)

3.2. Biochemical Analyses of Oxidative Stress in the Hippocampus of Alcohol-Exposed Adolescent Mice

To evaluate oxidative stress caused by alcohol and investigate the possible regulatory role of simvastatin, measurements of MDA levels along with GSH-Px and SOD enzyme activities were conducted in the hippocampi of male and female adolescent mice from all experimental groups.

3.2.1. Malondialdehyde (MDA) Levels

MDA levels in the hippocampus did not differ significantly across the experimental groups in either female or male adolescent mice (Fig. 3.1A). In female mice, alcohol exposure did not significantly increase lipid peroxidation, as no significant difference was found between the ALC and control groups (p = 0.667). Similarly, no significant differences were observed between the control group and the SIM (p = 0.144), ALC+SIM5 (p = 0.699), or SIM (p = 0.059) groups, suggesting that simvastatin treatment alone or in combination with alcohol did not alter MDA levels. Furthermore, no significant differences were found between the SIM and ALC+SIM5 (p = 0.283), ALC and ALC+SIM5 (p = 0.071), or ALC+SIM5 and ALC+SIM15 (p = 0.323) groups.

In male mice, alcohol and simvastatin treatments also did not significantly affect MDA levels in the hippocampus. Alcohol exposure did not result in a significant difference compared to the control group (p = 0.439), and simvastatin treatment alone (SIM, p = 0.143) did not significantly alter MDA levels. Additionally, the combination of alcohol and simvastatin (5 mg and 15 mg) did not produce any significant changes compared to alcohol alone or the control group (ALC+SIM5, p = 0.345; ALC+SIM15, p = 0.133). These results suggest that neither alcohol nor simvastatin, alone or in combination, had a significant impact on lipid peroxidation levels in the hippocampus of adolescent male or female mice.

3.2.2. Glutathione Peroxidase (GSH-Px) Enzyme Activity

In female mice (Fig. 3.1B), significant differences in GSH-Px activity were observed across the experimental groups (p = 0.004). Notably, the ALC group demonstrated significantly higher GSH-Px activity compared to the control (p = 0.007), ALC+SIM5 (p = 0.002), and ALC+SIM15 (p = 0.021), indicating an increase in antioxidant enzyme activity in response to alcohol-induced oxidative stress. Additionally, GSH-Px activity in the control group was

significantly lower than in the SIM group ($p = 0.032$), and the activity in the ALC+SIM5 group was comparable to that of the control, but lower than SIM ($p = 0.011$).

In male mice (Fig. 3.1B), GSH-Px activity also varied significantly across experimental groups ($p = 0.005$). The ALC group exhibited significantly higher GSH-Px activity compared to both the SIM group ($p = 0.006$) and ALC+SIM5 ($p = 0.001$), indicating an enhanced antioxidant response to alcohol exposure. In contrast, the GSH-Px activity in the control group was similar to that of the ALC+SIM5 group ($p = 0.020$), and the ALC+SIM15 group showed similar levels to SIM when compared to ALC+SIM5 ($p = 0.05$). These findings suggest that alcohol exposure leads to a significant increase in GSH-Px activity, while simvastatin, particularly at the lower dose (5 mg), modulates this effect.

3.2.3. Superoxide Dismutase (SOD) Activity

In both female and male mice, no significant differences in SOD activity were observed across the experimental groups (Fig. 3.1C). In female mice, no significant change in SOD activity was observed between the control and SIM groups ($p = 0.691$), indicating that simvastatin treatment alone did not alter SOD activity. Similarly, alcohol exposure did not significantly affect SOD activity compared to the control group ($p = 0.386$). The combination of alcohol and 5 mg simvastatin (ALC+SIM5) did not significantly alter SOD activity compared to alcohol exposure alone ($p = 0.171$). No significant differences were found between the SIM and ALC groups ($p = 0.251$), suggesting that simvastatin treatment alone did not influence SOD activity relative to alcohol exposure. Additionally, no significant differences were observed between the two doses of simvastatin (5 mg and 15 mg) combined with alcohol, as the comparison between ALC+SIM5 and ALC+SIM15 was not significant ($p = 0.068$).

In male mice, no significant differences were observed between any of the experimental groups. Simvastatin treatment alone (SIM) did not significantly affect SOD activity compared to the control group ($p = 0.889$). Similarly, alcohol exposure did not alter SOD activity compared to the control group ($p = 0.295$), and the addition of simvastatin (5 mg or 15 mg) to alcohol exposure did not result in significant changes in SOD activity. No significant differences were found between the SIM and ALC groups ($p = 0.299$), or between the ALC and ALC+SIM5 groups ($p = 0.880$), indicating that simvastatin did not significantly influence SOD activity in alcohol-exposed mice. Finally, no significant difference was observed between the

ALC+SIM5 and ALC+SIM15 groups (p = 0.631), suggesting that the two different doses of simvastatin did not lead to significant changes in SOD activity when combined with alcohol exposure.

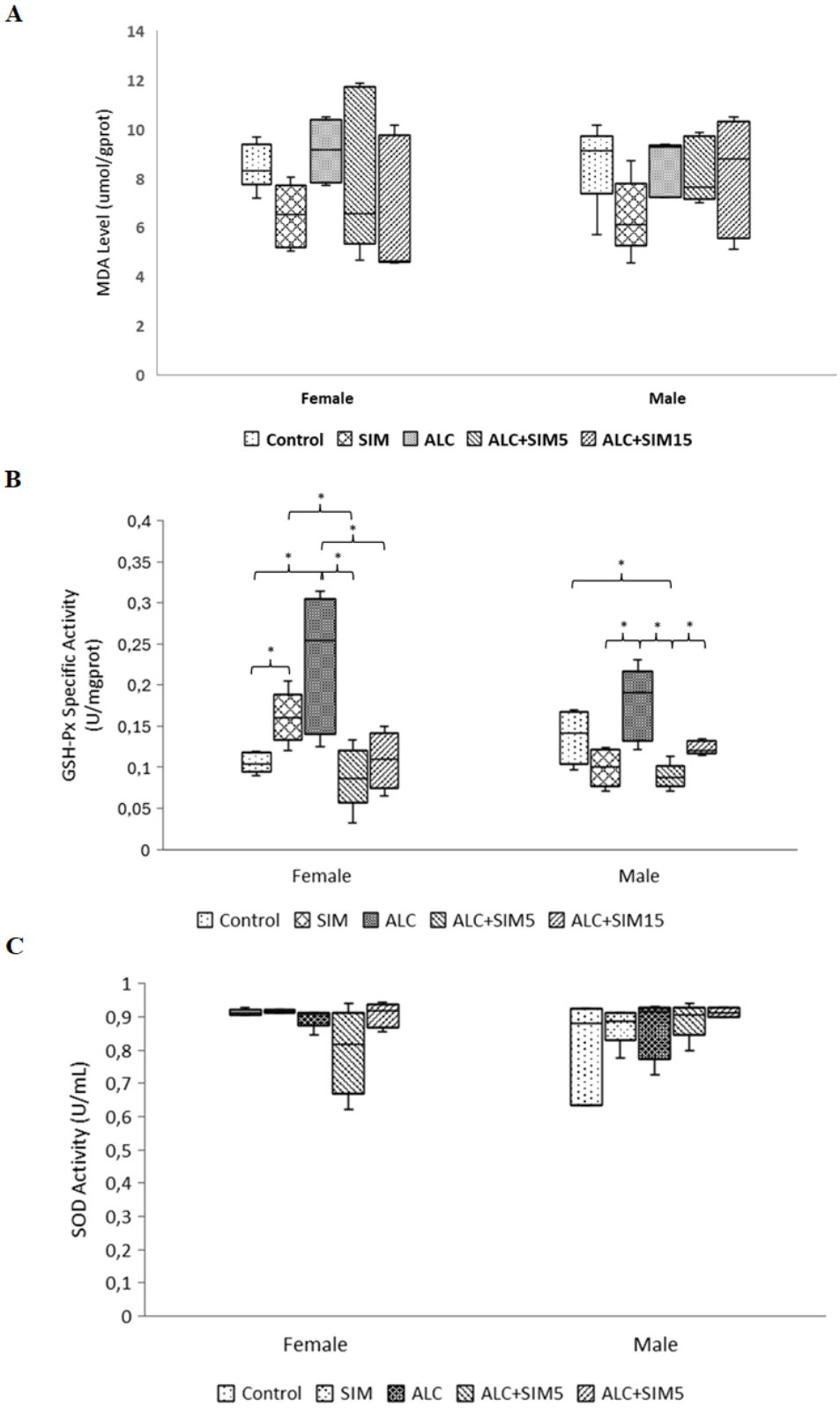


Figure 3.1: Effect of Alcohol and/or Simvastatin on Hippocampal MDA, GSH-Px, and SOD Levels in Adolescent Mice. Box plots presenting the statistical comparisons of (A) malondialdehyde (MDA) levels (µmol/g protein), (B) glutathione peroxidase (GSH-Px) activity (U/mg protein), and (C) superoxide dismutase (SOD) activity (U/mL) in the hippocampi of female and male adolescent mice

across the experimental groups. No significant differences in MDA levels were observed in either sex. In female mice, elevated GSH-Px activity was observed in the ALC group relative to the other groups, indicating increased oxidative stress due to alcohol exposure. In male mice, GSH-Px activity was similarly elevated in the ALC group compared to the other groups, with simvastatin treatment (5 mg) reducing alcohol-induced effects. No significant differences in SOD activity were observed among the experimental groups in either sex. Control – No treatment; SIM – 5 mg/kg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg/kg Simvastatin; ALC+SIM15 – Alcohol + 15 mg/kg Simvastatin. Females = 25; Males = 25. *Statistically significant at $p < 0.05$

3.3. Biochemical Analyses of Oxidative Stress in the Prefrontal Cortex of Alcohol-Exposed Adolescent Mice

To assess oxidative stress induced by alcohol and explore the possible regulatory effects of simvastatin, MDA levels and the activities of GSH-Px and SOD were measured in the prefrontal cortex of adolescent male and female mice across the treatment groups..

3.3.1. Malondialdehyde (MDA) levels

MDA levels in the prefrontal cortex of adolescent female mice did not differ significantly across the experimental groups (Fig. 3.2A). One-way ANOVA revealed no significant differences in MDA levels between groups ($F(4, 20) = 1.093$, $p = 0.387$). Pairwise comparisons using Tukey's post hoc test further confirmed the absence of significant differences between any of the groups (p-values > 0.05), indicating that the experimental conditions did not alter MDA levels in the prefrontal cortex of female mice.

Similarly, MDA levels in the prefrontal cortex of adolescent male mice did not differ significantly across the experimental groups (Fig. 3.2B). The Kruskal-Wallis test revealed no statistically significant differences in median MDA levels between groups ($p = 0.078$). Although pairwise comparisons using Dunn's post hoc test indicated potential differences between specific groups, including control vs. SIM ($p = 0.043$) and control vs. ALC+SIM15 ($p = 0.018$), these did not remain significant after correction for multiple comparisons. Overall, the results suggest that the experimental treatments did not significantly alter MDA levels in the prefrontal cortex of male mice.

3.3.2. Glutathione Peroxidase (GSH-Px) Enzyme Activity

In female mice (Fig. 3.2B), significant differences in GSH-Px activity were observed between the experimental groups ($p = 0.000$). GSH-Px levels in the control group did not differ significantly from those in the SIM ($p = 0.987$) or ALC groups ($p = 0.723$), indicating that neither SIM nor ALC altered GSH-Px activity. However, a significant reduction in GSH-Px activity was observed in the ALC+SIM5 group compared to the control ($p = 0.015$), SIM ($p = 0.005$), and ALC groups ($p = 0.001$), indicating a suppression of antioxidant enzyme activity. In contrast, the ALC+SIM15 group showed a significant increase in GSH-Px activity relative to the ALC group ($p = 0.008$), suggesting a compensatory upregulation in response to alcohol-induced oxidative stress. No significant difference was observed between the control and

ALC+SIM15 groups (p = 0.400), indicating that the higher dose of SIM may have restored GSH-Px activity to near-control levels.

In male mice (Fig. 3.2B), no significant differences in GSH-Px activity were observed across the experimental groups (p = 0.619), indicating that the treatments did not significantly affect antioxidant enzyme levels in the prefrontal cortex. Tukey's post hoc analysis confirmed the absence of significant differences between any of the groups, with all pairwise comparisons yielding p-values above 0.05. Although GSH-Px activity appeared slightly lower in the ALC+SIM5 group, this reduction was not statistically significant. Overall, these findings suggest that neither alcohol exposure nor its combination with simvastatin altered GSH-Px activity in the prefrontal cortex of adolescent male mice.

3.3.3. Superoxide dismutase (SOD) activity

Similar to the results of the hippocampus, no significant differences were observed in SOD activity in the prefrontal cortex of adolescent female mice across the different treatment groups (Fig. 3.2C). Specifically, there were no significant differences between the control and SIM (p = 0.637), ALC (p = 0.830), ALC+SIM5 (p = 0.302), or ALC+SIM15 (p = 0.830) groups, indicating that neither SIM, ALC, nor the combination of ALC+ SIM5 or ALC+SIM15 significantly altered SOD activity compared to the control group. Additionally, no significant differences were observed between SIM and ALC (p = 0.491), SIM and ALC+SIM5 (p = 0.577), SIM and ALC+SIM15 (p = 0.491), or ALC and ALC+SIM5 (p = 0.213), suggesting that alcohol and the combination treatments did not significantly affect SOD activity. Similarly, no significant differences were found between ALC and ALC+SIM15 (p = 1) or between ALC+SIM5 and ALC+SIM15 (p = 0.213), further indicating that none of the treatments had a notable impact on SOD levels.

Additionally, in male mice (Fig. 3.2C), no significant differences in SOD activity were observed between the experimental groups (F = 0.917, p = 0.474), indicating that none of the treatments had a substantial effect on SOD levels in the prefrontal cortex. Tukey's post hoc test confirmed this, showing no significant differences between the control, SIM, ALC, ALC+SIM5, or ALC+SIM15 groups. These findings suggest that oxidative stress, as measured by SOD activity, was not significantly altered by any of the treatments in the prefrontal cortex of adolescent female and male mice.

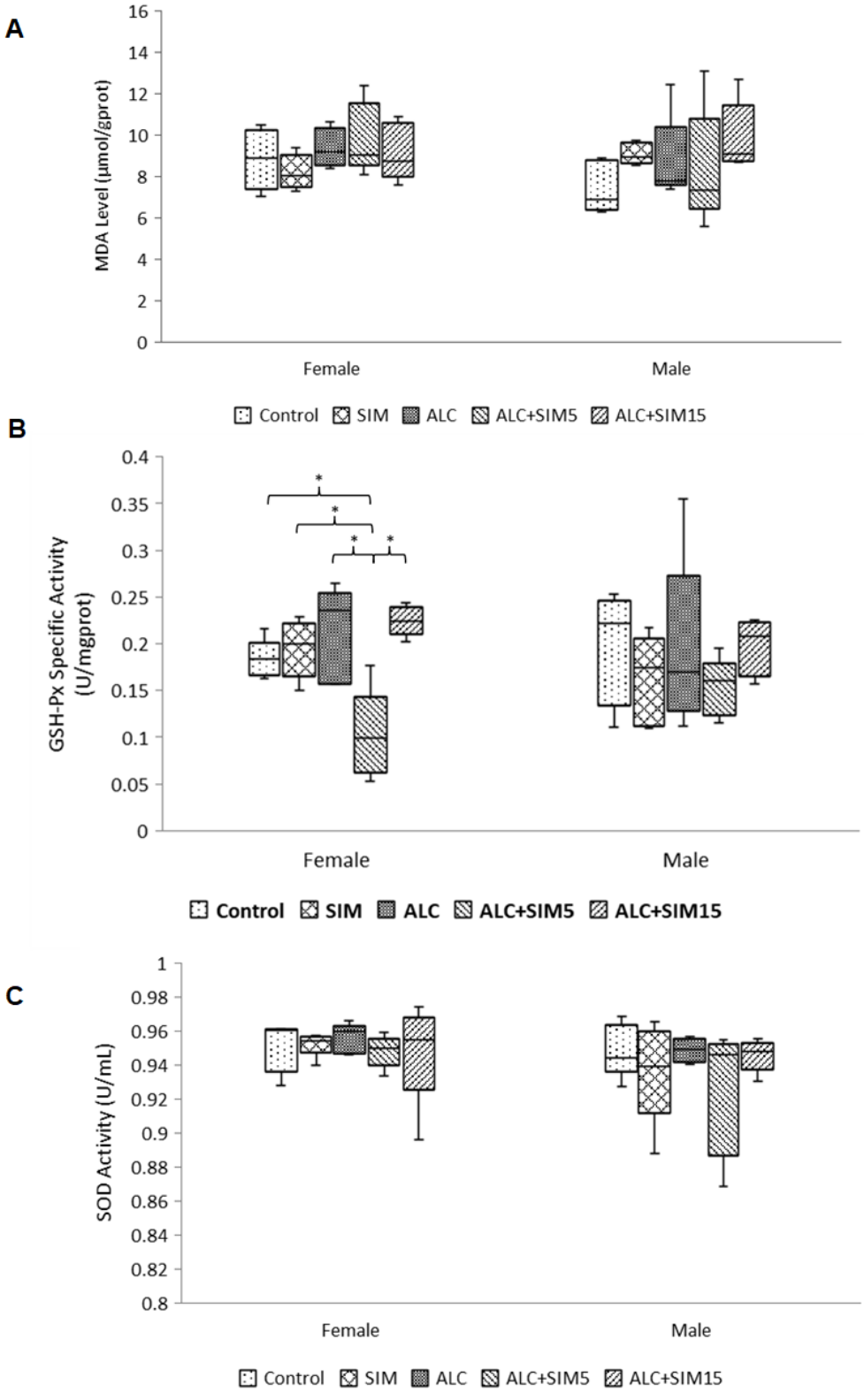


Figure 3.2: Oxidative Stress Markers in the Prefrontal Cortex of Adolescent Mice Following Alcohol and/or Simvastatin Treatment. Box plots illustrating (A) malondialdehyde (MDA) levels (µmol/g protein), (B) glutathione peroxidase (GSH-Px) activity (U/mg protein), and (C) superoxide dismutase (SOD) activity (U/mL) in the prefrontal cortex (PFC) of adolescent female and male mice

across treatment groups. In female mice, no significant differences in MDA levels or SOD activity were observed across the treatment groups. Significant differences in GSH-Px activity were found between the control and ALC+SIM5, SIM and ALC+SIM5, ALC and ALC+SIM5, and ALC+SIM5 and ALC+SIM15 groups, indicating that the combination of alcohol and 5 mg simvastatin had a distinct effect on antioxidant activity. In male mice, no significant differences were observed in MDA levels, GSH-Px activity, or SOD activity across any of the treatment groups, suggesting that neither alcohol exposure nor the combination with simvastatin altered oxidative stress or antioxidant enzyme activity in the prefrontal cortex. Control – No treatment; SIM – 5 mg/kg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg/kg Simvastatin; ALC+SIM15 – Alcohol + 15 mg/kg Simvastatin. Females = 25; Males = 25. *Statistically significant at $p < 0.05$

3.4. Immunohistochemical Analyses to Determine the Distribution of Proliferative and Immature Neurons in the Suprapyramidal Blade of the Dentate Gyrus of the Hippocampus

To investigate the impact of alcohol and simvastatin on neurogenesis within the hippocampus, immunohistochemical staining was used to examine the distribution of proliferating cells (PcNA-positive) and immature neurons (DCX-positive) in the suprapyramidal blade of the DG in adolescent male and female mice across all experimental groups.

3.4.1. General Morphology of the Hippocampus in Histological and Immunohistochemical Sections

No significant morphological alterations were detected across treatment groups in either sex, as assessed in both PcNA- (Fig. 3.3A) and DCX-immunolabeled sections (Fig. 3.4A). Specifically, the structural integrity of the dentate gyrus (DG) of the hippocampus remained consistent among all experimental conditions. Immunopositive PcNA and DCX cells were localized to the subgranular zone (SGZ), the principal site of neurogenesis within the DG, with no apparent disruptions in their distribution. Furthermore, the suprapyramidal and infrapyramidal blades of the DG were distinguishable and exhibited well-preserved anatomical boundaries across all groups.

3.4.2. Distribution of PcNA-Positive Cells in the Suprapyramidal Blade of the Dentate Gyrus

In the female mice (Fig. 3.3B), there was a significant difference in the distribution of proliferative cells across treatment groups ($p = 0.003$). Specifically, a significant reduction in the distribution of proliferative cells in the ALC group was revealed when compared to the control ($p = 0.015$), confirming alcohol's adverse effect on cell proliferation in the hippocampus. Additionally, the ALC+SIM15 group had a significantly higher distribution of PcNA-positive cells when compared to the ALC ($p = 0.000$), SIM ($p = 0.006$), or ALC+SIM5 ($p = 0.008$) groups. Additionally, SIM did not cause a significant change in the distribution of proliferative cells as evidenced by the lack of significance between SIM and control ($p = 0.183$) or SIM and ALC ($p = 0.220$). However, in the presence of alcohol, the higher concentration of SIM (15 mg) appeared to modulate alcohol-induced suppression of proliferation. In contrast, the lower dose of simvastatin (ALC+SIM5) did not display the same protective effects, suggesting a dose-dependent effect.

Similarly, in male mice (Fig. 3.3B) the distribution of PcNA-positive cells significantly differed across the experimental groups ($p = 0.000$). The ALC group exhibited a significant reduction in the distribution of proliferative cells compared to the control ($p = 0.024$), SIM ($p = 0.002$), and ALC+SIM5 ($p = 0.000$) groups, indicating alcohol-induced suppression of hippocampal proliferation. The distribution in the ALC+SIM5 group was comparable to the control group ($p = 0.017$), suggesting that the 5 mg dose of simvastatin mitigated the detrimental effects of alcohol. In contrast, the ALC+SIM15 group differed significantly from SIM ($p = 0.042$) and ALC+SIM5 ($p = 0.001$), with levels more closely resembling those of the ALC group ($p = 0.382$). No significant difference was observed between the SIM and control groups ($p = 0.392$), indicating that simvastatin alone did not affect proliferation. These findings indicate that chronic alcohol exposure disrupts hippocampal cell proliferation in adolescent mice, but simvastatin mitigates these effects in a dose- and sex-dependent manner. In females, a higher dose (15 mg) was needed for recovery, while in males, a lower dose (5 mg) was more effective.

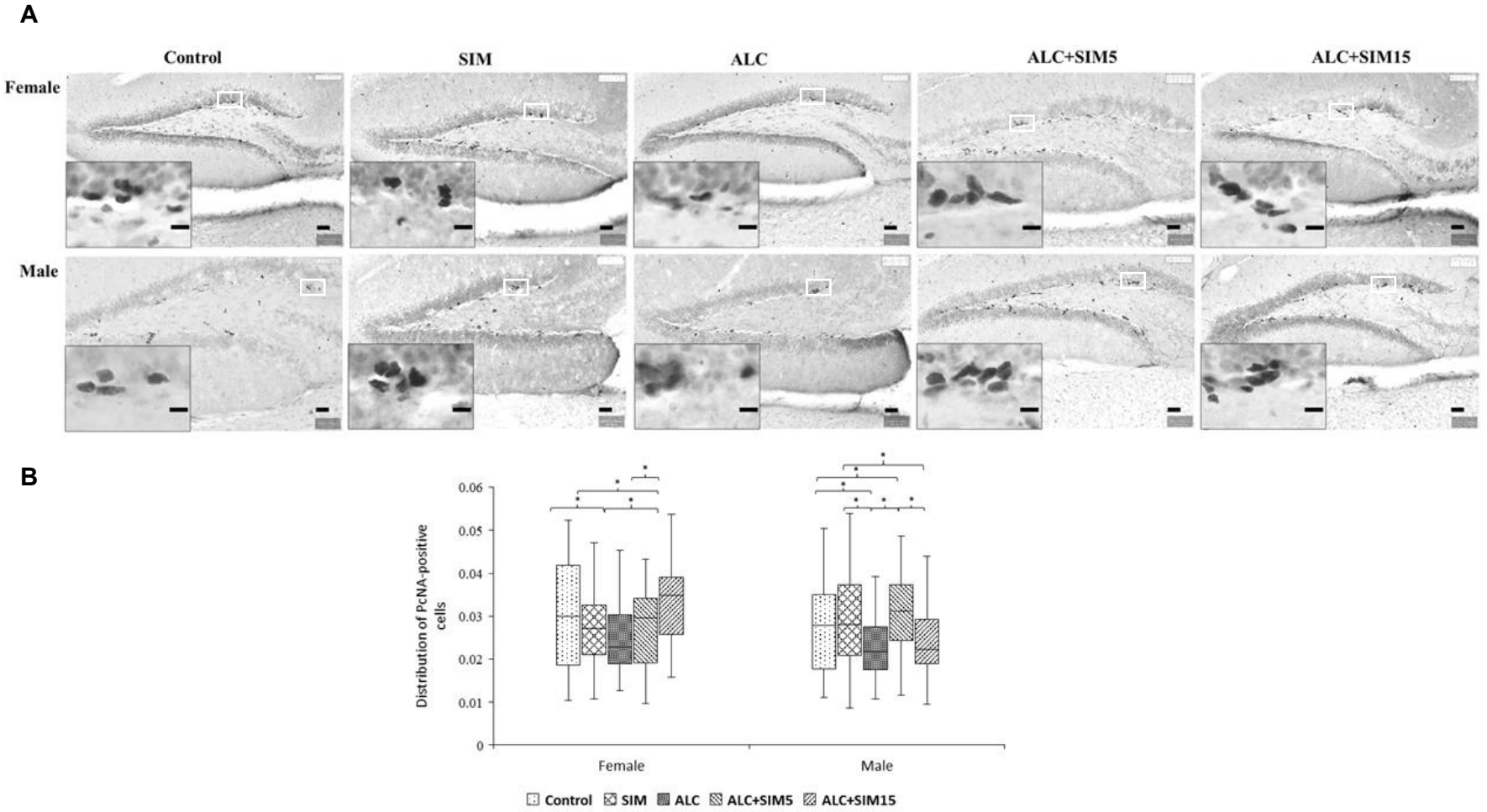


Figure 3.3: Distribution of Proliferating Cells (PcNA-Positive) in the Suprapyramidal Blade of the Dentate Gyrus of Adolescent Mice Following Alcohol and/or Simvastatin Treatment. (A) Representative photomicrographs showing PcNA-positive cells in the dentate gyrus (DG), and (B) boxplots illustrating the distribution of PcNA-positive cells within the suprapyramidal blade of the DG in adolescent female and male mice. PcNA-positive cells appeared dark and were primarily localized within the subgranular zone, consistent with the typical distribution of proliferative cells in the murine hippocampus. The white box in

the lower-magnification images indicates the region shown at higher magnification. In both sexes, the ALC group showed the lowest distribution of PcNA-positive cells. Simvastatin exhibited a dose- and sex-dependent neuroprotective effect, with the higher dose (15 mg) being more effective in females and the lower dose (5 mg) more effective in males. Simvastatin alone had no observable impact on proliferation compared to the control group. Control - No treatment (No of suprapyramidal blade assessed: F = 48; M = 64); SIM – 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 60; M = 70); ALC – Alcohol (No of suprapyramidal blade assessed: F = 49; M = 51); ALC+SIM5 - Alcohol + 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 58; M = 60); ALC+SIM15 - Alcohol + 15 mg Simvastatin (No of suprapyramidal blade assessed: F = 42; M = 44). Female = 25; Males = 25. *Statistically significant at $p < 0.05$. Scale bar of lower magnification = 100 μm; Scale bar of higher magnification = 20 μm.

3.4.3. Distribution of DCX-Positive Cells in the Suprapyramidal Blade of the Dentate Gyrus

In female mice, the distribution of DCX-positive cells significantly differed across experimental groups ($p = 0.000$; Fig. 3.4B). Specifically, alcohol exposure (ALC) significantly reduced the distribution of DCX-positive cells compared to the control ($p = 0.000$) group, confirming alcohol's detrimental impact on hippocampal neurogenesis. Similarly, the ALC+SIM15 group also showed a significant reduction in the distribution of DCX-positive cells compared to the control ($p = 0.000$), indicating that the higher dose of simvastatin did not fully mitigate the effects of alcohol. No significant differences were observed between the control group and either the SIM ($p = 0.546$) or ALC+SIM5 ($p = 0.082$) groups, suggesting that simvastatin alone or at a lower dose in combination with alcohol did not significantly alter DCX-positive cell distribution. However, the SIM group showed a significant increase in DCX-positive cells compared to the ALC ($p = 0.000$), ALC+SIM5 ($p = 0.015$), and ALC+SIM15 ($p = 0.000$) groups, suggesting that simvastatin alone may enhance or support hippocampal neurogenesis in the absence of alcohol. The ALC+SIM5 significantly increased the distribution of DCX-positive cells when compared to the ALC group ($p = 0.022$), indicating that the 5 mg simvastatin dose may partially counteract alcohol-induced neurogenesis suppression. In contrast, no significant difference was found between the ALC and ALC+SIM15 groups ($p = 0.944$), supporting the limited efficacy of the higher simvastatin dose in this context. A significant difference between ALC+SIM5 and ALC+SIM15 ($p = 0.034$) suggests a dose-dependent effect, with the lower simvastatin dose (5 mg) more effective at preserving hippocampal neurogenesis in alcohol-exposed female mice.

In male mice, a significant difference in the distribution of DCX-positive cells across treatment groups was observed ($p = 0.000$; Fig. 3.4B). Alcohol exposure (ALC) resulted in a significant reduction in the distribution of DCX-positive cells compared to the control ($p = 0.001$) group. Similarly, the ALC+SIM15 group exhibited a significant reduction in DCX-positive cells compared to control ($p = 0.026$), while no significant differences were found between ALC and ALC+SIM15 ($p = 0.358$). The SIM group did not significantly differ from control ($p = 0.465$), suggesting no impact of simvastatin alone on DCX-positive cells. However, the ALC+SIM5 group showed a significantly higher distribution of DCX-positive cells than the ALC group ($p = 0.000$), suggesting that the 5 mg simvastatin dose mitigates alcohol-induced damage. A significant difference was found between ALC+SIM5 and ALC+SIM15 ($p = 0.004$), indicating a dose-dependent effect, with the lower dose being more effective at preserving DCX-positive

cells in alcohol-exposed males. These findings show that alcohol significantly reduces DCX-positive cells in both female and male adolescent mice, indicating damage to hippocampal immature neurons, while simvastatin, particularly at a lower dose (5 mg), mitigates this effect in both sexes.

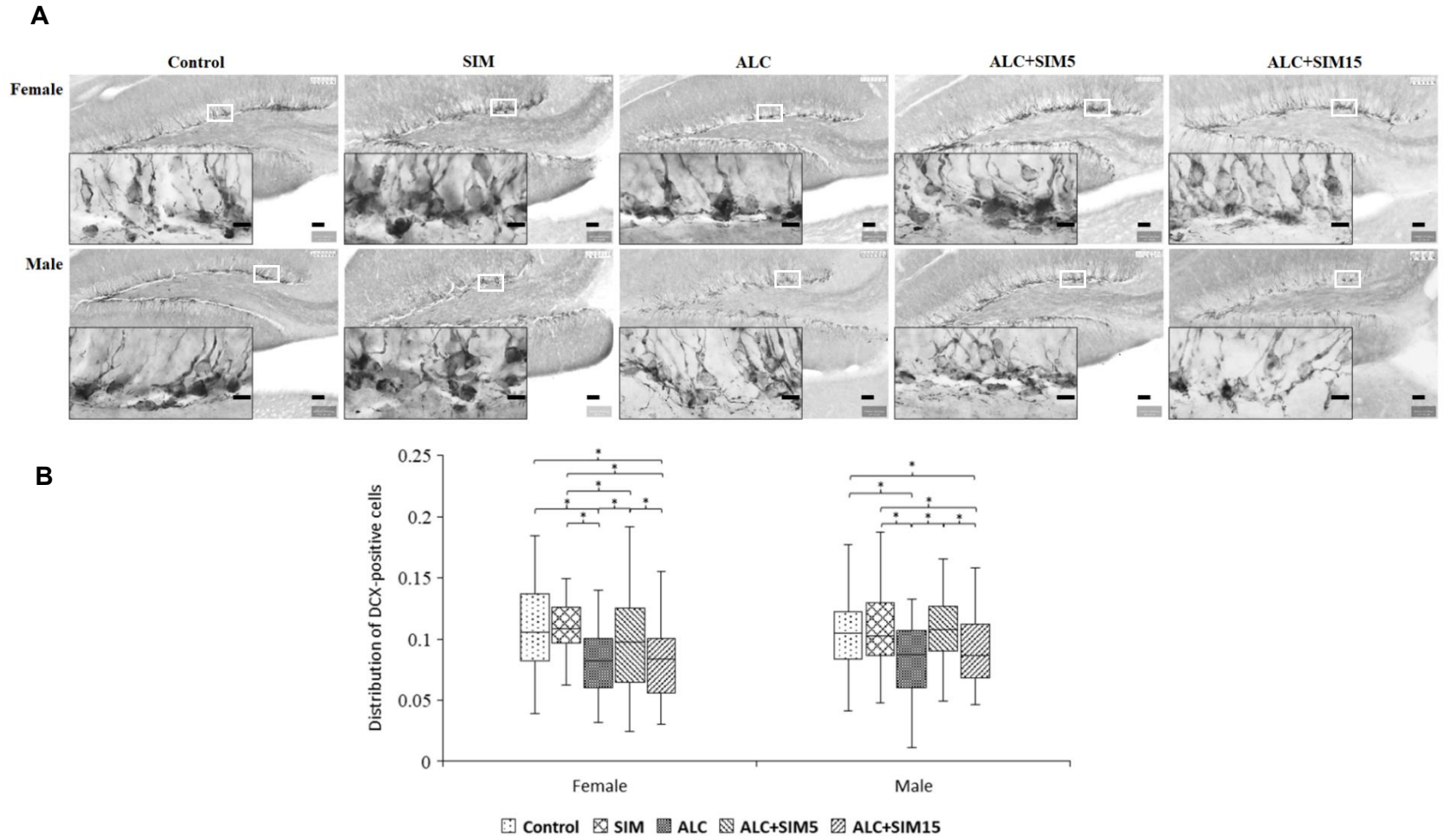


Figure 3.4: **Distribution of DCX-positive Cells in the Suprapyramidal Blade of the Dentate Gyrus of Adolescent Mice Following Alcohol and/or Simvastatin Treatment**. Illustrations of the (a) photomicrographs showing DCX-positive cells in the dentate gyrus (b) and the boxplots illustrating the statistical comparison of the distribution of DCX-positive cells in the suprapyramidal blades of the dentate gyrus in the hippocampus of both female and male adolescent

mice. The DCX-positive cells appeared dark, and no differences in their patterns were observed across the experimental groups. The white box in the lower-magnification images indicates the region shown at higher magnification. In both sexes, the distribution of DCX-positive cells was lowest in the ALC group. Simvastatin demonstrated a neuroprotective effect against alcohol, as shown by the increased distribution of DCX-positive cells in the SIM and ALC+SIM5 groups. The lower concentration of Simvastatin (5 mg) was more effective in counteracting the suppressive effects of alcohol in both sexes. Control - No treatment (No of suprapyramidal blade assessed: F = 58; M = 69); SIM – 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 69; M = 76); ALC – Alcohol (No of suprapyramidal blade assessed: F = 57; M = 52); ALC+SIM5 – Alcohol + 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 62; M = 65); ALC+SIM15 – Alcohol + 15 mg Simvastatin (No of suprapyramidal blade assessed: F = 48; M = 48). Female = 25; Males = 25. *Statistically significant at $p < 0.05$ Scale bar of lower magnification = 100 μm; Scale bar of higher magnification = 20 μm.

3.5. Effects of Alcohol Exposure and Simvastatin Treatment on the Estimation of the Population of Neuronal Cells, the Distribution of Astrocytic Cells, and the Hippocampal Volume of Adolescent Mice

Histological and immunohistochemical methods were utilized to estimate neuronal cell populations, evaluate the distribution of GFAP-positive cells, and measure hippocampal volume in both male and female mice across all experimental groups.

3.5.1. General Morphology of the Hippocampus in Histological and Immunohistochemical Sections

No significant morphological alterations were detected across treatment groups in either sex, as assessed in both GFAP- and NeuN-immunolabeled astrocytes and neurons (Fig. 3.5A, Fig. 3.6A), as well as Nissl-stained sections used for hippocampal volume estimations via Cavalieri (Fig. 3.7A). Specifically, the structural integrity of the DG of the hippocampus remained consistent among all experimental conditions. Immunopositive GFAP and NeuN cells exhibited typical morphology, with no notable changes in the shape, size, or distribution of astrocytes or neurons in any group. The Nissl-stained sections showed well-preserved cellular structures within the DG, providing clear delineation of the hippocampal regions. Immunopositive cells for GFAP and NeuN were detected in both the DG and cornu ammonis (CA) regions, exhibiting a uniform distribution without apparent structural abnormalities. Additionally, the suprapyramidal and infrapyramidal blades of the DG remained distinctly identifiable, with preserved anatomical integrity across all treatment groups.

3.5.2. Estimation of the Population of NeuN-Immunolabelled Neurons in the Suprapyramidal Blade of the Dentate Gyrus

An unbiased, design-based stereological approach using systematic random sampling was applied to estimate the total number of NeuN-positive neurons in the suprapyramidal blade of the DG in adolescent mice across different treatment groups and sexes. NeuN immunostaining specifically labelled mature neurons of small to medium size within various hippocampal subregions, including the DG, CA1, and CA3. These neurons displayed characteristic morphological features, such as distinct cell bodies, prominent nucleoli, and organised dendritic arbors. As expected, the DG exhibited high neuronal density, consistent with its tightly packed granule cell layer, and this pattern was observed uniformly across all

experimental groups. Importantly, no apparent differences were noted in the density or spatial distribution of NeuN-positive neurons between groups (Fig. 3.5A).

Optimal stereological parameters were applied to ensure reliable and unbiased sampling, including counting frame dimensions (45 µm × 45 µm), sampling grid spacing (100 µm × 100 µm), dissector height (10 µm), and section cut thickness (50 µm). Precision was assessed using the Gundersen coefficient of error (CE) with a smoothness factor of m = 1, indicating a uniform distribution of neurons. Across all groups and both sexes, the CE value was ≤ 0.10, which is within the acceptable range for ensuring the reliability and consistency of the NeuN cell estimates. This low CE indicates that the cell counts were highly reproducible and not significantly affected by sampling variability. Table 3.2 summarises key stereological parameters used to estimate NeuN-positive neuronal populations and their densities. The parameters recorded for each group included the average number of immunopositive markers counted, the number of tissue sections and sampling sites analysed, section thickness measurements, population estimates based on either section thickness or cell counts, coefficients of error (Gundersen m = 0 and m = 1), measured tissue volume, and calculated neuronal density (cells/mm³). Neuronal density values are presented as means ± standard error of the mean (SEM).

In female mice (Fig. 3.6B), no significant differences were revealed across the groups ($F = 0.729$, $p = 0.583$). The control group had a population of NeuN-positive cells with a mean of $4.88 \times 10^5 \pm 0.265 \times 10^5$. ALC treatment resulted in a reduction in the NeuN-positive cell population ($4.68 \times 10^5 \pm 0.251 \times 10^5$), although this difference was not statistically significant ($p = 0.583$). SIM treatment showed a slight increase in the NeuN-positive cell population ($5.27 \times 10^5 \pm 0.145 \times 10^5$), but again, this change was not significant. In the ALC+SIM5 and ALC+SIM15 groups, the populations were $4.87 \times 10^5 \pm 0.316 \times 10^5$ and $4.91 \times 10^5 \pm 0.254 \times 10^5$, respectively, with no significant differences observed when compared to ALC ($p = 0.747$ for ALC+SIM5 and $p = 0.911$ for ALC+SIM15). Overall, while slight variations in cell population density were observed, none of the differences reached statistical significance, indicating that neither alcohol nor simvastatin (at the doses tested) caused substantial changes in the NeuN-positive cell population in the suprapyramidal blade of the hippocampus.

In male mice (Fig. 3.5B), the analysis revealed a significant difference between the groups ($F(4, 20) = 3.107$, $p = 0.039$). The control group had a population of NeuN-positive cells with a

mean of $5.03 \times 10^5 \pm 0.454 \times 10^5$. ALC reduced the NeuN-positive cell population ($5.26 \times 10^5 \pm 0.280 \times 10^5$), although this difference was not statistically significant ($p = 0.560$). SIM treatment showed a significant increase in the NeuN-positive cell population ($5.98 \times 10^5 \pm 0.163 \times 10^5$), compared to the ALC+SIM5 ($p = 0.025$) group, indicating a positive effect on the density or survival of NeuN-positive neurons. In the ALC+SIM5 and ALC+SIM15 groups, the populations were $4.65 \times 10^5 \pm 0.209 \times 10^5$ and $4.97 \times 10^5 \pm 0.210 \times 10^5$, respectively, with no significant differences observed when compared to ALC ($p = 0.56$ for ALC+SIM5 and $p = 1.132$ for ALC+SIM15). Overall, while SIM showed a significant increase in the NeuN-positive cell population compared to ALC+SIM5, alcohol treatment did not cause a significant reduction, and no significant changes were observed with the combination treatments.

Table 3.2 Stereological Parameters and NeuN-positive Neuronal Density in the Hippocampus of Adolescent Female and Male Mice Across Treatment Groups

Treatment Group	N	Average Markers Counted	Total Number of Sections	Average Number of Sampling Sites	Average Measured Defined Mounted Thickness	Average Estimated Population using Mean Section Thickness (x 10^4)	Average Estimated Population using Mean Section Thickness with Counts (x 10^4)	Average Estimated Population using Number Weighted Section Thickness (x 10^4)	Average Coefficient of Error (Gundersen m=0)	Average Coefficient of Error (Gundersen m=1)	Average Measured Volume (x 10^8 μm^3)	Neuronal Density (x 10^5 cells/mm^3, Mean ± SE)
Female												
NT	5	1421	30	141	40	8.01	8.01	8.01	0.07	0.03	1.68	4.88 ± 0.265
SIM	5	1279	27	119	40	6.47	6.47	6.47	0.19	0.06	1.24	5.27 ± 0.145
ALC	5	1259	30	131	40	7.10	7.10	7.10	0.07	0.03	1.58	4.68 ± 0.251
ALC+SIM5	5	1462	29	142	40	8.26	8.26	8.26	0.07	0.03	1.79	4.87 ± 0.361
ALC+SIM15	5	1435	29	143	40	8.10	8.10	8.10	0.08	0.03	1.73	4.91 ± 0.254
Male												
NT	5	1098	30	111	41	6.36	6.36	6.36	0.08	0.03	1.30	5.03 ± 0.454
SIM	5	1371	30	116	40	7.73	7.73	7.73	0.10	0.03	1.30	5.98 ± 0.163
ALC	5	1182	30	107	40	6.64	6.64	6.64	0.08	0.03	1.28	5.26 ± 0.280
ALC+SIM5	5	1190	30	121	40	6.69	6.69	6.69	0.09	0.04	1.44	4.65 ± 0.209
ALC+SIM15	5	1161	30	108	39	6.48	6.48	6.48	0.08	0.034	1.30	4.97 ± 0.210

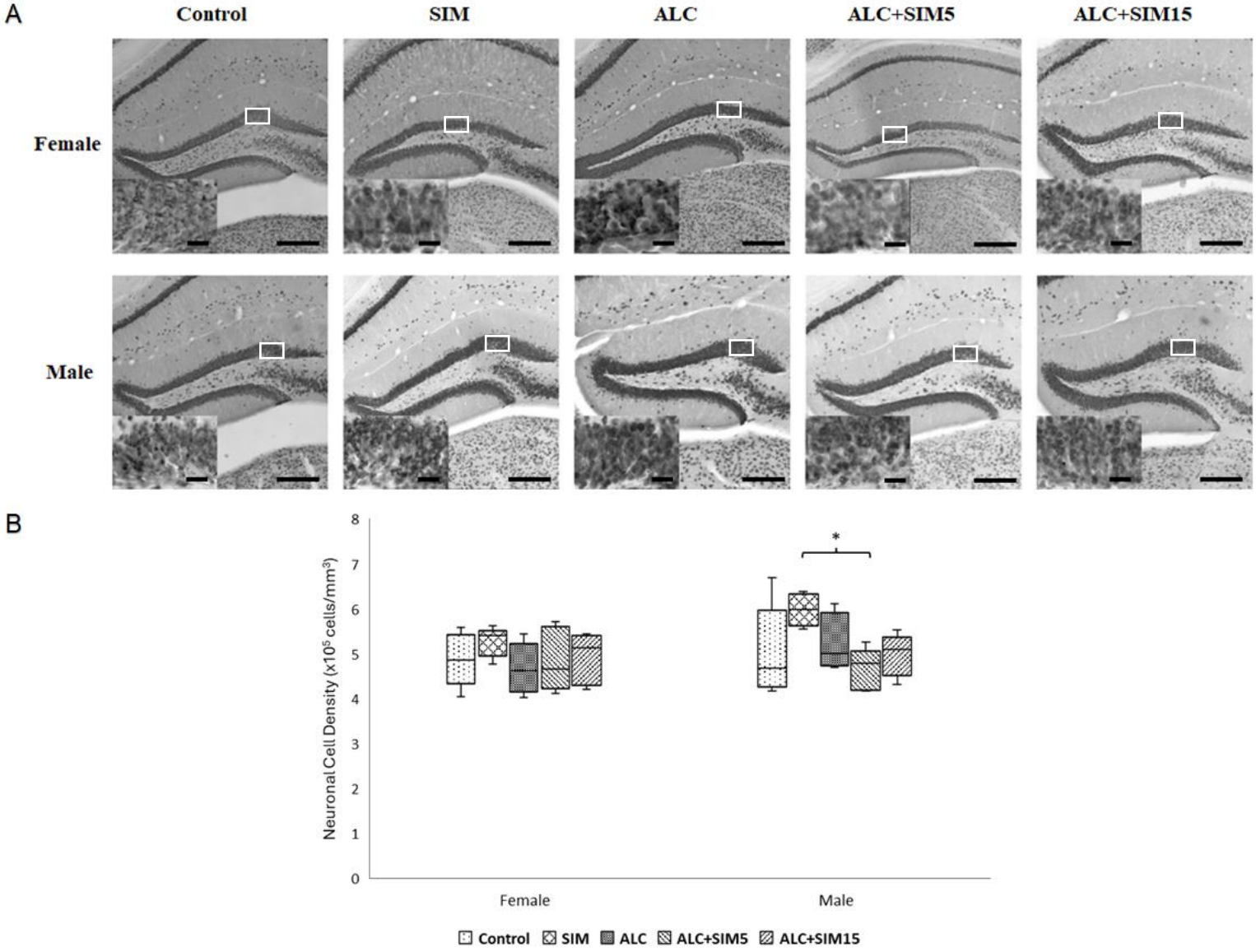

A
Control
SIM
ALC
ALC+SIM5
ALC+SIM15
Female
Male
B
Neuronal Cell Density (x10^5 cells/mm^3)
0
1
2
3
4
5
6
7
8
*
Female
Male
Control
SIM
ALC
ALC+SIM5
ALC+SIM15

Figure. 3.5: NeuN-positive Neuronal Density in the Suprapyramidal Blade of the Dentate Gyrus of Adolescent Mice Across Treatment Groups and Sexes. Illustrations of the A) photomicrographs showing NeuN-positive cells in the dentate gyrus, B) box plots showing the estimated NeuN-positive neuronal density (x 10^5 cells/mm^3) in the hippocampus of adolescent female and male mice across treatment groups. NeuN-immunolabeled cells in the dentate gyrus exhibited characteristic features such as prominent cell bodies, well-defined nuclei, and dendritic processes, consistent with normal neuronal architecture. The white box in the lower-magnification images indicates the region shown at higher magnification. No significant differences in neuronal density were observed among treatment groups in females, whereas a significant group effect was observed in males. A significant increase in neuronal density was found in the SIM group compared to the ALC+SIM5 group in males. Control - No treatment; SIM – 5 mg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg Simvastatin; ALC+SIM15 – Alcohol + 15 mg Simvastatin. Female = 25, Male = 25. *Statistically significant at $p < 0.05$. Scale bar of lower magnification = 200 μm; Scale bar of higher magnification = 20 μm

3.5.3. Distribution of GFAP-Positive Cells in the Suprapyramidal Blade of the Dentate Gyrus

GFAP immunolabelling was used to assess the effect of simvastatin on the astrocyte population in the suprapyramidal blade of the dentate gyrus following alcohol exposure in adolescent mice. All experimental groups and both sexes were included in the analyses (Fig. 3.6A). No significant morphological alterations were observed in GFAP-immunolabeled astrocytes across treatment groups. The structural integrity of the DG remained consistent, with GFAP immunopositive cells exhibiting typical morphology. There were no noticeable changes in the shape, size, or distribution of astrocytes in any group. GFAP-positive cells were observed in both the DG and CA regions, displaying a uniform distribution without any apparent disruptions. The suprapyramidal and infrapyramidal blades of the DG remained clearly defined, with well-maintained anatomical boundaries across all groups.

In the female mice (Fig. 3.6B), a significant difference ($p = 0.031$) was observed in the distribution of GFAP-immunolabeled cells across treatment groups. ALC significantly increased the distribution of GFAP-immunolabeled cells compared to the control ($p = 0.014$) and SIM ($p = 0.006$) groups, an indication of a negative impact of alcohol on neuroinflammation and glial cell populations in the hippocampus. However, co-administration of 5 mg simvastatin (ALC+SIM5) significantly reduced the alcohol-induced elevation of GFAP-positive cells ($p = 0.043$), suggesting a protective or regulatory role of simvastatin in mitigating alcohol-induced neuroinflammation. Conversely, no significant difference was observed between the control and SIM groups ($p = 0.921$), indicating that simvastatin alone does not notably affect GFAP cell distribution in the DG. Similarly, GFAP distribution in the ALC+SIM5 ($p = 0.609$) and ALC+SIM15 ($p = 0.106$) groups did not significantly differ from the control, implying a normalizing effect of simvastatin when administered alongside alcohol, returning the distribution of GFAP-positive cells to the level of control group. Comparisons between simvastatin-treated groups further highlight its potential dose-dependent effects. The difference between SIM and ALC+SIM5 ($p = 0.512$) was not significant, nor was the comparison between SIM and ALC+SIM15 ($p = 0.064$), though the latter approached significance, suggesting a potentially greater effect on GFAP modulation at the higher dose. Additionally, the difference between ALC and ALC+SIM15 ($p = 0.377$) and ALC+SIM5 and ALC+SIM15 ($p = 0.250$) groups was also not significant. These findings demonstrate that ALC significantly increased GFAP-positive astrocyte distribution in the DG, while simvastatin, particularly at 5 mg, mitigated this effect.

In the male mice (Fig. 3.6B), a significant difference (p = 0.000) was also observed in the distribution of GFAP-immunolabelled cells across treatment groups. Unlike in females, where alcohol increased GFAP-positive cell distribution, ALC significantly reduced the distribution of astrocytic cells compared to the control group (p = 0.000), suggesting a potential suppressive effect of alcohol on astrocyte populations in male mice. This reduction was further exacerbated by simvastatin treatment, with ALC+SIM5 (p = 0.000) and ALC+SIM15 (p = 0.000) showing significantly lower GFAP cell distributions than the control group. SIM treatment also resulted in a significant reduction compared to the control group (p = 0.042), but it was not significantly different from the ALC group (p = 0.130), indicating that alcohol and simvastatin may have overlapping effects in reducing the distribution of GFAP-positive cells. However, when simvastatin was combined with alcohol, a progressive reduction in GFAP-positive cell distribution was observed, with ALC+SIM5 showing significantly lower values compared to both SIM (p = 0.022) and ALC+SIM15 (p = 0.000). Additionally, the ALC+SIM15 group displayed significantly lower GFAP levels compared to both ALC (p = 0.002) and ALC+SIM5 (p = 0.023), suggesting a possible dose-dependent effect of simvastatin in further reducing GFAP levels in alcohol-exposed males.

In summary, ALC significantly altered the distribution of GFAP-positive astrocytes in both female and male mice, with a sex-specific pattern of response: alcohol increased GFAP distribution in females and decreased it in males. Simvastatin, particularly at 5 mg, demonstrated a protective effect in females by mitigating alcohol-induced increases in GFAP, while in males, simvastatin exacerbated alcohol-induced reductions in GFAP-positive cells, with a dose-dependent effect observed at higher doses.

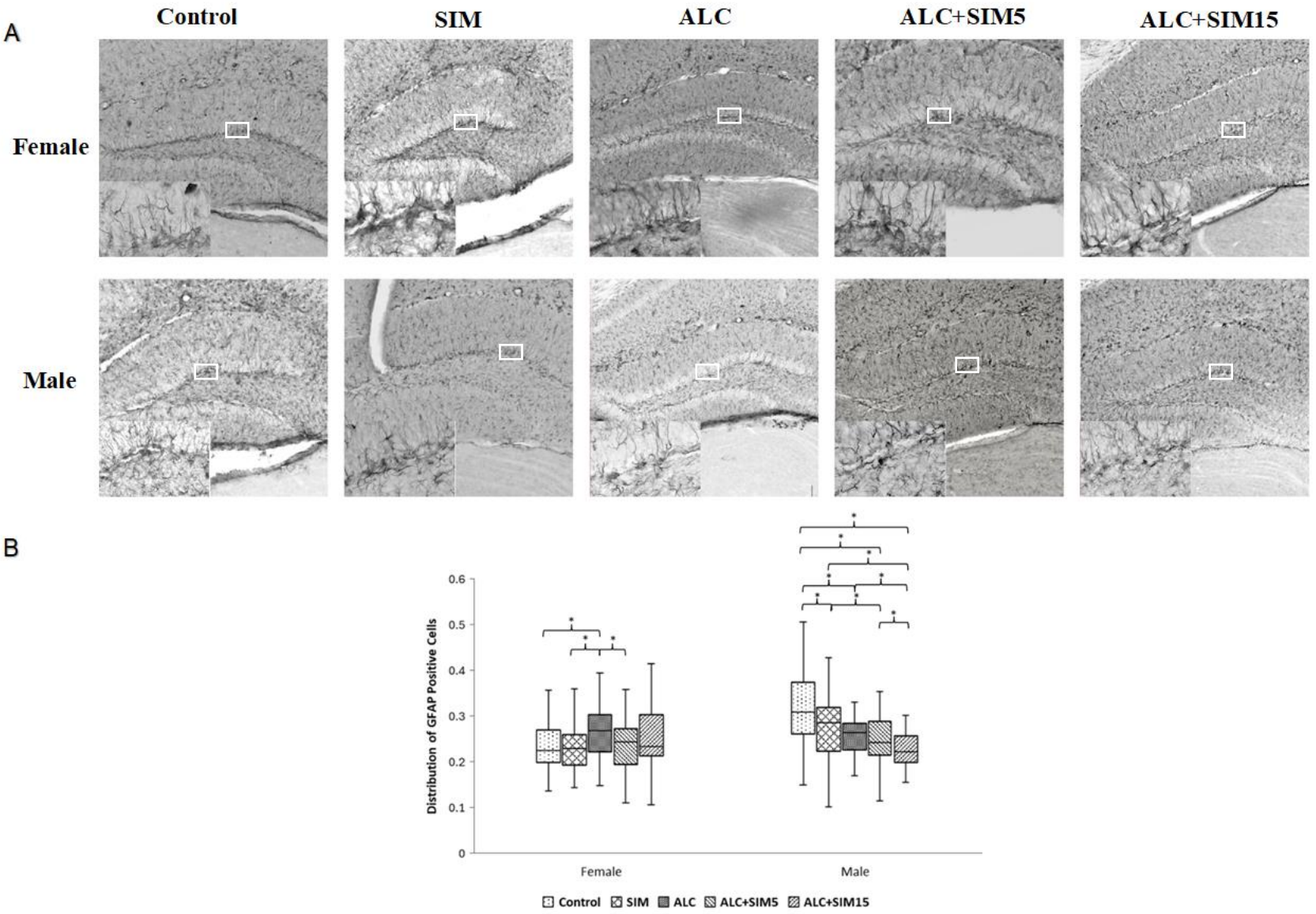

A
Control
SIM
ALC
ALC+SIM5
ALC+SIM15
Female
Male
B
Distribution of GFAP Positive Cells
0.6
0.5
0.4
0.3
0.2
0.1
0
Female
Male
Control
SIM
ALC
ALC+SIM5
ALC+SIM15

Figure 3.6: **GFAP-Positive Astrocyte Distribution in the Suprapyramidal Blade of the Dentate Gyrus of Adolescent Mice Across Treatment Groups and Sexes**. Illustrations of A) the photomicrographs showing GFAP-positive cells in the dentate gyrus and (B) the boxplots showing the statistical comparison of the distribution of GFAP-positive cells in the suprapyramidal blades of the dentate gyrus in the hippocampus of both female and male adolescent mice. GFAP-positive astrocytes appeared dark, with characteristic stellate morphology. The white box in the lower-magnification images indicates the region shown at higher magnification. ALC significantly increased GFAP-positive astrocyte distribution in females, while it decreased in males. SIM further reduced GFAP distribution in a dose-dependent way in both sexes, with 5 mg simvastatin effectively counteracting alcohol-induced gliosis in females. In males, simvastatin further suppressed GFAP levels in a dose-dependent fashion, suggesting a potential synergistic effect with alcohol. These results highlight the distinct, sex-dependent responses to alcohol and simvastatin treatment, the distribution of GFAP-positive cells in the dentate gyrus. Control - No treatment (No of suprapyramidal blade assessed: F = 41; M = 49); SIM – 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 56; M = 45); ALC – Alcohol (No of suprapyramidal blade assessed: F = 46; M = 53); ALC+SIM5 – Alcohol + 5 mg Simvastatin (No of suprapyramidal blade assessed: F = 48; M = 50); ALC+SIM15 – Alcohol + 15 mg Simvastatin (No of suprapyramidal blade assessed: F = 48; M = 53). Female = 25; Males = 25. *Statistically significant at $p < 0.05$. Scale bar of lower magnification = 100 μm; Scale bar of higher magnification = 10 μm.

3.5.4. Volume Estimation of Nissl-stained Sections using the Cavalieri Principle

In the present study, volumetric analysis was confined to the DG subregion, as the DG is particularly susceptible to alcohol-induced structural alterations and plays a pivotal role in hippocampal-dependent cognitive processes. This targeted approach enabled a more precise assessment of region-specific changes. Volume estimates of the entire DG, obtained using the Cavalieri principle on Nissl-stained hippocampal sections, were calculated using stereological sampling parameters of 50 µm section thickness and a 50 × 50 µm grid size. The Gundersen coefficient of error (CE) was ≤ 0.10 across all groups, indicating acceptable precision and reproducibility of the estimates. Summary data are presented in Table 3.3.

No significant morphological alterations were detected across treatment groups in either sex, as assessed in the Nissl-stained sections used for hippocampal (DG) volume estimations (Fig. 3.7A). Specifically, the structural integrity of the DG remained consistent among all experimental conditions. The Nissl-stained sections revealed well-preserved cellular architecture within the DG, with clear delineation of the hippocampal subregions. Additionally, the suprapyramidal and infrapyramidal blades of the DG were clearly identifiable and maintained intact anatomical boundaries in all experimental groups.

The estimated DG volume did not differ significantly among the treatment groups in either female or male adolescent mice (Fig. 3.7B). In both sexes, treatment did not have a measurable effect on DG volume (female: F (4, 20) = 0.56, p = 0.691; male: F (4, 23) = 0.60, p = 0.666). Post hoc comparisons confirmed that no significant pairwise differences were present between any treatment groups in either sex ($p > 0.63$). In females, the SIM group showed the highest mean volume estimate (422.65 mm^3), while the ALC+SIM5 group showed the lowest (339.08 mm^3). In males, the SIM group showed the highest mean volume estimate (384.03 mm^3), and the ALC+SIM15 group the lowest (335.00 mm^3). However, these differences did not reach statistical significance. These findings indicate that, under the conditions tested, alcohol and simvastatin, whether administered separately or together, did not significantly affect DG volume in either female or male adolescent mice.

Table 3.3 Cavalieri Method for Estimating Dentate Gyrus Volume from Nissl-Stained Sections in Adolescent Male and Female Mice Across Treatment Groups

Treatment Group	Number of Animals per Group	Total Number of Sections per Group	Average Coefficient of Error (Gundersen, m=1) per Group	Average Volume (mm^3) per Group
Female				
Control	5	47	0.013	357.28
SIM	5	51	0.011	422.65
ALC	5	53	0.012	358.98
ALC+SIM5	5	47	0.013	339.08
ALC+SIM15	5	45	0.013	377.13
Male				
Control	5	51	0.014	328.45
SIM	5	54	0.013	384.03
ALC	5	53	0.013	345.70
ALC+SIM5	5	44	0.033	332.84
ALC+SIM15	5	44	0.034	335.00

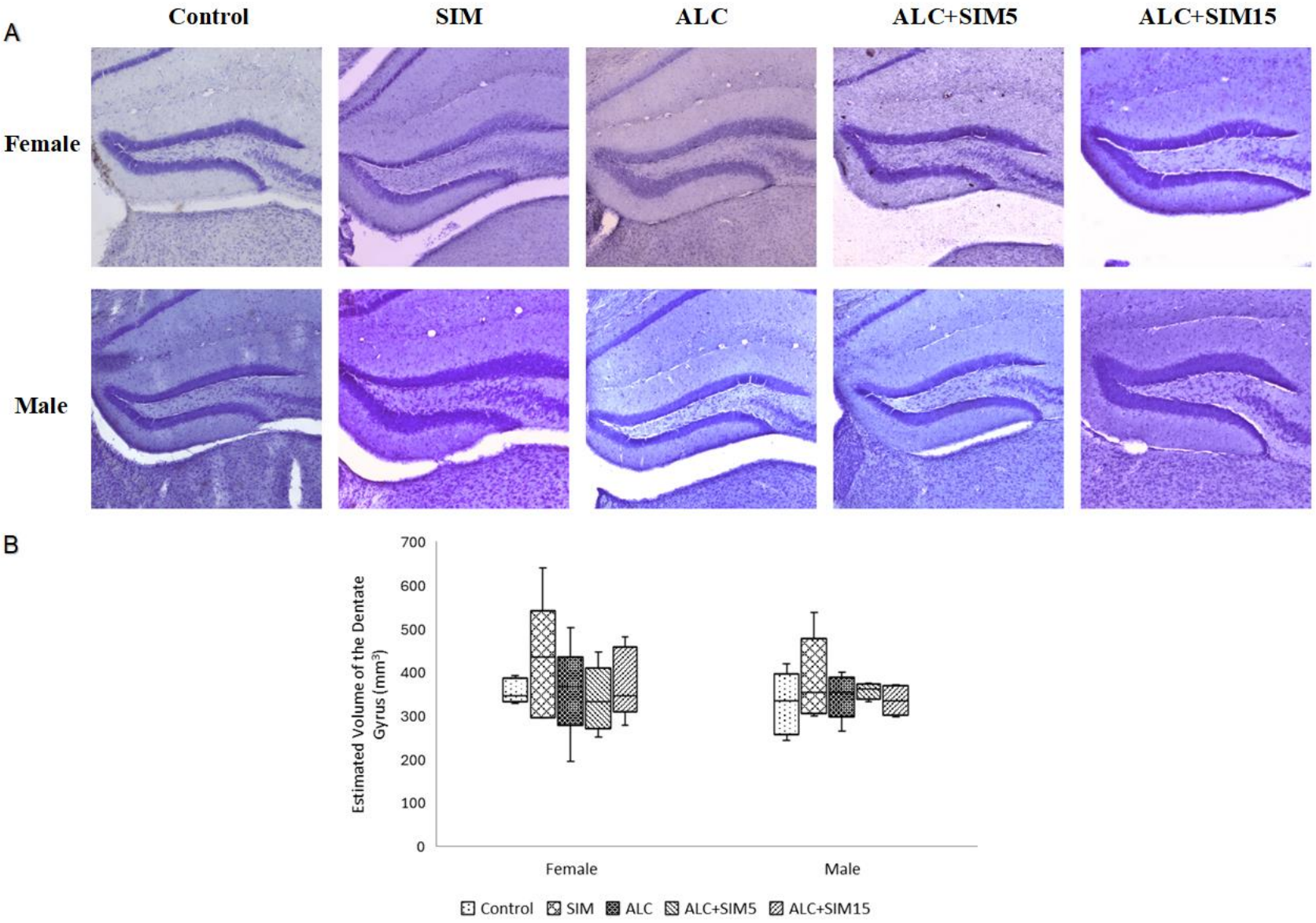

A
Control
SIM
ALC
ALC+SIM5
ALC+SIM15
Female
Male
B
Estimated Volume of the Dentate Gyrus (mm³)
700
600
500
400
300
200
100
0
Female
Male
Control
SIM
ALC
ALC+SIM5
ALC+SIM15

Figure 3.7: **Volume Estimates of the Dentate Gyrus in Adolescent Mice Across Treatment Groups and Sexes Based on the Cavalieri Principle**. Illustrations of A) photomicrographs showing Nissl-stained dentate gyrus and B) Box plots showing the estimated volume of the dentate gyrus (DG) in adolescent male and female mice across treatment groups. Volume estimates were obtained using the Cavalieri principle applied to Nissl-stained coronal sections. There were no statistically significant differences between the treatment groups in both sexes. Additionally, no significant morphological alterations were detected across treatment groups, with well-preserved cellular architecture within the DG. The suprapyramidal and infrapyramidal blades were distinguishable, exhibiting intact anatomical boundaries in all treatment conditions. Control - No treatment; SIM – 5 mg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg Simvastatin; ALC+SIM15 – Alcohol + 15 mg Simvastatin. Female = 25; Males = 25. *Statistically significant at $p < 0.05$. Scale bar of lower magnification = 200 μm

3.6. Estimation of Neuronal Density in the Prefrontal Cortex Using Nissl-staining and NeuN-immunolabelling Across Cortical Layers (I–IV)

The number of Nissl-stained and NeuN-immunopositive cells within cortical layers I-IV of the PFC was quantified in male and female mice across all treatment groups to assess the impact of alcohol (ALC), simvastatin (SIM), and their combined treatment on neuronal cell density.

3.6.1. Estimation of Neuronal Density Using Nissl-stained Cells in the Cortical Layers (I-IV) of the Prefrontal Cortex

In the Nissl-stained PFC sections (Fig. 3.8A), no morphological alterations were observed across cortical layers I through IV after treatment. Layer I continued to show a sparse presence of neuronal cell bodies and dendritic processes, while layers II and III maintained consistent densities of pyramidal neurons and their dendritic branches. The distribution of small, granule-like cells in layer IV remained unchanged. These results indicate that the treatment did not cause significant structural changes in the PFC, preserving the typical architecture of the cortical layers (Fig. 3.8B).

Neuron counts were obtained using the optical fractionator probe, with carefully optimized stereological parameters to ensure unbiased and efficient sampling. These parameters included a counting frame size of 50 × 50 µm, a sampling grid of 75 × 75 µm, a section thickness of 50 µm, and a dissector height of 10 µm, consistently applied to the Nissl-stained PFC sections. Table 3.4 summarises the key stereological metrics for both female and male mice across treatment groups. It includes the average number of markers counted per group, the total number of sections analysed, and the average number of sampling sites, reflecting the sampling scope and consistency. Additionally, the average measured mounted thickness and estimated neuronal population, calculated using mean section thickness alongside counts, are reported to provide insight into the accuracy of the estimates. The reliability of these estimates was assessed via the Gundersen coefficient of error (CE), with values for m = 0 and m = 1 shown to demonstrate data precision. Lastly, the average measured volume of the prefrontal cortex (in mm^3) per group is presented to contextualize the cell density findings.

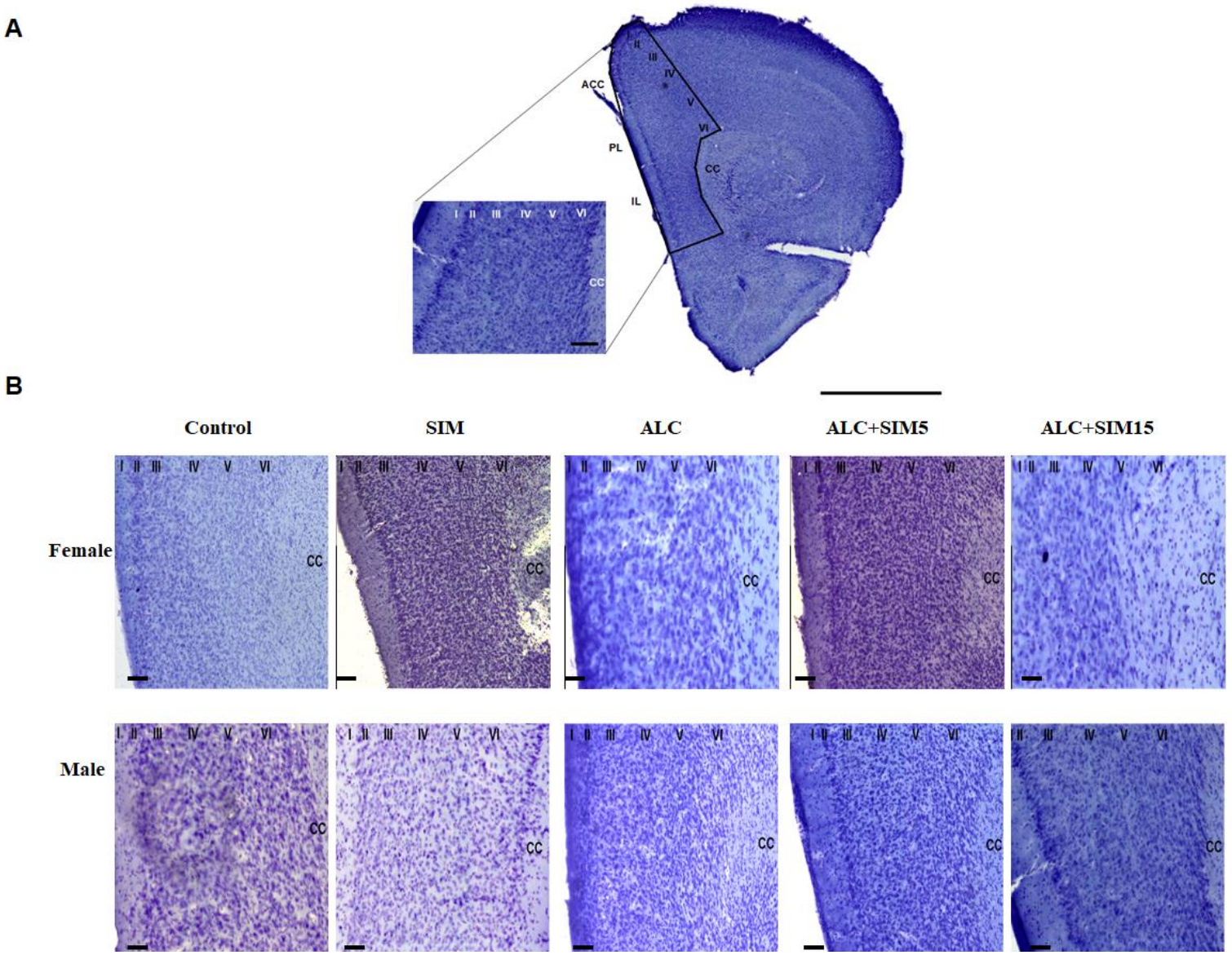
In the PFC of female adolescent mice across experimental groups (Fig. 3.8C), treatment had a statistically significant effect on overall cell density. The ALC group showed a significant

increase in cell density ($3.18 \times 10^5 \pm 4.96 \times 10^3$, $p = 0.009$) compared to the control group ($2.45 \times 10^5 \pm 9.24 \times 10^3$), suggesting that alcohol exposure may lead to a compensatory increase in overall cell population in the PFC. In contrast, the SIM group did not differ from the control ($2.75 \times 10^5 \pm 1.58 \times 10^4$, $p = 0.53$), indicating that simvastatin alone did not significantly affect the total cell density. Both the ALC+SIM5 and ALC+SIM15 groups exhibited significantly higher cell densities than the control, with the ALC+SIM5 group having a mean of $3.30 \times 10^5 \pm 2.62 \times 10^3$ ($p = 0.004$) and the ALC+SIM15 group showing a mean of $3.62 \times 10^5 \pm 2.26 \times 10^4$ ($p = 0.000$). The ALC+SIM15 group exhibited a particularly pronounced increase in cell density ($3.62 \times 10^5 \pm 2.26 \times 10^4$), significantly higher than the SIM group ($2.75 \times 10^5 \pm 1.58 \times 10^4$, $p = 0.002$). Additionally, the ALC+SIM5 ($3.30 \times 10^5 \pm 2.62 \times 10^3$, $p = 0.004$) and ALC+SIM15 ($3.62 \times 10^5 \pm 2.26 \times 10^4$, $p = 0.000$) groups had significantly higher cell densities compared to the ALC group ($3.18 \times 10^5 \pm 4.96 \times 10^3$), suggesting that the combination of alcohol exposure and simvastatin treatment led to a significant increase in total cell density.

In the PFC of male adolescent mice across experimental groups (Fig. 3.8C), treatment did not have a statistically significant effect on overall cell density. The mean cell density in the control group was $2.80 \times 10^5 \pm 1.31 \times 10^3$, and the SIM group showed a mean of $2.99 \times 10^5 \pm 1.96 \times 10^3$, with no significant difference between the two ($p = 0.53$). The ALC group had the highest mean density at $3.30 \times 10^5 \pm 1.65 \times 10^3$, though this did not differ significantly from the control ($p = 0.09$). Both the ALC+SIM5 ($3.11 \times 10^5 \pm 2.59 \times 10^3$, $p = 0.09$) and ALC+SIM15 ($3.28 \times 10^5 \pm 5.13 \times 10^3$, $p = 0.09$) groups also showed higher cell densities compared to the control, but these differences were not statistically significant. One-way ANOVA confirmed that there were no significant differences between the treatment groups ($F(4,19) = 0.64$, $p = 0.64$), and post hoc comparisons confirmed that no pairwise comparisons were significant ($p > 0.05$). These results suggest that, in male adolescent mice, the treatments tested did not significantly affect the overall cell density in the PFC under the conditions used in this study.

Table 3.4 Summary of Stereological Data for Nissl-stained Cells in the Prefrontal Cortex Across Treatment Groups and Sexes

Treatment Group	Average Markers Counted	Total Number of Sections	Average Number of Sampling Sites	Average Measured Defined Mounted Thickness	Average Estimated Population using Mean Section Thickness (x 10^4)	Average Estimated Population using Mean Section Thickness with Counts (x 10^4)	Average Estimated Population using Number Weighted Section Thickness (x 10^4)	Average Coefficient of Error (Gundersen, m=0)	Average Coefficient of Error (Gundersen, m=1)	Average Measured Volume (x 10^8 µm³)	Average Cell Density (x 10^5 cells/mm³)
Female											
NT	1664	15	213	32	3.97	3.97	3.97	0.29	0.07	1.64	2.43
SIM	2368	14	279	33	7.07	7.07	7.07	0.16	0.04	2.61	2.75
ALC	2626	15	271	34	8.04	8.05	8.05	0.14	0.04	2.53	3.18
ALC+SIM5	2982	15	293	35	9.35	9.35	9.35	0.15	0.04	2.71	3.46
ALC+SIM15	3306	15	293	33	9.73	9.73	9.73	0.14	0.04	2.70	3.62
Male											
NT	2082	14	233	32	5.34	5.34	5.34	0.25	0.06	1.89	2.80
SIM	2469	15	275	34	7.55	7.55	7.55	0.14	0.04	2.51	2.99
ALC	2779	15	275	33	7.83	7.83	7.83	0.18	0.05	2.38	3.30
ALC+SIM5	2731	15	287	33	7.60	7.60	7.60	0.18	0.05	2.49	3.11
ALC+SIM15	2867	12	304	36	9.47	9.47	9.47	0.13	0.03	2.87	3.28


A
ACC
II
III
IV
V
VI
PL
CC
IL
I II III IV V VI
CC
B
Control
SIM
ALC
ALC+SIM5
ALC+SIM15
Female
Male
CC

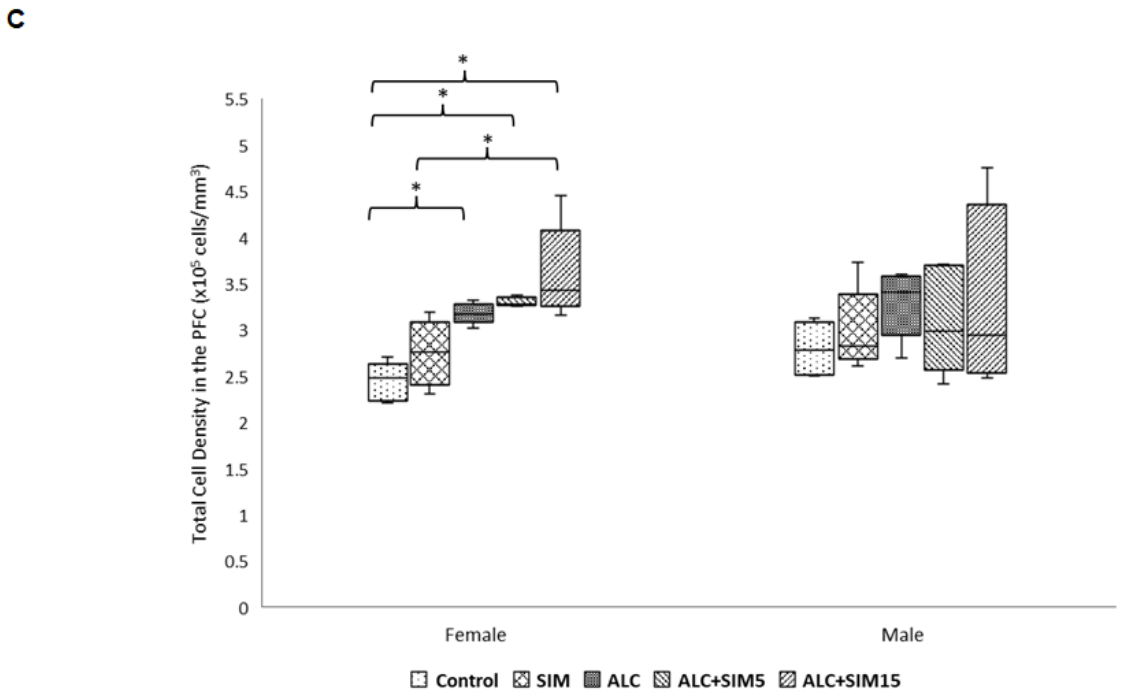


Figure 3.8: Effect of Alcohol and/or Simvastatin on Nissl-Stained Cell Densities in the Prefrontal Cortex of Adolescent Mice Across Sexes.

A) Representative low-magnification photomicrograph illustrating the prefrontal cortex (PFC) region of interest (ROI) and cortical layers (I–VI). B) Higher-magnification Nissl-stained images from each treatment group and sex showing the ROI and cortical layers (I–VI). C) Box plots presenting Nissl-stained neuronal cell densities ($\times 10^5$ cells/mm^3) in the PFC of female and male mice across treatment groups. In females, the ALC group exhibited significantly higher cell density compared to the control group. The ALC+SIM5 and ALC+SIM15 groups also showed significantly increased densities relative to the control, with ALC+SIM15 being significantly higher than SIM alone. In males, no significant differences were observed among groups, indicating no treatment-related effects on PFC cell density. Control – No treatment; SIM – 5 mg/kg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg/kg Simvastatin; ALC+SIM15 – Alcohol + 15 mg/kg Simvastatin. Female n = 25; Male n = 25. *$p < 0.05$. Scale bars: low magnification = 200 µm; high magnification = 20 µm

3.6.2. Estimation of the Neuronal Density Using NeuN-immunolabelled Positive Cells in the Cortical Layers (I-IV) of the Prefrontal Cortex

No notable morphological alterations were observed in the NeuN-immunolabelled PFC sections across cortical layers I through IV after treatment (Fig. 3.8A). Layer I remained sparsely populated with neuronal cell bodies, with minimal dendritic processes visible. In layers II and III, pyramidal neurons maintained consistent density, exhibiting well-defined cell bodies, prominent nuclei, and dendritic extensions characteristic of normal neuronal architecture. Layer IV showed no alterations in the distribution or morphology of small, granule-like cells, which exhibited the typical compact structure. These results indicate that the treatment did not cause significant morphological alterations in the prefrontal cortex, maintaining the normal organization and structure of the cortical layers (Fig. 3.8B).

Stereological analysis was performed using the optical fractionator probe, with optimized parameters applied to ensure efficient and unbiased sampling. The parameters consisted of a counting frame measuring 50 × 50 µm, a sampling grid of 75 × 75 µm, section thickness of 50 µm, and a dissector height of 10 µm. These settings were consistently applied to all NeuN-immunolabelled sections. Table 3.5 provides a summary of the stereological parameters for male and female mice across all treatment groups. This includes the average count of NeuN-positive markers per group, the total number of sections examined, and the average number of sampling sites, together representing the thoroughness and consistency of sampling. Additionally, the average mounted section thickness and estimated neuronal populations, calculated using mean section thickness and counts, are reported, offering valuable information on the quantification of mature neurons. To assess the precision of these estimates, average coefficients of error (Gundersen, m = 0 and m = 1) are included. Additionally, the average measured volume of the prefrontal cortex (mm^3) per group is provided to support the interpretation of calculated neuronal densities.

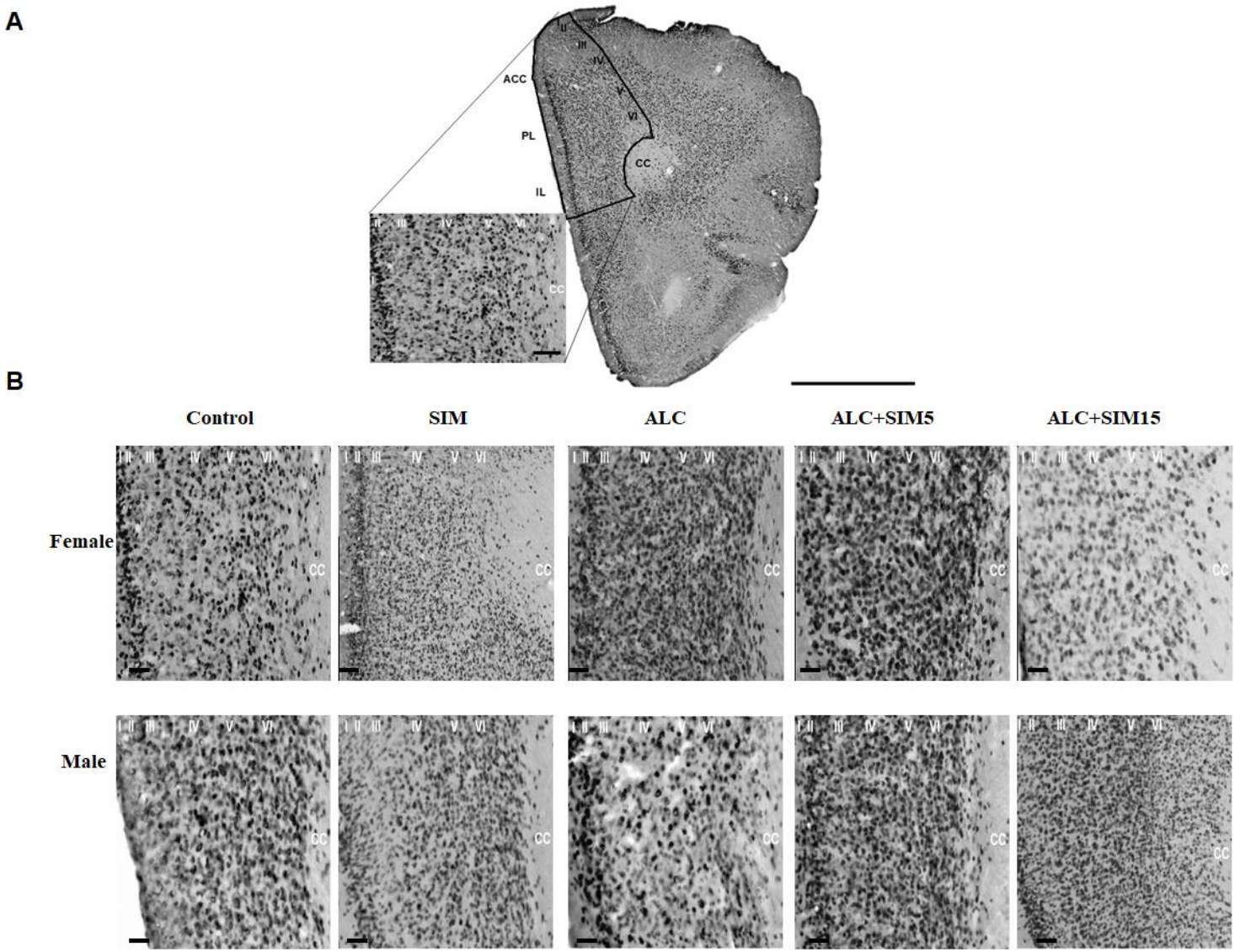
A one-way ANOVA was conducted to assess the effect of treatment on NeuN-positive cell density in the PFC of female adolescent mice (Fig. 3.9C) and revealed a statistically significant effect of treatment ($F(4, 20) = 5.022$, $p = 0.006$). Compared to the control group, the ALC+SIM15 group exhibited a significant increase in NeuN-positive cell density ($p = 0.002$). Although ALC and ALC+SIM5 resulted in numerical increases in NeuN-positive cell density relative to the control group, these differences were not statistically significant ($p > 0.05$).

Specifically, ALC increased the mean neuronal density from 1.65×10^5 cells/mm³ (control) to 2.19×10^5 cells/mm³ (ALC), while ALC+SIM5 further elevated the mean density to 2.30×10^5 cells/mm³. SIM also increased the mean neuronal density (mean: 2.11×10^5 cells/mm³) compared to the control group, although this was not statistically significant. Overall, these findings suggest that while alcohol exposure and simvastatin treatment individually increased NeuN-positive cell density to some extent, only ALC+SIM15 (mean: 2.68×10^5 cells/mm³) produced a statistically significant enhancement in neuronal density within the PFC of female mice.

In contrast to the significant treatment effects observed in female mice, analysis of NeuN-positive cell density in the PFC of male mice did not reveal a consistent pattern of significant treatment-related changes. ($F(4, 19) = 2.351$, $p = 0.091$; Fig. 3.9C). However, post hoc comparisons identified one significant pairwise difference: the ALC+SIM15 group exhibited a significantly higher NeuN-positive cell density than the ALC+SIM5 group ($p = 0.007$). Although ALC and ALC+SIM15 resulted in increases in NeuN-positive cell density relative to the control group, these differences were not statistically significant ($p > 0.05$). Specifically, ALC increased the mean neuronal density from 2.16×10^5 cells/mm³ (control) to 2.62×10^5 cells/mm³ (ALC), while ALC+SIM15 maintained a similarly elevated mean density of 2.63×10^5 cells/mm³. SIM decreased the mean neuronal density to 2.07×10^5 cells/mm³ compared to the control group, although this reduction was not statistically significant. The ALC+SIM5 group showed a moderate increase to 2.32×10^5 cells/mm³. Overall, these findings suggest that ALC modestly increased NeuN-positive cell density in male mice, particularly when combined with high-dose simvastatin, but the changes did not reach statistical significance, and treatment-related effects were less consistent than those observed in females.

Table 3.5 Summary of Stereological Data for NeuN-Positive Cells in the Prefrontal Cortex Across Treatment Groups and Sexes

Treatment Group	Average Markers Counted	Total Number of Sections	Average Number of Sampling Sites	Average Measured Defined Mounted Thickness	Average Estimated Population using Mean Section Thickness (x 10^4)	Average Estimated Population using Mean Section Thickness with Counts (x 10^4)	Average Estimated Population using Number Weighted Section Thickness (x 10^4)	Average Coefficient of Error (Gundersen, m=0)	Average Coefficient of Error (Gundersen, m=1)	Average Measured Volume (x 10^8 µm³)	Average Neuronal Cell Density (x 10^5 cells/mm³)
Female											
NT	1282	15	238	32	3.06	3.06	3.06	0.23	0.06	1.87	1.65
SIM	1637	14	250	33	4.91	4.91	4.91	0.16	0.04	2.30	2.11
ALC	1923	15	286	34	5.88	5.88	5.88	0.15	0.04	2.68	2.19
ALC+SIM5	2135	15	307	35	6.61	6.61	6.61	0.14	0.04	2.87	2.30
ALC+SIM15	2428	15	293	33	7.26	7.26	7.26	0.13	0.04	2.69	2.68
Male											
NT	1409	13	217	33	3.81	3.81	3.81	0.23	0.06	1.79	2.16
SIM	1612	15	258	34	4.42	4.42	4.42	0.24	0.06	2.14	2.07
ALC	2263	15	278	33	6.43	6.43	6.43	0.18	0.05	2.46	2.62
ALC+SIM5	1974	15	273	40	7.27	7.27	7.33	0.14	0.04	2.50	2.87
ALC+SIM15	2231	15	300	36	6.92	6.92	6.93	0.18	0.05	2.68	2.63


A
ACC
PL
IL
II
III
IV
V
VI
CC
B
Control
SIM
ALC
ALC+SIM5
ALC+SIM15
Female
Male

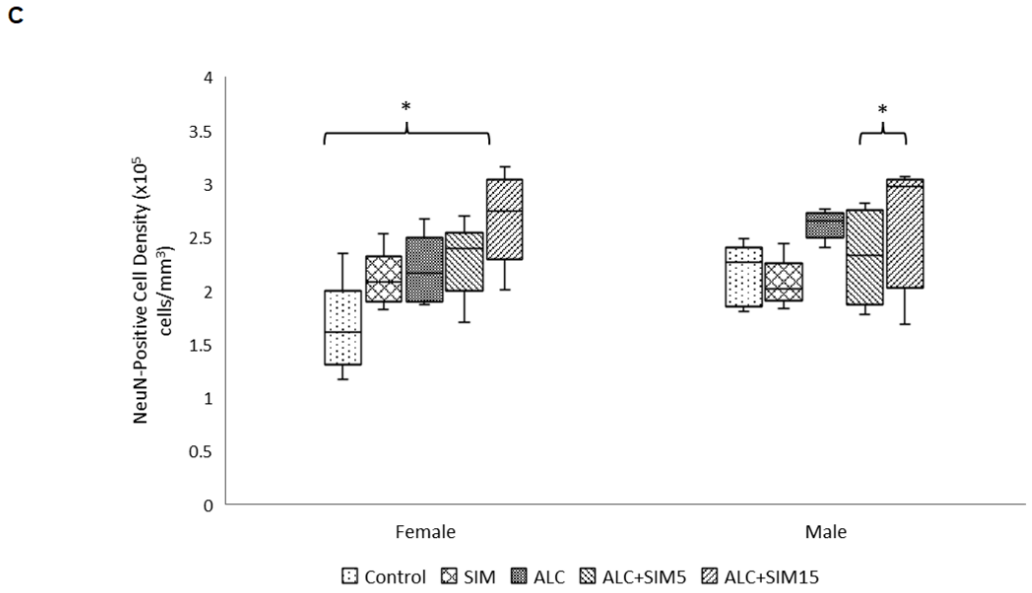


Figure 3.9: Effect of Alcohol and/or Simvastatin on NeuN-Positive Cell Densities in the Prefrontal Cortex of Adolescent Mice Across Sexes.

A) Representative low-magnification photomicrograph showing the prefrontal cortex (PFC) region of interest (ROI) and cortical layers (I–VI). B) Higher-magnification images of NeuN-immunolabelled sections from each treatment group and sex, illustrating NeuN-positive cells within the defined ROI and cortical layers. C) Box plots showing NeuN-positive neuronal cell densities ($\times 10^5$ cells/mm³) in the PFC of female and male mice across treatment groups. In females, the ALC+SIM15 group showed a significant increase in NeuN-positive cell density compared to the control group. While alcohol and simvastatin alone elevated neuronal density, only ALC+SIM15 induced a statistically significant increase. In males, no consistent treatment-related differences were observed. Although ALC and ALC+SIM15 groups showed elevated densities compared to control, these increases were not statistically significant. However, the ALC+SIM15 group had a significantly higher density than the ALC+SIM5 group. Treatment groups: Control – No treatment; SIM – 5 mg/kg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg/kg Simvastatin; ALC+SIM15 – Alcohol + 15 mg/kg Simvastatin. Female n = 25; Male n = 25. *$p < 0.05$. Scale bars: low magnification = 200 µm; high magnification = 20 µm

3.7. Quantification of the Expression Levels of Neurotrophic Markers (IL-6, IL-10, CREB1, and BDNF) in the Hippocampus Using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To assess neuroinflammatory and neuroplasticity-related alterations in the hippocampus, mRNA levels of IL-6, IL-10, CREB1, and BDNF were measured using reverse transcription polymerase chain reaction (RT-PCR). This analysis was conducted on both female and male mice across five treatment groups: control, SIM (5 mg), ALC, ALC+SIM5, and ALC+SIM15.

3.7.1. Neuroinflammatory Responses to Alcohol and Simvastatin Treatment as Indicated by IL-6 and IL-10 Expression in the Hippocampus

Table 3.6 summarises the statistics for IL-6, a pro-inflammatory cytokine, and IL-10, an anti-inflammatory cytokine, which were measured to assess the neuroinflammatory response to alcohol and simvastatin treatment in the hippocampus of adolescent female and male mice. IL-6 expression remained relatively stable in females but decreased in males following treatment, with the highest levels observed in male controls. IL-10 expression was more variable, especially in females, with the highest mean in the ALC+SIM15 group. In males, IL-10 peaked in the ALC group, but overall showed less variability than in females.

In female mice, ALC and SIM treatments did not significantly affect the expression of either IL-6 (Fig. 3.9A) or IL-10 (Fig. 3.9B) in the hippocampus. One-way ANOVA for IL-6 demonstrated a marginal effect that did not reach statistical significance ($F(4,20) = 2.37$, $p = 0.087$), with ALC suggesting a potential increase in IL-6 expression compared to the control, however this difference was not statistically significant ($p = 0.995$, Cohen's $d = 0.28$). SIM alone did not significantly alter IL-6 expression relative to the control ($p = 0.929$). Additionally, ALC+SIM5 showed a non-significant increase compared to ALC ($p = 0.425$; Cohen's $d = 1.35$). Similarly, no significant differences were observed with ALC+SIM15 ($p = 0.997$). For IL-10, there was also no significant effect of treatment on expression levels ($F(4,17) = 0.54$, $p = 0.709$). Similarly, ALC showed a non-significant increase in IL-10 expression compared to the control group ($p = 0.963$, Cohen's $d = 0.55$). SIM did not alter IL-10 expression relative to control ($p = 1.000$), and no significant differences were observed with ALC+SIM5 or ALC+SIM15 ($p = 0.999$ and $p = 0.976$, respectively). Overall, although both IL-6 and IL-10 expression levels varied in response to ALC and SIM, these changes were not statistically significant.

In male mice, ALC and SIM treatments did not significantly affect the expression of IL-6 (Fig. 3.9A) or IL-10 (Fig. 3.9B) in the hippocampus. For IL-6, a one-way ANOVA demonstrated a marginal effect that did not reach statistical significance ($F(4,19) = 2.36$, $p = 0.090$), with ALC showing a potential decrease in IL-6 expression compared to the control group, although this difference was not statistically significant ($p = 0.123$, Cohen's d = 3.59). SIM alone did not significantly alter IL-6 expression relative to the control ($p = 0.360$), and no significant differences were observed with ALC+SIM5 or ALC+SIM15 ($p = 0.431$ and $p = 0.654$, respectively). Additionally, the Welch F test revealed a significant difference in variance between groups ($p = 0.0096$), though post-hoc comparisons did not indicate any significant differences between treatments. For IL-10, a one-way ANOVA showed an effect that was close to, but did not reach, statistical significance ($F(4,15) = 2.36$, $p = 0.0999$), with ALC showing a potential increase in IL-10 expression compared to control ($p = 0.197$, Cohen's d = 3.26). SIM did not significantly alter IL-10 expression compared to control ($p = 0.842$), and no significant differences were observed with ALC+SIM5 or ALC+SIM15 ($p = 0.321$ and $p = 0.048$, respectively). While the overall data suggested modulation of both IL-6 and IL-10 expression, none of the observed differences reached statistical significance.

Taken together, while IL-6 and IL-10 expression levels indicated possible modulation by ALC and SIM treatments in both male and female mice, these changes did not reach statistical significance

Table 3.6 Summary Statistics for IL-6 and IL-10 Expression Across Treatment Groups and Both Sexes in Adolescent Mice.

Group	N	Mean ± SE	Std. Dev.	Min	Max	Shapiro Wilk	p-value	Coeff. Of Variation (%)
			IL- 6					
Female								
Control	5	1.01 ± 0.07	0.16	0.82	1.23	0.96	0.82	15.66
SIM	5	0.93 ± 0.09	0.20	0.73	1.16	0.85	0.19	21.78
ALC	5	1.05 ± 0.07	0.15	0.89	1.25	0.94	0.65	13.90
ALC+SIM5	5	1.22 ± 0.05	0.12	1.05	1.33	0.93	0.59	9.60
ALC+SIM15	5	1.01 ± 0.06	0.14	0.89	1.22	0.90	0.39	13.92
Male								
Control	4	1.08 ± 0.02	0.04	1.03	1.12	0.90	0.43	4.07
SIM	5	0.91 ± 0.07	0.15	0.67	1.04	0.89	0.34	16.75
ALC	5	0.85 ± 0.08	0.17	0.70	1.12	0.88	0.31	20.26
ALC+SIM5	5	0.82 ± 0.06	0.13	0.63	0.98	0.97	0.87	16.08
ALC+SIM15	5	0.89 ± 0.05	0.11	0.73	0.99	0.91	0.47	12.73
			IL-10					
Female								
Control	5	1.43 ± 0.64	1.42	0.33	3.84	0.81	0.09	99.18
SIM	4	1.60 ± 0.28	0.57	1.13	2.33	0.89	0.38	35.44
ALC	4	2.08 ± 0.33	0.65	1.30	2.70	0.92	0.54	31.38
ALC+SIM5	4	2.24 ± 1.25	2.49	0.64	5.92	0.76	0.05	111.16
ALC+SIM15	5	2.64 ± 0.59	1.32	0.86	3.94	0.88	0.31	49.94
Male								
Control	4	1.04 ± 0.16	0.32	0.68	1.44	0.98	0.92	30.68
SIM	5	1.66 ± 0.40	0.90	0.47	2.51	0.90	0.40	54.36
ALC	4	2.51 ± 0.60	1.21	1.27	4.17	0.92	0.52	48.10
ALC+SIM5	3	1.12 ± 0.47	0.81	0.53	2.04	0.87	0.30	71.92
ALC+SIM15	4	2.49 ± 0.50	1.01	1.69	3.97	0.82	0.14	40.53

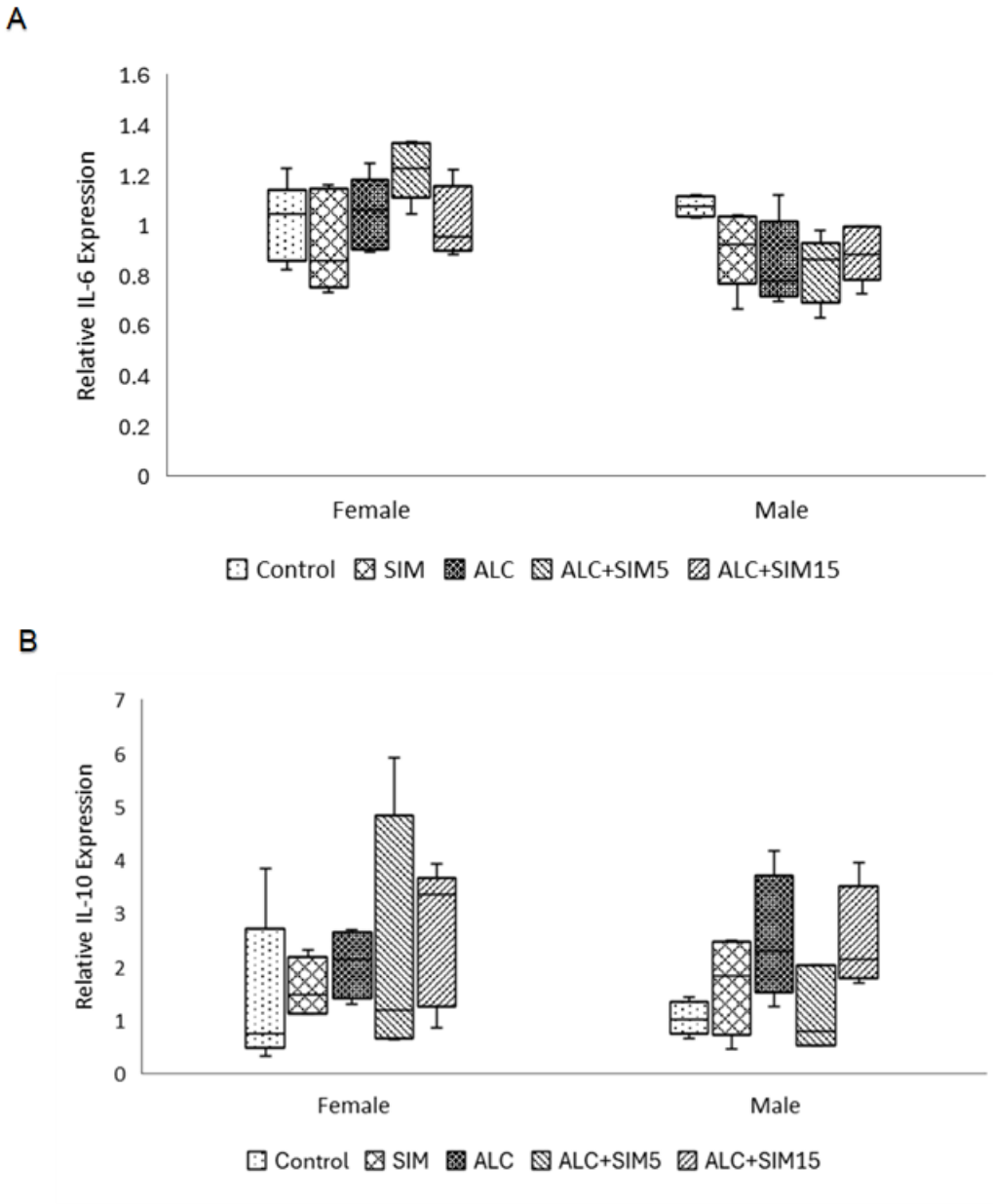


Figure 3.10: Hippocampal IL-6 and IL-10 Expression Following Alcohol and/or Simvastatin Treatment in Female and Male Adolescent Mice. Boxplots showing A) IL-6 and B) IL-10 expression in the hippocampus of female and male mice following alcohol and simvastatin treatment**.** No statistically significant differences were observed in IL-6 or IL-10 expression in either female or male mice across all treatment groups. ALC and co-treatment with SIM did not significantly alter expression levels of either cytokine in the hippocampus. Data are presented as mean ± SEM; n = 4–5 animals per group. Control - No treatment; SIM – 5 mg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg Simvastatin; ALC+SIM15 – Alcohol + 15 mg Simvastatin. *Statistically significant at $p < 0.05$.

3.7.2. CREB and BDNF Expression in the Hippocampus Indicating Neuroplasticity Responses to Alcohol and Simvastatin Treatment

Table 3.7 provides summary statistics for CREB, a transcription factor that regulates genes involved in synaptic plasticity, learning, and memory, as well as BDNF, a neurotrophic factor linked to neuronal survival and plasticity. Expression levels were measured to examine the effects of alcohol and simvastatin treatment on neuronal signalling and neuroplasticity within the hippocampus of adolescent female and male mice. In females, ALC and SIM treatments increased CREB expression compared to the control group, with some variability across groups. In males, CREB expression also rose following alcohol exposure and co-treatment with simvastatin, with the highest levels in the ALC+SIM15 group. BDNF expression in females was lowest in the control group and showed modest increases across treatment groups, peaking in the ALC+SIM15 group. In males, BDNF expression was similar to that of females at baseline, with slight increases following SIM administration and the greatest elevations after combined alcohol and simvastatin treatment. Overall, both CREB and BDNF showed treatment-related increases.

In the female mice, a one-way ANOVA revealed that differences in CREB expression among the groups did not reach statistical significance ($F(4, 20) = 2.779$, $p = 0.055$; Fig. 3.10A). However, the calculated omega squared ($\omega^2 = 0.2216$) indicated a moderate to large effect size, suggesting that the treatment may have a meaningful influence on CREB expression despite the lack of statistical significance at the conventional alpha level (0.05). Post hoc pairwise comparisons revealed no statistically significant differences between groups, although the comparison between the control and ALC groups approached significance ($p = 0.056$). Notably, the ALC and simvastatin-treated groups all showed slightly elevated mean CREB expression relative to the control group, with the highest levels observed in the ALC group (1.23 ± 0.06) and the ALC+SIM5 group (1.21 ± 0.08). Despite the lack of statistical significance, all pairwise comparisons between the control and treatment groups showed large effect sizes (Cohen's d ranging from -1.39 to -1.89). These findings suggest that alcohol exposure may enhance CREB expression in female mice, and that co-administration of simvastatin does not appear to attenuate this effect in a statistically significant manner.

In contrast, CREB expression levels in the hippocampus of male adolescent mice (Fig. 3.10A) differed significantly across treatment groups ($F(4,18) = 5.181$, $p = 0.006$). Post hoc

comparisons revealed that CREB expression was significantly increased in the ALC+SIM15 group compared to both the control ($p = 0.005$, Cohen's d = 1.30) and SIM ($p = 0.021$, Cohen's d = 1.16) groups. No significant differences were observed between ALC and ALC+SIM15 or between ALC and any other group, suggesting that simvastatin at a higher dose (15mg, ALC+SIM15) may have independently contributed to elevated CREB levels, rather than attenuating alcohol-related changes. These findings suggest that, while treatment significantly influenced CREB expression in male mice, with the ALC+SIM15 group exhibiting the greatest increase, the response to treatment in female mice was more subtle and did not reach statistical significance. This highlights a potential sex-specific modulation of CREB expression in response to alcohol and simvastatin treatment.

For BDNF expression, female adolescent mice (Fig. 3.10B) revealed no statistically significant differences in hippocampal BDNF expression across the treatment groups ($F(4,20) = 0.52$, $p = 0.722$). Consistently, post hoc comparisons did not identify any significant differences between treatment groups ($p > 0.05$). Although the ALC+SIM15 group exhibited the highest mean expression value, the difference relative to the control group was not statistically significant ($p = 0.686$, Cohen's d = 0.53). These results suggest that neither ALC, SIM, nor their combination significantly altered BDNF expression in the hippocampus of female mice. Similarly, there were no statistically significant differences in BDNF expression among treatment groups in male adolescent mice ($F(4,19) = 0.84$, $p = 0.516$; Fig. 3.10B). Post hoc comparisons supported this, with no pairwise comparisons reaching statistical significance ($p > 0.05$). Although the ALC+SIM15 group showed the highest mean BDNF expression, it did not significantly differ from the control group ($p = 0.546$, Cohen's d = 0.61) or other treatment groups. These findings suggest that neither alcohol exposure nor simvastatin treatment, alone or in combination, produced significant alterations in hippocampal BDNF expression in male mice.

Overall, the findings reveal sex-specific variations in how alcohol and simvastatin affect hippocampal CREB expression, showing significant changes in males but only minor, non-significant alterations in females. Additionally, BDNF expression did not differ significantly between treatment groups in either sex.

Table 3.7 Summary Statistics for CREB and BDNF Expression Across Treatment Groups and Both Sexes in Adolescent Mice

Group	N	Mean ± SE	Std. Dev.	Min	Max	Shapiro Wilk	p-value	Coeff. Of Variation (%)
			CREB					
Female								
Control	5	1.01 ± 0.05	0.12	0.82	1.11	0.89	0.37	11.45
SIM	5	1.18 ± 0.04	0.09	1.02	1.27	0.86	0.24	7.98
ALC	5	1.23 ± 0.06	0.12	1.04	1.36	0.92	0.52	10.14
ALC+SIM5	5	1.21 ± 0.08	0.17	1.06	1.48	0.89	0.36	14.16
ALC+SIM15	5	1.2 ± 0.04	0.09	1.06	1.26	0.78	0.06	7.17
Male								
Control	4	1.1 ± 0.03	0.06	1.04	1.18	0.94	0.65	5.31
SIM	5	1.1 ± 0.04	0.10	0.97	1.19	0.85	0.20	8.87
ALC	5	1.24 ± 0.04	0.10	1.09	1.34	0.92	0.55	7.94
ALC+SIM5	5	1.22 ± 0.02	0.05	1.16	1.27	0.83	0.13	4.33
ALC+SIM15	4	1.27 ± 0.02	0.04	1.23	1.32	0.97	0.84	3.15
			BDNF					
Female								
Control	5	1.01 ± 0.06	0.13	0.85	1.17	0.92	0.51	13.17
SIM	5	1.09 ± 0.07	0.16	0.83	1.27	0.92	0.54	14.82
ALC	5	1.13 ± 0.09	0.19	0.85	1.36	0.98	0.95	17.14
ALC+SIM5	5	1.09 ± 0.08	0.17	0.90	1.27	0.87	0.28	15.90
ALC+SIM15	5	1.14 ± 0.06	0.14	1.02	1.38	0.83	0.15	12.21
Male								
Control	5	1.01 ± 0.06	0.14	0.91	1.23	0.82	0.11	13.41
SIM	5	1.06 ± 0.10	0.23	0.78	1.38	0.99	0.98	22.00
ALC	5	1.01 ± 0.03	0.07	0.92	1.07	0.90	0.40	6.67
ALC+SIM5	5	1.09 ± 0.04	0.08	0.99	1.18	0.92	0.54	7.21
ALC+SIM15	4	1.15 ± 0.05	0.09	1.06	1.28	0.94	0.66	8.15

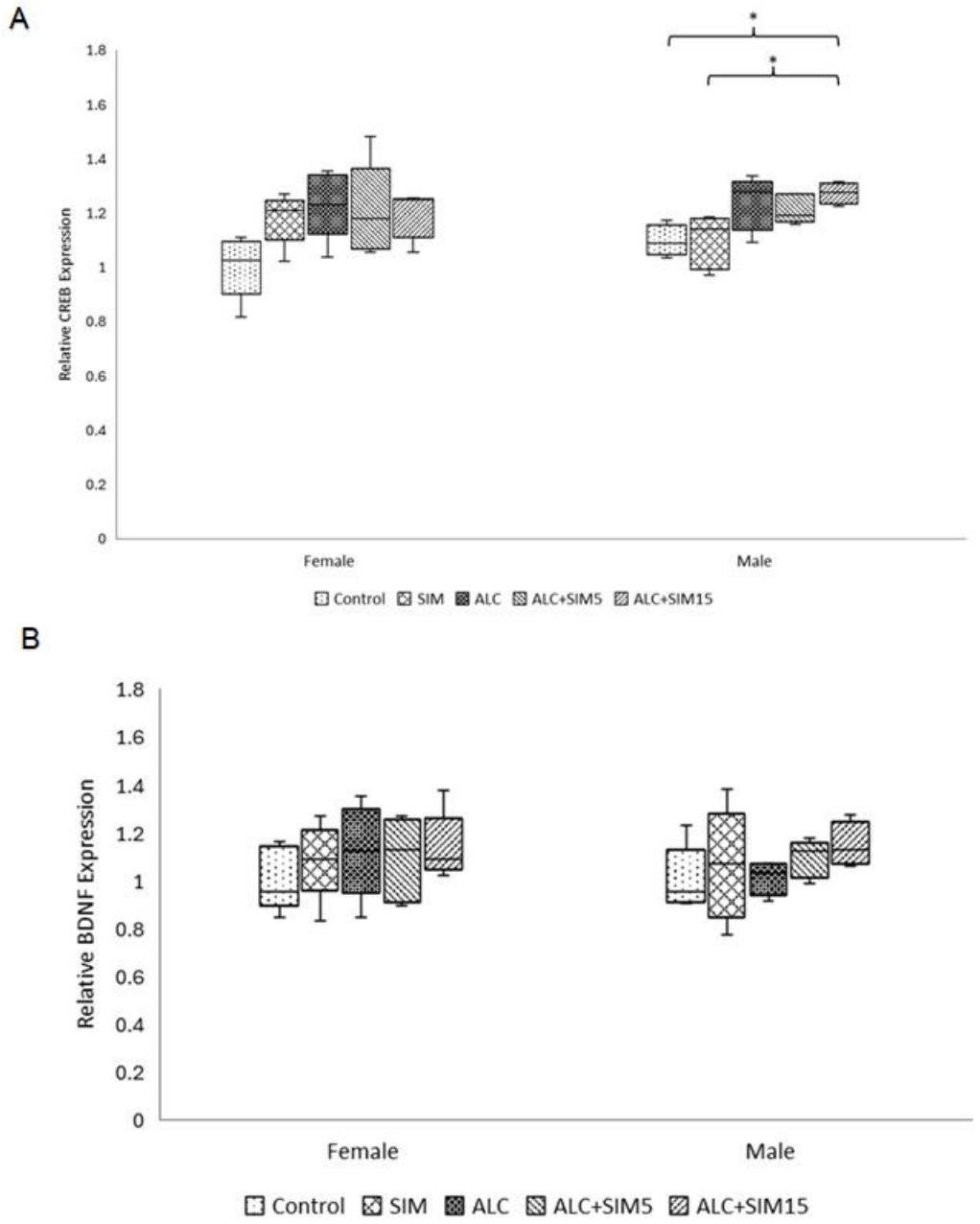


Figure 3.11: Hippocampal CREB and BDNF Expression Following Alcohol and/or Simvastatin Treatment in Female and Male Adolescent Mice. Boxplots showing hippocampal A) CREB and B) BDNF expression in adolescent male and female mice following treatment. In female mice, no significant differences in CREB expression were observed across the treatment groups. In male mice, CREB expression was significantly increased in the ALC+SIM15 group compared to the control and SIM groups. No significant differences in BDNF expression were found in either female or male mice across the treatment groups. Control - No treatment; SIM – 5 mg Simvastatin; ALC – Alcohol; ALC+SIM5 – Alcohol + 5 mg Simvastatin; ALC+SIM15 – Alcohol + 15 mg Simvastatin. *Statistically significant at $p < 0.05$

CHAPTER 4

DISCUSSION

Adolescence represents a critical developmental period characterised by ongoing structural and functional maturation of key brain regions, particularly the hippocampus and prefrontal cortex (PFC). This period is essential for the refinement of higher-order cognitive processes but also entails heightened vulnerability to external insults, such as alcohol exposure. This study examined the neuroprotective effects of simvastatin in counteracting the harmful impact of adolescent alcohol exposure on neurogenesis, oxidative stress, and neuroinflammation in the hippocampus and PFC. To analyse these parameters, a combination of biochemical, molecular, immunohistological, and histological techniques was employed. Oxidative stress in the hippocampus and PFC was assessed by quantifying malondialdehyde (MDA) levels and the enzymatic activities of key antioxidants, namely glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD). Neuronal populations in the PFC were assessed using Nissl staining and NeuN immunolabelling. Hippocampal neurogenesis was evaluated via immunolabelling for PcNA and DCX, while neuroinflammation was assessed using GFAP immunolabelling. In addition, the expression levels of inflammation-related genes (IL-6, IL-10) and neuroplasticity-associated markers (CREB, BDNF) were quantified using RT-PCR.

4.1. Effect of Alcohol Exposure and/or Simvastatin Treatment on Body Mass, Brain Mass, and Brain-to-Body Mass Ratio

Body and brain mass remained statistically unchanged across treatment groups in female mice. However, significant differences emerged in the brain-to-body mass ratio. Specifically, females treated with simvastatin (ALC+SIM5 and ALC+SIM15) exhibited a significantly higher brain-to-body mass ratio compared to the ALC group. Additionally, the ALC group showed a reduced ratio compared to the SIM-only and ALC+SIM groups, suggesting that simvastatin may have mitigated alcohol-related reductions in brain-to-body proportion. These findings point to a potential modulatory effect of simvastatin on brain development or body growth in alcohol-exposed female mice. The observed alterations in brain-to-body mass ratio in female mice are consistent with previous reports suggesting that simvastatin can influence neurodevelopmental processes, possibly through neuroprotective or anti-inflammatory mechanisms (Cimino et al., 2005; Chong et al., 2019; Lietzau et al., 2023). The more pronounced effects observed in females may reflect sex-specific sensitivity to pharmacological and toxicological interventions,

potentially mediated by hormonal regulation or differences in developmental timing although these factors were not directly measured in this study (Wolstenholme et al., 2011; Gelineau et al., 2017).

In contrast, no significant differences were observed in body mass, brain mass, or brain-to-body mass ratio among male mice across all treatment groups. These findings suggest that neither alcohol exposure nor simvastatin treatment significantly influenced these somatic parameters in males under the current experimental conditions. The absence of observable changes may reflect sex-specific physiological or metabolic responses to alcohol and simvastatin. Previous studies have reported divergent responses between sexes to similar interventions, potentially arising from differences in neuroendocrine regulation, baseline resilience, or pharmacokinetic profiles (Podhorna, McCabe and Brown, 2002; Collins et al., 2016). While simvastatin appeared to exert modulatory effects in females, its impact may extend to other biological domains not assessed in this study, such as behavioural, cellular, or molecular endpoints.

4.2. Effect of Alcohol Exposure and/or Simvastatin Treatment on Hippocampal Oxidative Stress and Antioxidant Defences in Adolescent Mice

The hippocampus, rich in polyunsaturated fatty acids and critical for cognitive function, is particularly sensitive to oxidative insults, especially under conditions such as chronic alcohol exposure (Singh et al., 2010; Patel et al., 2021; Ene et al., 2023). Alcohol metabolism promotes the generation of ROS, disrupts redox homeostasis, and impairs antioxidant enzyme activity, leading to lipid peroxidation and neuronal damage (Negre-Salvayre et al., 2008). Malondialdehyde (MDA), a key by-product of this process, serves as a widely used marker of oxidative damage (Ayala, Muñoz and Argüelles, 2014). In light of these mechanisms, this study investigated the extent of hippocampal oxidative stress following alcohol exposure in adolescent mice and evaluated the potential neuroprotective effects of simvastatin, a compound previously shown to modulate oxidative and inflammatory pathways.

In this study, hippocampal MDA levels did not differ significantly between experimental groups in either sex. This outcome contrasts with several previous studies in which chronic alcohol exposure reliably elevated MDA concentrations in rodent hippocampus, indicating increased lipid peroxidation and oxidative stress (Smith et al., 2005; Rajput et al., 2017;

Pamplona-Santos et al., 2019). The absence of significant group differences may reflect factors such as strain-specific baseline oxidative status or inter-individual variability, as C57BL/6J mice are known to exhibit inherently elevated oxidative markers due to mitochondrial susceptibility (Moreno-Loshuertos et al., 2006; Wang, Malo and Hekimi, 2009). While alcohol-related neurotoxicity has consistently been linked to oxidative damage and neuronal dysfunction (Zeigler et al., 2005; Tiwari and Chopra, 2013; Pant et al., 2017), the current findings did not replicate these effects in a statistically significant manner. A slight reduction in MDA levels was observed in females receiving alcohol and simvastatin (ALC+SIM15), suggesting limited but potentially sex-specific neuroprotection. Although this reduction did not reach statistical significance, it aligns with prior research showing that simvastatin can attenuate oxidative stress through the inhibition of NADPH oxidase and enhancement of endogenous antioxidant defences (Eger et al., 2016; Zhang et al., 2016; Jafari et al., 2021b).

The modest effect of simvastatin observed may be attributed to the severity of the alcohol-induced oxidative burden or the additional influence of neuroinflammatory pathways, which have been shown to impair antioxidant efficacy (Cimino et al., 2005; Lietzau et al., 2023). Furthermore, comparisons between the ALC and SIM groups approached significance, indicating that subtle treatment effects may exist. Notably, previous reports have indicated that females may be more vulnerable to alcohol-induced oxidative damage, possibly due to hormonal or neuroimmune differences (Alfonso-Loeches, Pascual and Guerri, 2013; Grace et al., 2021; Baweja et al., 2023), a trend partially reflected in the present dataset. However, other studies have reported non-significant MDA elevations in male rodents following alcohol exposure (Enache et al., 2008) highlighting the importance of considering sex as a biological variable in oxidative stress research.

Alcohol exposure is known to impair hippocampal antioxidant defences, primarily by reducing the activity of glutathione peroxidase, which leads to increased oxidative stress and heightened neurotoxicity (Herrera et al., 2003; Almansa et al., 2013; Tsermpini, Plemenitaš Ilješ and Dolžan, 2022). However, conflicting findings have emerged from clinical studies. For example, Guemouri et al. (1993), Chen et al. (2011), and Wu et al. (2020) reported elevated serum GSH-Px levels in individuals with alcohol use disorder (AUD) compared to healthy controls. These discrepancies could reflect compensatory upregulation in peripheral tissues or variations in the chronicity and intensity of alcohol exposure, highlighting the complexity of systemic antioxidant responses. Simvastatin, a compound with well-documented antioxidant properties,

has been shown to upregulate GSH-Px activity, providing neuroprotection by alleviating oxidative stress (Eger et al., 2016; Zinellu and Mangoni, 2021; Mansouri et al., 2022). In various pathological models, including traumatic brain injury (Lim et al., 2017), senile dementia (Liu et al., 2018), sepsis (Catalão et al., 2017), and alcohol-induced neurotoxicity (Jafari et al., 2021b), simvastatin has reduced oxidative damage and preserved neuronal integrity, further reinforcing its potential as a neuroprotective agent.

In the present study, both sexes showed the highest GSH-Px activity in the alcohol-treated group, which suggests a compensatory upregulation of antioxidant enzymes in response to alcohol-induced oxidative stress. This adaptive response is in line with previous research, which indicates that ROS can activate redox-sensitive transcription factors such as Nrf2, which promote the expression of antioxidant enzymes like GSH-Px (Ma, 2013; Pizzino et al., 2017; Zhang et al., 2017). However, under conditions of prolonged oxidative stress, ROS may also damage these enzymes, impairing their function and reducing their protective effect (Pizzino et al., 2017). Simvastatin administration in this study led to a significant reduction in GSH-Px activity in the SIM, ALC+SIM5, and ALC+SIM15 groups compared to the alcohol group, indicating its ability to attenuate alcohol-induced oxidative stress. This effect was consistent across both sexes, supporting the notion that simvastatin's antioxidant and anti-inflammatory properties (Eger et al., 2016; Mansouri et al., 2022) can modulate the oxidative stress response in the hippocampus. Notably, the observed decrease in GSH-Px activity, despite elevated ROS levels, suggests that simvastatin may reduce the oxidative burden and limit the need for excessive compensatory enzyme activity.

SOD plays a crucial role in maintaining cellular redox balance by catalysing the dismutation of superoxide anions (O_2^-) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), acting as a primary defence against oxidative stress (Younus, 2018; Chidambaram et al., 2024). Chronic alcohol consumption has been shown to impair SOD activity, thereby reducing the brain's antioxidant capacity and promoting oxidative damage, which may contribute to neurological dysfunction and cognitive deficits (Marklund et al., 1983; Huang et al., 2009; Yang et al., 2022; Kado et al., 2024). In contrast, simvastatin has been demonstrated to enhance SOD activity, providing neuroprotection by mitigating oxidative stress across various pathological models, including alcohol-induced neurotoxicity (Jafari et al., 2021b) and Alzheimer's disease (Adeli et al., 2017).

In the present study, chronic alcohol exposure did not significantly alter SOD activity in either male or female mice, a finding consistent with research suggesting that alcohol's impact on SOD can be tissue- and context-dependent. Enache et al. (2008), for example, observed no significant changes in brain SOD activity following chronic alcohol exposure in a prenatal restraint stress rat model, indicating that alcohol-induced oxidative stress does not always directly affect SOD levels. Similarly, simvastatin treatment did not significantly modify SOD activity in either sex, as no differences were detected between the SIM and control groups. Likewise, ALC did not significantly affect SOD activity compared to controls. Although a borderline increase in SOD activity was noted in female mice treated with low-dose simvastatin in combination with alcohol (ALC+SIM5), this was not statistically significant. No significant differences were observed between ALC and ALC+SIM5, or between ALC+SIM5 and ALC+SIM15 in either sex, suggesting that simvastatin, even at varying doses, did not substantially modulate SOD activity in alcohol-exposed mice. These findings may reflect a context-dependent effect of simvastatin on SOD, possibly influenced by factors such as the sensitivity of the hippocampal redox system during adolescence.

The literature on alcohol's effects on SOD activity is heterogeneous, with studies reporting increased (Haorah, Knipe and Persidsky, 2011), decreased (Kado et al., 2024), or unchanged (Marklund et al., 1983) SOD levels following alcohol exposure. In adolescent models, Kaptan et al. (2019) also reported no significant changes in hippocampal SOD activity, aligning with the present findings. On the other hand, simvastatin has been shown to enhance SOD activity and reduce oxidative stress in brain regions such as the hippocampus and cortex (Eger et al., 2016; Adeli et al., 2017; Tong et al., 2018), though these effects were not observed in the current study. The absence of significant changes in SOD activity across treatment groups suggests that SOD may not be a primary target of simvastatin's antioxidant effects in the adolescent hippocampus. However, the observed trend in female mice receiving low-dose simvastatin alongside alcohol suggests a subtle interaction that warrants further investigation. This variability emphasises the complexity of oxidative stress and antioxidant regulation, as noted by Singh and Singh (2008). It is possible that other antioxidant enzymes, particularly GSH-Px, were more prominently upregulated in response to alcohol-induced oxidative damage, reflecting a shift in the antioxidant defence system away from SOD. As noted by Won et al. (2012), compensatory mechanisms may obscure changes in individual enzymes. Additionally, the immaturity of the antioxidant system in adolescents (Rupérez et al., 2020; Samsonov and Urlacher, 2025) may have limited the detectable effects on SOD activity.

In summary, findings from this study provide insights into how chronic alcohol exposure and simvastatin treatment influence oxidative stress and antioxidant defences in the hippocampus. While alcohol exposure did not significantly alter the activity of key antioxidant markers such as MDA, GSH-Px, or SOD, some trends suggest a compensatory upregulation of antioxidant responses, particularly the elevation of GSH-Px activity, consistent with prior evidence of cellular adaptation to oxidative stress. However, simvastatin administration, despite its well-documented antioxidant properties, did not produce substantial changes in these enzymatic activities in the adolescent hippocampus under the present experimental conditions.

These findings further indicate that the regulation of oxidative stress in the adolescent brain may involve complex, tissue-specific mechanisms that are not fully captured by traditional antioxidant enzyme assays alone. To deepen our understanding of how simvastatin and similar interventions modulate oxidative pathways, future research should incorporate molecular techniques such as quantitative PCR and Western blotting to assess the gene and protein expression of key antioxidant regulators, including those in the Nrf2/ARE pathway. Additionally, the use of ELISAs to quantify specific oxidative damage markers, alongside immunohistochemistry to map their spatial distribution, would provide a more comprehensive profile of hippocampal oxidative stress and would be beneficial for evaluating the therapeutic potential of simvastatin in mitigating alcohol-induced oxidative damage during adolescence.

4.3. Effect of Alcohol Exposure and/or Simvastatin Treatment on Oxidative Stress and Antioxidant Defences in the Prefrontal Cortex of Adolescent Mice

Chronic and binge-pattern alcohol consumption has been consistently linked to increased oxidative stress in the PFC, with elevated levels of MDA frequently reported as a key biomarker of lipid peroxidation (Cortez, Brocardo and Leasure, 2021; Tsermpini, Plemenitaš Ilješ and Dolžan, 2022). The production of ROS during alcohol metabolism disrupts neuronal membrane integrity and impairs cellular function, potentially leading to structural and functional impairments in the PFC (Wu and Cederbaum, 2003). Given the PFC's role in cognitive processes such as decision-making, impulse control, and working memory, oxidative damage in this region has been associated with behavioural and cognitive deficits (Lee et al., 2014; Liu et al., 2017). Considering these findings, the current study examined oxidative stress markers and antioxidant defences in the PFC of adolescent mice following alcohol exposure and simvastatin treatment.

Several studies have confirmed that sustained alcohol exposure elevates MDA levels in the PFC, often in conjunction with neuroinflammation, mitochondrial dysfunction, and neurotransmitter imbalance (Ajayi et al., 2023; Lin et al., 2024). Alcohol has also been shown to reduce antioxidant enzyme activity, particularly GSH-Px and SOD, thereby weakening the brain's defences against oxidative damage (Fowler et al., 2014; Pahng et al., 2017). The PFC may be especially vulnerable to such damage due to its high metabolic demands and dense synaptic networks (Crews, He and Hodge, 2007). This susceptibility, coupled with the ongoing maturation of the adolescent brain, emphasises the importance of evaluating oxidative stress in this context.

Despite the established relationship between alcohol and oxidative damage, MDA levels in the PFC did not differ significantly across treatment groups in either sex. Although alcohol has been widely reported to increase MDA levels (Omotoso et al., 2022; Lin et al., 2024), the ALC group in female mice did not show a statistically significant rise and may reflect age-related resilience or compensatory antioxidant mechanisms within the female PFC. In male mice, a tendency toward increased MDA following alcohol exposure was noted, yet the variability in MDA levels, particularly in alcohol-exposed and low-dose simvastatin groups (SIM and ALC+SIM5), may have obscured consistent treatment-related effects. As Caballería (2003) and Cederbaum (2012) suggested, individual differences in oxidative response and antioxidant capacity may have contributed to the observed non-significant outcomes.

Simvastatin treatment did not significantly alter MDA levels in either sex. In female mice, neither 5 mg nor 15 mg doses of simvastatin produced measurable changes in lipid peroxidation, despite the drug's established antioxidant properties (Tong et al., 2018). This aligns with previous studies reporting inconsistent effects of simvastatin on MDA, including unchanged brain MDA concentrations in hyperlipidaemic rats (Vukšić et al., 2023) and limited reductions in lipid peroxidation despite evidence of anti-inflammatory activity (Dombrecht et al., 2006). In males, 15 mg simvastatin produced a slight, non-significant decrease in MDA levels in the ALC+SIM15 group compared to the ALC group, indicating a modest potential antioxidant effect. These observations suggest that the neuroprotective efficacy of simvastatin may be dose-dependent and modulated by sex-specific factors and the brain's baseline oxidative status (Catalão et al., 2017; Sardari et al., 2024).

Assessment of GSH-Px activity revealed a dose-dependent response to simvastatin in female mice. Specifically, the ALC+SIM5 group showed a significant decrease in GSH-Px activity compared to the control, ALC, and SIM groups. This reduction may indicate either a suppression of antioxidant defences or an increased oxidative burden at the lower simvastatin dose. In contrast, the ALC+SIM15 group exhibited significantly higher GSH-Px activity relative to the ALC+SIM5 group, suggesting a compensatory upregulation of antioxidant mechanisms at the higher dose. These findings suggest that 5 mg simvastatin may have been inadequate to support antioxidant function specifically in the PFC or may have interfered with endogenous defences, whereas the 15 mg dose appeared to partially restore GSH-Px activity under alcohol-induced oxidative stress. Importantly, no significant differences in GSH-Px activity were observed among the control, ALC, and SIM groups, indicating that neither alcohol nor simvastatin alone significantly altered this enzyme. Rather, their combined administration, particularly at different simvastatin doses, emerged as the critical factor influencing GSH-Px activity.

In male mice, GSH-Px activity did not significantly differ across treatment groups. The absence of statistically significant changes may reflect the male brain's capacity for compensatory antioxidant responses (Culbert et al., 2022) or inherently higher baseline GSH-Px activity (Brunelli et al., 2014). These results point to a sex-dependent regulation of oxidative stress and antioxidant enzyme activity, with male mice appearing more resilient to changes in GSH-Px levels following alcohol and simvastatin exposure. This outcome aligns with previous reports indicating that antioxidant responses can differ substantially between sexes, potentially due to differences in hormonal regulation, gene expression, or neurodevelopmental trajectories (Celestino et al., 2018; Steyn et al., 2019).

SOD activity in the PFC remained unchanged across treatment groups in both sexes. In female mice, neither alcohol nor simvastatin significantly changed SOD activity, indicating a preserved enzymatic antioxidant response. Similarly, in males, SOD levels appeared stable across treatment groups, except for a non-significant trend toward elevated activity in the ALC+SIM15 group compared to controls. This trend may reflect a modest modulatory effect of high-dose simvastatin when co-administered with alcohol. Overall, the observed stability in SOD activity suggests that this antioxidant defence mechanism is maintained in the PFC under the experimental conditions tested. These findings are consistent with previous studies reporting the relative resilience of SOD activity to moderate physiological stress in the PFC.

For instance, Götz et al. (2001) showed that chronic alcohol exposure impaired the glutathione redox system, evidenced by reduced GSH/(GSH+2GSSG) ratios, without significantly affecting SOD activity across multiple brain regions. Similarly, Aksu et al. (2009) reported no significant differences in SOD activity in the hippocampus and PFC following acute exercise in rats, highlighting the enzyme's consistent regulation under transient stress conditions. More recently, Wu et al. (2020) found that serum SOD activity remained stable in individuals with alcohol use disorder during detoxification, despite substantial alterations in other oxidative stress biomarkers.

These findings are consistent with the present study and reinforce the notion that SOD activity in the adolescent PFC remains relatively unaffected by alcohol exposure or simvastatin treatment. This apparent stability may be attributed to compensatory antioxidant mechanisms, including catalase and glutathione-dependent systems, which help preserve redox homeostasis under stress conditions (Morris et al., 2019; Trofin et al., 2025). Although SOD plays a critical role in mitigating oxidative stress, its unaltered activity across treatment conditions suggests that alternative enzymatic pathways may have effectively countered oxidative challenges, thereby sustaining antioxidant defence. The preservation of SOD activity under these conditions may hold neurobiological significance, as it reflects a retained capacity to prevent oxidative damage during adolescence, a developmental window characterised by increased susceptivity to neurotoxic insults and neurodegenerative processes.

From these findings, it is important to consider the biological and experimental factors that may have shaped the antioxidant response observed in this study. Several influences may account for the outcomes, including sex-specific metabolic and hormonal effects, as well as developmental differences in antioxidant capacity. Hormonal fluctuations during adolescence may have contributed to sex-dependent responses, as oestrogen and testosterone are known to modulate oxidative stress pathways and antioxidant enzyme expression (Franconi and Campesi, 2014; Agabio et al., 2017). Furthermore, the PFC's prolonged maturation during adolescence may have further influenced its vulnerability to oxidative damage and responsiveness to pharmacological intervention (Alfonso-Loeches, Pascual and Guerri, 2013; Fowler et al., 2014).

In summary, the findings from both the hippocampus and PFC highlight the adolescent brain's vulnerability to oxidative damage, particularly in the PFC, likely due to its ongoing maturation,

elevated metabolic demands, and dense synaptic activity (Venkateshappa et al., 2012; Fowler et al., 2014). Although alcohol exposure during adolescence produced limited and region-specific effects on oxidative stress markers, the hippocampus exhibited more pronounced compensatory increases in GSH-Px activity. In contrast, the PFC exhibited subtler, dose-dependent modulation, particularly in females treated with simvastatin. The relative stability of SOD activity across both regions suggests the presence of robust compensatory mechanisms, including catalase and glutathione-related systems, that help preserve redox homeostasis during this critical developmental window. These findings highlight the complexity of antioxidant defence regulation in the adolescent brain and suggest that intrinsic protective mechanisms may buffer against oxidative stress in a region- and sex-specific manner.

To further understand these dynamics, future studies should incorporate additional oxidative stress biomarkers and more detailed sex-based analyses. Importantly, integrating behavioural assessments could help establish functional correlates of biochemical alterations, while molecular approaches such as quantitative PCR may reveal underlying transcriptional changes in antioxidant pathways. In addition, immunohistochemical analyses would provide critical spatial resolution by localising enzyme activity to specific cell types and subregions within the hippocampus and PFC. Together, these complementary strategies will be essential for advancing our knowledge of how alcohol and simvastatin interact with the antioxidant system in the developing brain.

4.4. Neurogenic Capacity of Simvastatin Against Alcohol-Induced Brain Damage on the Distribution of Proliferative Cells and Immature Neurons in the Suprapyramidal Blade of the Dentate Gyrus

Neurogenesis, the generation of new neurons from proliferating neural stem cells, persists through adolescence primarily in the subgranular zone (SGZ) of the dentate gyrus (DG) within the hippocampus (Eriksson et al., 1998; Gage, 2002; Crews et al., 2006; Ortega-Perez, Murray and Lledo, 2007; Gil-Perotín, García-Verdugo and Álvarez-Buylla, 2009; Lazarov and Hollands, 2016; Kempermann, 2022). This process, which contributes to hippocampal plasticity and supports cognitive functions such as learning and memory, exhibits heightened activity during adolescence compared to adulthood (Sousa, Madeira and Paula-Barbosa, 1998; Hueston, Cryan and Nolan, 2017; Boldrini et al., 2018). Adolescence represents a critical window wherein neurogenic rates peak, rendering the hippocampus particularly sensitive to

environmental influences and pharmacological agents. Given that neurogenesis is modulated by both intrinsic factors and external stressors, including alcohol exposure, alterations during this period may have lasting implications for hippocampal structure and function (Åberg et al., 2005; Cameron and Glover, 2015; Opendak, Briones and Gould, 2016). The current findings are discussed within this developmental framework, focusing on how alcohol and simvastatin exposure might impact neurogenic processes and thereby influence adolescent brain maturation.

To assess cell proliferation, Proliferating Cell Nuclear Antigen (PcNA) was utilized as a marker due to its role in the G1/S phase of the cell cycle (Oliveira et al., 2008). Although Ki-67 has been noted for its higher sensitivity, many studies, including the present one, favour PcNA for its reliability in detecting proliferative activity (Bologna-Molina et al., 2013). Complementing this, doublecortin (DCX) was employed to identify immature and migrating neurons, reflecting ongoing neurogenic progression (Ayanlaja et al., 2017).

Importantly, at the morphological level, the dentate gyrus displayed a consistent structure across all experimental groups. Both the suprapyramidal and infrapyramidal blades were clearly distinguishable, and proliferative and immature cells remained confined to the SGZ. No gross structural alterations were observed following chronic alcohol exposure or simvastatin treatment. This morphological consistency indicates that, despite experimental interventions, the overall organization of the dentate gyrus was preserved, aligning with previous reports of structural resilience in adolescent rodent models (Crews et al., 2006; Broadwater et al., 2014).

Neurogenic activity in the DG is modulated by intrinsic factors such as growth factors and neurotransmitters, as well as extrinsic influences including alcohol, stress, and nutrition (Åberg et al., 2005; Cameron and Glover, 2015; Opendak, Briones and Gould, 2016). The adolescent brain is particularly vulnerable to alcohol-induced neurotoxicity, as shown in various animal models (Markwiese et al., 1998; Crews et al., 2000; Spear, 2014). Quantitative analyses revealed that chronic alcohol exposure significantly reduced the distribution of proliferative (PcNA-positive) and immature (DCX-positive) neurons in the hippocampi of both female and male adolescent mice, indicating impaired neurogenesis. This suppression may increase the proportion of immature excitatory synapses, potentially contributing to alcohol-related memory impairments (Slawecki et al., 2001; Silvers et al., 2003; Tapert, Caldwell and Burke, 2004; White and Swartzwelder, 2004; Zeigler et al., 2005; Crews et al., 2006; Hanson, Owens

and Nemeroff, 2011). Alcohol's neurodegenerative effects are likely mediated, in part, by oxidative stress, which compromises mitochondrial function and induces neural progenitor cell death (Nixon and Crews, 2002). This is supported by the current study's hippocampal biochemical findings, where elevated GSH-Px activity in the alcohol-treated group indicated oxidative stress, coinciding with fewer proliferative and immature neuronal cells. Inflammation may also play a role, as alcohol-induced activation of glial cells promotes the release of pro-inflammatory cytokines, including TNF-α and IL-6, which are known to inhibit progenitor cell proliferation and interfere with neuronal differentiation (Zonis et al., 2013, 2015; Walter and Crews, 2017). In addition, alcohol-related downregulation of neurotrophic factors like brain-derived neurotrophic factor (BDNF) may further impair hippocampal neurogenesis (Tateno et al., 2004; Silva-Peña et al., 2019).

These interpretations are supported by previous rodent studies showing reductions in PcNA- and DCX-positive cells in the DG following alcohol exposure (He et al., 2005; Morris et al., 2010; Tiwari and Chopra, 2013; Broadwater et al., 2014; Le Maître et al., 2018; Olateju et al., 2018). Nixon and Crews (2002) and Herrera et al. (2003) demonstrated that both acute and chronic alcohol exposure suppress progenitor cell proliferation in the DG of adolescent and adult rodents. Consistent with these findings, Crews, Nixon and Wilkie (2004) reported reduced hippocampal neurogenesis in individuals with alcohol use disorders, providing translational support from human studies. Morris et al. (2010) similarly observed significant reductions in both PcNA- and DCX-positive cells in the DG following alcohol exposure, although no such effects were noted in the subventricular zone (SVZ), suggesting a region-specific vulnerability. Taffe et al. (2010) further confirmed reduced neurogenesis in alcohol-dependent individuals. Finally, Broadwater et al. (2014) reported a decrease in DCX-expressing neurons in adolescent rodents following intermittent alcohol exposure, reinforcing the developmental sensitivity of hippocampal neurogenesis to alcohol-related disruption.

Simvastatin has previously been shown to enhance hippocampal neurogenesis in adult models (Wu et al., 2008; Robin et al., 2014; Wang et al., 2015), although studies examining its effects during adolescence remain limited. In the present study, simvastatin treatment exhibited a dose-dependent and sex-specific effect on neural proliferation. In female mice, both concentrations of simvastatin (5 and 15 mg) significantly counteracted the alcohol-induced reduction in proliferative cells. However, the higher dose (15 mg) mitigated alcohol's suppressive effects more effectively, as reflected by significant differences between the ALC and ALC+SIM15

groups, as well as between the ALC+SIM5 and ALC+SIM15 groups. Simvastatin alone did not significantly impact proliferation relative to controls in females, suggesting that its neuroprotective effects were primarily evident in the context of alcohol-induced damage. In contrast, male mice exhibited a different dose-response pattern. ALC+SIM5 resulted in a significantly higher distribution of proliferative cells compared to both the ALC and control groups, indicating that the lower simvastatin dose (5 mg) conferred neuroprotection. The significant difference between ALC+SIM5 and ALC+SIM15 further suggests a dose-dependent effect, where the higher dose of simvastatin (15 mg) was less effective in restoring proliferation. ALC+SIM15 did not differ significantly from the ALC group, reinforcing the notion that high-dose simvastatin may not exert the same protective effects in male mice as in females. Overall, these results highlight sex-dependent responses to simvastatin treatment, with males exhibiting greater neuroprotection at a lower dose (5 mg), whereas females required a higher dose (15 mg) for significant mitigation of alcohol-induced proliferation deficits. Sex differences have been shown to significantly influence hippocampal neurogenesis during adolescence. One study examining the impact of puberty on hippocampal morphology found that prepubertal males and females had similar hippocampal volumes. However, post-pubertal females exhibited significantly larger bilateral hippocampi compared to males, suggesting that puberty and sex hormones play key roles in shaping hippocampal structure and function (Satterthwaite et al., 2014).

Similarly, analysis of DCX expression revealed that alcohol exposure significantly reduced the number of DCX-positive cells in both sexes, indicating suppression of neuronal maturation. In female mice, the ALC+SIM15 group also exhibited a significant reduction compared to the control group, suggesting that high-dose simvastatin may exert a potentially inhibitory effect on the survival or maturation of immature neurons. In contrast, the SIM group showed a significant increase in DCX-positive cells relative to the ALC, ALC+SIM5, and ALC+SIM15 groups, indicating that simvastatin alone may enhance neurogenesis. Notably, no significant differences were observed between the control and SIM groups, or between the control and ALC+SIM5 groups, suggesting that simvastatin at 5 mg did not significantly alter DCX expression. Similar trends were observed in male mice, where the ALC+SIM15 group also showed a significant reduction in DCX-positive cells compared to the control group. These findings align with previous reports indicating that high doses of simvastatin can impair neural stem cell proliferation and differentiation by inducing cell cycle arrest and autophagy, particularly during the G1 phase of the cell cycle (Wang et al., 2016; Carson et al., 2018).

Therefore, while simvastatin may promote the proliferation of intermediate precursor cells at optimal concentrations, higher doses may attenuate this effect, potentially limiting the maturation of immature neurons.

Although SIM did not significantly differ from the control group, it significantly increased the distribution of DCX-positive cells compared to the ALC and ALC+SIM15 groups, reinforcing its neuroprotective potential. Interestingly, the ALC+SIM5 group showed no significant difference from control, suggesting that the lower simvastatin dose (5 mg) effectively mitigated alcohol-induced reductions in immature neurons. However, the ALC+SIM15 group did not significantly differ from the ALC group, supporting the notion that a higher simvastatin dose may not provide comparable protective effects. A significant difference between ALC+SIM5 and ALC+SIM15 further suggests a dose-dependent response, in which a higher dose may diminish the compensatory benefits observed at a lower dose.

Simvastatin has been shown to modulate the Wnt/β-catenin signalling pathway, a critical regulator of neuronal development (Robin et al., 2014). *In vivo* studies have reported that simvastatin increases the number of immature neurons in the DG by stimulating the proliferation of intermediate precursor cells in the SGZ, consistent with the findings of the present study. Its effect on the Wnt pathway is independent of cholesterol levels and is mediated through the inhibition of isoprenoid biosynthesis (Tong, Royea and Hamel, 2022). Additionally, simvastatin has been shown to upregulate DCX expression, thereby promoting neuronal differentiation and maturation of newly generated neurons (Wang et al. 2015; Xie et al. 2015). The attenuation of alcohol-induced suppression of neurogenesis by simvastatin is thought to involve its anti-inflammatory effects (Zhou et al. 2017), upregulation of neurotrophic factors (Wu et al. 2008) and enhancement of synaptic plasticity (Chen et al. 2016).

Overall, the present findings demonstrate that alcohol exposure significantly impairs hippocampal neurogenesis during adolescence by reducing the distribution of proliferating cells and immature neurons. Simvastatin treatment, particularly at the lower dose of 5 mg, mitigated these adverse effects, although its efficacy varied depending on sex and dosage. In female mice, the higher dose of 15 mg effectively counteracted alcohol-induced suppression of proliferation, whereas in males, the lower dose proved more beneficial, promoting greater neuronal survival and proliferation. Conversely, the higher simvastatin dose appeared less effective in males and may have exerted adverse effects on immature neurons. These sex-

dependent responses suggest underlying biological differences in hippocampal sensitivity to simvastatin during adolescence.

Consistent with previous reports that simvastatin enhances neural progenitor proliferation and increases DCX-positive immature neurons (Lu et al., 2007; Wang et al., 2015; Xie et al., 2015; Chen et al., 2016; Trigiani et al., 2019), the current findings reinforce simvastatin's neuroprotective potential. Specifically, simvastatin at a lower dose (5 mg) consistently counteracted alcohol-induced deficits by increasing the distribution of both PcNA- and DCX-positive cells in the adolescent hippocampus, while the higher dose (15 mg) was primarily beneficial in females. These results provide important new insights into simvastatin's capacity to protect against alcohol-induced impairments in adolescent hippocampal neurogenesis. Alcohol exposure during this critical developmental period significantly disrupts neural proliferation and maturation, processes essential for learning and memory. Notably, simvastatin, particularly at lower concentrations, enhanced antioxidant activity and promoted neurogenesis, exhibiting distinct dose- and sex-specific effects. Collectively, these findings highlight simvastatin's potential as a therapeutic intervention to mitigate alcohol-related brain damage during adolescence.

While the present study demonstrates dose- and sex-dependent neuroprotective effects of simvastatin against alcohol-induced neurogenic deficits, several areas warrant further investigation. Future studies could assess long-term functional outcomes, such as hippocampal-dependent cognitive behaviours, to clarify the translational relevance of the observed neurogenic improvements. Moreover, mechanistic investigations into specific signalling pathways, including Wnt/β-catenin and neuroinflammatory cascades, could elucidate the molecular basis for simvastatin's differential effects between sexes. Expanding analyses to other brain regions affected by alcohol exposure would provide a more comprehensive understanding of simvastatin's neuroprotective capacity. Finally, evaluating the combined effects of simvastatin with other antioxidant or neurotrophic agents may reveal synergistic strategies for mitigating adolescent alcohol-induced brain damage.

4.5. Effect of Alcohol Exposure and/or Simvastatin Treatment on the Estimation of the Population of Neuronal Cells, the Distribution of Astrocytic Cells, and the Hippocampal Volume of Adolescent Mice

Given the hippocampus's central role in learning and memory, and its heightened sensitivity to alcohol-induced neurotoxicity during adolescence, evaluating cellular and structural changes is essential for understanding long-term consequences. Building on previous findings of alcohol-induced disruptions in hippocampal neurogenesis, the present analysis expands upon these observations by evaluating broader indicators of structural and cellular integrity. Specifically, changes in the estimated number of NeuN-positive neurons, GFAP-positive astrocytes, and hippocampal volume were examined to assess the extent of neurodegeneration and glial response following adolescent alcohol exposure. These complementary markers provide insight into neuronal survival and the condition of the hippocampal microenvironment, offering a more comprehensive understanding of alcohol's impact on brain structure. Moreover, evaluating whether simvastatin modulates these parameters is essential for assessing its potential neuroprotective effects on tissue preservation and cellular resilience. Such insights are critical for determining the therapeutic potential of simvastatin in mitigating long-term alcohol-related damage in the adolescent brain.

4.5.1. Estimation of Neuronal Cell Populations Using NeuN Immunolabelling Following Alcohol Exposure and/or Simvastatin Treatment in Adolescent Mice

An unbiased stereological approach was employed to estimate the total population of NeuN-immunolabelled neurons in the suprapyramidal blade of the DG in adolescent mice following alcohol exposure and/or simvastatin treatment. NeuN immunostaining distinctly labelled mature granule cells with consistent distribution across groups. From the quantification, no significant alcohol-induced neuronal loss or simvastatin-mediated neuroprotection was detected in either sex, although modest group differences were observed in male mice. NeuN is a well-established marker for mature, postmitotic neurons (Mullen, Buck and Smith, 1992) and serves as a standard tool for assessing neuronal density, structural integrity, and treatment effects (Jacko et al., 2018). However, reductions in NeuN immunolabelling may reflect not only neuronal loss but also downregulation of the protein or antigenicity in response to neurotoxic stress (Unal-Cevik et al., 2004), thereby limiting its sensitivity to early-stage or non-lethal neuronal alterations (Spalding et al., 2013; Le Maître et al., 2018).

In female mice, alcohol exposure did not significantly alter the NeuN-positive cell population compared to controls, although a non-significant neuronal reduction was observed in the ALC group. Simvastatin, administered either alone or in combination with alcohol, similarly, did not produce significant alterations in NeuN-positive cell estimates. This may indicate the absence of overt neuronal loss or reflect the limited sensitivity of NeuN to detect early neuronal dysfunction (Spalding et al., 2013; Le Maître et al., 2018). Importantly, the preservation of NeuN immunoreactivity does not preclude underlying stress or sublethal injury, particularly in the context of adolescent vulnerability (Duan et al., 2016).

In male mice, a significant effect across the groups was detected, with post hoc analyses revealing a higher number of NeuN-positive neurons in the SIM group compared to the ALC+SIM5 group. While this may suggest a dose-dependent trophic influence of simvastatin, it is more likely that the observed effect reflects indirect mechanisms, such as modulation of glial activity, rather than direct neuronal preservation (Wu et al., 2010a; Peña and Vazquez, 2022; Bobermin et al., 2024). Neither alcohol nor the combination of alcohol and simvastatin (ALC+SIM5 or ALC+SIM15) resulted in significant changes in NeuN-positive cell estimates compared to controls. This aligns with previous findings that the adolescent brain may be relatively resilient to short-term alcohol-induced neuronal loss, particularly within tightly packed hippocampal regions such as the DG (Crews, He and Hodge, 2007; Coleman, Crews and Vetreno, 2021). Moreover, simvastatin's lack of detectable neuroprotective effects in the combined treatment groups suggests its efficacy may depend on the severity of neuroinflammation or oxidative stress, which may have been below threshold in the model used in the present study.

It is also plausible that simvastatin exerted neuroprotective effects through modulation of non-neuronal processes, such as astrocytic and microglial pathways, rather than directly preserving neuronal populations (Li et al., 2009b; Wu et al., 2010b; Xu et al., 2012; Turkin, Tuchina and Klempin, 2021). Simvastatin has been shown to suppress neuroinflammation, regulate oxidative stress, and modulate glial function, which are mechanisms that may maintain neuronal function without potentially or directly altering NeuN-positive cell counts (Paintlia et al., 2005; Roy et al., 2015). Additionally, its neuroprotective actions may be dose- and context-dependent, emerging more clearly under conditions of pronounced injury or inflammation (Li et al., 2009a; Wang et al., 2015; Chong et al., 2019). These alternative mechanisms are further explored in the context of GFAP immunoreactivity in the following section.

In summary, neither alcohol nor simvastatin (at the tested doses) caused substantial changes in the NeuN-positive neuronal population in the suprapyramidal blade of the DG in adolescent mice. The findings suggest that alcohol-induced damage may not have progressed to overt neuronal loss, and that simvastatin's potential neuroprotective effects may act via non-neuronal targets. Importantly, the absence of significant treatment effects highlights the importance of incorporating complementary markers of neuronal health and function to assess subtle or early-stage neurotoxic or neuroprotective outcomes in adolescent models.

4.5.2. Distribution of Astrocytic Cells in the Suprapyramidal Blade of the Dentate Gyrus Using GFAP Immunolabelling Following Alcohol Exposure and/or Simvastatin Treatment in Adolescent Mice

While GFAP immunolabelling was employed in this study as a widely accepted marker of astrocytes, it is important to acknowledge that GFAP expression does not uniformly represent astrocyte number or function and cannot reliably distinguish between quiescent and reactive states (Bernal and Peterson, 2011; Escartin et al., 2021). Under physiological conditions, GFAP is expressed at variable levels across brain regions, with many astrocytes in grey matter expressing low or undetectable levels of the protein (Bushong et al., 2002; Cahoy et al., 2008). In response to injury or inflammatory stimuli, astrocytes typically undergo reactive astrogliosis, characterized by hypertrophy and upregulation of GFAP and other cytoskeletal proteins (Sofroniew, 2009; Pekny and Pekna, 2016). However, astrocyte reactivity is highly heterogeneous, and some reactive phenotypes do not involve increased GFAP expression, limiting its sensitivity as a sole marker (Cunningham, Dunne and Lopez-Rodriguez, 2019). Additionally, GFAP cannot differentiate between potentially neurotoxic (A1-like) and neuroprotective (A2-like) reactive astrocyte subtypes, which require transcriptomic profiling or additional markers for distinction (Liddelow and Barres, 2017; Li et al., 2025). Therefore, although GFAP offers valuable insight into astrocyte distribution and general reactivity, relying on it as a sole indicator of astrocyte activation presents a methodological limitation. Future studies employing complementary markers such as vimentin, nestin, S100β, or Aldh1L1, alongside functional and morphological analyses, would enable a more detailed characterization of astrocyte status (Yang et al., 2011).

The current results demonstrate that alcohol exposure significantly altered the distribution of GFAP-positive cells in the suprapyramidal blade of the DG of adolescent mice in a sex-

dependent manner. In female mice, alcohol markedly increased the number of GFAP-immunolabelled cells, suggesting activation of astrocytes consistent with reactive gliosis. These findings align with previous research indicating that alcohol exposure can induce neuroinflammatory responses by promoting astrocyte reactivity and glial remodelling in the hippocampus (He et al., 2005; Chen, Ozturk and Zhou, 2013). Franke (1995) similarly reported region- and duration-dependent astrocytic hypertrophy and increased GFAP expression following intermediate-term ethanol exposure. After prolonged exposure, however, GFAP expression was downregulated and astrocyte numbers were reduced, indicating a time-dependent shift from reactive gliosis to astrocytic degeneration. Pekny and Pekna (2016) described reactive astrogliosis as a dynamic response that initially protects neural tissue but can become maladaptive if sustained, which may underlie the heightened astrocytic activation seen in the alcohol-exposed female hippocampus.

This type of astrocytic activation is often interpreted as an early indicator of neuroinflammation and has been implicated in the pathogenesis of alcohol-induced cognitive deficits and neurodegeneration. The augmented GFAP expression in females appears to reflect not only a general response to alcohol-induced injury but also underlying sex-specific mechanisms. Oestrogen has been shown to modulate astrocyte function, enhancing reactivity under inflammatory conditions (Brann et al., 2007; Villa et al., 2016). As astrocytes express oestrogen receptors, the hormone-mediated amplification of glial reactivity may account for the heightened GFAP distribution observed in female mice. Supporting this, astrocytes in female mice have demonstrated greater sensitivity to inflammatory insults, including alcohol, which may exacerbate neuroinflammatory responses in a sex-specific manner (Morken et al., 2014; Wilhelm et al., 2015; Gozlan, Lewit-Cohen and Frenkel, 2024). This is consistent with the findings of McGrath et al. (2017), who reported that under alcohol exposure, GFAP-positive cell numbers in the SGZ decreased in both sexes, but females displayed a higher baseline GFAP-positive population and a more pronounced reactive response, highlighting inherent sex differences in astrocyte biology and ethanol sensitivity.

Notably, simvastatin administration at 5 mg significantly attenuated the alcohol-induced increase in GFAP-positive cells in females, reducing levels to those comparable with the control group. However, comparisons with other groups did not reach significance. This suggests that simvastatin exerted a modulatory effect on astrocyte activation, potentially through its well-documented anti-inflammatory properties. Previous studies have shown that

simvastatin reduces reactive gliosis in models of TBI and neurodegeneration (Li et al., 2009a; Bowers, 2021; Salari, Roghani and Khalili, 2024). Mechanistically, simvastatin's ability to enhance Wnt/β-catenin signalling, an important pathway involved in neurogenesis and cellular repair, may underlie its capacity to reduce astrocytic reactivity following alcohol exposure (Robin et al., 2014). Additionally, sex-specific pharmacokinetics may contribute to simvastatin's greater efficacy in females, with differences in metabolism and blood-brain barrier permeability possibly enhancing the drug's central effects (Soldin and Mattison, 2009; Maggioli et al., 2016; Farkouh et al., 2021). Simvastatin on its own did not significantly alter GFAP expression in females, reinforcing the view that its effects are context-dependent and more pronounced in the presence of neuroinflammatory stimuli such as alcohol exposure (Jafari et al., 2021b; Nasef et al., 2021b). This aligns with findings from Lundgaard et al. (2018), who observed that low-dose ethanol reduced GFAP expression and improved glymphatic function, whereas high-dose ethanol increased GFAP and disrupted astrocytic AQP4 localization, indicating a dose-dependent switch from adaptive to maladaptive gliosis. These findings support the interpretation that simvastatin does not inherently suppress astrocytic function but instead acts to normalise glial responses when activated by pathological stimuli. Although the higher simvastatin dose (15 mg) did not significantly reduce GFAP-positive cell levels compared to the ALC group, the comparison between SIM and ALC+SIM15 approached significance, indicating a possible dose-related trend that warrants further investigation (Bowers, 2021).

In male mice, alcohol exposure significantly reduced the distribution of GFAP-positive astrocytes in the suprapyramidal blade of the DG, suggesting a sex-specific suppression of astrocytic reactivity. This is consistent with the sex-dependent effects reported by McGrath et al. (2017), where males exhibited a lower baseline of GFAP-positive cells and a marked reduction following ethanol exposure, in contrast to the higher and more reactive profile observed in females. This finding contrasts with the commonly observed astrocytic activation following alcohol-induced neuroinflammation (Crews and Nixon, 2009; Xu et al., 2021) and may reflect a blunted or maladaptive glial response unique to the male adolescent brain. Previous research has shown that adolescent males exhibit lower baseline astrocytic reactivity and attenuated neuroimmune responses relative to females (Koss and Frick, 2017), potentially influenced by circulating sex hormones such as testosterone and oestradiol, which modulate glial development and inflammatory signalling during adolescence (Duarte-Guterman et al., 2015; Villa et al., 2016).

The observed reduction in GFAP-positive cell distribution may also reflect alcohol-induced astrocytic degeneration or impaired GFAP transcription, rather than a true loss of astrocyte number (Adermark and Bowers, 2016). Further support comes from studies that investigated alcohol's effects on glial cells and found that ethanol metabolism in astrocytic mitochondria generates ROS that promote reactive gliosis. However, prolonged alcohol exposure led to a net reduction in astrocyte and oligodendrocyte numbers in the hippocampus, aligning with the decreased GFAP levels observed here (Franke, 1995; Salazar et al., 2008). Notably, simvastatin treatment further exacerbated this reduction, particularly in combination with alcohol, and a stepwise decline was observed across ALC, ALC+SIM5, and ALC+SIM15 groups. The significantly lower GFAP levels in the ALC+SIM15 group compared to both ALC and ALC+SIM5 indicate a dose-dependent suppression of GFAP expression, consistent with a cumulative or synergistic effect of alcohol and simvastatin in reducing astrocytic marker expression.

Although simvastatin is typically regarded as anti-inflammatory via attenuation of glial activation (Roy et al., 2015; Chong et al., 2019), its administration in an already glial-suppressed environment, such as the alcohol-exposed male hippocampus, may lead to further maladaptive suppression of astrocytic activity. This is further supported by the significant reduction observed in the SIM group compared to the control group, although no significant difference was found between the SIM and ALC groups. This statistical similarity suggests that alcohol and simvastatin may share overlapping pathways in modulating glial activity, potentially converging on transcriptional regulators of GFAP expression. Furthermore, the progressive reduction in GFAP distribution from SIM to ALC+SIM5 to ALC+SIM15 reinforces a dose-responsive effect of simvastatin when administered alongside alcohol. The marked decline in GFAP expression may represent not only astrocytic loss or dysfunction, but also phenotypic modulation, including a transcriptional downregulation of GFAP or a shift to a neuroprotective, non-reactive state, characterized by reduced intermediate filament expression (Escartin et al., 2021). Together, these findings reflect the dualistic nature of astrocytic reactivity described by Pekny and Pekna (2016), where early gliosis serves neuroprotective functions, but prolonged suppression or excessive reactivity may interfere with regeneration and tissue recovery.

Collectively, these findings suggest that in adolescent males, the vulnerability of astrocytes to alcohol and simvastatin may contribute to a maladaptive glial response, characterised by

suppressed astrocytic presence and diminished neuroprotective support. This pattern aligns with the notion that excessive or prolonged modulation of astrocyte function, whether through chronic alcohol exposure or pharmacological intervention, can disrupt homeostatic glial roles essential for synaptic regulation, metabolic support, and neuronal resilience (Sofroniew, 2015; Pekny and Pekna, 2016). The observed reduction in GFAP-positive cell density may thus reflect a shift from reactive gliosis towards astrodegeneration, with potential implications for impaired neuroplasticity and delayed tissue recovery during a critical developmental window.

In summary, these findings reveal sex-specific astrocytic responses to alcohol and simvastatin treatment in the adolescent hippocampus, with alcohol inducing pronounced reactive gliosis in females and suppressing astrocytic marker expression in males. Simvastatin exhibited a context-dependent modulatory role, attenuating alcohol-induced gliosis in females while further reducing GFAP expression in males, possibly reflecting divergent neuroimmune baselines and pharmacodynamic profiles between sexes. Together, these results highlight the complexity of astrocyte regulation during adolescence and emphasise the importance of considering sex as a biological variable in neuropharmacological research. While GFAP served as a reliable general marker of astrocytes, its inability to discriminate between reactive phenotypes or capture dynamic morphological and functional changes represents a methodological limitation. Future studies incorporating additional markers of astrocyte activation states (e.g., S100β, vimentin, Aldh1L1) would enable a more detailed interpretation of the glial response to alcohol exposure and simvastatin treatment. Moreover, the concurrent assessment of microglial activation using markers such as Iba1, CD68, P2RY12, or Tmem119 may offer valuable insight into astrocyte–microglia interactions, which are known to critically influence neuroinflammatory outcomes. Integrated analyses of astrocytes and microglia could enhance understanding of the cell-type-specific mechanisms underlying the neurobiological effects observed. Additionally, mechanistic investigations into hormone-mediated modulation and the dose-dependent effects of simvastatin on astrocyte function are warranted to clarify therapeutic windows and long-term outcomes. Despite these considerations, the current findings provide novel insights into the sex-specific neuroglial landscape shaped by alcohol exposure and statin treatment during a critical neurodevelopmental period.

4.5.3. Effect of Alcohol Exposure and/or Simvastatin Treatment on the Dentate Gyrus Volume of Adolescent Mice

No significant changes in DG volume were detected in either female or male adolescent mice following alcohol and/or simvastatin administration. These findings are consistent with previous reports suggesting that hippocampal volume may not be a sensitive indicator of early or subtle neuropathological changes induced by alcohol during adolescence. For example, Redwine et al. (2003) observed no significant hippocampal volume reductions during the early pathological stages in a rodent model of Alzheimer's disease, suggesting that cellular pathology can precede gross anatomical changes. Likewise, Schmitz and Hof (2007) reported stable DG volume in ageing rats despite underlying neurobiological alterations, supporting the notion that structural changes in this region may not always be evident at the macroscopic level. Notably, (Miki et al., 2000a, 2000b) demonstrated alcohol-induced neuronal loss specifically in the hilus of juvenile rodents, while the granule cell layer and overall DG volume remained unchanged, highlighting that significant cellular damage can occur in the absence of measurable volumetric loss. In line with this, Avchalumov et al. (2021) reported molecular and synaptic disruptions in the DG following chronic alcohol exposure without corresponding reductions in volume. Collectively, these findings emphasise the limitation of volumetric analysis alone and suggest that alcohol-related pathology during adolescence may be more accurately captured by cellular, molecular, or functional markers.

The potential for simvastatin to influence DG volume in the context of adolescent alcohol exposure has not been extensively explored. Indirect evidence from studies involving other statins, however, points to possible neuroprotective roles during brain development. For example, Tijsseling et al. (2013) demonstrated that pravastatin attenuated dexamethasone-induced reductions in cortical and hippocampal neuronal soma volume and prevented astrocytic proliferation in neonatal rats, suggesting that statins may counteract structural impairments resulting from neurotoxic insults. In the present study, simvastatin administration at either 5 mg or 15 mg did not result in detectable alterations in DG volume, indicating that its potential neuroprotective mechanisms may not involve gross structural preservation in this region. This interpretation is consistent with previous research showing that simvastatin can preserve neuronal integrity without affecting overall hippocampal volume. For example, Doraiswamy, Steffens and Mcquoid (2004) and Can et al. (2012) reported preserved neuronal populations and behavioural improvements in rodent models following simvastatin treatment,

despite unaltered hippocampal volume. Likewise, Samaras et al. (2019) observed cognitive benefits in human cohorts using statins, without concurrent changes in hippocampal volume. Taken together, these findings suggest that simvastatin's neuroprotective efficacy may manifest at the cellular or molecular level, potentially through modulation of inflammation, synaptic plasticity, or neurogenesis, rather than as measurable changes in regional brain volume.

The absence of volumetric alterations in the DG under the tested conditions contributes to a growing body of evidence suggesting that alcohol-induced changes during adolescence may manifest primarily at the cellular rather than structural level. Alcohol-related hippocampal atrophy is well documented in human studies, particularly during periods of rapid brain development such as adolescence. However, rodent studies have demonstrated that structural atrophy may not emerge unless experimental conditions exceed a critical threshold for disruption (Miki et al., 2000a; Redwine et al., 2003; Schmitz and Hof, 2007). Volumetric stability, therefore, does not preclude meaningful neurobiological changes, as reflected in the present study by sex-specific alterations in GFAP expression. Although previous clinical (De Bellis, 2001; Nagel et al., 2005; Medina et al., 2007) and preclinical studies (Spadoni et al., 2013) have reported sex differences in hippocampal vulnerability, no such differences were detected in DG volume here, potentially reflecting compensatory mechanisms that preserve gross structural integrity despite underlying cellular perturbations. Moreover, while Nissl staining combined with the Cavalieri method provides a robust and validated approach for estimating regional brain volume, this method may not capture microstructural or subregional alterations, such as those affecting CA1 or CA3, which have demonstrated greater susceptibility to alcohol-induced damage in other studies (Watson, Kirkcaldie and Paxinos, 2010; Ming and Song, 2011). This apparent dissociation between structural and cellular responses underscores the importance of integrating molecular and functional markers alongside volumetric measures to more comprehensively assess alcohol-related neuropathology. Supporting this interpretation, Avchalumov et al. (2021) reported functional disturbances in the DG following chronic alcohol exposure in the absence of measurable volume loss.

In summary, the absence of significant volumetric changes in the DG following alcohol exposure and simvastatin treatment in adolescent mice highlights the limitations of gross anatomical assessments in detecting early or subtle neurobiological alterations. While alcohol is known to induce hippocampal atrophy under certain conditions, the present findings align

with previous reports suggesting that such structural changes may not be detectable unless the experimental conditions produce a sufficient degree of cellular disruption. Similarly, the lack of volumetric effects following simvastatin treatment suggests that its neuroprotective actions may not involve macroscopic preservation of DG volume but rather operate through cellular or molecular mechanisms. This interpretation is supported by prior studies showing preserved neuronal integrity and cognitive benefits in the absence of detectable changes in hippocampal volume. Notably, the observed sex-dependent alterations in GFAP expression within the same experimental cohort point to underlying glial responses that may precede or occur independently of volumetric shifts. Together, these findings highlight the value of complementing stereological volume estimates with cellular, molecular, and functional markers to more accurately characterise the neurodevelopmental impact of alcohol and the modulatory potential of pharmacological interventions such as simvastatin.

4.6. Stereological Estimation of Neuronal Populations in Cortical Layers I–IV of the Prefrontal Cortex Following Alcohol Exposure and/or Simvastatin Treatment Using Nissl Staining and NeuN Immunolabelling

The PFC is integral to higher-order cognitive processes, including working memory, decision-making, and behavioural inhibition, and its protracted maturation renders it particularly susceptible to neurotoxic insults during adolescence (Fuster, 2001; Otero and Barker, 2014; Friedman and Robbins, 2022). To assess the impact of alcohol exposure and/or simvastatin treatment on cortical integrity, two complementary histological markers, Nissl staining and NeuN immunolabelling, were employed. While both techniques target cell bodies, they differ in cellular specificity: Nissl staining labels all cell somata, encompassing neurons, glia, and progenitor cells, whereas NeuN immunolabelling selectively identifies mature, postmitotic neurons. Therefore, these approaches yield distinct yet complementary insights into the cellular composition and potential neuroplastic adaptations within the PFC.

4.6.1. Stereological Estimation of Nissl-Stained Cells in Cortical Layers I–IV of the Prefrontal Cortex

To estimate the population of cells within cortical layers I–IV of the PFC, Nissl-stained sections were examined for morphological integrity and cell density across treatment groups. The analysis revealed a preserved laminar organization, with no overt morphological abnormalities in any group. The consistent appearance of pyramidal neurons in layers II–III and granule-like

cells in layer IV indicates that neither alcohol exposure nor simvastatin treatment induced gross neurodegeneration or disrupted cytoarchitecture in adolescent mice. These results align with prior studies demonstrating that Nissl staining can reveal both overt neuronal loss and more subtle morphological changes depending on alcohol exposure parameters (Crews et al., 2000; Crews and Nixon, 2009).

Neuron population estimates were obtained using rigorous, unbiased stereological methods, specifically the optical fractionator probe, which is widely regarded as the gold standard for quantitative histology (West, Slomianka and Gundersen, 1991). Consistent application of optimized parameters and thorough sampling across the PFC ensured accurate and reliable cell counts. The precision of these estimates was confirmed by low Gundersen coefficients of error, reflecting minimal sampling variance. This methodological robustness strengthens the validity of the observed sex- and treatment-dependent differences in Nissl-stained cell density, enabling confident interpretation of subtle structural alterations induced by alcohol exposure and simvastatin treatment.

In female mice, a significant increase in Nissl-stained cell density was observed following alcohol exposure. While seemingly paradoxical, this finding is consistent with evidence that adolescent alcohol exposure can induce gliotic responses or adaptive neuroplasticity rather than uniform cell loss (Vetreno et al., 2017; Meyer et al., 2019). As Nissl staining labels both neuronal and non-neuronal (glial) cell bodies, the increased density may reflect changes in glial cell number or size in addition to potential neuronal effects. This interpretation is supported by studies reporting that early-life stress or adolescent alcohol exposure can preserve or even enhance cytoarchitecture through compensatory mechanisms (Burda and Sofroniew, 2014; Montesinos et al., 2016). However, the precise cellular composition of the increased density cannot be determined with Nissl staining alone, and cell-specific immunolabelling is required to distinguish between neuronal and glial contributions.

Simvastatin treatment alone did not significantly alter cell density in female mice. However, when combined with alcohol, a dose-dependent increase in Nissl-stained cell populations was observed, most prominently in the ALC+SIM15 group. This pattern corresponds with simvastatin's pleiotropic effects, including anti-inflammatory and glia-modulatory actions (Liao and Laufs, 2005; Métais et al., 2014; Catalão et al., 2017; Sodero and Barrantes, 2020). Several studies have reported simvastatin's neuroprotective capacity in diverse

neurodegeneration models by reducing histopathological damage and preserving neuronal integrity (Wu et al., 2010b; Xu et al., 2012; Bhat et al., 2021). The observed increase in cell density may therefore reflect a combination of alcohol-induced structural plasticity and simvastatin's role in supporting cell survival, potentially involving both neurons and glia (Wang et al., 2005; Miron et al., 2007; Mackovski et al., 2016; Sushma et al., 2023).

In contrast, male mice did not show significant changes in cell density across treatments. This sex-specific divergence may stem from differences in hormonal regulation, baseline glial populations, or neuroimmune responsiveness, consistent with documented variations in male and female rodents' vulnerability and recovery following alcohol exposure (Cahill, 2006; Anderson et al., 2012; Vetreno and Crews, 2014; Becker, McClellan and Reed, 2017). Although Nissl staining cannot definitively identify the increased cell types, complementary NeuN and GFAP immunostaining suggest a mixed cellular contribution that may vary by sex and treatment.

Collectively, these findings highlight the use of Nissl staining in detecting both degenerative and compensatory structural changes within the adolescent PFC. The use of unbiased stereological approaches further strengthens the confidence in these data by minimizing sampling bias and ensuring consistent quantification. The observed sex-dependent and dose-responsive effects of simvastatin highlight its potential in modulating alcohol-induced cortical alterations. However, confirmation of the cellular identities and functional implications of these histological changes requires further investigation employing cell-specific markers and integrated analyses.

4.6.2. Stereological Estimation of NeuN-Positive Neuronal Populations in Cortical Layers I–IV of the Prefrontal Cortex

Despite the absence of overt morphological alterations across cortical layers I–IV, as confirmed by NeuN immunolabelling, stereological analysis of NeuN-positive cell distribution in the PFC revealed treatment-related changes in neuronal density, with notable sex-dependent differences. In female mice, a significant increase in NeuN-positive neuronal density was observed in the ALC+SIM15 group relative to controls, whereas ALC, SIM, and ALC+SIM5 yielded numerical but non-significant increases. These findings suggest a dose-dependent neuroprotective or pro-survival effect of simvastatin when administered concurrently with

alcohol, potentially mitigating alcohol-induced neurotoxicity and supporting neuronal preservation or compensatory neuroplastic responses.

Previous studies have demonstrated that adolescent alcohol exposure disrupts PFC structure and function through mechanisms involving excitotoxicity, oxidative stress, and apoptosis (Crews and Nixon, 2009; Vetreno and Crews, 2014). Interestingly, the mild, non-significant increase in NeuN-positive neuronal density following alcohol exposure may reflect a transient compensatory response to injury, potentially involving synaptic remodelling or increased neuronal activation (Vetreno and Crews, 2014; Chini and Hanganu-Opatz, 2021). This interpretation is supported by research indicating that neuroplasticity, including changes in dendritic complexity and spine density, can transiently counterbalance neuronal dysfunction or loss in the adolescent PFC (Hamilton, Whitcher and Klintsova, 2010; Frankfurt and Luine, 2015).

Simvastatin has been reported to exert neuroprotective effects in the cortex via anti-inflammatory, antioxidant, and synaptic modulatory mechanisms (Xavier et al., 2012; Roy et al., 2015). Its ability to enhance synaptic plasticity and modulate BDNF expression (Métais et al., 2014), alongside suppression of neuroinflammation and microglial activation (Zheng et al., 2018) may underlie the increased NeuN-positive neuronal density observed in the ALC+SIM15 group. The observed enhancement of neuronal density suggests that simvastatin may augment endogenous plasticity mechanisms in the alcohol-compromised PFC, particularly in female mice.

In contrast, treatment-related changes in male mice were less pronounced. While NeuN immunolabelling revealed modest, non-significant increases in neuronal density following alcohol and simvastatin exposure, a significant difference was detected between ALC+SIM15 and ALC+SIM5 groups, with the higher dose producing greater neuronal density. This dose-sensitive but subdued response in males may reflect sex-specific differences in statin pharmacodynamics, PFC plasticity, or neuroinflammatory responses (Whittle et al., 2011; Grissom and Reyes, 2019). Additionally, previous studies have highlighted differential hormonal regulation and metabolic processing of alcohol and statins between sexes, which may contribute to the more robust responsiveness observed in females (Whittle et al., 2011; Squeglia, Jacobus and Tapert, 2014).

Across all groups, NeuN-positive neurons morphologically exhibited characteristic features such as prominent cell bodies, well-defined nuclei, and dendritic processes, consistent with preserved cortical cytoarchitecture. The lack of observable structural disorganization suggests that the treatment-induced changes in neuronal density may reflect subtle neuroplastic or activity-dependent regulatory processes rather than overt neuronal degeneration or neurogenesis, in line with the limited neurogenic capacity of the adolescent PFC (Unal-Cevik et al., 2004).

Collectively, these findings indicate that adolescent alcohol exposure induces sex-specific alterations in PFC neuronal density and that simvastatin, particularly at higher doses, may confer neuroprotection or enhance plasticity-related mechanisms in female mice. The absence of gross structural disruptions alongside increased NeuN-positive neuronal density supports the interpretation that simvastatin modulates neuronal integrity under neurotoxic conditions, warranting further investigation into its therapeutic potential and sex-dependent effects during adolescent brain development.

Taken together, the combined analyses of Nissl-stained and NeuN-immunolabelled cells in the PFC revealed sex-dependent and treatment-related alterations in cellular density that may reflect adaptive neuroplastic responses to adolescent alcohol exposure and the modulatory effects of simvastatin. In female mice, both Nissl staining and NeuN immunolabelling indicated a dose-dependent increase in cortical cell populations following co-administration of alcohol and simvastatin, with the highest simvastatin dose (15 mg) producing the most pronounced effect. This pattern suggests that simvastatin may support neuronal survival or promote neuroplasticity in the context of alcohol-induced neurotoxicity, potentially through anti-inflammatory and glia-modulatory mechanisms. The increase in Nissl-stained cell density in alcohol-exposed females, in the absence of overt morphological disruption, may reflect gliosis or compensatory structural changes rather than neurodegeneration, a hypothesis supported by the cell-type specificity of NeuN labelling. In contrast, male mice exhibited more modest and inconsistent changes in both total and neuronal cell density, emphasising potential sex-specific differences in cortical vulnerability, neuroimmune responsiveness, or simvastatin efficacy.

Although the present study did not include behavioural assessments, the observed alterations in neuronal and cellular density within layers I–IV of the PFC may have functional

consequences, particularly given the role of this region in executive functions such as attention, decision-making, and behavioural inhibition. Adolescence represents a critical period of synaptic pruning and circuit refinement in the PFC, and even subtle disruptions in neuronal architecture during this window can lead to lasting impairments in cognitive flexibility and impulse control (Selemon, 2013; Drzewiecki and Juraska, 2020). The alcohol-induced increase in cell density observed in female mice may reflect reactive or maladaptive neuroplasticity, while the simvastatin-mediated enhancement, especially at the higher dose, could indicate a compensatory neuroprotective effect. These histological outcomes may have downstream effects on circuit function and behaviour, highlighting the importance of future studies incorporating behavioural phenotyping to validate the neurofunctional significance of these findings.

While the use of stereological methods strengthens confidence in these findings, the inability of Nissl staining to distinguish between neurons and glia limits definitive interpretation of cellular identity. Furthermore, the NeuN marker, while reliable for identifying mature neurons, may not capture dynamic changes in functional connectivity or glial contributions. Future studies incorporating additional cell-specific markers (e.g., GFAP, Iba1, and oligodendrocyte lineage markers), in combination with molecular and electrophysiological analyses, are needed to elucidate the mechanisms underlying these sex- and treatment-dependent effects and to further investigate the therapeutic potential of simvastatin in adolescent neuroprotection.

4.7. Molecular Mechanisms Regulating Neuroinflammation and Neuroplasticity in Adolescence Following Alcohol Exposure and/or Simvastatin Treatment

The adolescent hippocampus experiences dynamic structural and functional changes that make it especially vulnerable to disruptions in neuroimmune and neuroplastic processes. Cytokines such as IL-6 and IL-10 modulate neuroinflammation through distinct signalling pathways, with IL-6 mediating both pro- and anti-inflammatory effects, and IL-10 exerting predominantly anti-inflammatory actions via sustained STAT3 activation and SOCS3 upregulation (Ozaki and Leonard, 2002; Hutchins, Diez and Miranda-Saavedra, 2013; Hunter and Jones, 2015; Johnson, O'Keefe and Grandis, 2018). This regulatory balance is essential for maintaining neural homeostasis during critical periods of development (Green and Nolan, 2014). Concurrently, the expression patterns of CREB and BDNF in the hippocampus provide insights into the molecular mechanisms governing synaptic plasticity, memory formation, and neuronal

resilience (Vaynman, Ying and Gomez-Pinilla, 2003; Motaghinejad et al., 2021). These processes may be particularly susceptible to modulation by alcohol and pharmacological agents such as simvastatin during adolescence, emphasising the importance of examining both inflammatory and plasticity-related pathways.

4.7.1. Cytokine Balance and Neuroinflammatory Regulation in Adolescence following Alcohol Exposure and/or Simvastatin Treatment

This section addresses the objective of investigating how alcohol exposure and simvastatin treatment influence cytokine balance and neuroinflammatory regulation in the adolescent hippocampus. Alcohol exposure during adolescence disrupts the equilibrium between IL-6 and IL-10, promoting neuroinflammation that may contribute to the vulnerability of the developing brain (Crews et al., 2006; Vetreno, Qin and Crews, 2013). Sex-specific variations in this disruption are substantiated by evidence indicating more pronounced neuroinflammatory and cognitive consequences in adolescent females following binge ethanol exposure (Squeglia et al., 2012; Alfonso-Loeches, Pascual and Guerri, 2013). Against this background, simvastatin's neuroprotective potential emerges from its capacity to modulate inflammatory pathways, inhibit microglial activation, and suppress NF-κB signalling (Wang et al., 2014; Lietzau et al., 2023; Acuña et al., 2024).

In line with these regulatory mechanisms, hippocampal IL-6 expression was slightly elevated following alcohol exposure in female mice. Co-treatment with low-dose simvastatin (ALC+SIM5) further increased IL-6 levels relative to the control and SIM groups, whereas high-dose simvastatin (ALC+SIM15) normalised IL-6 expression to levels comparable to the control group. Although these differences were not statistically significant, the pronounced increase in the ALC+SIM5 group, coupled with reduced variability, suggests a consistent pro-inflammatory response at the lower simvastatin dose. This non-linear pattern aligns with reports that low-dose statins may exert limited or context-dependent anti-inflammatory effects in the CNS (Liao and Laufs, 2005; Stroes, 2005; Proute et al., 2021). Moreover, the dual role of IL-6 as both a pro-inflammatory and neurotrophic cytokine (Erta, Quintana and Hidalgo, 2012) may underlie its variable expression following alcohol exposure and pharmacological modulation. Alcohol-induced neuroinflammation has previously been linked to hippocampal cytokine dysregulation, including IL-6 upregulation (Jiang et al., 2021a), further supporting the findings of this study. In contrast, male mice exhibited the highest IL-6 expression in the control

group, with all treatment conditions showing decreased levels. This uniform reduction suggests a sex-specific suppressive response to alcohol and simvastatin, possibly reflecting differences in microglial activation, IL-6 signalling, or statin metabolism. Evidence suggests that oestrogen may potentiate, while testosterone may attenuate IL-6 expression in the brain (Villa, Della Torre and Maggi, 2019; Farkouh et al., 2021; Cruz et al., 2023), which could partially account for the divergent cytokine profiles observed between sexes.

To determine whether simvastatin also modulated anti-inflammatory pathways, IL-10 expression was evaluated as a potential compensatory response to the observed IL-6 elevations. In female mice, IL-10 expression appeared modestly elevated in response to alcohol exposure and simvastatin co-treatment, with the highest mean levels observed in the ALC+SIM15 group. Although none of the comparisons reached statistical significance, these trends may suggest a potential enhancement of anti-inflammatory signalling at the higher simvastatin dose. Prior studies have shown that alcohol exposure can stimulate IL-10 production as part of a compensatory neuroimmune response (Qin et al., 2008; Suryanarayanan et al., 2016). Furthermore, simvastatin has been reported to upregulate IL-10 expression and promote an anti-inflammatory phenotype in microglia under inflammatory conditions (Zheng et al., 2018; Bagheri et al., 2020). However, marked variability in the ALC+SIM5 group complicates interpretation and may reflect individual differences in treatment response or developmental stage. Given the absence of statistically significant changes, these observations remain speculative but are consistent with the hypothesis that simvastatin may modulate neuroimmune homeostasis in a dose-dependent manner.

In male mice, IL-10 expression was elevated in the ALC group relative to controls, though this difference was not statistically significant. This trend is consistent with evidence that alcohol may initially trigger anti-inflammatory responses through IL-10 upregulation as a protective mechanism (Qin et al., 2008; Suryanarayanan et al., 2016). Interestingly, co-treatment with low-dose simvastatin (ALC+SIM5) reduced IL-10 levels compared to ALC, while high-dose simvastatin (ALC+SIM15) preserved IL-10 expression, showing a near-significant difference relative to the control group. These findings align with reports that the anti-inflammatory effects of statins are dose-dependent, with lower doses potentially exerting less immunomodulatory efficacy (Liao and Laufs, 2005; Stroes, 2005). The observed sex differences may reflect differential sensitivity to alcohol-induced neuroinflammation and statin

metabolism during adolescence (Villa et al., 2016; Farkouh et al., 2021), highlighting the importance of sex-specific considerations in therapeutic strategies.

Overall, the results indicate that alcohol exposure produces modest changes in hippocampal cytokine expression in adolescent mice, and that simvastatin modulates this response in a manner dependent on both dose and sex. The dose- and sex-specific modulation of IL-6 and IL-10 by simvastatin, though not statistically significant, aligns with known pharmacological properties of statins and highlights their potential as modulators of neuroimmune activity during adolescent brain development. The sex-specific differences observed align with previous reports suggesting that male and female brains engage distinct inflammatory pathways during adolescence (Schwarz and Bilbo, 2012; Pyter et al., 2013; Hanamsagar and Bilbo, 2016; McCarthy, Nugent and Lenz, 2017). While statistical significance was not achieved, the observed biological patterns highlight the necessity for continued investigation into the neuroimmune effects of alcohol and statins during brain development. Future studies incorporating validation at the protein level (e.g., via ELISA) and an expanded panel of inflammatory markers (such as TNF-α and NF-κB) will be critical for elucidating these complex interactions.

4.7.2. Molecular Regulation of Neuroplasticity in Adolescence Following Alcohol Exposure and/or Simvastatin Treatment

The expression of CREB and BDNF in the adolescent hippocampus demonstrated sex-specific responses to alcohol and simvastatin treatment. In female mice, CREB expression peaked in the ALC group and showed a modest, non-significant reduction after simvastatin co-treatment. Although these changes did not reach statistical significance, a moderate-to-large effect size suggests potential biological relevance. This pattern may reflect compensatory CREB upregulation in response to alcohol-induced stress, potentially enhancing synaptic plasticity or memory-related processes. These findings differ from studies reporting alcohol-induced CREB suppression in adults (Pandey, Roy and Mittal, 2001; Pandey et al., 2005; Zuo et al., 2018; Motaghinejad et al., 2021), suggesting a possible compensatory response unique to adolescent females.

Oestrogen may underlie this pattern, as it enhances CREB signalling via the cAMP/PKA pathway without altering total CREB levels (Zhou et al., 2005). Additionally, oestrogen has

been shown to activate CREB/BDNF/TrkB signalling, contributing to neuroprotection (Jiang et al., 2021b). Given the heightened neuroplasticity of the adolescent brain, these pathways may be selectively recruited in females to preserve synaptic function. Notably, simvastatin at the tested doses did not significantly alter CREB expression in females, suggesting limited interaction with oestrogen-mediated signalling within this context.

In males, CREB expression was significantly influenced by treatment, with the ALC+SIM15 group showing the most robust increase compared to control and SIM groups. The low variability in this group reinforces the reliability of this effect. While the ALC group also exhibited elevated CREB, differences between ALC and ALC+SIM15 were not statistically significant, indicating a possible additive or synergistic effect of alcohol and simvastatin. These findings align with previous reports showing that simvastatin enhances CREB phosphorylation via PI3K/Akt and ERK1/2 activation, promoting neuroplasticity-related gene expression (Carloni et al., 2009; Métais, Hughes and Herron, 2015; Wang et al., 2015). The greater sensitivity of males to pharmacological modulation of CREB may reflect sex differences in intracellular signalling or hormonal regulation (Maynard et al., 2018; Flores-Bonilla and Richardson, 2020).

In contrast to CREB, BDNF expression remained stable across all groups and sexes, despite its well-established regulation by CREB. This lack of change was unexpected, given reports of alcohol-induced BDNF suppression and simvastatin-induced enhancement in pathological contexts (Silva-Peña et al., 2019; Peregud et al., 2023; Shafiee et al., 2023). Previous studies have shown that simvastatin can enhance BDNF expression, particularly under conditions of neuroinflammation, oxidative stress, or ischemia, likely via both CREB-mediated transcription and the attenuation of pro-inflammatory and oxidative factors (Wu et al., 2008; Mahmood et al., 2009; Liu et al., 2018; Manickavasagam, Lin and Oyewumi, 2020; Nasef et al., 2021). The absence of a significant BDNF response in this study suggests that the adolescent hippocampus may respond differently to simvastatin compared to adult or pathological models. This dissociation may arise because CREB activation did not coincide with peak BDNF transcription (Lonze and Ginty, 2002), or because alternative downstream CREB targets were preferentially activated (Sakamoto, Karelina and Obrietan, 2011). Additionally, post-transcriptional regulation mechanisms, including mRNA stability and protein turnover, may have buffered changes in BDNF protein levels (Taliaz et al., 2010; Ruiz, Shi and Meffert, 2014). Notably, the relatively high coefficient of variation observed in some groups,

particularly the male SIM group (~22%), may have further limited the detection of statistically significant changes and should be considered when interpreting these findings.

Overall, these findings highlight the complex, context-dependent regulation of CREB and BDNF in the adolescent hippocampus. CREB expression was sensitive to both sex and treatment, with males showing a particularly robust response to alcohol and simvastatin co-administration. In contrast, BDNF levels remained stable, suggesting that CREB activation did not translate into increased BDNF expression under current experimental conditions. This dissociation may reflect temporal mismatches, post-transcriptional control, or subregion-specific effects not captured in whole homogenates. These results underscore the importance of considering biological sex, developmental stage, and temporal resolution in future investigations of neuroplasticity-related pathways during adolescence.

CHAPTER 5

CONCLUSION

5.1. Overview

Adolescence represents a critical neurodevelopmental window marked by ongoing maturation of the hippocampus and prefrontal cortex, regions essential for learning, memory, and executive function, and highly susceptible to the neurotoxic effects of alcohol. This study examined simvastatin's potential to counteract alcohol-induced brain damage in adolescent C57BL/6J mice, with a focus on oxidative stress, neuroinflammation, and neurogenesis. A multimodal experimental approach was employed, integrating biochemical assays, molecular analysis, immunohistochemistry, and stereology to comprehensively assess brain responses to alcohol and simvastatin treatment. Key parameters evaluated included markers of oxidative stress (MDA), antioxidant defence (GSH-Px, SOD), neurogenesis (PcNA, DCX), neuroinflammation (GFAP, IL-6, IL-10), and neuronal integrity (Nissl, NeuN, CREB1, BDNF). Given the growing interest in the pleiotropic actions of statins, beyond their lipid-lowering effects, this study explored whether simvastatin could mitigate alcohol-induced neurotoxicity and thereby offer a potential therapeutic avenue for managing alcohol-related brain damage. This concluding chapter synthesises the main findings, highlights the study's contributions to the field, and discusses implications for future research and clinical translation.

5.2. Summary of Key Findings

In the hippocampus, alcohol exposure did not significantly elevate MDA levels in either sex, contrasting with previous reports and potentially reflecting strain-specific responses or adaptive mechanisms in the adolescent brain. Alcohol increased GSH-Px activity, suggesting a compensatory antioxidant response. Simvastatin reduced GSH-Px activity relative to the alcohol-only group, implying a reduction in oxidative burden. SOD activity remained unchanged across all groups, indicating it was not a primary target of modulation. These results suggest that simvastatin exerted modest regulatory effects on hippocampal redox homeostasis, within a context of complex interactions between alcohol-induced oxidative stress and intrinsic antioxidant defences.

In the prefrontal cortex, MDA levels were not significantly elevated by alcohol, though a slight increase was observed in males. Simvastatin had no significant effect on MDA levels, although a non-significant reduction was noted in males at the higher dose. GSH-Px activity exhibited a dose-dependent pattern in females: it decreased with the lower simvastatin dose but increased with the higher dose, indicating potential compensation. In males, GSH-Px activity remained stable. SOD activity did not change significantly across treatment groups. These findings underscore the influence of sex and brain region on antioxidant responses and indicate that simvastatin's modulatory effects are both dose- and sex-dependent.

Hippocampal neurogenesis was significantly impaired by alcohol, with reduced distribution of PcNA- and DCX-positive cells in the suprapyramidal blade of the dentate gyrus. Simvastatin partially restored proliferative activity in a dose- and sex-dependent manner: both doses were effective in females, with the 15 mg dose more efficacious, while males responded more robustly to the 5 mg dose. Simvastatin alone did not alter proliferation relative to controls, suggesting its benefits are most evident following alcohol-induced disruption. DCX expression also improved with 5 mg simvastatin, particularly in females, whereas the 15 mg dose was less effective. These results indicate that simvastatin supports hippocampal neurogenesis in a sex- and context-sensitive manner.

Stereological assessment of NeuN-positive neurons in the hippocampus showed no significant changes across groups. Neuronal counts were stable in females, while a modest increase was observed in males treated with simvastatin alone. This suggests that alcohol-induced hippocampal dysfunction does not necessarily involve detectable neuronal loss and that simvastatin's effects may act through non-neuronal mechanisms. Supporting this, GFAP immunolabelling revealed sex-dependent astrocytic responses: in females, alcohol increased GFAP expression, which was attenuated by 5 mg simvastatin, suggesting anti-inflammatory effects; in males, alcohol decreased GFAP-positive cell density, with simvastatin further reducing levels in a dose-dependent manner. These contrasting patterns suggest that simvastatin influences glial function in a sex-dependent manner, potentially conferring neuroprotection in females while intensifying vulnerability in males. Hippocampal volume remained unchanged across groups, reinforcing the idea that simvastatin's effects were more cellular than structural.

In the prefrontal cortex, Nissl-stained cell density in cortical layers I–IV remained morphologically intact across groups. In females, alcohol exposure significantly increased Nissl-stained cell density, further enhanced by simvastatin in a dose-dependent manner, most notably at 15 mg. This may reflect alcohol-induced plasticity combined with simvastatin's neuroprotective and glia-modulatory effects. In contrast, the male mice did not demonstrate significant changes. These sex-dependent effects suggest distinct cortical adaptations to alcohol and simvastatin, detectable through unbiased stereological methods.

Neuroinflammatory cytokine profiles in the hippocampus were also affected in a sex-specific manner. In females, alcohol elevated IL-6 levels; this effect was amplified by low-dose simvastatin but attenuated at the higher dose. IL-10 levels increased modestly in co-treatment groups, particularly at the higher dose, indicating possible anti-inflammatory compensation. In males, IL-6 levels decreased across all treatment groups, while IL-10 remained relatively unchanged. These findings suggest that simvastatin modulates hippocampal cytokine expression in a dose- and sex-specific fashion.

Molecular markers of neuroplasticity also demonstrated sex-specific regulation. In females, CREB expression increased with alcohol and was modestly reduced by simvastatin, suggesting a compensatory plasticity mechanism. In males, CREB expression was significantly elevated in the alcohol and high-dose simvastatin group, indicating heightened CREB sensitivity. BDNF expression, however, remained stable across all groups, suggesting a disconnect between CREB activity and BDNF transcription, potentially due to temporal dynamics, brain region specificity, or post-transcriptional regulation.

Taken together, these findings illustrate the intricate and sex-dependent nature of neurobiological responses to alcohol and simvastatin during adolescence. Alcohol induced broad yet variable disruptions in redox balance, neurogenesis, glial activity, and plasticity-related signalling. Simvastatin demonstrated selective modulatory effects that differed by sex, dose, and brain region. In the hippocampus, simvastatin alleviated oxidative stress, restored neurogenic markers, and modulated glial responses, particularly in females. In the prefrontal cortex, its influence was more evident in enhancing structural integrity and potentially supporting compensatory plasticity. The dissociation between molecular and cellular outcomes, especially in CREB and BDNF regulation, further underscores the complexity of neurodevelopmental adaptations under pathological conditions.

By applying a multimodal approach, spanning biochemical, molecular, and stereological analyses, this study advances understanding of the adolescent brain's vulnerability to alcohol and the neuroprotective potential of simvastatin. The findings highlight the importance of sex-specific approaches and point to glial modulation as a key mechanism. Future research should continue to explore simvastatin's therapeutic promise in adolescent alcohol-related neurotoxicity.

5.3. Contributions to Knowledge

This study advances our understanding of adolescent brain vulnerability to alcohol-induced neurotoxicity and the modulatory potential of simvastatin by providing novel, sex-specific insights into neurodevelopmental, biochemical, cellular, and molecular outcomes. One of the key contributions is the demonstration of a complex interaction between alcohol, oxidative stress, and antioxidant defences in adolescent brain regions. Contrary to expectations, chronic alcohol exposure did not consistently elevate MDA levels in the hippocampus or prefrontal cortex, suggesting the presence of adaptive or strain-specific resilience mechanisms. The region-specific and differential modulation of antioxidant enzymes, particularly the selective regulation of GSH-Px activity and the absence of changes in SOD, offers new insight into the dynamic redox balance during adolescence and the important role of simvastatin in modulating these defences.

Another significant contribution lies in the discovery of dose- and sex-dependent effects of simvastatin on hippocampal neurogenesis. Simvastatin was found to mitigate alcohol-induced suppression of PcNA- and DCX-positive cell populations, with optimal efficacy varying by dose and sex, highlighting the need for tailored therapeutic approaches. Additionally, the dissociation between preserved NeuN-positive neuronal populations and pronounced, sex-specific changes in astrocytic responses provides compelling evidence that early alcohol-induced neuropathology may be driven more by glial dysregulation than by overt neuronal loss. Simvastatin's attenuation of reactive gliosis in females, contrasted with its exacerbation of astrocytic reductions in males, highlights its divergent glial modulatory actions.

In the prefrontal cortex, the sex-specific enhancement of Nissl- and NeuN-positive cell densities following simvastatin treatment in alcohol-exposed females suggests that the drug may promote cortical neuroplasticity in a sex-dependent manner. Furthermore, the study

identifies divergent neuroinflammatory and neuroplasticity pathways, as evidenced by sex- and dose-dependent regulation of IL-6, IL-10, and CREB expression. The decoupling of BDNF expression from CREB activation suggests complex molecular regulation influenced by temporal or region-specific factors, expanding our understanding of the interplay between neuroimmune and plasticity mechanisms during adolescence.

Finally, this study introduces methodological advances by integrating stereological analysis, immunohistochemistry, and molecular assays within the same adolescent cohort, enabling a comprehensive assessment of alcohol and simvastatin effects across multiple biological levels. The combined focus on sex differences, regional specificity, and dose-response relationships fills important gaps in preclinical neuropharmacological research. In summary, these findings contribute new knowledge regarding the sex- and dose-specific neurobiological effects of alcohol and simvastatin during adolescence, emphasise the central role of glial modulation in early alcohol-induced brain changes, and support the potential of simvastatin as a sex-sensitive neuroprotective intervention targeting antioxidant, anti-inflammatory, and neurogenic pathways. To our knowledge, this is the first study to integrate biochemical, molecular, immunohistochemical, and stereological approaches within the same adolescent cohort to examine simvastatin's neuroprotective potential against alcohol-induced brain alterations. By doing so, it fills a critical gap in preclinical research, providing novel evidence that simvastatin's effects are sex-, dose-, and region-specific rather than uniformly protective.

This study contributes novel, sex-specific insights into the neurobiological effects of alcohol and simvastatin during adolescence, highlighting the complex interplay between oxidative stress, neuroinflammation, neurogenesis, and glial responses. Key findings include the region- and dose-dependent modulation of antioxidant defences, differential effects of simvastatin on neurogenesis and astrocytic activity, and sex-specific alterations in cortical neuroplasticity markers. By integrating stereological, molecular, and immunohistochemical techniques within a single adolescent cohort, this research advances methodological approaches and fills critical gaps in understanding the mechanisms underlying adolescent alcohol-induced neurotoxicity and the potential for simvastatin as a targeted, sex-sensitive neuroprotective strategy.

5.4. Study Limitations

Several design constraints and methodological limitations should be considered, as they may influence the interpretation and scope of the study's conclusions. First, the biomarkers employed, such as MDA and SOD, while widely used, possess intrinsic constraints related to sensitivity and specificity that may have influenced the detection of oxidative stress changes. Similarly, immunolabelling with NeuN and GFAP, though established indicators of mature neurons and astrocytes, respectively, does not encompass the full spectrum of neuronal functionality or astrocytic reactivity, thereby limiting insights into early neuronal dysfunction or subtle glial phenotypes. The reliance on Nissl staining and NeuN immunohistochemistry restricted cellular resolution, precluding detailed cell-type-specific analyses and the evaluation of additional neurogenic or inflammatory markers, such as microglial or oligodendroglial markers, that could have enriched mechanistic understanding.

Another limitation of this study is the absence of behavioural or cognitive assessments, which precludes direct evaluation of whether the observed neuroanatomical and molecular alterations translate into functional impairments or recovery. This is particularly significant given that the hippocampus and prefrontal cortex are critically involved in learning, memory, and executive function, domains often disrupted by adolescent alcohol exposure. Behavioural assessment of the impact of the neuroprotective potential of simvastatin will be beneficial to better understand the efficacy of these intervention. Future studies incorporating a battery of behavioural tests will provide additional information on the impact of the drug on cognitive function.

Morphological analyses focused on the hippocampus and prefrontal cortex which are few of the subregions of the brain. This specificity limit the generalisability of results across the broader neural circuitry which are potentially affected by alcohol or simvastatin. Additionally, the pharmacological characterisation of simvastatin was limited by the absence of pharmacokinetic data in adolescent mice, and the alcohol exposure paradigm may not fully replicate the diversity of clinically relevant drinking patterns. Although inclusion of both sexes is a strength, the lack of direct measures of hormonal status or pubertal development introduces complexity in interpreting sex-dependent effects. Furthermore, histological and stereological assessments were not performed blinded to treatment allocation. Although standardized protocols and objective counting criteria were applied to reduce bias, this omission is acknowledged as a limitation, and future studies should incorporate blinded analyses to

enhance rigor. Lastly, volumetric assessments using Cavalieri estimates, while methodologically robust, may lack the resolution to detect subtle microstructural changes, and species- and strain-specific factors inherent to the C57BL/6J mouse model warrant cautious extrapolation of these findings to human adolescent neurobiology.

Despite these limitations, the study provides important insights into the neurobiological consequences of adolescent alcohol exposure and the potential neuroprotective effects of simvastatin. Through region-specific analyses of oxidative stress, neuroinflammation, and neuronal integrity, the findings contribute to a growing understanding of how pharmacological interventions may mitigate alcohol-induced damage during critical windows of brain development. By integrating molecular, anatomical, and sex-specific observations, this research lays the groundwork for future studies that incorporate broader cellular profiling, behavioural outcomes, and pharmacokinetic validation to more fully characterise therapeutic mechanisms and inform translational applications.

5.5. Recommendations for Future Research

To build upon the findings and address the limitations identified in this study, the following research directions are recommended:

Firstly, future research should incorporate longitudinal, multi-timepoint studies that go beyond simply mapping temporal changes, aiming instead to delineate critical windows during adolescence when alcohol exposure most profoundly disrupts neurodevelopmental trajectories and when simvastatin intervention yields maximal neuroprotection. Such designs should integrate multimodal assessments, combining molecular, cellular, and functional readouts, to resolve the progression of oxidative stress, neuroinflammation, and neurogenesis alterations over time. Additionally, employing advanced in vivo imaging or minimally invasive biomarkers could provide dynamic insights into neuroplasticity and glial responses in real time, thereby informing the timing and dosing of therapeutic strategies. This approach would address mechanistic gaps regarding the persistence versus reversibility of alcohol-induced damage and the mechanisms underlying sex-specific responses to simvastatin, ultimately refining targeted interventions for adolescent alcohol-related brain injury.

A critical next step involves incorporating comprehensive functional and behavioral assessments alongside biochemical and cellular analyses to establish the translational relevance of neurobiological findings. Animal studies provide a unique opportunity to link molecular and histological changes directly to cognitive, emotional, and executive functions mediated by the hippocampus and prefrontal cortex. Including electrophysiological recordings and validated behavioral paradigms, such as spatial learning, memory retention, anxiety-like behaviors, and decision-making tasks, would clarify how alcohol-induced neurotoxicity and simvastatin's modulatory effects translate into altered brain function. This is essential because structural or molecular alterations alone cannot fully predict functional outcomes or behavioral impairments relevant to human adolescent alcohol exposure. Moreover, functional validation in animal models strengthens the mechanistic interpretation of observed changes, facilitates the identification of biomarkers of impairment or recovery, and enhances the development of targeted therapeutic interventions. Investing in these methodologies will deepen understanding of the neurocognitive impact of alcohol during critical developmental periods and the potential of simvastatin to preserve or restore function, ultimately improving clinical relevance and informing future translational research.

To gain a more comprehensive mechanistic understanding of alcohol-induced neurotoxicity and simvastatin's neuroprotective effects, future research should expand molecular profiling to include critical signalling pathways and cell-type-specific markers. Investigating pathways involved in redox homeostasis (such as Nrf2), inflammatory regulation (NF-κB), and neurogenic signalling (Wnt/β-catenin) will elucidate the intracellular processes governing oxidative stress, neuroinflammation, and neuronal survival. Additionally, assessing mitochondrial function through key enzymes can reveal metabolic impairments linked to alcohol exposure. Importantly, these analyses should be region-specific to capture differential vulnerability and responses: for example, Nrf2 and mitochondrial markers in the hippocampus to address oxidative stress and neurogenesis; NF-κB in the prefrontal cortex to investigate inflammatory modulation; and oligodendrocyte marker Olig2 in both regions to evaluate myelination and glial support. Incorporating these diverse molecular targets will clarify the cellular and molecular cascades mediating the damaging and protective effects observed, thereby advancing understanding of simvastatin's mechanisms and identifying novel therapeutic targets within discrete adolescent brain regions.

Complementing this molecular approach, future studies should specifically broaden immune cell profiling to include microglia and peripheral immune populations, which play pivotal roles in neuroinflammatory processes beyond those mediated by astrocytes. Employing markers such as Iba1 to characterize microglial activation states and assessing peripheral immune cell infiltration or activation will provide a more complete picture of neuroimmune interactions during adolescent alcohol exposure and simvastatin treatment. Understanding how simvastatin modulates these diverse immune populations could uncover additional neuroprotective mechanisms, as microglia critically influence synaptic pruning, neurogenesis, and inflammatory signalling during brain maturation. This focused neuroimmune investigation will enhance mechanistic insights into how immune cell dynamics contribute to both alcohol-induced damage and therapeutic recovery.

To deepen mechanistic understanding further, subsequent studies should examine the influence of alcohol and simvastatin on gene expression profiles and epigenetic modifications within the hippocampus, prefrontal cortex, and other vulnerable brain regions. Investigating alterations in DNA methylation, histone modifications, and non-coding RNA expression could reveal how these exposures modulate neurodevelopmental trajectories and contribute to lasting changes in neuronal and glial function. Such molecular-level insights would elucidate the persistence of alcohol-induced neurotoxicity and the potential of simvastatin to reverse or mitigate these epigenetic marks, thereby informing novel strategies for early intervention.

Given the pronounced sex-specific differences observed in neurobiological responses to alcohol and simvastatin, future research should systematically assess circulating sex hormones, pubertal development stages, and the pharmacokinetics of both substances in male and female adolescent animals. Hormonal fluctuations during adolescence profoundly influence brain maturation, neuroinflammatory responses, and antioxidant capacities, potentially shaping vulnerability or resilience to neurotoxicity. Moreover, sex differences in drug absorption, distribution, metabolism, and elimination can lead to distinct pharmacodynamic effects, which are critical to understanding the efficacy and safety of simvastatin as a neuroprotective agent. Integrating these measures will enable clarification of the biological mechanisms underlying sex-specific outcomes, allowing for the development of personalized therapeutic interventions tailored to hormonal and metabolic profiles. This approach is fundamental to bridging preclinical findings with clinically relevant sex-informed treatment strategies during the sensitive adolescent period.

Additionally, future studies should broaden the anatomical scope by including additional hippocampal subfields such as CA1 and CA3, alongside relevant cortical regions like the orbitofrontal cortex and anterior cingulate cortex. This expanded focus will enhance the anatomical resolution of alcohol-induced neurotoxicity and simvastatin's neuroprotective effects. Moreover, employing complementary imaging modalities, such as high-resolution MRI and confocal microscopy, can improve sensitivity for detecting microstructural and synaptic changes beyond traditional volumetric analyses. Such comprehensive morphometric approaches will deepen understanding of region-specific vulnerability and therapeutic responses during adolescent brain development.

Lastly, future research should explore a broader range of simvastatin doses and extend treatment durations to more precisely delineate dose-response relationships and therapeutic windows. By including lower and higher doses as well as varying lengths of exposure, such studies could identify the optimal dosing regimens that maximize neuroprotective efficacy while minimizing potential adverse effects in adolescent animals. This approach would also clarify the temporal dynamics of simvastatin's actions, distinguishing acute from chronic effects, and provide critical insights for translating findings into clinically relevant treatment protocols tailored for the adolescent brain.

In summary, future research should adopt an integrative, longitudinal, and sex-informed approach to deepen understanding of alcohol-induced neurotoxicity and simvastatin's neuroprotective potential during adolescence. This includes incorporating behavioural and functional assessments, expanding molecular and immune profiling, examining gene expression and epigenetic regulation, and refining dose-response analyses. Broadening anatomical focus and leveraging advanced imaging and in vivo techniques will enhance the resolution of region-specific effects. Together, these strategies will strengthen mechanistic insight, improve translational relevance, and inform the development of targeted interventions for adolescent brain health.

5.6. Conclusion

Overall, this study supports the potential of simvastatin as a targeted intervention for alcohol-related brain damage during adolescence, with particularly promising effects observed in

females. Simvastatin demonstrated beneficial outcomes on oxidative stress regulation, neurogenesis, and glial modulation; however, these effects were not consistent across sexes or brain regions. In males, simvastatin appeared to exacerbate certain alcohol-induced glial alterations, highlighting the need for caution in its generalized application. Thus, while simvastatin is not universally protective, it emerges as a sex-sensitive therapeutic candidate whose efficacy may be enhanced through dose optimisation and tailored targeting of alcohol-disrupted neurobiological pathways. These findings emphasise the importance of incorporating sex as a biological variable in therapeutic research and encourage further investigation into simvastatin's mechanisms of neuroprotection, particularly in female adolescent populations vulnerable to alcohol-induced brain injury. Importantly, this work provides the first demonstration that simvastatin can partially restore hippocampal neurogenesis and modulate astrocytic responses following adolescent alcohol exposure, thereby directly addressing a major gap in understanding the pleiotropic actions of statins in the developing brain.

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APPENDIX A:

PHD PROTOCOL APPROVAL

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Ms Sandra Munesar
E-mail: Sandra.munesar@wits.ac.za

24 April 2025
Person No: 2287758
PAG

Miss R du Preez
11 Galena Avenue
Helderkruin
1724
South Africa

Dear Miss Robin du Preez

Doctor of Philosophy: Approval of Title

We have pleasure in advising that your proposal entitled *Neuroprotective effects of simvastatin pre-treatment against alcohol-induced brain damage in adolescent mice* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Please take note that any study involving human or animal participants must receive ethics clearance from the relevant Research Ethics Committee before the study commences. It is the student's responsibility to apply for ethics clearance before starting any data collection/research activity involving human or animal participants.

Yours sincerely

Ms Sandra Munesar
Faculty Registrar
Faculty of Health Sciences

APPENDIX B:

ANIMAL ETHICS AND M&E'S

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2019/11/63/C

APPLICANT: Dr O Olateju
School: School of Anatomical Sciences; Department: N/A;
Location: CAS

PROJECT TITLE: Does simvastatin have neuroprotective effects against Alcohol-induced brain damage in C57BL/6J

Category: C; Species and Numbers involved: *150X 3 Weeks old (9-15g) male Mus musculus C57BL/6J*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 1 Oct 2019. This approval remains valid until 20 Jan 2022.

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

An annual progress report must be provided to the AREC.

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

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

Signed: _____ Date: 22/1/2020
(Chairperson of the AREC)

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

Signed: _____ Date: 22 January 2020
(Registered Veterinarian)

CC: Student supervisor: N/A N/A
Director Central Animals Service: Dr Kim Jardine

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr. Oladiran Olateju

b. Department: Anatomical Sciences

c. Experiment to be modified / extended

		AESC NO	
Original AESC number	2019/11	63	C
Other M&Es :			0

d. Project Title: Does simvastatin have neuroprotective effects against Alcohol-induced brain damage in C57BL/6J mice?

		No.	Species
e.	Number and species of animals originally approved:	300	*Mus musculus*
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	300	*Mus musculus*
h.	Number of animals used to date:	0	

i. Specific modification / extension requested:

1. To increase the alcohol concentration from 1 g/Kg to 2.5 g/Kg.

k. Motivation for modification / extension:

To maximise the scientific value of the study, we like to increase the alcohol concentration from 1 g/Kg to 2.5 g/Kg. This is to ensure that the blood alcohol concentration is within the range reported by Olateju et al. (2018) (i.e. 184 mg/dL) and the alcohol-induced brain damage is significant in the alcohol-treated mice. Ensuring a significant alcohol-induced brain damage is vital for the study. More so, 2.5 g/Kg is often considered a moderate dose of alcohol. This concentration is chosen based on studies on long-term alcohol effects that used alcohol concentrations above 1 g/Kg (Masur et al., 1986; Lê and Israel 1994; Bell et al., 2000; Chuck et al., 2006; Brabant et al., 2014; Karlsson and Erika 2016; Olateju et al., 2018).

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Signature: 

Date: 06 June 2020

RECOMMENDATIONS
Approval of the higher concentration of 2.5 g/kg alcohol to be evaualted in the study.

Date: 13 June 2020

Signature: 

Deputy-Chairman, AESC

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND

ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Dr. Oladiran Olateju

b. Department: Anatomical Sciences

c. Experiment to be modified / extended

			AESC NO
Original AESC number	2019/11	63	C
Other M&Es :			2

d. Project Title: Does simvastatin have neuroprotective effects against Alcohol-induced brain damage in C57BL/6J mice?

		No.	Species
e.	Number and species of animals originally approved:	300	*Mus Musculus*
f.	Number of additional animals previously allocated on M&Es:	0	
g.	Total number of animals allocated to the experiment to date:	300	*Mus musculus*
h.	Number of animals used to date:	0	

i. Specific modification / extension requested:

1. To add Ms Makgotso Nchodu (Student no: 1325876) (MSc student) as a co-worker.

k. Motivation for modification / extension:

1. To add Ms Makgotso Nchodu (Student no: 1325876) (MSc student) as a co-worker for purpose of higher degree.

Date: 17 April 2021 Signature:

RECOMMENDATIONS

Approval of co-worker Makgotso Nchodu to the study. Please ensure that new co-workers attend the WRAF orientation course prior to intiating any animal work. Please contact Dr K. Jardine for details (Kim.Jardine@wits.ac.za)

Date: 20 April 2021 Signature:

Deputy Chair, AESC

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: AREC23-12-004C

APPLICANT: Dr O Olateju
School: Anatomical Sciences; Department: N/A; Location: WRAF

PROJECT TITLE: Does Simvastatin have neuroprotective effects against Alcohol-induced brain damage in C57BL/6J mice?
Category: C; Species and Numbers involved: 50X, Male, 3-week old, 9-15g, C57BL/6J, Mus musculus and 50X, Female, 3-week old, 9-15g, C57BL/6J, Mus musclus

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 27 February 2024. This approval remains valid until 14 March 2026 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

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

An annual progress report must be provided to the AREC.

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

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

Signed: _____ _____ Date: __18/03/2024_____

(Chairperson of the AREC)

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

Signed: ____ _____ Date: __18/03/2024_____

(Registered Veterinarian)

CC: Student supervisor: N/A
Acting Director Wits Research Animal Facility (WRAF): Sr Amelia Rammekwa

APPENDIX C:

SECTION 20 APPROVAL AND AMENDMENT

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Marna Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MarnaL@dalrrd.gov.za
Reference: 12/11/1/1/20 (2622SR)

Ms. Robin du Preez
School of anatomical Sciences,
Faculty of Health Sciences,
University of the Witwatersrand,
Johannesburg, 2193

E-mail: 2287758@students.wits.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Ms. Robin du Preez

Your fax / memo / letter/ Email received 15 August 2022, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form received 15 August 2022 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study using the specified waste contractor (Averda Waste Company). Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
4. Permission in terms of the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act, 1947 (Act No 36 of 1947) and/or the Medicines and Related Substances Control Act, 1965 (Act No 101 of 1965) may be needed prior to the start of the study;
5. Ethics approval must be obtained prior to the start of the study;

6. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
7. This Section 20 approval is valid until 30 November 2025.

Title of research/study: *"Does Simvastatin have neuroprotective effects against alcohol-induced brain damage in C57BL/6J?"*
Researcher (s): Ms. Robin du Preez
Institution: University of the Witwatersrand (WITS)
Your Ref./ Project Number: 2019/11/63/C
Our ref Number: 12/11/1/1/20 (2622SR)

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date:
2022 -11- 2 1

SUBJECT: RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001
Enquiries: Ms. Mama Laing · Tel: 012 319 7442 · Fax: +27 12 319 7470 E-mail: MamaL@dalrrd.gov.za
Reference: 12/11/1/1/20 (6320 CM)

Ms. Robin du Preez
University of the Witwatersrand (WITS)
School of anatomical Sciences,
Faculty of Health Sciences,
University of the Witwatersrand,
Johannesburg, 2193
E-mail: 2287758@students.wits.ac.za; gladiran.olateju@wits.ac.za

Dear Ms. Robin du Preez,

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Title of research project / study: "Does Simvastatin have neuroprotective effects against alcohol-induced brain damage in C57BL/6J?"

Your application requesting an amendment of the Section 20 permit issued by the Director of Animal Health on 2022-11-21 for the study mentioned above refers. I am pleased to inform you that the amendment is hereby granted with the following conditions:

Conditions:

1. A veterinary import permit must be obtained prior to importation of the following samples:
   1.1 Anti-Ki-67 rabbit polyclonal antibody (ab15580) (ABCAM) and
   1.2 Anti-Doublecortin (DCX) (ab18723) (ABCAM) from the United Kingdom.
   1.3 Anti-Serotonin antibody (ab125) (Merck) and
   1.4 Anti-GFAP antibody (ab5804) (Merck) and
   1.5 Normal Rabbit Serum (#31883) (Thermofischer Scientific) from the United States of America.

2. The above-mentioned samples may only be handled at the School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.
3. This amendment does not relieve the researcher of any of the other conditions as contained in the Section 20 permit issued on 2022-11-21 for this study.
4. Written permission from the Director of Animal Health must be obtained prior to any deviation from the conditions approved for this study under the Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za

Kind regards,

Dr Mpho Maja

DIRECTOR: ANIMAL HEALTH

Date:

2024 -03- 12

APPENDIX D:

MANUSCRIPT SUBMISSION FOR PUBLICATION

Metabolic Brain Disease: Decision on your manuscript

MB

Metabolic Brain Disease <sandra.flores@springernature.com>
To Oladiran Olateju

You replied to this message on 28 May 2025 14:17.

Reply | Reply All | Forward

Sat 17 May 2025 04:28

CAUTION: External email - verify sender before opening links or attachments. Report issues to ithelp@wits.ac.za.

Ref: Submission ID 8efaa234-ea00-4ee4-b347-45690eb67694

Dear Dr Olateju,

Your manuscript, "Neuroprotective Effects of Simvastatin Against Alcohol-Induced Oxidative Stress and Neurodegeneration in the Hippocampus of Adolescent Mice", has now been assessed.

We invite you to revise your paper, carefully addressing the comments from the reviewers and the editor. Please ensure the results are accurately reported, any overstated conclusions are rewritten and the limitations of the work fully explained. When your revision is ready, please submit the updated manuscript and a point-by-point response. This will help us move to a swift decision.

We recommend submitting all revisions within 14 days of the corresponding author receiving a revision request email.

If you need more time, please contact us and include your submission ID.

Kind regards,

Mei Liu
Editor
Metabolic Brain Disease

Reviewer Comments:

Reviewer 1
I appreciate the author's efforts for their contribution.
I have a question about the selection of the statins. Why only statins?
what are the author's expectations towards dose-related changes in male and female?

Reviewer 2
I think this is a well thought through research with a strong scientific significance and translational promise. The choice of experiments carried out effectively tests the authors hypothesis in a justifiable manner.

With the increasing number of exogenous substances capable of causing brain toxicity, the concept of testing existing and safe drugs for their neuroprotective potential is imperative and will foster drug development and repurposing, especially for neurodegenerative diseases. Also, alcohol abuse and misuse among the younger population is a global issue and requires urgent attention by the scientific community. The manuscript reads well and is professionally put-together. I would recommend that this research be published but with minor revisions.

Comments on the methods:
1) In the introduction, it is noted that findings from this study might reveal more insights on how simvastatin could prevent alcohol-induced brain damage. My emphasis here is on "prevention". Although this could be negligible, I do not think that the experimental design completely depicts a prevention protocol. In the methods, for a 28th-day period, ethanol was administered (via I.P) to the animals shortly after treatment with simvastatin (5 or 15mg). For a 28th day course prevention strategy, maybe mice could have been pretreated with simvastatin alone for the initial 14 days, then followed by simvastatin + ethanol or ethanol alone for the later 14 days.
In addition, it may be interesting to see what a reversal protocol might show. A reversal protocol is important because in real time, most people get intoxicated with ethanol (from adolescent years) before the occurrence of brain damage and search for therapeutic options. In this case, mice would be pre-exposed to ethanol (maybe for the initial 14 days) before the introduction of treatment with simvastatin in the later days. Findings here would tell us if simvastatin reverses or ameliorates neurotoxic injury due to ethanol.

2) Behavioral memory assays: Although the focus of this study was on the probable neuroprotective effect of simvastatin on biomarkers of neurodegeneration, It will be interesting to see if simvastatin protects against alcohol-induced memory deficits. This would enhance the overall depth of discoveries in this study. Moreover, one of the early symptoms of alcohol-induced neurotoxicity is cognitive decline which is also associated with a reduction in hippocampal neurogenesis and increase in oxidative stress. Please, I suggest that authors further check to see if simvastatin protects against ethanol induced spatial and non-spatial memory loss in mice. This specific aim is relevant to the overall significance of the study.

Thank you very much!

APPENDIX E:

TURNITIN

Robin_Thesis for Submission_Turnitin_Final_May 2025.docx

ORIGINALITY REPORT

14%	11%	13%	3%
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