

UNIVERSITI TEKNOLOGI MARA

**DELTA-9-
TETRAHYDROCANNABINOL
(Δ^9 -THC) MODULATES
COGNITION, HIPPOCAMPAL
CELLULAR INTEGRITY AND
ALZHEIMER-RELATED PROTEIN
MARKERS IN A D-GALACTOSE +
 AlCl_3 -INDUCED WISTAR RAT
MODEL**

ARISHA BT ABDULLAH MAS'OD

MSc

May 2026

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ARISHA BT ABDULLAH MAS'OD

Thesis submitted in fulfilment
of the requirements for the degree of
**Master of Science
(Physiology)**

Faculty of Health Sciences

May 2026

CONFIRMATION BY PANEL OF EXAMINERS

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ABSTRACT

Alzheimer's disease (AD) is a complex, progressive neurodegenerative disorder characterized by a gradual decline in cognitive functions, especially memory and learning, due to synaptic dysfunction and neuronal loss in the hippocampus and associated cortical regions. Central pathological features include extracellular deposition of amyloid-beta ($A\beta$) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau. Despite extensive research, current pharmacological interventions are largely palliative, underscoring the need for novel therapeutic strategies capable of modifying disease progression. Delta-9-tetrahydrocannabinol (Δ^9 THC), the primary psychoactive constituent of *Cannabis sativa*, has garnered increasing interest for its neuroprotective, anti-inflammatory, and neuromodulatory properties. Importantly, recent findings suggest that Δ^9 THC may also support adult hippocampal neurogenesis, a process closely linked to learning, memory, and cognitive resilience. This study aims to elucidate the neurotherapeutic potential of Δ^9 THC in AD-induced Wistar rats animal model via administering 200mg/kg of $AlCl_3$ and 60 mg/kg of D-galactose to induce Alzheimer-like degenerative properties in a total of 10 weeks. Upon treatment, behavioural results demonstrated by the Δ^9 THC-treated AD rats exhibited significantly improved spatial learning and memory retention compared to untreated AD counterparts. At the cellular level, Δ^9 THC treatment was associated with a substantial reduction of apoptotic neuronal cells. Crucially, Δ^9 THC also promoted the expression of neurogenesis markers and improved synaptic protein levels, suggesting an enhancement in both neuronal regeneration and synaptic plasticity. Along with that, Δ^9 THC has further proved its ability through the significant downregulation of protein levels BACE1 and p-tau Thr231 in AD-induced brains as portrayed in the western blot protein bands. In conclusion, this study provides compelling evidence that Δ^9 THC exerts multifaceted neurotherapeutic effects in an Alzheimer's disease model, improving cognitive outcomes by mitigating neuronal death through preserving neuronal integrity, and potentially enhancing adult neurogenesis and reducing key pathological hallmarks, BACE1 and p-tau Thr231 that lead to formation of plaques and tangles throughout the disease progression. From this study, the outcome emphasised the therapeutic promise of Δ^9 THC in targeting not only pathological hallmarks of AD but also in promoting mechanisms essential for cognitive recovery and neural enhancement for learning and memory.

ACKNOWLEDGEMENT

My deepest gratitude is extended to the blessings of Allah s.w.t for giving me the opportunity to embark on my master's journey together in completing this long and challenging journey successfully. Not to forget the sacrifices that my supervisor, Prof. Dr. Zukhairi Amom have given along this unforgettable path together with the expertise of a neuropathologist and a great researcher in the field of brain and learning memory, Dr. Fatin Nadzirah Zakaria.

My appreciation goes deeper to the professors, lecturers and esteemed mentors whose invaluable guidance, scientific expertise, and constructive feedback have been instrumental in shaping this research. Their encouragement and critical insights strengthened the investigation into the potential role of cannabis-derived compounds, particularly Δ^9 THC, in addressing the complexities of Alzheimer's disease, while broadening understanding of neurodegenerative disorders and therapeutic innovation. Appreciation is also conveyed to the laboratory staff whose assistance with experimental procedures, technical expertise, and data collection greatly enhanced the scientific integrity of this work.

Finally, this thesis is dedicated to the unwavering support from both of my lovable parents; Abdullah Mas'od bin Ismail and _____ that gave never-ending morale boosts in this research experience along with the continuous encouragement from my dear significant other, Muhamad Arif Ang throughout these few memorable years in master's research. Their beliefs and kindness have provided the motivation and resilience required for the successful completion of this research until the very end. This would never been completed without their support and love. This such blessing is dedicated to them. Alhamdulillah.

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LIST OF SYMBOLS

SYMBOLS

α	Alpha
A β	Amyloid-beta
β	Beta
Cd	Cadmium
Cu	Copper
γ	Gamma
Fe	Iron
κ	Kappa
kg	Kilogram
Pb	Lead
Hg	Mercury
mg	Milligram
NO ₂	Nitrite ion
pH	Potential of Hydrogen

LIST OF ABBREVIATIONS

ABBREVIATIONS

Al	Aluminium
α	Alpha
β	Beta
Cd	Cadmium
Cu	Copper
γ	Gamma
Fe	Iron
κ	Kappa
kg	Kilogram
Pb	Lead
Hg	Mercury
mg	Milligram
pH	Potential of Hydrogen

LIST OF NOMENCLATURES

NOMENCLATURES

Aluminium Chloride	AlCl_3
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
β -actin	Beta-actin
C.pneumoniae	Chlamydia pneumoniae
C.sativa	Cannabis sativa
Cis p-T231	Cis-phosphorylated Tau at Threonine 231
D-Gal	D-Galactose
ER α	Estrogen Receptor Alpha
ER β	Estrogen Receptor Beta
GSK-3 β	Glycogen Synthase Kinase 3 Beta
IL-1B	Interleukin-1 Beta
NF- κ B	Nuclear Factor Kappa B
p-Tau	Phosphorylated Tau
Ser	Serine
Ser202	Serine 202
Ser396	Serine 396
Ser404	Serine 404
THC	Tetrahydrocannabinol
Thr	Threonine

Thr205	Threonine 205
Thr231	Threonine 231
TNF- α	Tumor Necrosis Factor Alpha
Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

1.1 Research Background

In the 90s, advancements in genetic evolution aided geneticists in progressing to discover the root cause of AD. Over a hundred other hypotheses that have been proposed and most corresponding pharmacotherapies yielded negative results (except for a few drugs acting on neurotransmitter brain systems), AD has been redefined in a narrow perspective at which its biological mechanism is based only in biomarkers (amyloid and tau) to enhance development of new therapies for mild or preclinical stages specifically (Bermejo-Pareja & Teodoro, 2024). During the last third of 20th century, it was then neuropathological foundation for AD research was established after discovering brain alterations in mental disorders and new brain fixation histological staining techniques enabled enhanced study of brain tissue (Bermejo-Pareja & Teodoro, 2024). Alzheimer's disease (AD) is an age-dependent neurodegenerative process distinct from normal aging, which is associated with memory and cognitive decline. Prevalence is expected to grow in coming decades and it is considered as a growing public health fear with huge social and economic burdens. Based on population projections from 2010 to 2040, Malaysia is projected to become an ageing nation where the proportion of people 60 and older will exceed 15.3% of the total population in 2030 (Department of Statistics Malaysia, 2022). National Health and Morbidity Survey (NHMS) stated that the overall prevalence of probable dementia in Malaysia was 8.5%.

With an ageing population, the number would most likely to increase over the years if there is no intervention taken (National Institutes of Health, 2018). This provides an urgent need to discover novel targets and compound in developing new approaches to delay and retard the disease process targeting various aspects of AD pathology. *Cannabis sativa* (C.sativa) is a medicinal plant that contains diverse group of bioactive compounds known as phytocannabinoids, terpenes and flavonoids. Cannabinoids have been the centre of attention as it displays interactions with endogenous regulatory system involving in maintaining neural homeostasis. Despite increasing preclinical evidence supporting the role of cannabis-derived compounds in

neurodegenerative disorders, Δ^9 THC in clinical settings focusing AD patients remains incompletely understood. Concerns regarding psychoactive effects, dosage, routes of administration, long-term safety continue being the limitations to its clinical translation. Despite the use of Δ^9 THC is increasing in some regions, the widespread of therapeutic effectiveness was halted as in some regions, its prevalence remains low and lacks standardized clinical guidelines. Clinical findings indicated precise monitoring is needed especially when dealing with elderly patients, in this case AD individuals as the tolerance level might be compromised. Thus, these variabilities underscore the need for controlled experimental investigations to better elucidate the role of specific cannabinoids, particularly Δ^9 THC, in the context of AD pathology.

1.2 Justification for This Research

There are numerous practical challenges that are yet to be solved. Repeated attempts through understanding, designing, writing papers, discussing certain problems will aid in understanding the problem to find ways in enlighten the research problems. The expanding of knowledge allows opportunities to communicate in our inter-related fields to rise solutions to important research niches. This research was done based on the curiosity and the drive to solve never-ending development of AD enhancing future directions of neurotherapeutic solutions. Furthermore, this research aims to publish new findings in this area of research that are useful for the scientific community by filling the gaps in previous researches.

1.3 Problem Statement

Alzheimer's Disease (AD) is the most common form of dementia especially among elderly and it is a progressive neurological disorder characterised by presence of plaques and tangles in the brain. Currently, World Health Organization (WHO) has stated that around 55 million people have dementia globally and this number is expected to rise to 78 million in 2030 and 139 million in 2050 (World Health Organization, 2022). The overall prevalence of probable dementia in Malaysia was 8.5% as reported in NHMS (National Institutes of Health, 2018). Prevalence is expected to grow worldwide in the upcoming years due to the increasing proportion of older people in the population in nearly every country and if there is no new development of pharmacological

approach. Pharmacotherapies for AD are still limited as in the case of multi-factorial neurological disorders which require a higher degree of complexity in treating brain-related cases. Hence, researchers aim to discover new therapeutic ways to alleviate the symptoms associated with neurological impairments.

The urgency is enhanced when World Alzheimer Report (2022) has stated that there is no new drug that has been introduced and used widely in the last 20 years for AD (Gauthier et al., 2022). This may be due to most treatments available are symptomatic that focused on ameliorating progressive memory-declining aspects in the disease such as reduction of natural cholinergic degradation and compensation of abnormal glutamate neurotransmission. The strongest available evidence approved by U.S. Food and Drug Administration (FDA) which are used as pharmacological approaches include acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine and combination of memantine and donepezil), N-methyl-D-aspartate (NMDA) receptor partial antagonist (memantine) and recently discovered; amyloid-targeting antibody drug (lecanemab) (U.S Food and Drug Administration, 2022).

These six drugs increase the communication between the nerve cells and may improve the ability to think or slow the loss of these ability in people living with AD. However, these will not cure or stop the disease from getting worse in the future. The first approved-drug discovered that addresses reduction in beta-amyloid plaques was aducanumab. Recently, lecanemab was discovered and approved to be more effective in reducing beta-amyloid plaques for mild AD patients with mild cognitive impairments. However, based on clinical trials conducted there is a possibility to develop adverse events of ARIA (amyloid related imaging abnormalities). Researchers still lack information on its drug effectiveness and safety and evidence of clinical efficacy (U.S Food and Drug Administration, 2022). There is still no existing approved alternatives that aims for neurodegenerative processes by protecting against neuronal cell death, oxidative stress and inflammation events which counteract and reversing the process.

The intervention must begin at an early stage to target simultaneously multiple substrates in order to stop or slow down the degradation process. Hence, combination of $AlCl_3$ and D-gal was used in this study to exert neurodegenerative effects in the cognitive-sensitive areas of the brain without interfering other irrelevant areas. Considering that progressive memory-declining of AD is characterized by $A\beta$ and tau aberrant to different biological substrates causing neuronal cell death, targeting the

multiple substrates simultaneously will aid in stopping or slowing down the development of disease. The ability of cannabinoid agonists in interfering the underlying pathophysiological of the neurodegenerative processes has sparked the interest of many current researchers. Thus, this approach targets to explore efficacy at non-psychoactive doses uplifting its potential superiority in specific domains targeting in bringing effectiveness rather than only symptomatic relief, which most AD therapies aim for. As AD is associated with learning and memory mainly expressed in the hippocampus and cortex, interference in its regulation of the disease might bring consequent reductions in neurobehavioural dysregulation. The outcomes are vital to serve as a foundation for future researchers aiming to explore deeper niches focusing on managing neuropsychiatric symptoms. Hence, Δ^9 THC exerts multi-modal effects in the endocannabinoid system (ECS) rather than single-targeted therapeutic mechanism. To further explore the potential of Δ^9 THC, this research targets to study its preservative effects on synaptic integrity, modulation in neurogenic cellular differentiation and determine relevant effects on the hallmark markers of AD.

1.4 General Research Objectives

To identify and evaluate the potential of novel Δ^9 THC in a multi-targeted approach to simultaneously modulate neurobehavioural, neuronal survivability and development along with ameliorating AD hallmarks to develop a more effective disease-modifying therapies for future prospective.

1.5 Specific Research Objectives

- a) To examine the effects of Δ^9 THC on improving memory retention and spatial learning in an AD-induced Wistar rat model via neurobehavioural study.
- b) To investigate the effects of Δ^9 THC ameliorating neuronal apoptosis in CA1, CA3 and Dentate Gyrus (DG) regions in AD-induced Wistar rat model through histological study.
- c) To evaluate the effect of Δ^9 THC on GFAP and Calbindin as neurogenesis protein markers in AD-induced Wistar rat model through immunofluorescence (IF).

- d) To determine the capabilities of Δ^9 THC in reducing Beta-site APP Cleaving Enzyme 1 (BACE1) and Phospho-tau (Thr231) protein band intensity in AD-induced Wistar rat model through western blot.

1.6 Research Question

- a) How does Δ^9 THC administration affects memory retention and spatial learning in an AD-induced Wistar rat model?
- b) How does Δ^9 THC administration ameliorates neuronal apoptosis in CA1, CA3 and DG regions in AD-induced Wistar rat model?
- c) Did Δ^9 THC administration affects GFAP and calbindin as neurogenesis protein markers in AD-induced Wistar rat model?
- d) Is Δ^9 THC able to reduce Beta-site APP Cleaving Enzyme 1 (BACE1) and Phospho-tau (Thr231) protein band intensity in halting AD progression?

1.7 Significance of Study

The use of Δ^9 THC in rodent models of Alzheimer's disease (AD) holds significant scientific and translational value, particularly in exploring novel therapeutic avenues that go beyond conventional amyloid- or NFT-targeted treatments. The results from this study will prompt more pharmacological intervention research for AD and will lead to more investigations on development of Δ^9 THC as an alternative medication in clinical settings for Alzheimer therapeutic purposes. Targeting AD at early stage might be one of the early interventions that is beneficial for future healthcare. Furthermore, animal models provide a controlled environment in which the multifaceted effects of Δ^9 THC can be systematically studied. This model allow researchers to investigate optimal dosing, timing, and molecular pathways involved in cannabinoid activity, establishing proof-of-concept evidences before conducting clinical translation among AD patients in the medical field. Importantly, these results also help identify potential side effects and pharmacodynamic properties in a mammalian system. Given the increasing societal and regulatory interest in medical cannabinoids, findings from rodent studies offer foundational support for advancing Δ^9 THC-based interventions into human clinical trials. Thus, despite translational limitations, these animal models are essential in validating mechanistic hypotheses,

refining therapeutic targets, and accelerating the development of cannabinoid-based neurotherapeutics for Alzheimer's disease.

1.8 Limitation of Study

While preclinical studies investigating the effects of Δ^9 THC in rodent models of AD have reported promising neurotherapeutic outcomes, findings in this study must be interpreted with caution due to several inherent limitations (Aso & Ferrer, 2014; Cao et al., 2014; Scuderi et al., 2018). Notably, significant translational gaps exist in this research methodology as only a few neurogenesis markers responsible for certain neurogenesis stages were included and only limited areas of the brain were included in studying its potential effects. Complexity of the human endocannabinoid system (ECS), higher-order cognitive architecture, and multifactorial AD pathology cannot be fully recapitulated in rodent models, potentially resulting in an oversimplification of therapeutic mechanisms (Cummings et al., 2014; Martorana & Koch, 2014). This might inadequately reflect the sporadic, late-onset, and multifactorial nature of AD in human populations (Drummond & Wisniewski, 2017). In addition, behavioural paradigms employed in the neurobehavioural test in this study may insufficiently capture the complexity of executive dysfunction, episodic memory impairment, and progressive cognitive decline characteristic of clinical AD (Webster et al., 2014).

Another critical consideration is the psychoactive profile of Δ^9 THC, which may modulate anxiety-related behaviours, locomotor activity, or sedation in rodents, thereby confounding the interpretation of cognitive outcomes (Renard et al., 2016). Moreover, prolonged Δ^9 THC exposure in animal models may not accurately predict long-term safety or efficacy in aged human populations, where neurodevelopmental history, psychiatric vulnerability, or prior cannabis exposure may interact with AD pathology (Volkow et al., 2016). Finally, many preclinical investigations are constrained by limited sample sizes and short experimental durations, reducing statistical power and limiting the generalizability of findings (Begley & Ioannidis, 2015). Consequently, although animal studies provide valuable mechanistic insights into the neurotherapeutic potential of Δ^9 THC, rigorously-designed human clinical trials are essential to establish its therapeutic viability for AD.

1.9 Scope of Research

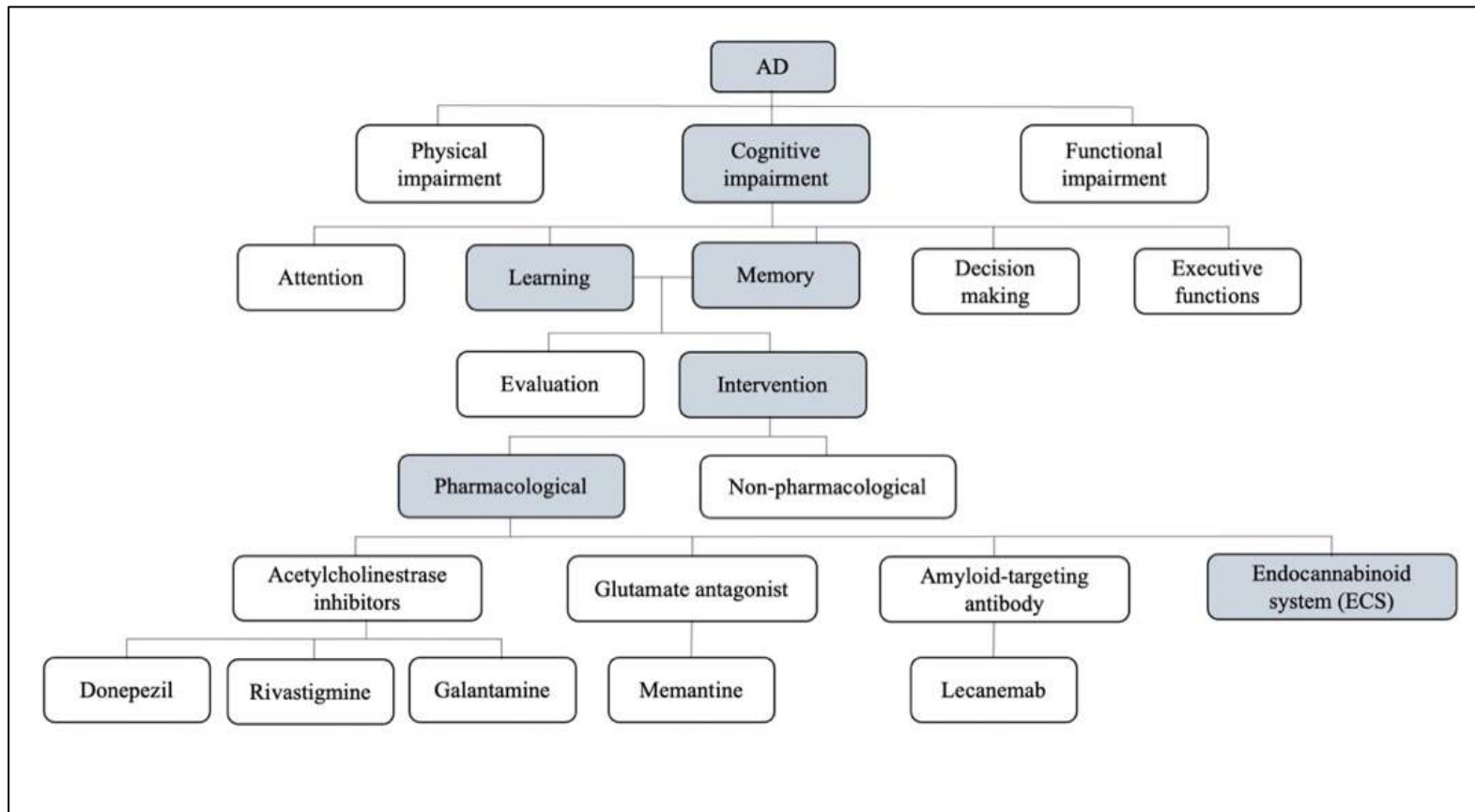


Figure 1 Flowchart Showing Scope of Research

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Dementia has emerged as a global health challenge as according to the World Health Organization's 2022 blueprint for dementia research, an estimated 55.2 million individuals globally affected (World Health Organization, 2022). The rates of incidence of dementia is reported to be generally increasing in the lower income countries while reducing in the higher income countries (Knopman et al., 2021). This is more alarming when the number of people living with dementia is expected to surge to 78 million by 2030 (World Health Organization, 2022). This case would not only be a burden to the community living with the disease, but there will be an urgent call as it affects economically leading to financial burdens associated with medical care, social services and informal caregiving for the affected individuals (World Health Organization, 2022). AD, is the predominant form of dementia exhibits similar epidemiological trends and represents the worldwide urgency with an escalating challenges for its care (Quefurth & LaFerla , 2010).

Other forms of dementia consists of younger-onset AD, vascular dementia, frontotemporal dementia and dementia with Lewy bodies (Queensland Brain Institute, 2025; World Health Organization (WHO), 2023). Pharmacological interventions for AD are still limited yet there are few approaches that were approved as therapies to ease down the symptoms. This includes the current six drugs available which are symptomatics as it reduces memory-declining aspects of AD. None of these treatments prevent or reverse the progression of disease but rather they treat the disease symptoms with limited efficacy (Cummings et al., 2023; Alzheimer's Association, 2024). Δ^9 THC at non-psychoactive doses has been gained interest to be explored due to its pleiotropic activities. This research tries to explore the potential therapeutic effects of Δ^9 THC by investigating potential cognitive improvements and its capabilities in reducing major

neuropathological hallmarks of AD such as A β accumulation, tau pathology, neurobehavioural and neuronal apoptosis (Aso & Ferrer, 2023; Cassano et al., 2020).

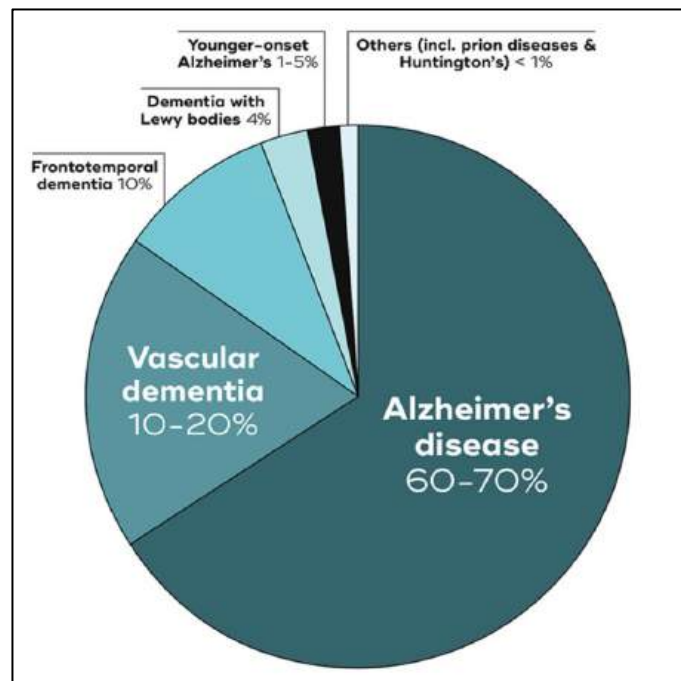


Figure 2.1 Types of Dementia

Figure 2.1 shows the types of dementia available and AD has scored the highest percentage among the six types of dementia. Percentage of AD is at 60-70% meanwhile vascular dementia is only around 10-20% that were known in the clinical settings. However, there are other reasons related to its pathological condition such as frontotemporal dementia (10%), dementia with Lewy bodies (4%), younger-onset Alzheimer's (1% until 5%) and other possible causes including prion diseases and Huntington's disease which scored less than 1% (Alzheimer's Association, 2024; Nichols et al., 2022). This shows AD is the major type of dementia among older people. Further in-depth researches have distinguished the causes of AD into two types, either concrete or associated type of causes. Concrete causes are the factors that are deterministically causal meanwhile associated causes are the factors that increase risk or modify disease progression without being independently sufficient. This is vital as it provides critical values in determining disease progression according to the AD subtypes while understanding the mechanistic heterogeneity underlying the clinical phenotypes (De Strooper & Karran, 2016; Scheltens et al., 2021).

Concrete causes of AD refer to well-established etiological factors that directly cause pathological effects, typically through genetic mutations with high penetrance allowing it to produce core neuropathology even in the absence of additional risk factors. Meanwhile associated causes of AD refer to the risk factors that influence the likelihood or severity of AD but are neither nor sufficient enough to cause disease pathology in the brain. These factors include genetic variants with incomplete penetrance, vascular and metabolic dysfunction, neuroinflammation and lifestyle or environmental exposures that are capable in inducing late-onset AD and significantly increases susceptibility via mechanisms that include impaired amyloid and beta clearance, however do not guarantee manifestation of the disease (Livingston et al., 2020; Leng & Edison, 2021; Chen et al., 2024).

Thus, to further elucidating pathophysiology of AD, animal models are categorized based on its capability on replicating concrete causative mechanisms or associated, multifactorial contributors to the disease. The types of AD animal models can be divided into two; spontaneous and induced models which differ in the way of integrating AD pathology either through via genetic, chemically-induced, surgical, vascular, metabolic or combined (multifactorial) models (Long & Holtzman, 2019; Saito et al., 2021). Meanwhile, the development of AD progression via spontaneous animal models is done through normal aging without any artificial manipulation whereas induced models typically involve external stimuli to create similar pathology as AD individuals. The induced models are further divided into transgenic or non-transgenic models depending on the type of AD and parameters of research being studied. Development of early onset AD (familial AD) only accounts for 5% to 6% of overall AD patients causing the use of non-transgenic induced models more preferable. Non-transgenic models represent the natural development of AD in human (sporadic AD) that includes neuropathological hallmarks of AD-like senile plaques, neurofibrillary tangles, oxidative stress and cognitive impairment (Kaushal et al., 2013). To date, rat models have been extensively used in research because it is able to mimic AD changes (Braidy et al., 2012).

To induce AD-like behavioural symptoms, a combination of D-gal and $AlCl_3$ may be used in experimental designs as D-galactose (D-gal) is a reducing sugar that easily reacts with the free amines of amino acids in peptides and proteins, forming advanced glycation end products (AGEs) (Vlassara et al., 1994). Neurotoxicity induced by chronic systemic exposure to D-gal in rats have been commonly used as a model for

studying the mechanism of AD (Gao et al., 2015). Chronic administration of D-gal at low doses has induced changes that mimic natural aging processes in animals including cognitive impairment changes in gene transcription and oxidative stress (Ho et al., 2003; Wei et al., 2005; Tian et al., 2005). These changes are considered to cause biochemical alterations similar to those seen in human brain (Ullah et al., 2015). Another inducing agent to create AD-like environments in the brain is aluminium (Al). It is a well-known neurotoxin being used widely in experimental models due to its ability to induce are symptoms linked to AD (Wang et al., 2018). Mechanistically, upon entering the human brain, Al affects both fast and slow axonal transport, induces inflammatory responses and causes synaptic and structural abnormalities which result in neurodegeneration (Syed Umesalma, 2015). Thus, this proves the relationship between the neurotoxicity of Al in animals and its involvement in creating the AD-like environment to replicate the pathogenicity of neurodegeneration in human brains (Ali et al., 2016).

Along with that, excessive exposure to Al can lead to the over expression of A β PP which lead to deposition of A β plaque on the brain cells, thus making Al a potential alzheimerogenic chemical contributing towards dysfunctional learning abilities (Dumont, 2011). In this study, we used the combination of D-gal and AlCl₃ as the chemical inducer to develop AD rat model (Chiroma et al., 2018). Recently, cannabis has gained interest among researchers especially in medical use. Δ^9 THC is the main psychoactive compound found in *Cannabis Sativa* plant. Pharmacologically, Δ^9 THC acts on cannabinoid receptor; CB1 and CB2 receptors. Previous studies have shown that Δ^9 THC is used therapeutically as an analgesic, antiemetic and anti-convulsant (Shahbazi et al., 2020). Researchers have sparked their interests in finding new pharmacological alternatives to treat neurodegenerative diseases. In this study, we investigate the potential neurotherapeutic effects of Δ^9 THC at non-psychoactive doses in animal model. This new informative database could serve as the basis for the development of Δ^9 THC as new therapeutic approach, thus can enhance the knowledge on clinical settings.

2.2 Alzheimer's Disease (AD)

The term 'Alzheimer's disease' has been used for over 100 years since first it was used in 1910 (Yang et al., 2016). It is a progressive neurological disorder characterised by presence of plaques and neurofibrillary tangles in the brain (Weston-

Green, 2019) causing gradual degeneration and death of cortical cells (Kim et al., 2019). There are two types of AD, the first one is the early-onset familial AD or known as EOAD (< 65 years of age) which is termed after its pathology of the brain changes up to 15-20 years before the onset of the symptoms. Second type is the late-onset sporadic AD or LOAD (> 65 years of age). AD presents a progressive declining course which is further being divided into few different stages namely; early, middle or late. The stages differ depending on the cognitive status of the patients, especially based on the degree of brain disabilities (Bringas et al., 2020). The early stages of the disease are typically characterized by short term memory loss. However, as the disease progresses, the characteristics are more defined as it is presented by language problems, spatial disorientations, mood swings and behavioural issues including long term memory loss (Waldemar et al., 2007). Eventually, the disease would progress towards loss of bodily functions and ultimately total failure.

To date, AD has no cure, although it has high incidence rates posing a huge economic burden. Anyhow, the aetiology of AD needs to be precisely discovered first on how it could be prevented, stopped or slowed before engaging with potential treatments (Gaugler et al., 2018). AD is sporadic in nature. Hence, sporadic AD is the most common form of AD and it is widely being studied with relation to a specific genetic risk factor which is the gene that encodes apolipoprotein E (Liu et al., 2013). Only approximately 5% to 6% of AD cases are being represented by familial AD due to inherited autosomal dominant mutations in the genes encoding amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2). The mutations will cause aberrant cleavages resulting A β peptides of 40 residues (A β 40) or 42 residues (A β 42) that aids in the formation of A β plaques contributing to neuronal cell death in the brain (Hardy & Higgins, 1992).

2.3 Epidemiology of AD

According to the National Institute of Health (NIH), epidemiology is a branch of medical science that investigates all the possible related factors in determining presence or absence of diseases and disorders (National Institute of Health, 2011). The incidence and global prevalence of AD was significantly increased to 147.95% which is estimated to rise reaching a 4-fold increase, 150 million by 2050 (Li et al., 2022). In 2019, the estimated total of prevalent cases of AD and other dementias is 51.6 million. This is not

oddly surprising as the incidence of AD is seen to double every 5 years after the age of 65. This is due to AD being an age-related condition and the chances to develop brain deterioration gets higher as age increases. The incidence rate of age-specific has increased from 1% yearly (before the age of 65 years old) to 6% yearly (after the age of 85 years old). To further comprehend this alarming issue, it is beneficial to know its relevancy to age as it might be effective to recognize therapeutic targets to ease down the development of apparent AD symptoms and its downstream disease progression (Li et al., 2022).

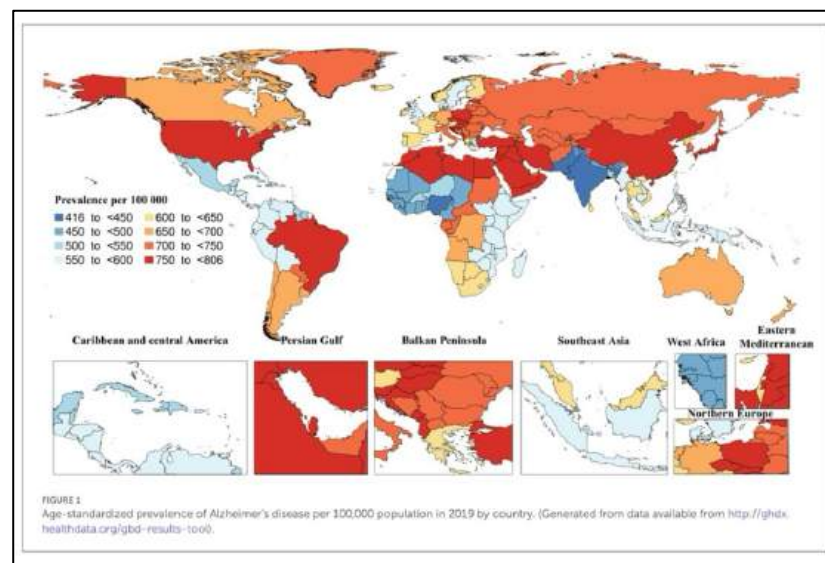


Figure 2.2 Age-standardized Prevalence of Alzheimer's Disease Per 100,000 Population in 2019 by Country (Amiri et al., 2022).

Figure 2.2 shows age-standardized prevalence of Alzheimer's disease per 100,000 population in 2019 by country (Amiri et al., 2022) in a recent prevalence analysis. Based on a recent prevalence analysis, the results showed AD cases had a large gap of increase as it went higher from 10% (after the age of 65) to 40% (after 85 years old) and in 2019, there were 1.6 million death cases due to AD and other dementias meanwhile the age-standardized prevalence rate that possesses the 95% Uncertainty Interval (UI) due to AD has the highest record in East Asia [781.4 (95% UI: 660-904.1)], North Africa and Middle East [(777.6 (95% UI: 660.8-896)] while South Asia [(428.4 95% UI 365-494)] and Western Sub-Saharan Africa [469.8 (95% UI: 402.1-539.4)] had the lowest rates (Amiri et al., 2022). In 2019, comparing specifically on national level, there are a few concerning regions that possess the highest national age-standardized prevalence rates;

Turkey [805.1 (95% UI: 686.6-926.2)] and Bahrain [801.1 (95% UI: 684.2-925.8)] while the lowest rates of age-standardized prevalence rate due to AD were found to be in India [416.4 (95% UI: 354.9-479.8)] and Nigeria [441.4 (95% UI:379.6-503.6)]. Statistically, China [29.2% (95% UI: 26-32.5)] and Japan [(22.3% (95% UI:20-24.9)] had the largest increases in national age-standardized prevalence rates since 1990 and the lowest increases were recorded by Spain [(-13% (95% UI:-18.2 to -7.2)] and Luxembourg [-10.7% (95% UI: -16.1 to -5.7)] (Safiri et al., 2024).

2.4 Stages of AD

In AD, the symptoms vary depending on the stage of disease which can be classified according to the level of cognitive impairment and disability experienced by the individuals. The classification of AD stages is divided into three stages that were categorized depending on the severity of the disease progression; Stage 1 (presymptomatic or preclinical), stage 2 (mild cognitive impairment known as MCI) and lastly, stage 3 (moderate to severe dementia) (Dubois et al., 2024). The common symptoms of typical AD present include difficulty in retaining new information despite able to recall long-term past memories. However, it may disturbs a person's abilities to make judgements, problem-solving, executive functioning or even organizational skills that lead to difficulty in managing tasks requiring multitasking and abstract thinking.

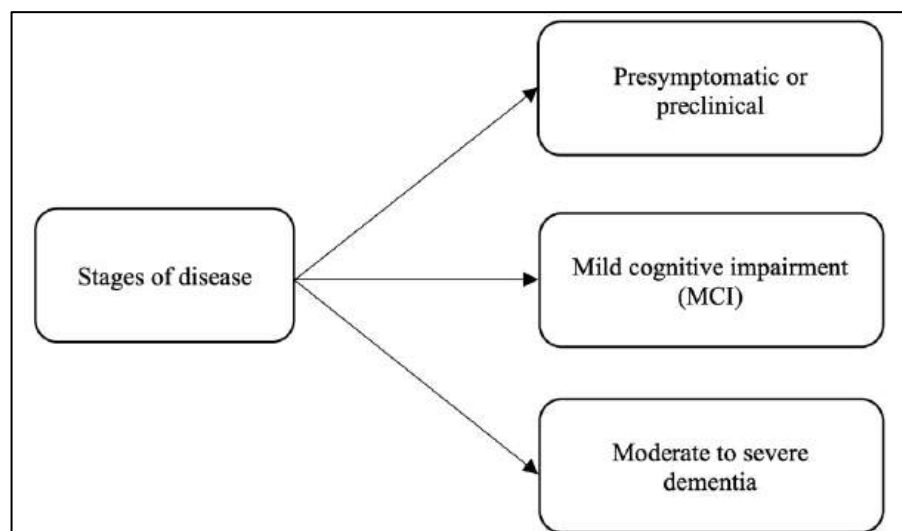


Figure 2.3 Stages of Alzheimer's Disease

Figure 2.3 shows the stages of AD depending on the severity of the progression of disease. Other than that, the early signs of cognitive decline may appear as language disorder, impaired visuospatial skills or both at the same time. Thus, the disease would deteriorate these brain functions even further if there is no effort to interfere in halting disease progression (Kumar et al., 2024). Furthermore, aging is the most significant biological risk factor for acquiring AD, either late-onset AD (LOAD) or early-onset AD (EOAD) as it has been found out that 10% of AD patients have EOAD and among these individuals, between the range of 35-60% are listed to have relatives who have dementia (Di Marzo et al., 2004; Dixit et al., 2008; Dumont, 2011; Elahi et al., 2019). Between these two, memory seems to be relatively well-preserved in the early stages of EOAD with respect to LOAD as LOAD was observed to have greater impact on memory whether recent events or well-consolidated information exhibiting impaired recognition (Tellechea et al., 2018). Thus, LOAD patients are in need of disease-modifying therapies to be able to minimize or comprehend the effects of disease deterioration.

2.4.1 Presymptomatic or Preclinical AD

Presymptomatic or preclinical AD or may have been known as the ‘silent stage’ of AD is defined as the asymptomatic stage since the individuals affected may experience measurable biomarker changes in the brain years before the symptoms affect the downstream memory, thinking and behaviour which are insufficient for physicians to further identify its diagnostic criterias (Sperling et al., 2011). This would lead to individuals carrying the defining pathology of AD but has yet to develop the clinically recognizable symptoms. However, presymptomatic AD differs from preclinical AD at which presymptomatic stage refer to individuals carrying the developing genes of AD but have yet to possess symptoms of AD (Sperling et al., 2011). Preclinical states of AD require the absence of both typical or atypical phenotypes and the presence of at least one biomarker of Alzheimer’s pathology (Dubois et al., 2024). The potential biomarkers recognized in this stage include low levels of amyloid and increased tau proteins in cerebrospinal fluid (CSF), however, these have yet to be enough to confirm AD specificity. More in-depth specific tests such as outcomes scores of cognitive tests; paired associates immediate recall test and digits symbol substitution test, increased tau proteins, measurements of right entorhinal cortex intensity and right hippocampal

volume on MRI might aid in predicting the progression of the disease at this preclinical stage (Dubois et al., 2024).

2.4.2 Mild Cognitive Impairment (MCI)

The middle stage of AD is known as the mild cognitive impairment (MCI) with either memory or non-memory domain impairment, such as executive ability or language function. Despite the cognitive declines, the affected individuals are still able to function social-independently which allows them to continue working as normal. This would lead to difficulty planning or performing familiar tasks, short-term memory loss, poor orientation and changes in visual perception (Johns Hopkins Medicine, 2024). As research suggested when there is an accelerated cognitive loss in the last 3 to 4 recent years before the diagnosis of MCI, this would increase the risk of progressing to AD at the rate of 10% to 12% per year (Howieson et al., 2008). However, interestingly MCI would not necessarily develop into AD as MCI generally can be due to variety of disorders. The reasons may vary including due to vascular dementia, frontotemporal dementia or even dementia with Lewy bodies (Budson & Solomon, 2012). Hence, it is important to identify definite laboratory evidences which can aid in diagnosing AD. correctly.

2.4.3 Moderate and Severe Dementia

Continuing with the disease progression, the last stage of AD can be further break down into = 'dx;'/wmoderate and severe stage. The staging system that were outlined was distinct according to the diagnostic criteria in Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) for AD (Porsteinsson, et al., 2021; Therriault, et al., 2022). To our concerns, the disease further progresses from MCI to moderate and severe dementia stage about 10% per year that includes increased severity of impairment and may develop other established risk factors for AD (Kumar et al., 2024). In this stage, the affected individuals would most likely to experience incapacitating memory impairment with notable language changes; paraphasic errors, reduced spontaneous verbal output, tendency to use circumlocution to compensate forgotten words and a decline in visuospatial abilities disturbing one's navigation with the surroundings (Kumar et al., 2024). For moderate to late stages, the common symptoms

include neuropsychiatric symptoms such as social withdrawal, agitation, psychosis and extrapyramidal motor signs like dystonia (painful and prolonged muscle contractions) and akathisia (inability to remain still) (Balint et al., 2018; Takeshima et al., 2018). However when the memory deterioration becoming worse, the plaques and tangles will slowly spread throughout the areas of the brain causing brain shrinkages (National Institute on Aging, 2023).

2.5 Risk Factors of AD

2.5.1 Genetics

2.5.1.1 Apolipoprotein E

Apolipoprotein E (APOE) is a lipid transport protein in the brain, significantly is needed neuronal repair and synaptic plasticity. APOE is a gene located on chromosome 19 that has three common alleles; $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ which encodes for ApoE2, ApoE3 and ApoE4 protein isoforms, respectively (Zhao et al., 2020). In contrast with $\epsilon 2$ that has protective effect against the disease, individuals that possess $\epsilon 4$ (ApoE4) have an increased risk in developing AD (Zhao et al., 2020). Specifically, allele $\epsilon 4$ has been identified as a significant genetic risk factor for LOAD. This is because the presence of APOE $\epsilon 4$ allele causes damage to the neurons and increases 3 times the risk in developing AD when an individual who is an heterozygous carriers of $\epsilon 4$ allele, However, homozygous carriers has 15 times increased risk, which is a fifth-fold compared to the normal condition.

Primarily, $\epsilon 4$ (ApoE4) is found to be contributing to AD through its affinity towards beta-amyloid protein and the corresponding impacts on its aggregation and clearance. ApoE4 is in charge to transport A β peptides across the blood-brain barrier and further enhancing its degradation. Compared to ApoE2 and ApoE3, due to ApoE4 less efficiency in clearing A β accumulation in the brain, this still results in the high levels of extracellular beta amyloid plaques (Huynh et al., 2019). Not only on A β , ApoE4 has showed the ability to influence tau pathology and neurofibrillary tangles formation as suggested in recent studies that ApoE4 promotes tau-related neurodegeneration by enhancing microglial activation and inflammation creating an environment that accelerates neuronal dysfunction (Dias et al., 2025). Hence, this

provides the insights at which ApoE4, neuroinflammation and tauopathy are interrelated in terms its central role in disease progression.

2.5.1.2 ATP Binding Cassette Transporter A1 (ABCA1)

ATP-binding cassette transporter A1 (ABCA1) is categorized as a crucial membrane transporter involving in lipid metabolism (cholesterol and phospholipid transport). ABCA1 facilitates the efflux of ApoE to regions with less lipid apolipoproteins forming high-density lipoproteins (HDL) essential for maintaining neuronal and synaptic integrity. However, genetic mutations and dysfunction in ABCA1 are considered as genetic risk factor for AD as it will affect downstream deteriorations such as disturbance in ApoE lipidation, amyloid-beta clearance and neuroinflammation (Kim et al., 2019). Proper lipidation of ApoE is beneficial for its functional properties in clearing toxic A β peptides from the brain. In the absence or lesser levels of functional ABCA1, ApoE remains poorly lipidated and disrupt its ability to transport and clear A β peptides. Hence, this still allows formation of amyloid plaques to increase gradually.

Another factor in amyloid clearance, ABCA1 is also needed for regulation of neuroinflammation and microglial function properties. Microglia are responsible for engulfing and degrading A β aggregates, however, dysfunctional of ABCA1 impairs the regular mechanism of cholesterol homeostasis leading to excessive inflammatory responses and impaired A β clearance. This gets worsen when it stimulates pro-inflammatory environment, exacerbating neuronal damage and cognitive decline (Fitz et al., 2021). Neurodegeneration is further enhanced when dysfunctional of ABCA1 causes increase levels of oxidative stress and dysfunctional of mitochondria promoting neurodegeneration. There are recent discoveries aiming to mitigate AD progression in strategizing to increase ABCA1 expression via genetic interventions or lipid-based therapies (Tanaka et al., 2023). Overall, ABCA1 indeed has a key role in AD pathogenesis, thus making it a promising therapeutic target for at least, delaying and even preventing AD.

2.5.1.3 Bridging Integrator 1 (BIN1) and Clusterin Gene (CLU)

As for Bridging Integrator 1 (BIN1) genetic risk factor, it has been recognized as the second most significant risk factor for late-onset AD, following APOE. This was proved through the identification of BIN1 variants associating with increased susceptibility to AD (García-Agudo et al., 2024). This gene is able to produce multiple isoforms via alternative splicing across neuronal, microglial and oligodendroglial cells, suggesting its diverse cell-specific functions (McAvoy et al., 2021). Other than that, BIN1 functions in endocytic trafficking and any dysregulation of these might impair normal processing of APP by causing endosome enlargement and facilitating accumulated A β (Calafate et al., 2021). Another hallmark of AD specifically tau proteins, have been proven to be interacting with BIN1 causing it to go into its pathological behaviour. Overexpressed predominant neuronal BIN1 isoform is able to cause tau-dependent neuronal hyperexcitability by disrupting the chemical equilibrium between excitatory and inhibitory synaptic signalling and lead to synaptic network dysfunctions (De Rossi et al., 2020).

Additionally, BIN1 is highly expressed in microglia and oligodendrocytes at which this contributes in disease-associated microglia through Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and Apolipoprotein E (APOE) as BIN1 influences inflammatory responses and synaptic homeostasis in glial cells proving its multiple significances (Patel et al., 2022). Interestingly, there were studies that suggested that BIN1 may have exert sex-specific effects in AD progression, particularly in Genome-Wide Association Study (GWAS) that was used to identify genetic variations that are associated with specific diseases or traits by scanning the entire genome of many individuals. As for AD, GWAS was used to identify key risk genes including Apolipoprotein E (APOE), Bridging Integrator 1 (BIN1), Clusterin (CLU), Phosphatidylinositol-binding Clathrin Assembly Protein (PICALM) and Triggering Receptor Expressed on Myeloid Cells 2 (TREM2), among others. From this, GWAS-based neuropathological study has reported that BIN1-associated risk is more pronounced in females, affecting mechanisms such as endocytosis, amyloid processing, tau propagation and synaptic transport (García-Agudo et al., 2024). Thus, BIN1 might be a possible multi-faceted contributor to AD, affecting multiple pathways including endosomal-lysosomal function, tau toxicity, neuroinflammation and synaptic dysregulation in neuronal cells.

CLU primarily affects amyloid-beta metabolism, neuroinflammation, and synaptic function, whereas BIN1 is more strongly linked to tau pathology, endocytosis, and synaptic dysfunction. While both genes play distinct roles, their dysfunction leads to overlapping consequences, including neuronal loss, synaptic deficits, and cognitive decline. CLU or also known as Apolipoprotein J, is an important factor especially for late-onset AD (LOAD). Chaperone proteins which are encoded by CLU are widely expressed in the brain significantly play its role in lipid transport, apoptosis regulation, immune modulation and A β clearance mechanism. To initiate in A β aggregation and clearance, CLU will firstly bind to soluble A β peptides to directly prevent its conversion in toxic oligomers and fibrils. Thus, any impairment or dysfunctional CLU expression will affect the ability to undergo these processes and leads to accumulation and soon, amyloid plaques (Yin et al., 2021).

Furthermore, CLU is known to be functional in facilitating transportation of A β across blood-brain-barrier (BBB), so any disturbance in this mechanism will result in reduced CLU functions and increased amyloid burden contributing to neuronal toxicity. Another function of CLU is to modulate glial cell activity to maintain the homeostasis in the neurons, particularly microglia and astrocytes. As AD progresses, CLU gets even more dysfunctional so this leads to excessive glial activity increasing chances for neuronal damage (Pimenova et al., 2021). There were even proofs that CLU is involved in synaptic plasticity and has neuroprotective qualities as it can regulate synaptic pruning; one of the processes to maintain optimum functions of neural circuits in the brain (Foster et al., 2022). Despite the positive values and insights that researchers are keen in focusing on CLU as a potent neurotherapeutic target, there is a controversial factor. Excessive CLU activity in the neurons are found to possess potential in promoting tau aggregations, one of pathological features of the disease (Shi et al., 2023). Still, CLU has been a complex area to work way through it due to this probability. All in all, both Clusterin Gene (CLU) and Bridging Integration 1 (BIN1) are important genetic risk factors for Alzheimer's disease, contributing to different aspects of disease pathology.

2.5.1.4 Evolutionarily Conserved Signalling Intermediate Toll (ECSIT Pathway)

ECSIT or Evolutionarily Conserved Signalling Intermediate Toll pathways is a multifunctional adaptor protein identified as a component of the Toll-like receptor

(TLR) signalling pathways. This pathway is crucial in immune signalling, mitochondrial function and oxidative stress regulation that are consisted as important mechanisms in AD. In a further prospect, ECSIT has a role in maintaining mitochondrial homeostasis and reactive oxygen species (ROS) production that are important for cellular survivability as it is localized in mitochondria at which it regulates the components of electron transport chain (ETC) (Roh et al., 2020; Misawa et al., 2022). Specifically, dysfunctional of ECSIT impairs the activity of mitochondrial Complex I causing increased production of mitochondrial ROS, a driver of oxidative damage in neurons (Graham et al., 2019; Wang et al., 2020; Swerdlow et al., 2022). Oxidative stress has proven to enhance both A β production and tau hyperphosphorylation creating a feedback loop that amplifies neurodegeneration. Previous research has demonstrated that ECSIT deficiency in neurons will lead to elevated ROS level and increased susceptibility to A β toxicity, both *in-vitro* and *in-vivo* (Fang et al., 2022). Thus, proving ECSIT has a vital role in mediating mitochondrial resilience in neurodegenerative settings.

Aside from oxidative stress, ECSIT is crucial for mitophagy, selective degradation of damaged mitochondria. This is to maintain and regulate mitochondrial function in highly-energy and long-lived neurons. This is because impaired mitophagy has seen to contribute in the accumulation of dysfunctional mitochondria and exacerbation of neuronal stress in both, AD models and patients (Fang et al., 2019; Lautrup et al., 2023). These were further proved when ECSIT has been shown to interact with essential proteins for mitophagy. Aside from mitophagy, ECSIT influences inflammatory signalling and thus, its impacts on neurons and glial cells (Cenini & Voos, 2019). The mechanism is due to Toll-like Receptors (TLR)-mediated signalling ECSIT pathways activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and production of inflammatory cytokines like interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) (as seen in its elevated levels in AD brains contributing to synaptic damage and A β toxicity (Heneka et al., 2015). If the mechanism disrupted, the vital roles of ECSIT in maintaining basal immune surveillance and cellular stress responses may change to overactivation or dysfunction leading to chronic inflammation and oxidative stress, the factors of neurodegeneration (Heneka et al., 2020; Leng & Edison, 2021).

Another significant finding showed ECSIT represents a convergence point for AD-related pathways may be due to the involvement of ECSIT in the regulation of

neuronal survival. In animal models, impaired ECSIT pathways were proven to lead to synaptic memory impairments and hippocampal synaptic dysfunction even when it is not affected by amyloid pathology (Fang et al., 2022). Hence, the degradation processes might become worse with the presence of AD hallmarks. Furthermore, the interdependence relationship between ECSIT and AD was exhibited in knocked-out APP/PS1 transgenic mice (common AD model); accelerated cognitive decline, A β burden increased and tau pathology enhanced. This emphasises the inflammatory hypothesis caused by mitochondrial dysfunction cascade in AD involving all the mentioned factors including ROS regulation, mitophagy and inflammation. So, targeting ECSIT or its downstream signalling partners may provide novel avenues to restore and maintain mitochondrial homeostasis thereby slowing AD progression.

2.5.1.5 Estrogen Receptor Gene (ESR)

Over the past decade, estrogens have received considerable attention in the context of AD as they play significant roles in synaptic plasticity, mitochondrial function and anti-inflammatory responses which are very critical in AD pathology. Estrogen being an important hormone in women contributes in the rising cases between increased incidence and faster progression of AD in females especially (Mielke et al., 2018). The estrogen gene receptor (ESR) specifically ESR1 and ESR2, are responsible in encoding estrogen receptor alpha (ER α) and estrogen receptor beta (ER β), both are vital nuclear hormone receptors mediating levels of estrogen in the brain (Mosconi et al., 2021; Rahman et al., 2023). Furthermore, these genes were expressed in regions that are AD-vulnerable such as hippocampus, cortex and basal forebrain. When these receptors are activated, this will regulate the expression of genes involved in neuronal survival, synaptic formation and plasticity (Brinton et al., 2021; Arevalo et al., 2015). Optimum signalling between these receptors helps in synaptic maintenance and plasticity by enhancing brain-derived neurotrophic factor (BDNF) expression (Spencer-Segal et al., 2020). However, impaired estrogen levels in the brain will affect this signalling and at the same time causing more susceptible to amyloid beta toxicity and tau pathology as proved by a previous study on modulation of estrogen responsiveness in the brain (Sundermann et al., 2020).

Another crucial point in AD pathogenesis, estrogen signalling is mediated through ESR and it involves in the regulation of amyloid precursor protein (APP) processing

(Li et al., 2013). Estrogens are able to shift APP metabolism from amyloidogenic to non-amyloidogenic pathway by upregulating α -secretase activity and reducing the production of A β peptides (Zhao et al., 2020; Li et al., 2013). However, when the maintenance of the mechanism is impaired, hence, ESR functional properties are compromised leading to higher chances in developing accumulation of A β . ESR receptors, specifically ER α will directly interact with mitochondrial proteins in maintaining mitochondrial integrity, reducing developed ROS and promoting ATP production for cellular energy consumption (Irwin et al., 2021; Nilsen & Brinton, 2022). So, if these functions are disturbed, this would contribute to the progression of the disease.

Through the same receptors mentioned above, ER α and ER β , their roles are proved to be involved in regulation of one of the key drivers in AD neuroinflammation (Arevalo et al., 2015; Brinton et al., 2021). This is due to estrogen can inhibit the activation microglia and astrocytes, consequently lowering the levels of produced pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6. So, if there are impairments, especially decreased expression of ESR genes, this exacerbates chronic neuroinflammation and promotes neuronal damage (Vegeto et al., 2016; Vegeto et al., 2020). Between the two receptors (ER α and ER β), it has been proved that the latter, ER β has been involved more prominently in anti-inflammatory and neuroprotective roles in the aging brain proved in preclinical AD models (Arevalo et al., 2015; Sávári et al., 2022). Despite previous research showed beneficial outcomes in terms of cognitive preservation and minimal detrimental effects, it still important to highlight the importance of approaches that focus on personalizing treatments by leveraging estrogen signalling as AD treatments (Ryan et al, 2020). However, there are some limitations to this approach; genetic variation and reduced expression of ESR genes that can diminish the neuroprotective estrogen signalling. The interplay that targets ESR-mediated pathways have to be uncovered before developing specific therapeutic strategies to prevent or slow AD progression, especially when dementia-affected individuals are expected to rise from the current prevalence of 55 million to 78 million in 2030. If there are no strategies in tackling the issue, the numbers would even rise to 139 million in 2050 (World Health Organization, 2022) due to increasing population of older people in nearly every country.

2.5.2 Other Factors

2.5.2.1 Cardiovascular Diseases (CVD)

Beyond the well-known obvious risk factors of AD, there are still several factors overlapped and potent to be discovered even deeper. One of it is the relationship between cardiovascular disease (CVD) and AD that were proven to have biological connections causing vascular dysfunction and neurodegeneration specifically. This might occur due to the contributable risk caused by the downstream effects of strokes or vascular dementia such as those involved in lipid metabolism, blood pressure regulation and inflammation (Sweeney et al., 2019; Montagne et al., 2020). Even worse, chronic vascular insufficiency can lead to intense oxidative stress, inflammation and BBB disruption facilitating accumulation of A β and tau proteins. Due to this, this was termed as the ‘vascular hypothesis’ of AD (Sweeney et al., 2018). This can exacerbate and spread through mechanisms like glycogen synthase kinase-3 β (GSK-3 β) activation. Moreover, vascular dysfunction also impairs glymphatic system of the brain, further reducing the clearance mechanism of neurotoxic proteins and amplifying neuronal injury (Rasmussen et al., 2022; Tarasoff-Conway et al., 2020). Based on a genome-wide association studies (GWAS), the interplay between CVD and AD was proved stronger with the involvement of genes such as clusterin (CLU), ATP-binding cassette transporter 7 (ABCA7) and phosphatidylinositol binding clathrin assembly protein (PICALM), respectively. This aims to aid in regulating cholesterol efflux in the brain and microglial phagocytosis of A β and its dysfunction may simultaneously promote atherogenesis and impair neuronal survivability (Bellenguez et al., 2022). This may be due to ABCA7 functions in lipid homeostasis and immune cell differentiation meanwhile PICALM plays a role in clathrin-mediated endocytosis associated with AD (Kunkle et al., 2019).

This leads to growing interest in targeting vascular health via statins, antihypertensives and anti-inflammatory agents as therapeutic efforts. Beyond the genetic overlap, CVD can cause a cascade of vascular dysfunctional properties such as hypertension, atherosclerosis and atrial fibrillation leading to chronic cerebral hypoperfusion due to deprived level of oxygen and nutrients in the brain (Iadecola et al., 2016). Consequently, hypoperfusion will further affects neuronal metabolism by

promoting increased levels of oxidative stress, mitochondria dysfunctional and failure which contribute to damages occur among synapses and impairments in cognitive abilities (Iadecola, 2019; Snyder et al., 2023). Another CVD-related potential cause that might be reliable factor for AD is chronic ischemia. Chronic ischemia refers to a long-term reduction in blood flow to a particular tissue or organ, leading to insufficient oxygen and delivery of nutrients over time. In the context of brain, it is defined as prolonged reduced blood flow to brain regions causing neurodegeneration. For example, white matter damage and impaired clearance across blood-brain barrier (BBB) which are associated with vascular dementia and the downstream processes of AD. This would cause accelerated accumulation of A β in the BBB by depositing its deposits in the cerebral vessels (cerebral amyloid angiopathy (CAA)), weakening the vessel walls even further and increasing the risk in microbleeds in the brain (Charidimou et al., 2022).

In CVD, it is proven that the integrity of BBB is compromised, allowing influx of peripheral immune cells, toxins, and inflammatory mediators into the brain regions. The sudden increased exacerbates neuroinflammation, facilitates the accumulation of neurotoxic proteins, impairs regulation of cerebral blood flow and thus, able to increase neuronal vulnerability to injury (Sweeney et al., 2019; Greenberg et al., 2020). The neuroinflammation might lead to another biological mechanism; damaged vasculature stimulates glial cells (microglia and astrocytes) which in return will secrete pro-inflammatory cytokines (TNF- α and IL-1 β). These cytokines will then affect neuronal survivability. Other than that, these molecules are capable in production of hyperphosphorylated tau via GSK-3 β and MAPK contributing to one major AD hallmark, neurofibrillary tangles (Nation et al., 2019). In summary, the relationship between these two factors are proven as CVD contributes to AD through genetic vulnerabilities (GSK-3 β , CLU, ABCA7 and PICALM) and vascular-mediated biological mechanisms. This highlights the importance in converging these factors in establishing a strong foundation in understanding AD pathophysiology for further therapeutic interventions.

2.5.2.2 Obesity and Diabetes

Other modifiable risk factors for AD, that may be overlooked by the society are obesity and diabetes. These two are capable in increasing the risk of getting AD as

obesity can impair glucose tolerance and soon, causing in the development of type II diabetes (Arnold et al., 2018; Kumar et al., 2024). Type I diabetes is due to the pancreas is unable to generate insulin caused by immune system of own body attacks islet cells in the pancreas that makes insulin (DeFronzo et al., 2021). Meanwhile type II diabetes is the common type at which the pancreas produce less insulin than normal making the body become resistant towards it and unable to use for metabolic activities. Hence, chronic hyperglycemia or defined as persistently high blood sugar levels can lead to cognitive impairments and promote beta-amyloid plaques and neuroinflammation (Biessels & Despa, 2018; Barbagallo & Dominguez, 2022). This condition occurs when pro-inflammatory cytokines are triggered causing a higher insulin resistance (Saltiel & Olefsky, 2017; Kumar et al., 2024) at which the secreted pro-inflammatory cytokines (e.g., IL-6, TNF- α) from the surrounding adipose tissues leads to chronic low-grade inflammation. If the condition persists, the cytokines might cross blood-brain barrier (BBB) and lead to further degenerative events progressing with the disease development (Wang et al., 2021; Banks et al., 2021).

This insulin resistance in the AD brain is considered as ‘type 3 diabetes’ or ‘type 3 diabetes mellitus’ (T3DM) which is a term used by some researchers describing the link between AD and the metabolic processes associated with type II diabetes (Tumminia et al., 2018; Kellar & Craft, 2020). Despite the non-officially recognized medical condition, it is a theoretical concept highlighting the role of insulin resistance, impaired glucose metabolism, accumulation of neurotoxins and neuronal stress as contributing factors to AD. Under these conditions, neurons exhibit a diminished capacity to utilize glucose efficiently, leading to intracellular metabolic stress and energy deficits (Talbot & Wang, 2021). The functional neurons are unable to effectively convert glucose into energy, hence increasing the amount of glucose among the neuronal cells. This dysfunction adversely affects the activity of insulin-degrading enzyme (IDE), which under physiological conditions facilitates the clearance of A β peptides from the brain (Qiu & Folstein, 2006; Kellar & Craft, 2020). Consequently, impaired IDE function promotes A β accumulation and sustained neuroinflammation, reinforcing the core pathological hallmarks of AD (De Felice & Ferreira, 2014; Arnold et al., 2018). Moreover, insulin resistance is closely associated with elevated oxidative stress and mitochondrial dysfunction in neurons, further compromising synaptic integrity and accelerating disease progression over time (Butterfield & Bold-Kimball, 2020; Nguyen-Thi et al., 2024).

Progressing from diabetes, this may concurrently lead to obesity itself. In the context of obesity, the excess body fat will alter lipid profiles, increases the levels of low-density lipoprotein (LDL) and decreases high-density lipoprotein (HDL), both have been proved to be associated with AD progression (Anstey et al., 2017; Gratuze & Planel, 2022). When there is a high level of circulating saturated fats and cholesterol, this may disrupt the brain lipid homeostasis affecting the membrane fluidity, promoting amyloidogenic processing of APP and subsequently increasing A β production (Gratuze & Planel., 2022; Yassine et al., 2023). Certain apolipoprotein alleles, specifically APOE ϵ 4 may synergistically interact with obesity to amplify its risk (Willette & Kapogiannis., 2021; Yamazaki et al. 2019). Hence, diabetes and obesity were proved to have a interconnected relationship with AD, so targeting these two conditions might be useful to minimize the risk in getting AD or slowing down the disease neurodegenerative effects.

Not only in human settings, for *in-vitro* and animal studies have vastly supported and proved that insulin resistance can contribute to the pathogenesis. The therapeutic counteracts might be useful if there are approaches targeting several processes in the brain; memory processing (recognition and retrieval), morphology of the brain (brain atrophy) and enhancement of synaptic communication (Bilkei-Gorzo et al., 2017; Craft et al., 2023). There are many recent studies have indicated that impairments in insulin signalling and concurrent development of insulin resistance in the hippocampus, especially affecting the key areas needed for learning and memory can further accelerate the downstream neurodegeneration in AD individuals (Arnold et al., 2018; Nguyen-Thi et al., 2024). This can be seen through the effects of peripheral insulin resistance leading to a decrease insulin signalling in the central nervous system (CNS) in the brain, further altering the brain metabolism. Physiologically, the effects can even be notably seen among the neuronal cells; A β toxicity, tau hyperphosphorylation, oxidative stress and neuroinflammation (Talbot & Wang, 2021; Nguyen-Thi et al., 2024).

2.5.2.3 Infections

In recent years, infectious agents are increasingly recognized as significant contributors to the development and progression of AD. There were mounting evidences that suggest chronic or latent infections which can exacerbate AD pathologies including implications due to viruses, bacteria and fungi. For example, chronic or latent

infections with Herpes Simplex Virus Type 1 (HSV-1), *Chlamydia pneumoniae*, *Porphyromonas gingivalis* (a key oral pathogen) and even SARS-CoV-2 have been found to be associated with increased risk of developing AD (Dominy et al., 2019; Heneka et al., 2020; Itzhaki, 2022). These infections are considered as potential environmental risk factors with its central mechanism involving activation of neuroinflammation, disruption of blood-brain barrier (BBB) and even direct influence on protein misfolding and aggregation. This concept might be known as pathogen hypothesis of AD to some researchers which underlies microbial pathogens triggering neuroinflammatory responses and promote A β deposits in the brain. However, in the contrary, there is a possibility that the A β deposits might be due to the act of antimicrobial peptides and its accumulation is considered as a defense mechanism towards microbial agents (Kumar et al., 2015; Itzhaki et al., 2016).

Firstly, AD is believed to possess a viral relationship with some specific viruses as it is interestingly intercorrelated with its pathogenesis as the DNA of HSV-1 has been found in amyloid plaques. The viral reactivation may promote tau hyperphosphorylation, the prominent feature of AD hallmarks. Among the viral agents, the most studied pathogen is the Herpes Simplex Virus Type 1 (HSV-1) as it can establish latency in the nervous system and reactivate intermittently. The reactivation of HSV-1 in the brain have been shown to initiate an increased A β production and tau hyperphosphorylation specifically, kinases such as GSK-3 β and CDK5, both vital for tau phosphorylation possibly due to the innate immune response in the brain (Piacentini et al., 2019; De Chiara et al., 2022). Moreover, the virus also stimulates chronic microglial activation that may lead to further adverse effects; neuroinflammation and subsequently, neuronal death. Even though, the brain has protective mechanisms against pathogens, but there is a limit. The repeated reactivation may corrupt the neuroprotective properties and eventually submit to the progressive neurodegeneration. According to previous findings, relationship between HSV-1 and AD has arisen at which it involves the possibility of individuals carrying APOE ϵ 4 allele may facilitate viral entry or impair immune responses (Itzhaki et al., 2020; Linard et al., 2023). This has come up with the support to the claim of “antimicrobial protection hypothesis”, where A β is increased to entrap pathogens that entered and neutralize it (Itzhaki et al., 2014). However, when this chronic production of A β coupled with its impaired clearance mechanism, this leads to aggregation subsequently forming plaques.

Beside viral infections, another key pathogen in increasing the risk in developing AD are chronic bacterial infections, particularly *Chlamydia pneumoniae* and *Cytomegalovirus* (CMV). Both of these are capable in infecting the central nervous system (CNS) and provoking chronic immune activation. If this condition persists, the presence of the pathogens might cause a continuous flow of pro-inflammatory cytokines, prolonged activation of microglia and may further disrupt the blood-brain barrier (BBB) (Fulop et al., 2018; Little et al., 2021). Other than that, chronic infections also impair lymphatic system in clearing A β and other neurotoxic waste products from the brain. The common bacterial infection is caused by *C. pneumoniae*, which is an obligate intracellular bacterium primarily known for causing respiratory tract infections in the pathogenesis of AD. It has been proved to contribute to the disease progression through complex host–pathogen and immune-genetic interactions (Balín et al., 2018; Gérard et al., 2020). Although its role is less genetically defined than APOE ϵ 4, mounting evidence suggests that chronic bacterial infection may act as a disease modifier that accelerates neurodegenerative cascades.

Another pathogen related to AD is *Porphyromonas gingivalis* or known as *P. gingivalis* is a gram-negative bacterium implicated in chronic periodontal disease that invades the brain and is capable in triggering inflammation and neurodegenerative pathways. *P. gingivalis* is a primary pathogenic AD factor and defined as cysteine proteases that can be classified into arginine-specifics; arginine-specific gingipain A (RgA), arginine-specific gingipain B (RgpB) and lysine-specific (Kgp) variants (Dominy et al., 2019). These enzymes are central to the ability of the bacterium to invade host tissues, evade immune responses and modulate inflammatory pathways. These pathogens are able to enter the brain via systemic circulation or cranial nerves before triggering microglial activation and cytokine release creating a well-sustained neuroinflammation among the brain tissues (Poole et al., 2015; Haditsch et al., 2020). In particular, these infections caused will stimulate bodily counteract mechanism by activating microglia, increasing cytokines production and disrupting the blood-brain barrier. Interestingly, this bacterium is normally found in gum diseases, are also detected in the brains of AD patients.

The chronic effect known to be affected by *P. gingivalis* is the ability of the pathogen to cross blood-brain barrier (BBB) by degrading tight junction proteins, allowing bacterial components and toxins enter CNS as shown in animal and *in-vitro* studies (Poole et al., 2015). These neurotoxicity activities of *P. gingivalis* have been

detected in the hippocampus and cortical regions of AD patients (Dominy et al., 2019). Furthermore, *P. gingipains* were found to co-localized with amyloid plaques, suggesting a direct relationship between amyloid formation and stabilization (Dominiy et al., 2019). Gingipains, toxic cysteine proteases (proteolytic enzymes) were proved to induce the overproduction of A β because *P. gingivalis* infection stimulates APP processing through amyloidogenic pathway leading to an increased level of A β 42. Thus, inhibition of gingipains in AD animal models has successfully reduced A β production and improved consecutive cognitive functions that sums up the potential causal role of bacterial infections in AD pathogenesis due to chronic periodontal diseases.

Aside from that, *P. gingivalis* also influence tau pathology by releasing toxic proteases known as gingipains mainly Kgp (lysine-specific) and Rgp (arginine-specific). These gingipains can directly cleave tau proteins, producing abnormal tau fragments prone to misfolding and aggregation. The cleavage is able to alter the tau structure and expose the phosphorylation sites to promote its pathological transformation (Dominy et al., 2019). Cleavage of tau proteins is initiated when there is an inflammatory response activating kinases involved in tau phosphorylation such as glycogen synthase-3 β (GSK-3 β), cyclin-dependent kinase 5 (CDK5) and mitogen-activated protein kinase (MAPK) pathways such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) (Dominy et al., 2019). These enzymes were used to catalyze the hyperphosphorylation of tau reducing its affinity for microtubules and increase its tendency to form insoluble paired helical filaments (PHFs), which in turn aggregate into NFTs (Poole et al., 2015; Haditsch et al., 2020; Liu et al., 2022).

Another scope that needs as much attention in AD research is fungal infections or mycobiome-related mechanism that has emerged as a novel and intriguing contributor to its pathology. Fungi or fungal components have been suggested to be potentially involved in triggering neuroinflammation and amyloid plaques. The growing interest in this area focuses on the potential role of mycobiome in developing chronic inflammation, protein misfolding and immune activation in CNS. This hypothesis emerged due to the presence of fungal components (such as DNA, proteins or whole intact cells) can be found in AD brains and none in healthy normal brains (Pisa et al., 2015; Alonso et al., 2018). This may be due to the persistent fungal infections that can survive in the environments to trigger or accelerate the neurodegenerative changes in the brain (Alonso et al., 2018; Sochocka et al., 2022). Based on previous researches,

pattern recognition receptors (PRRs) such as Dectin-1, Toll-like receptors (TLRs) and NOD-like receptors (NLRs) were able to recognize fungal components such as β -glucans, mannans, and chitin which can be found on microglia and astrocytes. When these receptors are activated, this would release of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 which in turn will activate nucleotide-binding domain, leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome, disrupt neuronal function and create a conducive environment for tau hyperphosphorylation and synaptic dysfunction (Alonso et al., 2018; Li and Wu, 2021; Tetz et al., 2023).

Interestingly, in response to the fungal infection, A β is upregulated acting as an innate immune peptide with antimicrobial properties that capable of binding and neutralizing fungi. This defensive mechanism might eventually lead to an aggregation of A β into extracellular plaques when the body A β clearance processes are disturbed. The formation of plaques will cause microglia to be activated and forcing the neuron to undergo inflammatory cascade leading to neuronal toxicity and finally, death (Kumar et al., 2015; Pisa et al., 2017). However, the fungal hypothesis of AD remains controversial and requires further research to confirm the mechanistic pathways related caused by chronic fungal infections and needs an urgent compelling shift towards understanding AD as a potentially immune-mediated disease or potentially infectious leading to new development of antifungal therapeutic interventions.

2.5.2.4 Gut-Brain-Axis Dysregulation

The gut-brain axis (GBA) is a bidirectional communication network that requires a synergy of communication network among central nervous system (CNS), enteric nervous system (ENS), immune system and the gut microbiota (Carabotti et al., 2015; Cryan et al., 2019; Liu et al., 2020). This interplay of axis is needed to maintain the optimum regulation of neural, immune and endocrine signalling. In previous studies, dysregulation in the gut microbiota composition (dysbiosis) has emerged as an increasing evidence that has showed significantly contributing to AD (Vogt et al., 2017; Quigley, 2017). Dysbiosis is defined as the imbalance in the composition and function of the gut microbiome at which the levels of beneficial bacteria are reduced and potent harmful bacteria or fungi is increasing. Dysbiosis contributes in AD progression as it

alters gut barrier integrity, increases systemic inflammation and affects neuronal signalling (Vogt et al., 2017).

For instance, AD individuals were found to be consistently suffer from a reduction in beneficial gut bacteria (e.g., *Bifidobacterium* and *Lactobacillus*) and an increase in pro-inflammatory taxa of bacteria (e.g., *Escherichia/Shigella*, *Proteobacteria*) (Zhuang et al., 2018; Liu et al., 2019). These microbial alterations may lead to an increase production of lipopolysaccharides (LPS) and short-chain fatty acid (SCFA) imbalances (Zhao et al., 2017; Dalile et al., 2019). LPS is a potent endotoxin from Gram-negative bacteria which has the ability to translocate from the gut into the bloodstream and even worse, able to cross a compromised BBB causing an induction of microglial activation and pro-inflammatory cytokine release in the brain and lead to subsequent brain damage such as neuronal injury and synaptic loss (Banks et al., 2015; Zhao et al., 2017). Thus, chronic activation of the immune cells might jeopardize the neuronal cells in the hippocampus, a region essential for learning and memory making it less efficient or even death of neurons (Heneka et al., 2015; Heppner et al., 2015).

Meanwhile, aside from triggering inflammation and neuronal deaths, emerging evidence suggests that gut microbiota may directly interfere the amyloid beta dynamics. Based on studies conducted, some gut bacteria may naturally produce amyloid-like proteins, which are fibrillar structures some of which are similar to those found in human neurodegenerative diseases, like AD (Friedland & Chapman, 2017; Kowalski & Mulak, 2019). For example, *Escherichia coli*, *Salmonella enterica*, *Salmonella typhimurium* and *Bacillus subtilis*. Some of these gut bacteria might naturally produce amyloid-like proteins. These bacterial amyloids may further trigger cross-seeding and amplify beta amyloid aggregation and misfolding through molecular mimicry (Chen et al., 2016; Kowalski & Mulak, 2019). However, its seeding and amyloid spreading may occur at different levels of the brain-gut-microbiota axis. The mechanism at which the amyloid spreads includes interaction between neuron-to-neuron or distal neuron spreading, direct BBB crossing or via other cells such as astrocytes, fibroblasts, microglia and immune system cells (Jucker & Walker, 2018; Kowalski & Mulak, 2019).

Another possible pathway is when the combination of aging and gut dysbiosis impairs intestinal epithelial barrier leading to a condition called “leaky gut”. When this occurs, microbial metabolites, toxins and inflammatory mediators might compromise the functional properties of BBB itself and making the harmful substances enter CNS in the brain (Heneka et al., 2015; Tran & Mohajeri, 2021). Prolonged flow of these

substances will result in microglial priming, increased oxidative stress and neuroinflammation which are all present in early AD. This is worrying because these downstream effects will slowly but surely lead to tau phosphorylation, accumulation of A β and degradation of neurons (Long & Holtzman, 2019). Despite that, animal models of AD have showed that there are potential possibilities in remediating the degeneration through modifying the gut microbiota via antibiotics, probiotics or fecal microbiota transplantation (FMT) (Harach et al., 2017; Kim et al., 2020; D'Amato et al., 2020). These approaches focus on altering the A β build-up and targeting inflammatory markers in the brain as shown in a model that used germ-free or antibiotic-treated mice with the results of reduced A β plaque formation and lower inflammation among the neuronal cells. Interestingly, when microbiota from AD patients were transplanted into wild-type mice, the cognitive abilities were impaired and A β levels in recipient animals were increased (Harach et al., 2017; Kim et al., 2020). Compared to humans, clinical studies among AD patients have successfully found the correlations between gut microbiota profiles and CSF biomarkers of AD as well as seen in cognitive performances (Voght et al., 2017; Zhuang et al., 2018).

This is interesting as there is already proven research that summarizes the possible key role of gut dysbiosis and gut microbiota-host interactions in neurodegeneration especially in terms of gut microbial imbalance, increased intestinal permeability and systemic inflammation. There are even recent animal studies that further support the gut-brain link in AD at which the models used either germ-free mice or those treated with antibiotics that target in depleting gut microbiota in making sure no microbe interactions could occur causing interferences (Kim et al., 2020; D'Amato et al., 2020). Furthermore, the relationship between gut microbiota and AD pathology was proved to be potent as recolonization of microbiota from AD patients led to worsened plaque deposition, increased tau phosphorylation and neuroinflammations compared to control models. In the real clinical settings, fecal microbiota transplantation (FMT) from healthy donors to AD mouse models has showed positive outcomes; A β levels reduced and cognitive abilities improved (Vogt et al., 2017; Kim et al., 2020; D'Amato et al., 2020). Hence, this provides a brighter future for therapeutic approaches to converge gut-derived inflammatory targets together with the well-known contributors to open up promising avenues for biomarker development and novel therapeutic strategies including other possible factors such as probiotics, dietary interventions and gut-targeted drugs aimed in modulating the natural microbiota.

2.5.2.5 Metals and Environmental Pollutants

Other than the usual risk factors, environmental exposure to metals and airborne particulate matter has emerged as one of the significant contributing factors in the development and progression of AD. These hazardous chemicals are able to disrupt the neutral homeostasis through oxidative stress, chronic inflammation and protein misfolding (Cacciottolo et al., 2021; Walton, 2022). Heavy metals such as lead (Pb), iron (Fe), Cadmium (Cd), mercury (Hg), aluminium (Al) and copper (Cu) has been proven to enter the brain through inhalation, ingestion or dermal exposure. Once accumulated, these neurotoxic metals would act as catalysts for the generation of ROS via Fenton and Haber-Weiss reactions creating lipid peroxidation, protein carbonylation and DNA damage (Wu et al., 2020; Zhao et al., 2020). In neurons, oxidative stress is a strong inducer of A β generation through upregulating BACE1, an enzyme needed for cleavage of amyloid precursor protein (APP) into pathogenic A β 42 isoforms (Tong et al., 2018; Zhao et al., 2019). However, among the metals, aluminium and iron are the most studied in relation with amyloid aggregation and tauopathy. Aluminium has the ability to cross BBB and thus, accumulate in brain tissues and bind to phosphates in hyperphosphorylated tau and A β peptides. These peptides will facilitate the formation of plaques and tangles (Yokel & Battacharyya, 2020; Walton, 2022). The condition worsen when reactive oxygen species produced leads to DNA damage, lipid peroxidation and finally, neuronal apoptosis (Yokel & Bhattacharyya, 2020).

Despite being essential for cellular activities, an abundant of iron and copper could lead to neurodegeneration in excess through redox cycling and Fenton chemistry, creating hydroxyl radicals (-OH) that damage neuronal components (Singh et al., 2021). If the level is kept at minimum or in trace amounts, there would be no harm done. However, if there is an accumulation of the metals, hence creating a hazardous environment for neurons to thrive. For instance, lead (Pb) exposure during the development of neurons has long-lasting epigenetic effects that might develop degenerative mechanism later in life as the metals persist inside the body. This would cause downstream effects like alterations in genetic expressions involving A β and tau metabolic processing (Bihaqi et al., 2014; Wu et al., 2022). This differs when individuals are affected by mercury. Usually, infected individuals were affected from ingesting contaminated fish containing mercury, particularly in the form of

methylmercury. This leads to inability of the neuronal cells to sustain living due to excessive oxidative stress and cellular apoptosis (Farina et al., 2011; Tian et al., 2021).

Specifically, aluminium has been shown to enhance tau phosphorylation and enhance aggregation of A β peptides. Tau phosphorylation is done through activation of kinases (GSK-3 β and CDK5) and these post-translational modifications destabilize microtubules and lead to NFTs formation. Aside from that, aluminium is able to impair synaptic function by downregulating NMDA receptor subunits and promoting calcium dysregulation, essential components for learning and memory. Similar to iron and copper, although it is essential for brain functions, but it may turn toxic when available in abundant. The metal-A β complexes are surprisingly more resistant to degradation and can perpetuate cycles of oxidative damage and neuroinflammation. This was proved according to the post-mortem analyses showing elevated levels of redox-active metals in the hippocampus and cortex of AD brains (Zhao et al., 2019; Du et al., 2018).

Beyond metal pollutants, airborne pollutants specifically fine particulate matter (PM_{2.5}), ultrafine particles (UFPs) and nitrogen dioxide (NO₂) have received increasing attention as major AD modifiable risk factors. These particles are fine enough to be inhaled through and can translocate from the nasal epithelium to the brain via olfactory nerve as the particles' size are small enough to enter bloodstream through the lungs and eventually BBB. The pollutants would be directly exposed to the CNS and may trigger systemic inflammation and oxidative stress (Cacciottolo et al., 2021; Underwood et al., 2021). The particles that enter systemic circulation will induce peripheral inflammation and stimulating a central neuroinflammation through cytokine signalling across a compromised BBB. Once in the brain, microglia and astrocytes will be activated and lead to chronic inflammation and increased A β deposits. This was proved when studies using human cohorts have resulted the relationship between people living in high pollution areas tend to have higher plasma and cerebrospinal fluid (CSF) levels of A β and tau, and surprisingly, causing a reduction in the cortical intensity in some brain regions (Calderón-Garcidueñas et al., 2018; Killian et al., 2018). Thus, it is health-risking as the exposure to fine particles are able to enter the body via respiratory tract and be translocated to elsewhere through the olfactory bulb or bloodstream (Underwood et al., 2021).

Blood-brain barrier (BBB) pathway of airborne pollutants is stimulated when the fine particulate matters (PM_{2.5} and ultrafine particles) firstly evade pulmonary filtration due to its small size. Then, the particles would travel and enter systemic

circulation and ultimately reaching the BBB. However, once the particles reach the nervous system, there will be interactions with the immune cells (e.g. microglia) to activate recognition receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) causing upregulation of NF- κ B signaling, resulting in the release of pro-inflammatory cytokines like TNF- α , IL-1 β , and IL-6. The inflamed neurons would subsequently lead themselves to neuronal death creating a neurotoxic environment (Cacciottolo et al., 2017; Underwood et al., 2021). According to real-life settings, human studies revealed that pollution-induced changes may initiate neurodegenerative cascades way before clinical symptoms appear. This has made humans to neglect the possible on-sight symptoms to be normal as their everyday lives. In animal models, an experimental design exposing to diesel exhaust or with an environment that has ambient PM_{2.5} created a sped up formation of A β plaques, induction of tau hyperphosphorylation and impairments in spatial memory. The movement of increased expression of RAGE (receptor for advanced glycation end products) was defined as well as facilitated transportation of circulating A β across BBB into the brain (Calderón-garcidueñas et al., 2018; Peters et al., 2022).

In addition, in terms of long term effects, metals and pollutions might induce epigenetic changes throughout life as the neuronal gene expression has been disrupted. The mechanism initially starts in the early-life exposure causing hypomethylation of APP promoter that leads to persistent overexpression of APP and increased A β levels in aging brains (Wu et al., 2020; Senut et al., 2021). Metals entered might as well impair mitochondrial function that downregulates affecting and stimulating the mitochondria-dependent apoptotic pathways via the release of cytochrome c and activation of caspase-3 (Tian et al., 2021; Singh et al., 2022). However, when there is a dysfunctional changes that causes neuronal energy deficits and synaptic activity interventions, this will accelerate cell deaths. Together, the molecular and cellular processes affected by metal and environmental pollutants in the brain will disrupt the redox balance, activity of glial cells and epigenetic reprogramming and facilitate A β accumulation along with tau pathology. In the light of this, these environmental exposures are modifiable and become targets at reducing the globally-affected neurodegenerative diseases.

2.6 Pathophysiology of AD

The pathophysiology of AD includes irreversible cognitive and behavioural deficits such as memory loss, spatial disorientation and language impairments (Goncalves et al., 2019). Diagnosis of AD can only be done by postmortem with two main pathological hallmarks determined; (1) densely packed extracellular accumulation of amyloid-beta ($A\beta$) protein fragments around the neurons in the brain ($A\beta$ plaques) blocking the cell signalling and trigger neuroinflammation, and (2) intracellular accumulation of hyperphosphorylated microtubule-associated protein tau (MAPT) forming neurofibrillary tangles (NFT) during oxidative stress and block transport nutrients and other essential neuronal functions (Kim et al., 2019; Weston-Green, 2019). This will lead to neuronal cell death and destruction of functional synapses which change the neurotransmitter levels causing memory loss, cognitive dysfunction and behavioural disorders (Goncalves et al., 2019). Figure 2.4 shows factors affecting mechanism of AD (Rawat et al., 2022).

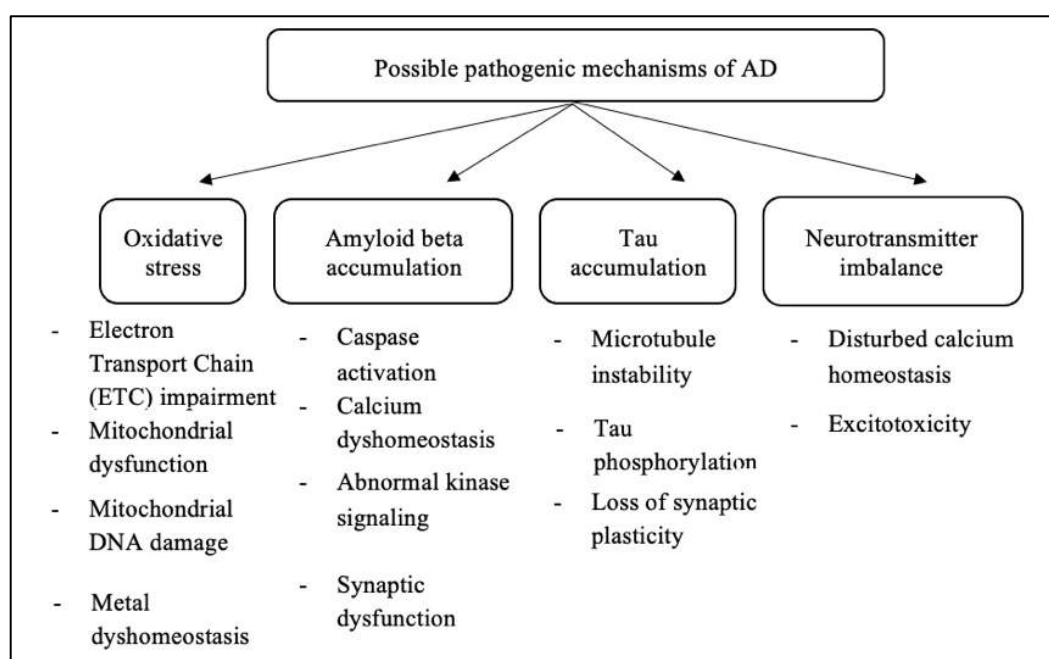


Figure 2.4 Factors Affecting Mechanism of AD

(Rawat et al., 2022)

2.7 AD Hypotheses

2.7.1 Amyloid Beta Hypothesis

This disease often categorized by the two pathological hallmarks consisting the appearance of a build-up of proteins that are commonly known as amyloid plaques and neurofibrillary tangles in the brain. This biological process is progressively getting worse over time, which means the symptoms that develop will eventually become severe. In the beginning, the production of amyloid begins with cleavage of amyloid precursor protein (APP) on the plasma membrane by β -secretase (BACE1) and γ -secretase to produce insoluble $A\beta$ fibrils. The insoluble $A\beta$ fibrils will then form oligomers before diffusing into the synaptic cleft interfering the synaptic signalling (Chen & Yan, 2010). Due to high concentrations of $A\beta$, kinases will be hyperphosphorylated activating microtubule-associated tau proteins to aggregate into insoluble NFTs (Li et al., 2023). This will produce a build-up of plaques and tangles which interestingly, gathering microglia around the plaques as well (Beeri et al., 2004). Activation of these microglia and consequent inflammatory responses by the localized neuronal cells will eventually aggravates neurotoxicity associated with an early loss of synaptic proteins at which the severity of AD progression depends on the levels and distributions of the synaptic proteins as learning and memory deficits in AD is proportional to the degree of neuronal degeneration whereby deposits of $A\beta$ is a measure of disease severity (Selkoe, 2004). Figure 2.5 shows ApoE and $A\beta$ plaque formation (Anu et al., 2020).

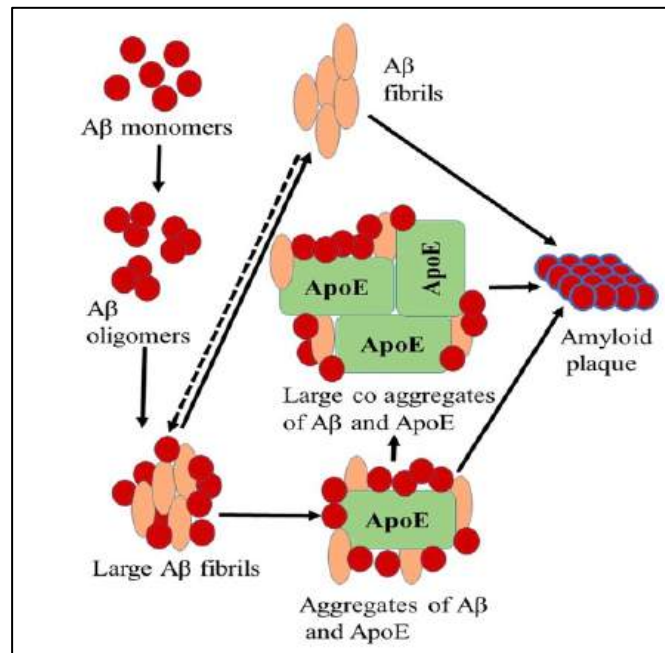


Figure 2.5 ApoE and Aβ Plaque Formation (Anu et al., 2020)

Beta amyloid, which also known as (amyloid beta Aβ), is a short peptide that is an abnormal proteolytic by product of Amyloid Precursor Protein (APP). APP is a single-pass transmembrane protein which is expressed at high levels in the brain mainly in neuronal and glial cells. Aβ fragments exist due to a set of enzymes cleave this protein sequentially at different sites (Zheng & Khoo, 2011) forming variety of fragments which involves in synaptic plasticity, regulates the excitability of neurons and protects neurons from oxidative and metabolic stress (Mattson & Chan, 2003). APP can be cleaved by two pathways, specifically either non-amyloidogenic by α secretases, and γ secretases or via the β- and γ-secretases producing several species of Aβ fragments. Neurotoxic forms of Aβ is formed by cleavage of APP initially by β-Site APP-cleaving enzyme 1 (BACE1), a major β-secretase in the brain (Cole & Vassar, 2007) followed by cleavage via γ-secretase which is carried out by an aspartyl proteolytic enzyme complex composed of presenilin 1 (PSEN1) and presenilin 2 (PSEN2).

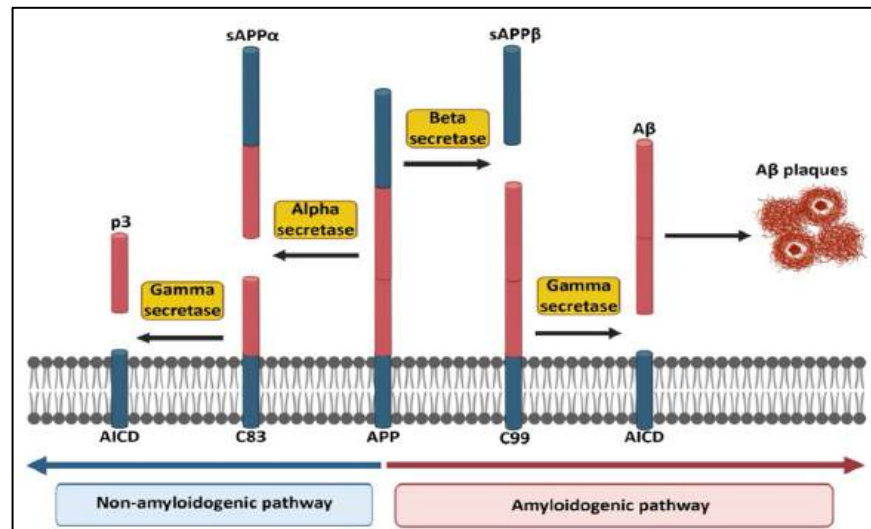


Figure 2.6 The Non-amyloidogenic and Amyloidogenic Pathways in the Cleavage of Amyloid-beta Precursor Protein (APP) (Mercan & Heneka, 2022)

Mutations in the proteins will alter the activity of γ -secretase and increase the ratio of A β in early-onset forms of AD (Cheng et al., 2011; Ridge et al., 2013). Under normal conditions, production and elimination of A β is at equilibrium regulating the A β at constant levels (Shankar & Walsh, 2009). However, in aging and pathological conditions such as excitotoxicity, the regulation of A β formation and clearance is disturbed that causes a build up of A β and senile plaque formation (Harkany et al., 2000). Accumulation of amyloid plaques in synapses will hinder the communication between adjacent neurons resulting loss of signal transmission from one neuron to another connecting neuron. This will trigger immune response in the brain to activate inflammation cascade which eventually damage the brain cells and lead to apoptosis (De-Paula et al., 2012; Sierra-Fonseca & Gosselink, 2018).

2.7.2 Tau Protein Hypothesis

Another distinctive pathophysiological characteristic of AD can be observed through tau protein. Tau protein is a microtubule-associated protein that plays a crucial role in neuronal cytoskeleton stabilization, maintenance of neuronal structure, axonal transport and synaptic plasticity (Dixit et al., 2008; Mietelska-Porowska et al., 2014). High expression of tau stabilizes and assembles the microtubule proteins in non-myelinated cortical axons, especially regions vital for learning and memory (Goedert et al., 2005; Reddy, 2011). Not only the axonal part, there are tau proteins found

redistributed into the soma and dendrites, some in glia and astrocytes as well (Reddy, 2011). Tau, just like other microtubule-binding proteins binds to the microtubules are affected by several factors that ensure the proper functional system of tau phosphorylation and dephosphorylation (Ramkumar et al., 2018).

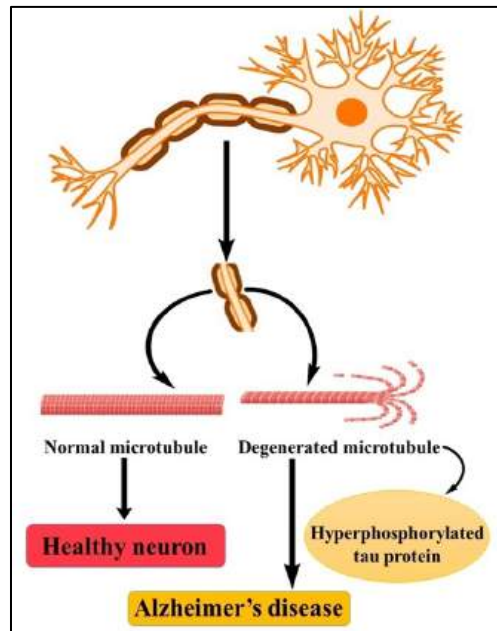


Figure 2.7 Flow of Hyperphosphorylated Tau Protein (Mercan & Heneka, 2022)

Tau can be phosphorylated and dephosphorylated by regulating the kinase and phosphatase activities respectively. In pathological state, hyperphosphorylated tau occurred causes dissociation of protein from microtubules and compromising axonal transport by accumulating as paired helical filaments which in turn aggregate into neurofibrillary tangles (NFTs) (Goedert & Spillantini, 2006) and formation of NFTs will lead to inflammation, synaptic impairment, neuronal loss and further decline of cognitive functions that could eventually lead to death (Roda et al., 2022; Sinsky et al., 2021). In the figures below, it shows the flow of hyperphosphorylated tau protein (Mercan & Heneka, 2022) in Figure 2.7 meanwhile Figure 2.8 shows formation, processing and possible interplay between extracellular amyloid plaques and intracellular neurofibrillary tangles (NFT) (Mercan & Heneka, 2022).

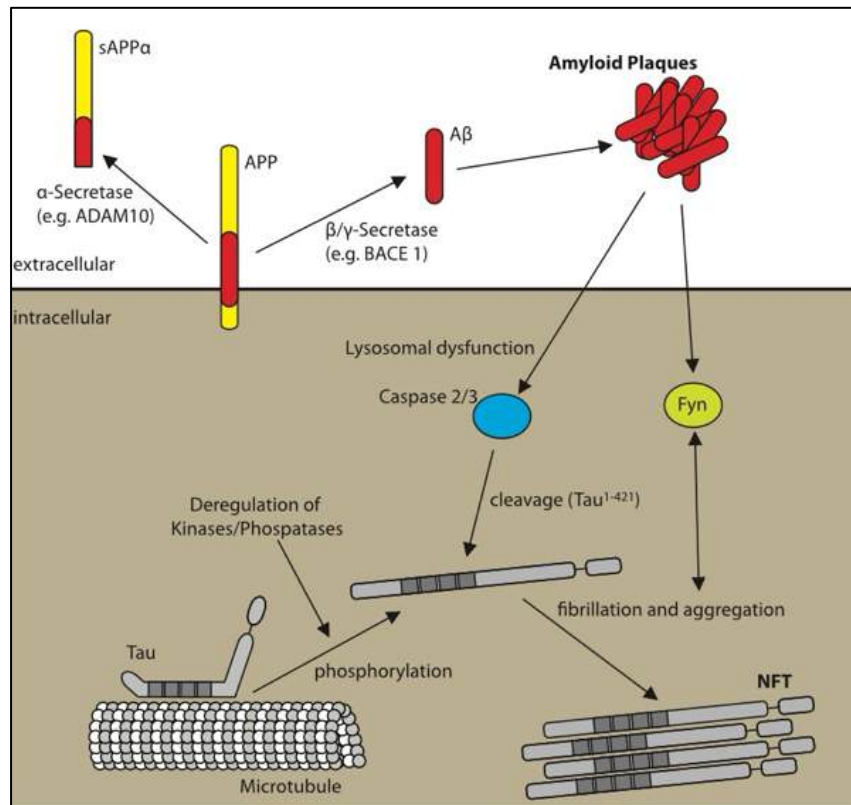


Figure 2.8 Formation, Processing and Possible Interplay between Extracellular Amyloid Plaques and Intracellular Neurofibrillary Tangles (Mercan & Heneka, 2022).

2.7.3 Cholinergic Hypothesis

Cholinergic Hypothesis of AD is the first proposed by Perry et. al (1999) that postulates the relationship between cholinergic neurotransmission altering the levels of acetylcholine (Ach), choline and choline acetyltransferase (ChAT) as AD progresses (Bekdash et al., 2021). The areas of the brain that are involved in the cholinergic transmission include basal forebrain, neurons projections to neocortex, hippocampus and amygdala (Bekdash et al., 2021). More importantly, there was a reduced level of expressions of these Ach receptors in the brains of postmortem AD patients (Sabri et al., 2018). The reduced levels of acetylcholine (Ach) in the brain was postulated due to the neuronal death in the Nucleus Basalis of Meynert (Cohen & Weinstein, 2018). In AD brains, there are two primary acetylcholine receptors (AChRs) levels that were reduced; nicotinic receptors (nAChRs) and muscarinic receptors (mAChRs) (Nyakas et al., 2011; Vema et al., 2018). In cholinergic presynaptic neurons, acetylcholine is synthesized by the enzyme choline acetyltransferase (ChAT) from choline and acetyl-coenzyme A, and will be transported to the synaptic vesicles via vesicular acetylcholine

the site of release, affinity to the receptors (ACh nicotinic receptors or muscarinic receptors), elimination from synapses by acetylcholinesterase and lastly, the type of neuronal targets). Cholinergic signalling depends on these factors as it will determine the excitability of the target neurons (Kamkwala et al., 2017). This further justifies the role of acetylcholine with its excitatory effect in the periphery at the neuromuscular junction at which acetylcholine modulates the neurons in the brain by activating or inhibiting the action potentials depending the surrounding stimuli it receives (Picciotto et al., 2012). As for mAChRs, distinguished as G-protein coupled receptors are vital to mediate acetylcholine-induced neurotransmission across synapses (Vema et al., 2018). If these pathways are disturbed or there is an imbalance of receptor activities specifically the regulation of cholinergic signalling, thus normal functions of neurons and learning and memory processes will be impaired (Ferreira-Vieira et al., 2016; Jiang et al., 2014).

Furthermore, the AD models have successfully showed promising results including a reduction in accumulation of amyloid plaques and hyperphosphorylated tau, subsequently improving in the cholinergic activities (Ferreira-Vieira et al., 2016). To modulate these neurotoxicity factors, any potential efforts in mediating the agonists or antagonists affecting the AChR-beta amyloid interactions would be relevant. Aiming for these would require the understanding of modulation activity AChRs and ACh levels across the brain specifically hippocampus, a prominent area related to AD to bring possible new outcomes and treatment pathways in treating it (Kamkwala et al., 2017). Towards its downstream mechanism, the modulation of AChRs and ACh levels was found to be effective in subsequently preventing beta amyloid-induced neuronal cell death since A β deposition induces glutamate release in the hippocampus via the increase in the activity of $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) (Hascup et al., 2016). The relationship between these two was further proven when decreased levels of $\alpha 7$ nAChRs-A β complex formations has resulted improvements in learning and memory whereas when there is a higher level of these complexes, the opposite effects occur; adverse effects in cholinergic signalling and cognitive functions (Takata et al., 2022). Also, in AD, the necessary regulation of its associated neurodegeneration requires the role of glial cells (microglia and astrocytes) which need to express nAChRs first to activate its functional properties (Takata et al., 2021). Once the activation has occurred, microglia will start to accumulate around the plaques facilitating clearing mechanism through phagocytosis or neuronal death via local inflammations at the

specific targets (Takata et al., 2021). This way, the neuronal inflammations can be hindered to spread more thoroughly affecting the neighbouring areas causing higher degeneration of neurons.

2.7.4 Glutamatergic Hypothesis

Excitatory glutamatergic neurotransmission occurs via N-methyl-d-aspartate receptor (NMDAR) and is critical for synaptic plasticity and neuronal survival. However, excessive levels of NMDAR activity can lead to excitotoxicity and even worse, cell death underlying a potential mechanism of neurodegeneration in AD. (Wang & Reddy, 2018). Physiologically, along the progression of AD, the overproduction of soluble A β peptides are known to predominantly affect glutamatergic terminals in the hippocampus (Pereira et al., 2019). This leads to hyperexcitability at early stages of the disease which causes downstream effects on the glutamatergic homeostasis in the central nervous system since glutamatergic synapses are the neurophysiological basis of memory processes (Tarkowski et al., 2003; Gaugler et al., 2018). Glutamatergic homeostasis is vital to make sure glutamate regulation in the hippocampus are at par and on balance (Sanchez-Fernandez et al., 2024). Factors affecting the regulation includes pre- and post- synaptic neuronal compartments along with glial cells (tripartite synapse). Tripartite synapse is a process that involves modulation of glutamate neurotransmission via vesicular glutamate transporters by storing glutamate into vesicles and mediating its neurotransmitter release after depolarization of neurons (Sanchez-Fernandez et al., 2024).

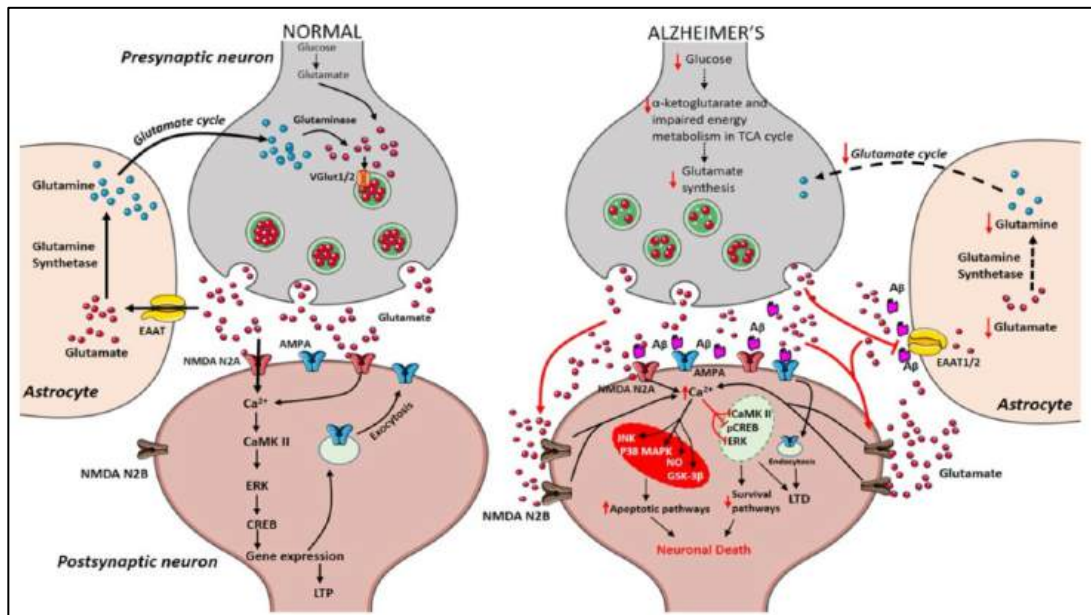


Figure 2.10 Glutamatergic Hypothesis of AD (Bukke et al., 2020)

Glutamate (L-glutamate, L-glutamic acid) is the most abundant and primary excitatory neurotransmitter in the vertebrate nervous system playing its key role as building blocks of peptides and proteins. This is because glutamate, an essential amino acid is a major excitatory neurotransmitter synthesized from glucose (Bukke et al., 2020; Christian Behl., 2023). As previous research suggested, it was proved that there is a strong relationship between glutamate alterations and glucose utilization in AD. As age increases, glucose utilization decreases causing a reduction in glutamate levels in the brain. Over 40% of neuronal synapses are glutamatergic and has been greatly affected in AD (Bukke et al., 2020). Physiologically, glutamate and its receptors, NMDA receptors are involved higher functional of the brain, specifically learning and memory processes. Despite that, excessively increased levels of extracellular glutamate of the brain can induce neurotoxicity and may cause neuronal death (Christian Behl., 2023). This excitotoxicity will lead to further disturbances in glutamate receptor signalling causing acute (e.g., stroke) and chronic neurodegeneration (e.g., AD) (Christian Behl., 2023). This process will continuously deteriorate neuronal abilities and functions as age increases unless there are efforts to hinder it from progressing worse. Figure 2.10 shows glutamatergic hypothesis of AD (Bukke et al., 2020).

2.7.5 Adrenergic Hypothesis

Another hypothesis that are considered related to AD is the adrenergic hypothesis which involves receptors responding towards adrenaline (epinephrine) respectively in the autonomic nervous system, specifically sympathetic nervous system (Millitou et al., 2025). In the brain, this mechanism is important in regulating brain functions such as attention, arousal, stress response, synaptic plasticity and neuroinflammation. Not only noradrenaline (norepinephrine) plays a key role in AD but adrenaline and its adrenergic receptors are able to influence cognitive deficits as well. Cognitive deficits in AD may result due to the disruption of adrenaline-mediated signalling pathways that acts on β -adrenergic receptors and α -adrenergic receptors expressed in hippocampus, prefrontal cortex and amygdala (Zhang et al., 2005). Among the both receptors; receptors are divided into alpha (α) and beta (β) or known as $\alpha 1$ and $\alpha 2$, while beta is categorized as $\beta 1$, $\beta 2$ and $\beta 3$. These receptors are G-protein coupled receptors (GPCRs) has primary functions to respond towards epinephrine in the brain to regulate cognitive, vasculature and inflammation. The relationship between these receptors and the disease progression can be seen when there is a dysregulation caused by early degeneration of brain regions (Gannon et al., 2019; Holland et al., 2021; Li et al., 2023).

Adrenergic receptors (categorized as α and β) are present mostly in the CNS (Milliotou et al., 2025). However, the locus coeruleus (LC) region in AD brain is greatly affected due to the enhanced hypofunction of norepinephrine signalling that would potentiate the neurotoxicity of $A\beta$, accumulation of tau and neuronal damages. Among the receptors, Beta 2 ($\beta 2$) has a higher affinity towards adrenaline while alpha receptors are more responsive and has a higher affinity towards noradrenaline. Interestingly, $\alpha 1$ and $\beta 1$ are responsible for excitation of neurotransmitters while $\alpha 2$ and $\beta 2$ are more towards its inhibition. These adrenergic receptors are Gs coupled receptor that may influence $A\beta$ production in two ways; (1) APP may be internalized with $\beta 2$ following periods of prolonged stimulation, therefore allowing APP to act as a substrate for b-secretases and g-secretases in the endosome, (2) $\beta 2$ may interact with the β -arrestin 2 protein that directly interacts with the adenosine-1A (a-1a) subunit of gamma-secretase, increasing its catalytic activity. The adenosine-2A (a-2a) receptor is a G-coupled receptor that influences $A\beta$ production by disrupting the interaction of APP with sorting-related receptor with A-type repeats (SorLA or known as SORL1), a protein

that acts as a sorting receptor for APP that protects APP from proteolytic cleavage influencing its trafficking within the cell.

SorLA achieves this by binding to APP in the trans-Golgi network (TGN) and slowing its exit into the secretory pathway, thus reducing its exposure to secretase. This plays a protective role by preventing APP from being cleaved by secretases which would otherwise lead to the production of A β . Figure 2.11 shows adrenergic hypothesis of AD and its correlated mechanistic pathways. Low levels of SorLA or mutations occurring in its retromer-binding site may lead to consequent mislocalization of APP, increasing its exposure to secretases and resulting in elevated A β production. Whereas, retromer is a protein complex involved in retrograde transport, interacts with SorLA and is crucial for accurate localization to the TGN. To maintain its functionalities at its peak, vacuolar protein sorting-associated protein 26 (VPS26) subunit of retromer will bind to a specific sequence of SorLA ensuring SorLA is recycled back to the TGN, enabling it to protect APP. Thus, given the extensive expression of adrenergic receptors with its multifunctional roles, it might be a significant contributing factor to further develop promising drugs for AD (Bekdash et al., 2021; Perez et al., 2023; Le et al., 2023). There are few therapeutic targets available now that may aid in AD pharmacotherapies such as salbutamol (targeting β 2-adrenergic receptor agonists) and propranolol (beta-blockers) (Zhang et al., 2005; Gannon et al., 2015).

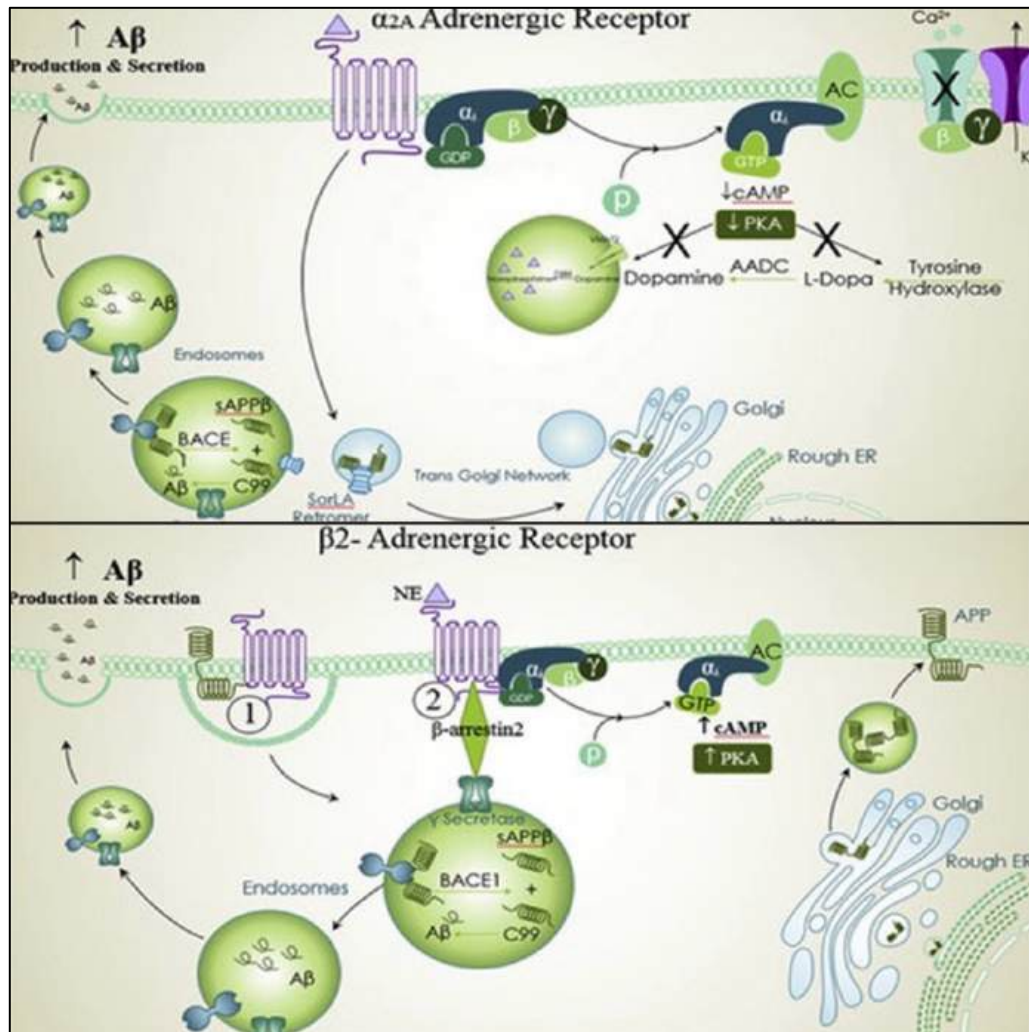


Figure 2.11 Adrenergic Hypothesis of AD (Bukke et al., 2020)

2.7.6 Monoaminergic Hypothesis

While trying to delineate the causes of AD, there were evidences related to deficits in the monoaminergic neurotransmission such as dopaminergic, noradrenergic and serotonergic (Pan et al., 2019; Krashia et al., 2019; Zhang et al., 2024). According to its mechanistic pathway, monoamine neurotransmitters will bind to its corresponding receptors after being synthesized and released into the postsynaptic membrane. When the levels of these neurotransmitters are higher than normal, either monoamine oxidase (MAO) or catechol-O-methyltransferase (COMPT) will aid in degradation or presynaptic terminals will reuptake the extra neurotransmitters via monoamine transporters.

2.7.6.1 Dopaminergic Hypothesis

Dopamine is a major monoamine neurotransmitter in the brain that engages in higher functions including spatial memory, motivation, arousal, reward and pleasure, in addition regulating motor neurons, lactation, sexual behavior and nausea (Krüger, et al., 2005; Berridge & Kringelbach, 2008; Nakagawa, et al., 2008; Salamone & Correa, 2012). These vital functions of dopamine lead to the importance of maintaining dopaminergic signalling in the brain and prevent it from being implicated. In various mental disorders such as addiction, attention deficit/hyperactivity disorder (ADHD), Huntington's disease, Parkinson's disease and schizophrenia, there were signs of affected mechanistic pathways in dopaminergic signalling as well (Klein et al., 2019). In its metabolism processes, the uptake of dopamine is done by the respective dopamine transporters (DAT). Dopaminergic neurons produce dopamine due to the action of tyrosine hydroxylase (TH) and aromatic amino acid decarboxylase (AADC). These newly synthesized dopamine is stored in vesicles with the aid of vesicular monoaminergic transporter-2 (VAMPT2). Cytosolic dopamine can be degraded in neurons or glial cells by catechol-o-methyltransferase (COMPT) or monoamine oxidase (MAO) to form homovanillic acid peroxide (H_2O_2).

Despite dopaminergic deficits were seen in other diseases, focusing on AD only, it was listed more than 50% of patients with mild cognitive impairment (MCI) or mild AD were seen to possess dopaminergic deficits associated with cognitive dysfunctions in AD individuals (Koch et al., 2014; Cacabelos, 2017). Meanwhile, AD mouse models have proved the relevancy in rescuing neurotransmission alleviating pathologies and cognitive deficits (Cordella et al., 2018). Although dopaminergic is often being related to being one of the ways in targeting AD, but in the human physiological bodily functions, the concept of dopaminergic system is related to brain reward (Mitchell et al., 2011). Rather than affecting learning and memory, disturbed levels of dopamine might account for neuropsychiatric symptoms in AD according to the results proved in clinical trials targeting dopamine agonists and dopamine reuptake inhibitors (Mitchell et al., 2011).

Recent research suggests that disrupted dopaminergic pathways, particularly in the mesocorticolimbic system and nigrostriatal pathway, contribute to synaptic dysfunction, amyloid-beta, tau pathology and neuroinflammation in AD. Particularly,

AD causes reduced levels of dopamine being transmitted to brain regions such as prefrontal cortex, hippocampus and striatum critical. As shown in Figure 2.12, dopamine is being regulated via interactions with receptors (D1, D2, D3, D4, and D5) that involves in synaptic plasticity regulation, long-term potentiation and memory learning. However, through the reduction in dopamine receptor density (specifically D1 and D2 receptors) impairs neurotransmission among neurons (Riek-Burchardt et al., 2010). This dopamine dysfunction also promotes tau hyperphosphorylation that eventually forms NFTs (Chen et al., 2014). Dopamine also crucial for modulation neuroinflammation via regulation of microglial activity thus reduced levels of dopamine cause overactivation of microglia and enhanced release of pro-inflammatory cytokines such as IL-1 β , TNF- α , IL-6 (Cai & Ratka, 2012). In the context of learning and memory, dopamine is essential synaptic plasticity and dysfunctional of long-term potentiation of neurons would lead to weaken synaptic connections and reduced neuronal resilience (Martorana & Koch., 2014). Hence, there would be an increase in synaptic loss and cognitive deficits along the disease progression.

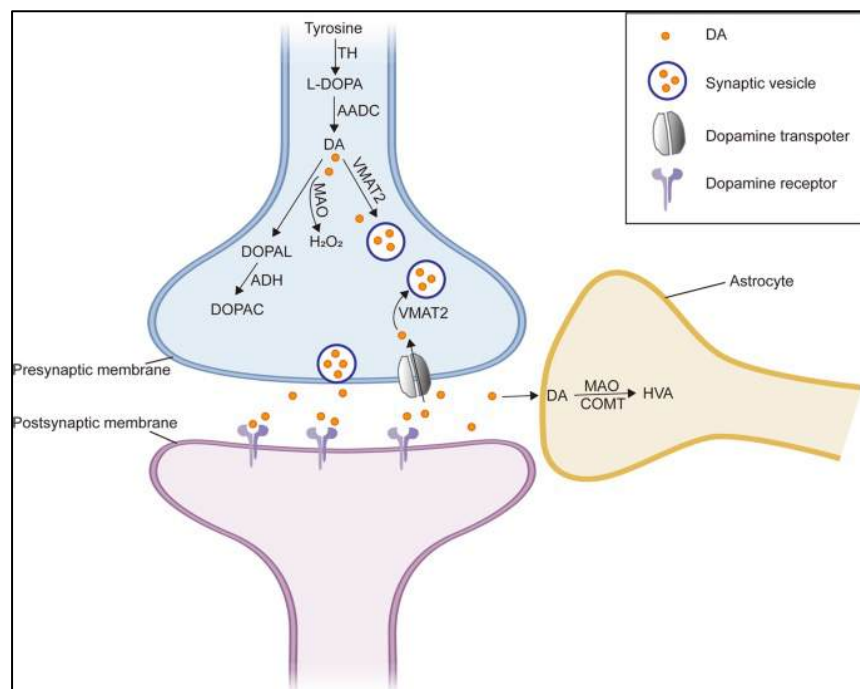


Figure 2.12 Dopaminergic Hypothesis of AD (Xu & Yang, 2022)

2.7.6.2 Noradrenergic Hypothesis

Another niche emerged as a crucial yet complex area is noradrenergic-related neurodegeneration associated with the severity of cognitive dysfunction in AD (Theofilas et al., 2017). This may be due to its enhanced level which can cause AD dysfunction; long-term potentiation deficits, cognitive decline and neuroinflammation in AD animal models (Ardestani et al., 2017). The noradrenergic hypothesis of AD focuses on the role of norepinephrine, a neurotransmitter released by the locus coeruleus (LC) which will be degenerated as AD becomes worse. The locus coeruleus is the primary source of norepinephrine (NE), loss of neurons in this area decreases norepinephrine release in cortex, hippocampus and regions involved in memory and cognition. Reduction of NE will affect synaptic plasticity, neuroprotection and brain's immune response. LC is a region projecting to several brain regions and related to few different pathways respectively; cognitive impairment pathway, neuroinflammation pathway, vascular dysregulation pathway, stress and resilience pathway. Interestingly, LC is among the earliest brain regions to show tau pathology and neuronal loss. Tau accumulation and loss of functional neurons disrupts the LC-NE mechanism and decreases the NE release.

In AD, neuronal cognitive ability is reduced severely when there is a reduction in NE impairing long-term potentiation (LTP) specifically in both hippocampus and prefrontal cortex. As in Figure 2.12, NE has shown its vital role in regulating the mechanism in memory consolidation and memory formation (Heneka et al., 2010). Hence, reduced NE signalling causes hyperactivation of microglia, resulting chronic inflammation exacerbating amyloid-beta plaques and hyperphosphorylated tau proteins (Zhang et al., 2013). The relationship between LC and AD supporting the noradrenergic hypothesis have been supported by various previous studies studying different aspects; postmortem studies showed LC degeneration and reduced NE levels were found in the brains of AD patients (Grudzien et al., 2007), animals studies revealed the role of LC-NE system in AD animal models (Heneka et al., 2010); and clinical studies showed pharmacological treatments aiming on NE reuptake inhibitors revealed potential to improve cognition function and reduce neuroinflammation in AD patients (Weinshenker, 2018).

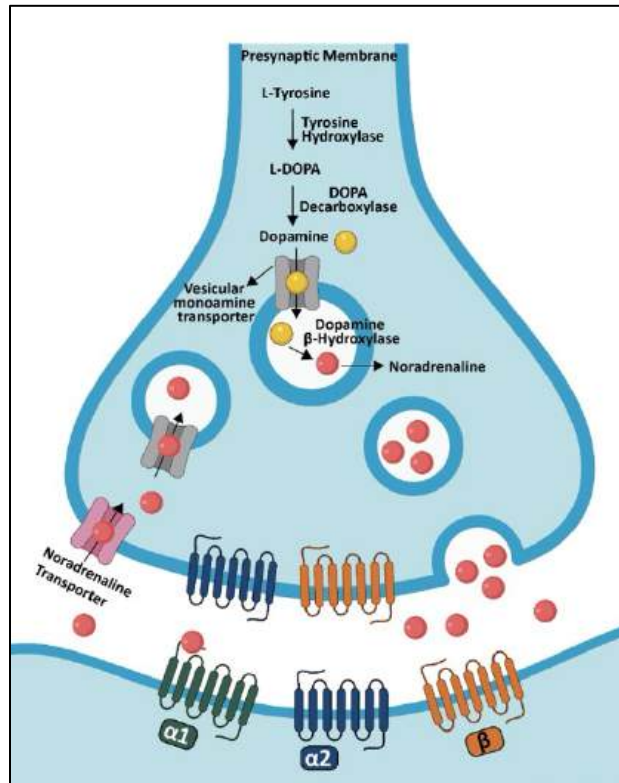


Figure 2.13 Noradrenergic Hypothesis of AD (Mercan & Heneka, 2022).

2.7.6.3 Serotonergic Hypothesis

While previous findings evidently showed the relationship between AD progression and noradrenergic modulation, there is another alteration that can be observed in both AD patients and AD animal models which is serotonergic deficits (Ramos-Rodriguez, 2013). Serotonergic hypothesis of AD suggests that serotonin-producing neurons in the raphe nuclei are important in regulating mood, memory, cognition and synaptic plasticity. Serotonin (5-HT) receptor modulation influences both A β and tau pathologies via several pathways; (1) extracellular signal-regulated kinase (ERK)/ α -secretase-mediated A β reduction via selective serotonin reuptake inhibitors (SSRIs) and 5-HT₄ to receptor subtypes that target effects on tau phosphorylation (5-HT_{2B/2C}), (2) neurotransmitter balance (5-HT₆) (3) synaptic plasticity. This proves serotonergic pathways as compelling, multi-modal targets for ameliorating Alzheimer's disease progression. This was proved when serotonin receptors (5-HT_{1A}, 5-HT_{2A}, and 5-HT₄) receptors in AD patients were significantly reduced.

Based on the diagram 2.14, it is shown that 5-HT₄R and 5-HT₆R-mediated signalling has crucial effects on the APP that might lead to the impairments of the

disease. Reduction of 5-HT_{1A} signalling especially, has been affected due to impairment of long-term potentiation (LTP) involved in learning and memory processes. Normally, serotonin signalling modulates APP processing as normal level of 5-HT_{1A} reduces beta-amyloid generation by promoting amyloidogenic APP cleavage (Sheline et al., 2014). Also, serotonin has neuroprotective properties and reduced levels increase the neuronal cells to oxidative stress and excitotoxicity (Cirrito et al., 2011). From the proven effects occurred in the brain, serotonin signalling has been proved by previous researches in clinical settings as well by using transgenic mice as animal models administered with selective serotonin reuptake inhibitors (SSRIs) or direct hippocampal serotonin infusion has resulted positive effects in lowering interstitial fluid A β levels by approximately 25%. Crucially, the experimental design has successfully decreased plaque burden by roughly 50% through α -secretase-mediated processing at which these mechanisms depend on extracellular signal-regulated kinase (ERK) activity.

There were extensive serotonergic denervations and serotonergic alterations has been observed associating with AD pathogenesis. Serotonergic dorsal raphe nuclei are one of the few that were first affected by tau protein abnormalities (Šimic' et al., 2017). This was proved further when there were neurofibrillary changes due to possible initial source of transneuronal spreading across brain (Grinberg et al., 2009). This is because the result of neuromodulation of cortical synaptic strength is associated with formation of memories for events (Vakalopoulos, 2017). These lead to efforts in restoring serotonergic function by selectively-targeting either serotonin reuptake inhibitors (eg. fluoxetine), serotonin receptor agonists or antagonists that aim 5-HT₄ receptors to modulate behavioural and cognitive symptoms and anti-inflammatory targets by restoring serotonin receptors (Sheline et al., 2014; Bianco et al., 2016; Bostancıoğlu, 2020).

Serotonin receptors play a pivotal role in modulating tau pathology in Alzheimer's disease (AD) through multiple signalling cascades that influence tau phosphorylation, aggregation, and clearance. According to Butzlaff and Ponimaskin (2016), serotonin denervation in AD disrupts receptor-mediated regulation of kinases such as GSK-3 β and CDK5, both central to tau hyperphosphorylation (Butzlaff and Ponimaskin, 2016). Based on the Figure 2.15, it shows how tau protein is regulated at which 5-HT₇ receptors are able to directly activate CDK5 in a G-protein-independent manner, promoting tau aggregation and neurodegeneration, whereas blocking 5-HT₇

activity in tauopathy mouse models (e.g., Tau[R406W]) significantly reduces tau phosphorylation and improves synaptic plasticity and memory (Ackmann et al., 2024). Similarly, 5-HT_{1A} receptor activation triggers the PI3K/Akt pathway, leading to GSK-3 β inhibition and subsequent attenuation of tau phosphorylation, particularly in the presence of amyloid- β or forskolin (Tian & Li, 2020).

As for the 5-HT₄ receptor, when activated, enhances proteasomal degradation of tau and promotes non-amyloidogenic APP processing by increasing α -secretase activity; an effect observed in PS19 mice at which 5-HT₄ agonists reduced tau burden and improved cognition (Jiang et al., 2024). Conversely, antagonism of 5-HT₆ receptors has been shown to affect the gamma-aminobutyric acid (GABA) mechanisms as it will dampen the GABAergic transmission and reduce γ -secretase activity, thereby lowering both tau and amyloid pathologies and restoring synaptic function. Furthermore, serotonin has been reported to inhibit tau kinases such as microtubule affinity-regulating kinase 4 (MAPK4) expanding the neuroprotective profile of serotonergic modulation (Smith & Wang, 2023). Together, these findings highlight that serotonergic receptors regulate tau pathology through both kinase inhibition and proteostatic enhancement, positioning them as viable, multi-target therapeutic nodes in the treatment of AD.

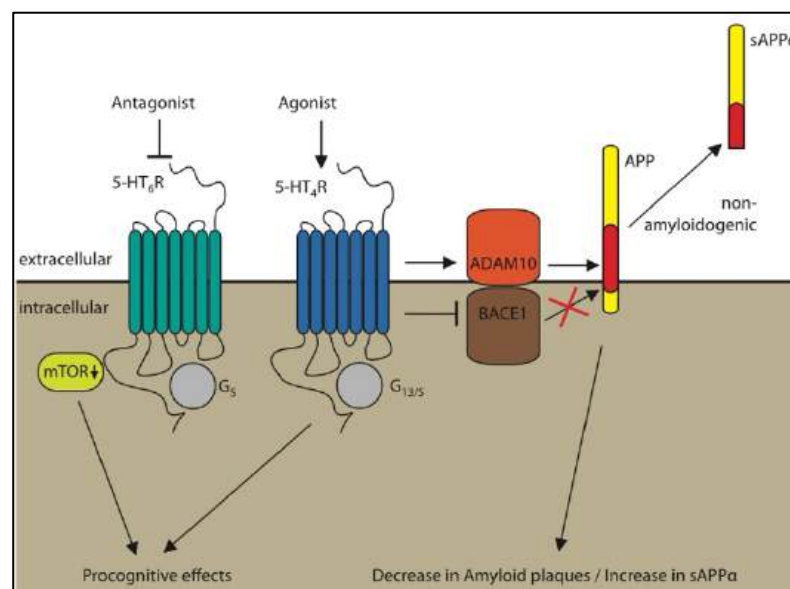


Figure 2.14 The Impact of 5-HT₄R and 5-HT₆R-mediated Signalling on the APP Degradation. While Stimulation of the 5-HT₄R Drives APP towards the Beneficial Non-amyloidogenic Path via Direct Interaction with α -secretase ADAM10 or β -secretase BACE1, Inhibition of the 5-HT₆ Receptor Promotes Precognitive Effects via Reduction of mTOR Activity (Butzlaff & Ponimaskin, 2016)

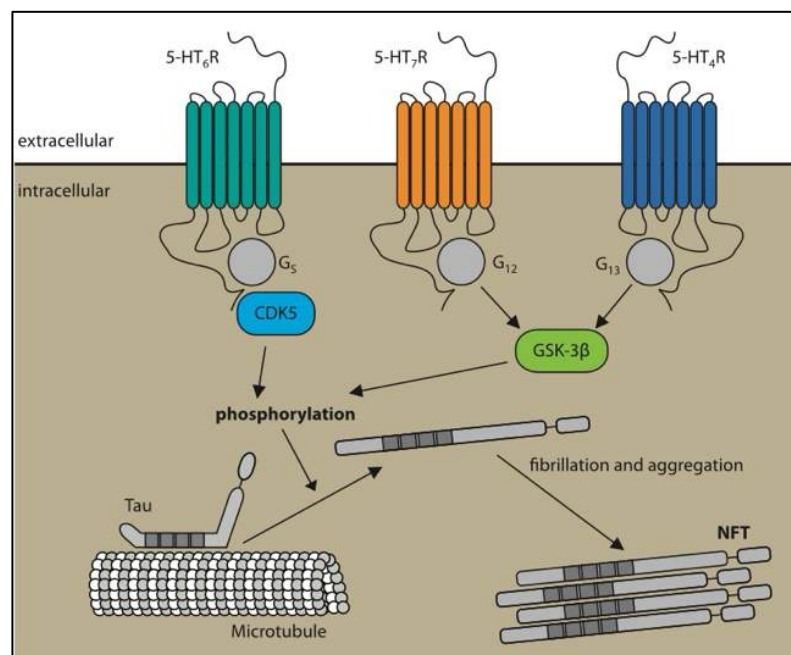


Figure 2.15 Modulation of 5-HT₄R and 5-HT₇R as Serotonin Receptors in Tau Pathology Regulating Glycogen Synthase Kinase 3β (GSK-3β) via Activity of G12/13 Proteins meanwhile 5-HT₆R Activates Cyclin Dependent Kinase 5 (CDK5) via Direct Interaction (Butzlaff & Ponimaskin, 2016)

2.7.7 Free Radical Damage Hypothesis

The free radical damage hypothesis proposes that AD is due to the interplay of presence of oxidative stress caused by imbalance of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the antioxidant defense mechanism in the brain. Figure 2.16 shows the flow of free radical damage hypothesis in AD and how it affects the consequent factors leading to severe damage in the brain. Mitochondrial dysfunction is a key source of ROS at which the electron transport chain activity is impaired leading towards excessive superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) production, this causes oxidative damage to lipids, proteins and even DNA (Swerdlow et al., 2019). DNA and RNA oxidation causes mitochondrial DNA mutations and impaired protein synthesis, driving neuronal apoptosis and cognitive decline (Guedes et al., 2023).

Neuroinflammation that occurs is able to amplify oxidative stress due to activation of microglia and astrocytes that produce ROS and nitric oxide (NO) leads to chronic neuronal damage (Zhang et al., 2024). The oxidative stress build-up might be due to excess iron (Fe^{2+}) and copper (Cu^{2+}) that promotes metal ion dysregulation that exacerbates oxidative stress generating highly reactive hydroxyl radicals promoting Aβ

aggregation and neuronal toxicity (Butterfield et al., 2020). Thus, the cellular damage and synaptic dysfunction causes the brain to require high oxygen demand. In ROS and RNS production, mitochondria are the primary sources that generate both of the reactive compounds due to electron leakage from electron transport chain (ETC). Furthermore, an increase of amyloid beta aggregates would cause the peptides to react and induce high levels of ROS production and the oxidative stress leads to widespread cellular damage; lipid peroxidation, protein oxidation, DNA damage and mitochondrial dysfunction in particular. ROS-mediated lipid peroxidation disrupts neuronal membranes causing neurotransmitter dysregulation while protein oxidation accelerates tau hyperphosphorylation and neurofibrillary tangle formation (Liu et al., 2022).

Other than damaging neuronal survivability, oxidative stress and free radical damage severely impairs neurogenesis in the brain of AD, contributing to declined cognitive abilities (Boldrini et al., 2022). This can be seen at which the excessive ROS generated can cause oxidative damage to neural stem cells (NSCs) by reducing its proliferation and differentiation (Boldrini et al., 2022). This disruption leads to premature apoptosis and declined neurogenesis that affects downstream learning and memory processes (Ohta et al., 2021). The build-up in tau hyperphosphorylation and beta amyloid aggregates would inhibit NSC function and neuronal maturation (Baydas & Tüfek, 2022). Also, the increase in chronic neuroinflammation driven by activated microglia and astrocytes among the neuronal cells would lead to suppression of neurogenic signalling pathways such as the canonical Wnt pathway (wingless-related integration site) or distinguished as Wnt/ β -catenin and the brain-derived neurotrophic factor (BDNF) (Llorens-Martín et al., 2023). The downstream effects from these mechanisms accelerates cumulative oxidative stress-induced neurogenic impairment such as hippocampal atrophy and cognitive decline in AD patients. The emerging pharmacotherapies with high potential in treating these effects include targeting oxidative damage through antioxidant therapies and mitochondrial protective agents for neurogenesis restoration and slowing down neurodegeneration (Mota et al., 2024).

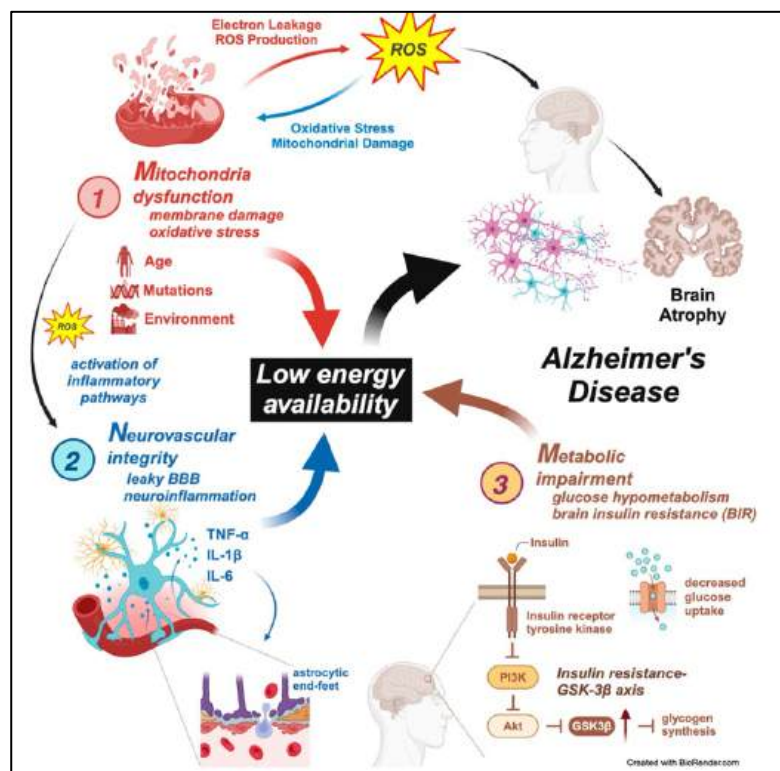


Figure 2.16 Flow of Free Radical Damage Hypothesis in AD (Kazemeini et al., 2024)

2.8 Pharmacotherapeutics of AD

To get a better understanding on the mechanism of action of interventions available to optimize current pharmacotherapeutics of AD, the degree of efficacy of relative drugs needs to be fully determined before releasing it commercially for clinical use. The drugs treatment for AD can be divided into few categories; discontinued, in-trial and approved as shown in the Figure 2.17. Meanwhile its therapeutic targets were summarized in Table 2.1 describing the current available pharmacotherapies of AD include cholinesterase inhibitors such as donepezil, rivastigmine, galantamine, memantine+donepezil, those that target to reduce A β plaques; aducanumab, lecanemab, gantenerumab. Meanwhile, memantine is used as a NMDA receptor partial antagonist. Recently, there are two new drugs that FDA approved recently that can be used as a treatment for AD, namely donanemab and gantenerumab. These two recent approaches focus on targeting A β epitopes that present only in established amyloid plaques thus this will increase precision and avoid any off targets binding to other A β species (Bukke et al., 2020).

In China, there is a new marine oligosaccharide drug developed named as sodium oligomannate (GV-971) to treat AD (Zhang et al., 2024). The mechanism of the drug

involves inhibition of neurotoxicity caused by A β aggregation in a way by preventing A β accumulation and declining A β polymerization. Interestingly, sodium oligomannate (GV-971) acts upon the brain by directly blocking the development of A β fibrils while destabilizing the already-existing fibrils into non-toxic molecules (Zhang et al., 2014). However, the available interventions can temporarily alleviate or stabilize symptoms, but it is still a challenge to stop long-term progression of the disease. There is still an urgent need for a highly functioning drug with low risk for the treatment of people living with early symptomatic AD, including cognitive impairments at an early or mild dementia stage.

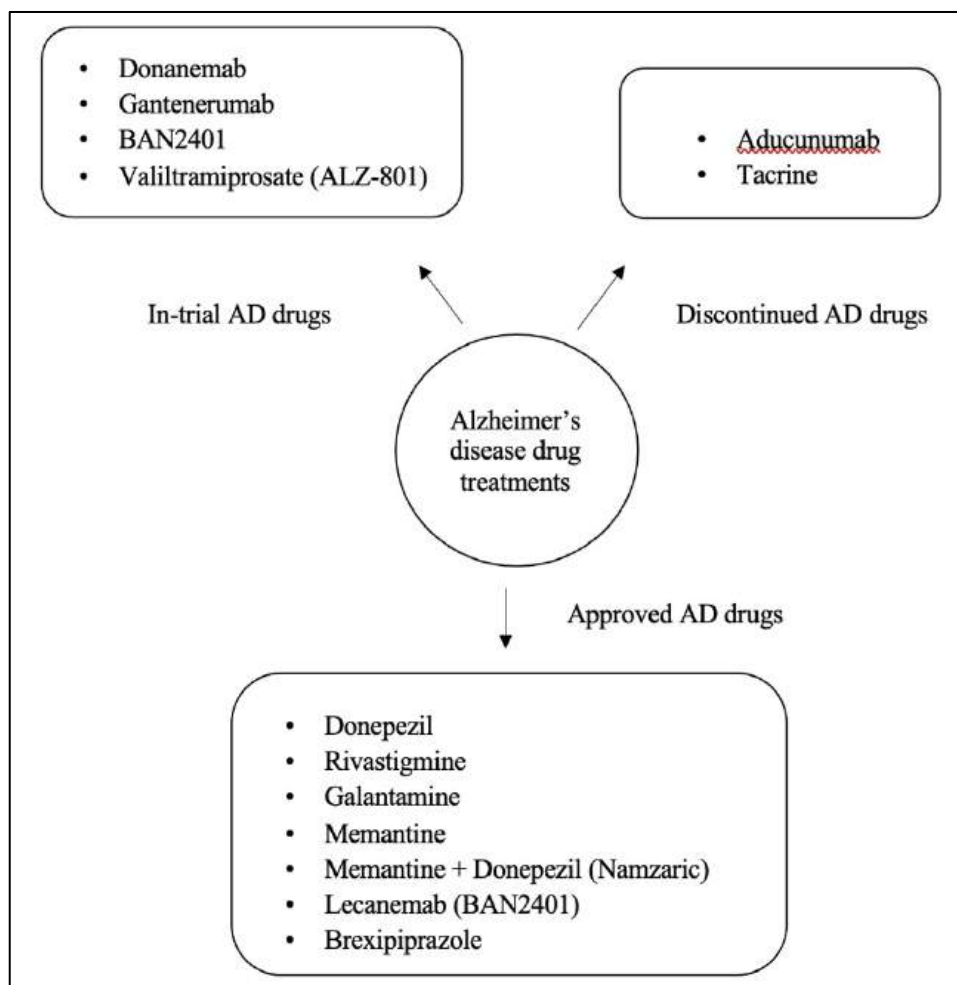


Figure 2.17 Drug Treatments for AD

Table 2.1 Summary of Current Pharmacotherapies Available for Alzheimer's Disease

Type of drugs	Mechanism of action	Limitation	Drug status	Reference
Donepezil	Acetylcholinesterase inhibitors, upregulates nicotinic receptors in cortical neurons, downregulates neuroinflammatory responses, activates microglia and astrocytes	Gastrointestinal reactions; nausea, diarrhea and vomiting	Active	Kumar et al., (2023)
Rivastigmine	Acetylcholinesterase inhibitors; reversibly binds to and inhibits cholinesterase enzymes	Severe gastrointestinal reactions and allergic cutaneous reactions	Active	Patel & Gupta (2023)
Galantamine	Acetylcholinesterase inhibitors, lowering oxidative neuronal damage	Extensive gastrointestinal metabolism	Active	Kalola et al., (2024)
Memantine	NMDA receptor partial antagonist, prevents overactivation of glutamate receptors	Adverse reactions including constipation, confusion, dizziness, headache and hypertension	Active	Kuns et al., (2024)
Memantine +Donepezil (Namzaric)	Acetylcholinesterase inhibitors	Higher rates of side effects compared to memantine as monotherapy	Active	Yaghmaei et al., (2024)

Lecanemab (BAN2401)	Amyloid-targeting antibody drug reducing A β plaques. Targets soluble protofibrils (large oligomers) over plaques	Risk of amyloid-related imaging abnormalities (ARIA). Increased risk of vasogenic edema	Active	Tolar et al., (2020)
Aducunumab	Reduce A β plaques	Slight benefit in slowing cognitive decline. However, side effects include brain edema, microhemorrhages and superficial siderosis	Discontinued	Vaz et al., (2022)
Donanemab (2024)	Targets A β plaques	Risk of amyloid-related imaging abnormalities (ARIA)	Under review	Song et al., (2024)
Gantenerumab	Targets A β plaques and does not bind to soluble A β	Risk of amyloid-related imaging abnormalities (ARIA)	Under review	Sobral et al., (2024)
Valiltramiprosate (ALZ-801)	Stabilize monomers and inhibit their misfolding and aggregation into toxic oligomers and improved gastrointestinal tolerability	High intersubject pharmacokinetic variability	Under review	Abushakra et al., (2024), Tolar et al., (2020)

Brexpiprazole	Antipsychotic that acts on noradrenergic, serotonergic, and dopaminergic neurotransmitter systems	Targets to reduce agitation but not improving learning and memory	Approved	Zhang et al., (2024), Lee et al., (2023)
Tacrine	Acts by inhibiting the metabolism of acetylcholine, prolonging its mechanism and increasing its levels in cerebral cortex	Targets to improve brain functions in mild-to-moderate AD patients. Resulted in adverse effects including hepatotoxicity, diarrhea, nausea and vomiting	Discontinued since 2013	Zhang et al., (2024), U.S. National Library Medicine (2020)

2.9 Cannabis

In the 19th century, pharmacological effects of cannabis have proven to provide evidences on the growth of cannabis research and its medicinal value (Stasilowicz et al., 2021; Walsh et al., 2021). Cannabis or generally known as marijuana is derived from the female plant, *Cannabis sativa* (Cohen & Weinstein, 2018) and it is an annual dioecious herb which belongs to the genus Cannabaceae family. The current accepted classification of cannabis can be distinguished into two subspecies; ssp. *Sativa* and ssp. *Indica*. Each subspecies further divides into two main varieties namely wild and cultivated (Stasilowicz et al., 2021). *C.sativa* ssp. *Sativa* var. *sativa* (*C.sativa*) and *C.indica* ssp. *Indica* var. *indica* (*C.indica*) are two variants of cannabis commonly grown for recreational use. With the advancement of medical technology and deeper understanding on one of its variants namely *C.sativa*, it has recently gained attention and been a topic of interest. This is due to its potential therapeutic effects to treat various diseases, particularly neurological disorders.

Cannabinoids, especially phytocannabinoids represent a major class of biologically active secondary metabolites produced by *C.sativa*. These compounds constitute a structurally related group of terpenophenolic molecules, biosynthesized from monoterpenoid and polyketide precursors (Hanus et al., 2016; Atalay et al., 2019). Phytocannabinoids are characterized by a dibenzopyran as a core structure, conferring high lipophilicity and the ability to cross blood–brain barrier (Hanus et al., 2016). Herbal cannabis contains over 550 identified chemical constituents, including more than 120 distinct phytocannabinoids, however Δ^9 THC remains most pharmacologically prominent and extensively investigated due to its abundance and potent biological effects (Hanus et al., 2016; Hanuš et al., 2020). The advancement in pharmacology of cannabis was based on its successful isolation, structural elucidation and synthesis of Δ^9 THC and CBD enabling in-depth systematic investigation of their molecular targets. Interestingly, Δ^9 THC is the principal psychoactive constituent and acts primarily as a partial agonist at cannabinoid receptors, thereby modulating intracellular signalling pathways involved in neurotransmitter release, synaptic plasticity, neuronal excitability and cell survival. Through these actions, Δ^9 THC directly influences neural processes that are disrupted in neurological and neurodegenerative conditions including neuronal

resilience in forming learning and memory processes (Lu & Mackie, 2021; Kendall & Yudowski, 2022).

In recent years, increasing preclinical and clinical evidences has supported the therapeutic use of cannabis-derived compounds in neurological conditions such as epilepsy, neuropathic pain, multiple sclerosis, and neurodegenerative diseases. For example, cannabinoid-based formulations have demonstrated efficacy in reducing seizure frequency in treatment-resistant epilepsy and alleviating chronic neuropathic pain through modulation of neuronal excitability and synaptic transmission (Li et al., 2021; Stasilowicz et al., 2021). These clinical successes have further intensified interest in phytocannabinoids as neuromodulatory agents capable of targeting multiple pathological mechanisms simultaneously. Despite these benefits, the therapeutic application of Δ^9 THC remains constrained by its psychoactive properties and dose-dependent effects. While low dose of Δ^9 THC has demonstrated neuroprotective, anti-inflammatory, and anti-amyloidogenic properties in preclinical models, higher doses may impair cognition leading to cause adverse neurobehavioural outcomes. Consequently, rigorous evaluation of Δ^9 THC under controlled experimental conditions is essential to define its optimal dosage range, treatment duration and therapeutic window.

2.10 Cannabinoids Receptor

The endocannabinoid system (ECS) is a neuromodulatory network critically involved in synaptic function, neuronal survival, and cognitive processes, primarily through the activation of cannabinoid receptors. The first cannabinoid receptor was discovered in 1988 which was named as cannabinoid type 1 receptor (CB1), followed by a plasma membrane G-protein-coupled receptor cloned in 1993, cannabinoid type 2 receptor (CB2) (Devane et al., 1988; Felder et al., 1993). Interestingly, these two cannabinoid receptors are recognized as key neuromodulators of brain homeostasis. The localization of CB1 receptors is concentrated in the central nervous system (CNS), particularly in brain regions governing learning and memory, including the hippocampus, prefrontal cortex and amygdala. CB1 is predominantly expressed at presynaptic terminals, especially on GABAergic terminals and glutamatergic neurons, at which their activation suppresses neurotransmitter release through the inhibition of adenylate cyclase activity and reduction of intracellular calcium influx (Xiong & Lim.,

2021). Through this mechanism, CB1 plays a central role in regulating short- and long-term synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD) which form the cellular basis of learning and memory (Zhang et al., 2024).

In the cannabinoid-AD interplay, CB1 receptor signalling has been shown to influence APP processing and A β production. Activation of CB1 has been proved to reduce BACE1 activity leading to decreased A β production and accumulation, thereby preserving synaptic integrity essential for memory formation (Chen et al., 2010; Aso & Ferrer, 2014). Postmortem analyses of AD patient brains revealed a marked reduction in CB1 expression, particularly in the frontal cortex, correlating with disease severity and cognitive impairment (Solas et al., 2013). Consistently, experimental studies using APP/PS1 transgenic mice demonstrate that CB1 downregulation exacerbates learning and memory deficits, whereas preservation of CB1 signalling mitigates synaptic dysfunction and cognitive decline (Aso et al., 2018). Furthermore, CB1 receptors are functionally associated with mitochondrial regulation, where their activation reduces oxidative stress and enhances mitochondrial efficiency, processes that are essential for sustaining synaptic energy demands during learning and memory consolidation (Hebert-Chatelain et al., 2006; Gugliandolo et al., 2020).

In contrast to CB1, CB2 receptors are minimally expressed in neurons under physiological conditions and one of the significant components for neural precursor cell proliferation, axonal guidance and synaptic transmission (Cassano et al., 2017). Abundance of CB2 is shown selectively in the plaques and surrounding areas hence indicating a correlation between endocannabinoid system (ECS) and plaque deposition (Xiong & Lim., 2021). This was proved by CB2 that has a relatively low expression contradicting with it being markedly upregulated in glial cells, particularly microglia in response to neuroinflammation and neurodegeneration. Thus, CB2 are selectively abundant around the amyloid plaques (Benito et al., 2008; Xiong & Lim, 2021). Given that CB2 activation lacks the psychoactive effects associated with CB1 stimulation, selective targeting CB2 is increasingly viewed as a promising therapeutic strategy to attenuate neuroinflammation-driven cognitive decline in AD (Atwood & Mackie, 2010; Cassano et al., 2017; Lu et al., 2016). All in all, restoration of cannabinoid receptor functions both CB1 and CB2 either through agonism or modulation of endogenous cannabinoids, therefore characterize the potential mechanistically targets for preserving cognitive function and slowing the progression of disease (Bilkei-Gorzo, 2012; Aso &

Ferrer, 2016). These support its potential role as complementary or disease-modifying strategy aimed at preserving learning and memory functions in AD (Bilkei-Gorzo, 2012; Cassano et al., 2017).

2.11 Δ^9 THC and its Pharmacological Actions

Δ^9 THC or commonly known as delta-9-tetrahydrocannabinol has the ability to cause intoxication and has antiemetic, analgesic effects which may be potentially neuroprotective and anti-inflammatory. Δ^9 THC acts as a partial agonist on cannabinoid receptors with a very high binding affinity towards both for the cannabinoid receptors (CB1 and CB2) regulating its psychoactive properties such as changes in mood or consciousness, memory processing and motor control (Vučković et al., 2018). When cannabinoid signalling is primarily disrupted, it will affect the downstream neuronal communications and neural plasticity. Collectively, Δ^9 THC represents as a biologically relevant ligand which is capable in engaging both CB1- and CB2-mediated signalling pathways implicated in learning, memory and AD pathology.

Stimulation of CB1 and CB2 receptors by Δ^9 THC has a broader spectrum of pharmacological effects as it is used therapeutically as an analgesic, antiemetic and anti-convulsant (Shahbazi et al., 2020). For Δ^9 THC, the receptors are often associated with adverse effects such as reduction in most cognitive functions, learning, memory, attention and executive function due to chronic and acute intoxication of cannabis for recreational purposes (Battisti et al., 2010). However, low-dose Δ^9 THC has been shown to restore synaptic homeostasis by suppressing excessive glutamatergic signalling, reducing intracellular calcium overload and protecting neurons against excitotoxic damage (Marsicano et al., 2003; Scotter et al., 2010). Thus, the dosage and duration of treatment are important factors to be determined prior implementing Δ^9 THC as one of the interventions for AD. Despite that, most Δ^9 THC-focused intervention studies were done on young adults or adolescents at which the age range has a high vulnerability to central effects of the drug, whereas only few studies were related to older adults or AD individuals.

2.12 Previous Research Findings of Δ^9 THC

Preclinical studies using AD-relevant animal models have demonstrated that Δ^9 THC exerts direct effects on neuronal survival, plasticity, and molecular pathology. This displays Δ^9 THC as a potential disease-modifying agent rather than a purely symptomatic treatment. Several *in vivo* studies have shown that chronic low-dose Δ^9 THC administration improves hippocampal-dependent learning and memory in AD-related models. In aged mice and transgenic models that exhibit amyloid pathology, Δ^9 THC treatment significantly have proven that it has the ability to restore spatial learning and memory proved by the positive outcomes regarding performance as measured by tasks such as the Morris Water Maze and object recognition tests (Bilkei-Gorzo et al., 2017; Aso et al., 2018).

These behavioural improvements were accompanied by preservation of neuronal structure and synaptic integrity within the hippocampus, suggesting that Δ^9 THC directly supports neuronal networks essential for memory encoding and retrieval. According to another previous study conducted, it was proven that pro-long exposure of low dose Δ^9 THC (3mg/kg/day) for a total of 4 weeks has significantly improved cognitive performances in old mice. Interestingly, the results were on par with the control mice group (Nidadavolu et al., 2021). However, when the same dose of Δ^9 THC was used on younger mice, the results were adversely affected. Cognitive impairments were observed rather than the estimated improvements post- Δ^9 THC treatments in neurobehavioural abilities. This further implies that there is a potential risk if Δ^9 THC is being administered in young age individuals in clinical settings as it might be deteriorating. Thus, more in-depth clinical research needs to be done especially in the aspect of dose-response relationship within different age individuals. From this, it can be deduced that Δ^9 THC possess a great potential despite concentration and duration of treatment may need to be explored further to fully comprehend the possibility of psychoactiveness according to different stage of age.

Meanwhile, other than restoring behavioural abilities, Δ^9 THC has been shown to affect the physiology of AD-affected regions at the cellular level, particularly through modulation of neuronal apoptosis. In AD transgenic models such as 5XFAD mouse, which recapitulates early and aggressive amyloid deposition, chronic low-dose Δ^9 THC administration resulted in marked reductions in neuronal degeneration and apoptosis within hippocampal subfields when compared with untreated controls at which the

changes were evident through decreased staining for markers signifying neuronal deaths, meanwhile there was a rise in preservation of cytoarchitecture integrity, suggesting Δ^9 THC mitigates amyloid-associated neuronal loss responsible for cognitive decline (Chen et al., 2012). For example, activation of CB1 receptors by Δ^9 THC in hippocampal neurons enhances pro-survival pathways leading to inhibition of molecular shifts creating a cellular environment that is less susceptible to oxidative stress and excitotoxicity (Galve-Roperh et al., 2013). Hence, by targeting neuronal apoptosis, Δ^9 THC contributes to the maintenance of hippocampal circuitry and there is a potential in delaying neurodegenerative pathology throughout the disease progression.

In addition to preserving existing neurons, Δ^9 THC was observed to enhance hippocampal neurogenesis, a process that is essential for learning and memory which was markedly impaired in AD (Jin et al., 2004; Wolf et al., 2010). Previous outcomes have demonstrated that activation of cannabinoid receptors promotes proliferation and survival of neural progenitor cells in the dentate gyrus, suggesting that cannabinoids can directly influence the generation of new neurons in adult brains (Jin et al., 2004). Extending these findings, previous results have demonstrated that administration of Δ^9 THC at low dosage specifically at 1.5 mg/kg in male Sprague-Dawley rats significantly increased the expression of multiple neurogenesis markers, including bromodeoxyuridine (BrdU), doublecortin (DCX), and brain-derived neurotrophic factor (BDNF), relative to controls (Suliman et al., 2017). These molecular changes coincided with improved performance in hippocampal-dependent cognitive tasks such as the novel-object discrimination test, indicating that Δ^9 THC-induced neurogenesis supports functional memory enhancement (Suliman et al., 2017).

Another evidence to prove ability of Δ^9 THC in stimulating neurogenesis was through the administration of ultra-low doses of Δ^9 THC in the hippocampal cells at which this resulted in protecting the brain from neuroinflammations that might induce cognitive damage (Ibork, 2025). Importantly, Δ^9 THC also modulates core molecular hallmarks of AD within neuronal populations. Previous experimental studies have demonstrated that Δ^9 THC reduces amyloid- β accumulation by downregulating BACE1 expression and activity, thereby suppressing amyloidogenic processing of amyloid precursor protein (Chen et al., 2010; Cao et al., 2014). For instance, a transgenic 5xFAD AD mouse model have proved significant reduction in A β plaque and neuronal degeneration revealing potential therapeutic strategy can be implemented on

hippocampal neurons as potentially disease-modifying rather than purely symptomatic (Chen et al., 2012).

In another in-vitro study, Δ^9 THC demonstrated a direct anti-amyloidogenic effect by inhibiting A β aggregation, a critical early event in AD pathogenesis (Cao et al., 2014). Via results from the thioflavin fluorescence assay test that was conducted in a dose-dependent manner, Δ^9 THC significantly reduced fluorescence intensity reflecting diminished aggregation. Findings from this provides strong evidence that Δ^9 THC is able to directly interfere with the conformational transition of soluble A β peptides into neurotoxic oligomers and fibrils. This is further supported by other evidences that Δ^9 THC has the potential to affect A β fibril formation and aggregation stimulating the removal of intracellular A β leading to hinder neuroinflammatory responses (Janefjord et al., 2014; Currais et al., 2016; Schubert et al., 2019). Collectively, Δ^9 THC proves it possess great capability in being multifactorial to target amyloid plaques deposition in AD models counteracting further impairments.

In addition to preserving hippocampal neurons and enhancing cognitive functions, Δ^9 THC has been shown to influence another molecular hallmark, aside from amyloid beta plaques, which is responsible for tau phosphorylation as one of the major AD pathologies. In aged APP/PS1 transgenic mice, low-dose Δ^9 THC (0.02-0.2 mg/kg) has significantly reduced the levels of phospho-tau and total tau proteins in brain tissue, suggesting a lowering of pathological tau burden (Wang et al., 2022). Other than that, mechanistically Δ^9 THC has been seen to attenuate tau hyperphosphorylation, including phosphorylation at threonine 231 (p-tau Thr231), a modification closely associated with synaptic dysfunction and neurofibrillary tangle formation (Esposito et al., 2007; Aso et al., 2016). These reductions suggested modulation of tau-related kinase activity and preservation of neuronal cytoskeletal stability aiming to further comprehend AD progression. Taken together, evidences from the animal studies indicate that Δ^9 THC can directly target neuronal mechanisms central to AD pathology. These combined effects provide a mechanistic basis for the observed improvements in learning and memory and highlight Δ^9 THC as a multi-target, neuron-focused therapeutic candidate for slowing or modifying AD progression.

2.13 Neurogenesis

In the context of memory functions, hippocampus primarily plays a key role in its mediation associated with dentate gyrus, entorhinal cortex and hippocampus (Wang and Morris, 2010). Neurocognitive symptoms of AD include memory and spatial learning deficits that worsen as the disease progresses, hence learning and memory consolidation targeting neurogenesis will aid as a potential therapeutic approach (Suliman et al., 2018). Neurogenesis is a process to produce new neurons from neural progenitor cells (NPCs) or neural stem cells, critically involved only during brain development (Kempermann et al., 2015). The multi-step of adult neurogenesis occurs in two main regions; subventricular zone (SVZ) and subgranular zone (SGZ) of the hippocampus (Sung et al., 2020). However, SVZ contributes to olfactory neural circuitry limited to early childhood (Sung et al., 2020). Hence, proving the significance of the hippocampus in mediating memory and spatial learning. Figure 2.18 shows depiction of the neurogenesis stages and its markers in the neurogenic process in the hippocampus (Kozareva et al., 2019).

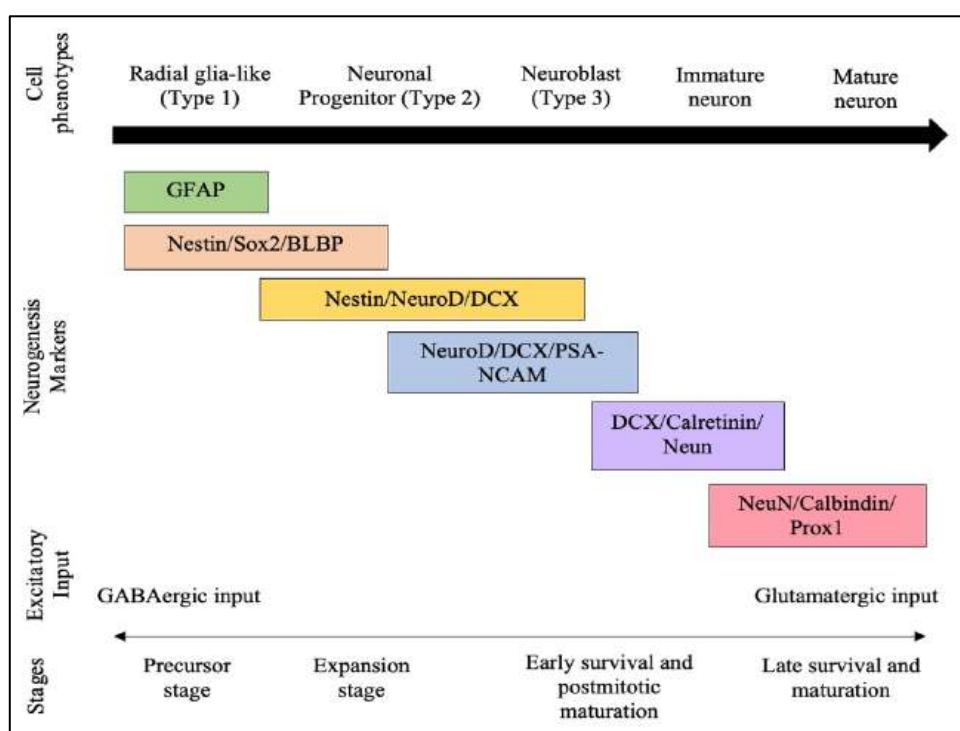


Figure 2.18 Neurogenesis Stages and its Markers

Adult neurogenesis in SGZ can be categorized into a few stages; precursor stage, expansion stage, early survival of postmitotic maturation and late survival maturation. The early precursor stage consists of proliferation of neural progenitors in the hippocampus such as radial glia-like cells (RGLs), also known as radial astrocytes or Type 1 cells (Filippov et al., 2003; Seri et al., 2004). These neural progenitors express GFAP, nestin, Sox2 (SRY-Box Transcription Factor 2) and brain lipid binding protein (BLBP) which play a role in radial processes in specialized irregular areas of the basement membrane (Licht et al., 2020). Consequently, the neural progenitors will rise into intermediate progenitors which later transform into neuroblasts (Kempermann et al., 2015). Next, the process continues into expansion stage that occurs when differentiation of intermediate progenitors (known as Type 2 cells) happens and it leads to high levels of expression of doublecortin (DCX), neurogenic differentiation factor (NeuroD) and polysialylated neural cell adhesion molecule (PSA-NCAM) (Kozareva et al., 2019; Sun et al., 2015).

After finally reaching the next stage, migration of Type 3 cells occurs when immature neurons migrate into the inner granule cell layer before differentiating into dentate granule cells in hippocampus. This is when doublecortin (DCX), calretinin and neuron-specific nuclear protein (NeuN) or also known as Fox-3 are expressed until it reaches the late survival and maturation stage at which NeuN, calbindin and Prospero-related homeobox 1 (Prox1) are expressed specifically (Kozareva et al., 2019). This leads to neuronal migration; dentate granule cells form extending dendrites and projecting axons through the hilus towards CA3 (Kozareva et al., 2019). Eventually, this leads to formation of a synaptic integration between new neurons and existing circuitry of mature neurons, or known as (Kempermann et al., 2015).

2.13.1 Glial Fibrillary Acidic Protein (GFAP)

GFAP is a principal intermediate filament protein of the astrocytic cytoskeleton and is prominently expressed in astrocytes within the adult hippocampus, a brain region critically affected in AD due to neuroinflammations. This occurs due to the formation of mature astrocytes and its GFAP-expressing radial glia-like neural stem cells persist in the adult brain contributing to gliogenesis, including the generation of astrocytes and

oligodendrocyte lineage cells in regions (Alvarez-Buylla & Lim, 2004; Sofroniew & Vinters, 2010). The regions include olfactory bulb and corpus callosum which are essential for brain homeostasis, synaptic support and repair processes following neurogenesis (Molofsky & Deneen, 2015). Functionally, upregulation of GFAP reflects profound astrocyte cytoskeletal remodelling, which alters astrocyte morphology, motility and interactions of neurons with microglia and its vasculature (Ben Haim et al., 2015; Verkhratsky et al., 2023). However, based on accumulating preclinical evidences, the findings indicate that manipulating GFAP expression suggest that astrocytes can adopt distinct functional phenotypes, ranging from neuroprotective to neurotoxic, depending on disease stage and microenvironmental cues (Sofroniew, 2020).

Other than related with astroglial cells, these highlight GFAP not only act as a passive indicator of astrocyte activation, but as a potential regulator of neurogenic outcomes. In AD mouse models, cannabinoid treatment has been shown to enhance GFAP expression, and partially restore hippocampal neurogenesis alongside improvements in cognitive performance (Aso et al., 2015; Moreno-Gonzalez et al., 2024). These observations suggest that GFAP may serve as a critical molecular interface linking cannabinoid pharmacology, astrocyte state, and neurogenic capacity. Moreover, Δ^9 THC has also been implicated in the regulation of adult neurogenesis, particularly within the hippocampal dentate gyrus, a brain region critically involved in learning and memory. Studies indicated that cannabinoid receptor specifically GFAP activation by Δ^9 THC promotes neural progenitor cell proliferation, differentiation, and survival. These enhancements would greatly contribute to hippocampal circuit remodelling and cognitive resilience from neurodegenerative effects (Jin et al., 2004; Wolf et al., 2010).

Despite these advances, the precise role of GFAP in regulating neurogenesis under pathological conditions remains incompletely understood. Most existing studies focus on GFAP as a biomarker of astrocyte activation leading to potential inflammations rather than as a functional participant in neurogenic signalling pathways. Further investigation into GFAP-associated enhancements, particularly in response to Δ^9 THC-mediated immunomodulation, could clarify whether modulating neurogenesis reactivity can shift towards a reparative state. Such insights would be highly relevant for developing specific-targeted therapeutic strategies aimed at preserving or restoring neurogenesis in AD. This includes the importance of exploring more animal models targeting other biomarkers for improving the accuracy of treatment. A combination of biomarkers will probably render the highest accuracy for A β pathology compared to

single-targeted therapies, at which it will aid in improving the statistical significance among pre-clinical AD studies (Huebschmann et al., 2020).

For example, convincing results from AD mouse model (PS2APP and hAPP-J20) have demonstrated that elevated GFAP expression was accompanied with both morphological changes and transcriptomic shifts in hippocampal astrocytes correlating with neurogenesis and neuronal survivability (Kraft et al., 2013; Heneka et al., 2015). Furthermore, it has been previously observed that effects of Δ^9 THC in upregulating GFAP has been interpreted as an expansion or activation of radial glia-like progenitors rather than pathological astrocytosis. This interpretation is supported by concomitant increases in downstream neurogenesis marker that involves in matured stage of neurogenesis specifically calbindin. This further indicates enhanced neuronal lineage commitment and maturation (Jin et al., 2004; Wolf et al., 2010). Importantly, the dual role of GFAP in both astrocytic reactivity and neurogenesis underscores the need for context-dependent interpretation of GFAP upregulation. This regenerative astrocytic response is particularly relevant in AD, where neurogenesis is suppressed, and highlights GFAP-positive astrocytes as active contributors to neural repair mechanisms rather than passive markers of pathology (Sofroniew & Vinters, 2010; Verkhratsky et al., 2010). Thus, targeting GFAP plays a part to target reduction in neuronal deaths with A β -related oxidative stress and mitochondrial dysfunctions exacerbating neuroinflammatory responses (Sun et al., 2014).

2.13.2 Calbindin

In early neurodegenerative process, AD is accompanied by calcium dysregulation, neuronal death, and mitochondrial and synaptic dysfunction (Hirai et al., 2001; Hardy & Selkoe, 2002). Calbindin is one of the major calcium-binding proteins that play its role as a buffer for calcium (Ca^{2+}) level and transport of calcium-binding proteins (Baimbridge et al., 1992). The altered levels of Ca^{2+} have been reported in AD patients resulting an impaired calcium-binding proteins homeostasis which will subsequently disrupt calcium signalling. Thereby, leads to neuronal loss and dysfunctional neural transmission (Foster & Kumar, 2002; Palotás et al., 2002). Hippocampal and cortical neurons that express high levels of calbindin exhibit increased resistance to A β -induced excitotoxicity, whereas calbindin-deficient neuronal populations show earlier synaptic dysfunction and degeneration (Palop et al.,

2003; Palop & Mucke, 2010). This pattern suggests that calbindin expression is a determinant of neuronal resilience, influencing why specific circuits deteriorate preferentially in AD.

Between neuronal synapses, calbindin is an important factor that aids in regulating short-term synaptic plasticity by influencing the amplitude and duration of intracellular Ca^{2+} transients following neuronal firing. However, mechanistic calbindin-dependent pathways were proved to be disrupted by $\text{A}\beta$ oligomers which in turn affects the stability of Ca^{2+} signalling leading to aberrant long-term potentiation (LTP) and enhanced long-term depression (LTD) (Rodríguez et al., 2009). Reduced calbindin exacerbates these effects by prolonged Ca^{2+} elevations which impair synaptic transmission and concurrently, accelerate spine loss (Mattson et al., 2000; Palop et al., 2003). This indicates the importance of calbindin as its loss contributes directly to early synaptic failure, a hallmark of AD progression.

Beyond neuronal potentiation and synaptic integrity, calbindin is also expressed during neuronal differentiation and maturation, making it a relevant marker and potential regulator of adult hippocampal neurogenesis. Newly generated neurons in the dentate gyrus undergo tightly-controlled calcium signalling to support proliferation, migration, synaptic integration, and survival, processes in which calbindin-mediated calcium buffering is critically involved (Brandt et al., 2003; Schwaller, 2010). In AD models, impaired neurogenesis has been linked to disrupted neuronal renewal and plasticity as calbindin correlates strongly with learning and memory integrative processes (Harkany et al., 2008; Prenderville et al., 2015). This was shown in the reported analysis on familial AD pathology that reflects strongly with a decreased levels of calbindin in the dentate gyrus of the brain (Palop et al., 2003).

Aside from affecting the neuronal signalling, implicated calbindin among the neuronal cells were observed to have correlated mechanisms with tau pathology in AD. There were previous experimental evidences showing the relationship between neurogenesis with tau pathology at which neuronal cells possess preserved calbindin expressions alongside with reduced tau pathology compared to the ones with calbindin-negative neurons in AD brains (Berridge, 2010). This proves there is a link between calbindin deficiency to both accumulation of amyloid and tau-driven neurodegenerative cascades that may cause cytoskeletal instability leading to hyperphosphorylations. Thus, upregulation of enhanced firing of intracellular Ca^{2+} will cause increase in

calbindin to counteract the degenerative processes hence aiding in limiting further tau phosphorylation and aggregation in AD brains.

Another underappreciated relationship in AD involves the influence of calbindin on neuronal lifespan and apoptosis. Sustained Ca^{2+} elevation activates apoptotic pathways through caspase signalling and calbindin acts by dampening Ca^{2+} -dependent enzyme activation, thereby promoting neuronal survival. This was proved by previous experimental studies that focus on knockdown of calbindin resulted with increased susceptibility to apoptosis under oxidative or amyloid stress, reinforcing its role as an intrinsic neurotherapeutic factor (Schwaller, 2010; Mattson, 2012). Either than that, calbindin also interacts with inhibitory network stability. GABAergic interneurons in the brain express calcium-binding proteins and calbindin-positive interneurons contribute to network synchronization and oscillatory activity critical for memory-encoding. As observed in AD models, reduced calbindin expression affects by disrupting excitation-inhibition balance, leading to hippocampal hyperexcitability recognized in early AD stages (Palop & Mucke, 2016). This dysfunctional of cognitive networks further impairs cognition and may contribute further towards neurodegeneration.

Evidences from human post-mortem studies and animal models consistently demonstrates a selective loss or downregulation of calbindin expression in AD, particularly in hippocampal neurons that are critical for learning and memory (Iacopino et al., 1992; Palop et al., 2003). Importantly, neurons that retain calbindin expression appear more resistant to AD-related pathology, supporting a neuroprotective role for this protein (Palop & Mucke, 2010). Despite that, calbindin remains underexplored as a functional contributor to neurogenesis and therapeutic response in AD. Most studies have focused on calbindin loss as a pathological marker rather than examining its dynamic regulation during disease progression or in any potential pharmacological intervention. Integrating calbindin as a molecular target alongside GFAP may contribute to discover potential enhancements in different stage of neurons in AD brains. Therefore, this offers a more comprehensive framework for understanding neuron-to-neuron interactions and therapeutic effectiveness in cannabinoid-based AD interventions for future perusal.

2.14 Neuroanatomy of the Hippocampus

The hippocampus is a major component of the brains of humans and other mammals. Hippocampus belongs to the limbic system and plays vital roles in consolidation of information from short-term memory to long-term memory and spatial navigation (Soya et al., 2011). Like the cerebral cortex, it is closely-associated as a paired structure with mirror-image halves in the left and right sides of the brain. In humans and other primates, the hippocampus is located inside the medial temporal lobe, beneath the cortical surface consisting Ammon's horn and the dentate gyrus (DG). Hippocampal formation is located deep within the parahippocampal gyrus of the temporal lobe and the floor of the third ventricle of the brain which consists of three parts mainly; hippocampus (hippocampal proper or Ammon's horn), dentate gyrus (DG) and subiculum (Andersen et al., 2006). The primitive form of three-layered cortical tissues play a role in mediating learning and formation of new memories with its primary cells are pyramidal cell with basal dendrites extending the ventricular surface and apical dendrites that extend towards the DG (Amaral & Lavenex, 2007; Spruston, 2008).

The pyramidal cells are arranged into a C-shaped orientation that is interlocked with another C shaped structure which is the DG. The hippocampal subfields are distinct sectors or regions classified as cornu ammonis 1 (CA1), cornu ammonis 2 (CA2) and cornu ammonis 4 (CA4) making up the whole structure of the hippocampus. These regions including DG, presubiculum and subiculum are the main parts responsible in creating a neural circuit, also known as trisynaptic circuit. The pyramidal cells located the closest to the subiculum are CA1, a region receiving collateral axons from CA3 pyramidal cells. CA4 is the last region located within the hilus of the DG with CA2 and CA3 regions are located in between CA1 and CA4 (Andersen et al., 2006; Amaral & Lavenex, 2007). However, learning and memory requires more interrelated components to transfer information. Thus, hippocampus is interconnected to the other regions of the brain through trisynaptic pathway consisting perforant and fornix pathways. Figure 2.19 shows the stages of neurogenesis consisting of proliferation, differentiation, migration, synaptic integration and survival phases. Meanwhile, Figure 2.20 shows the anatomy of hippocampus showing Ammon's horn (CA1-CA3) and dentate gyrus (DG).

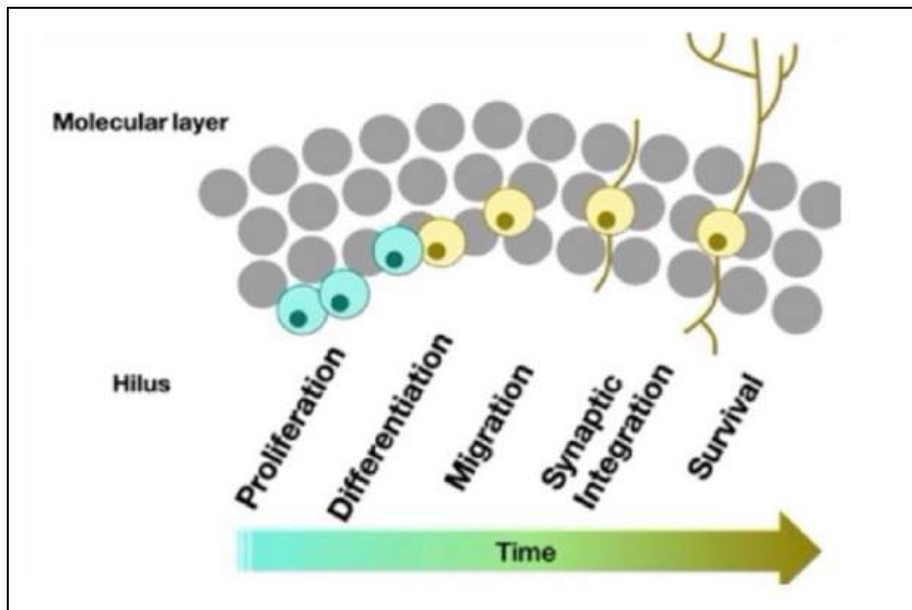


Figure 2.19 Stages of Adult Neurogenesis

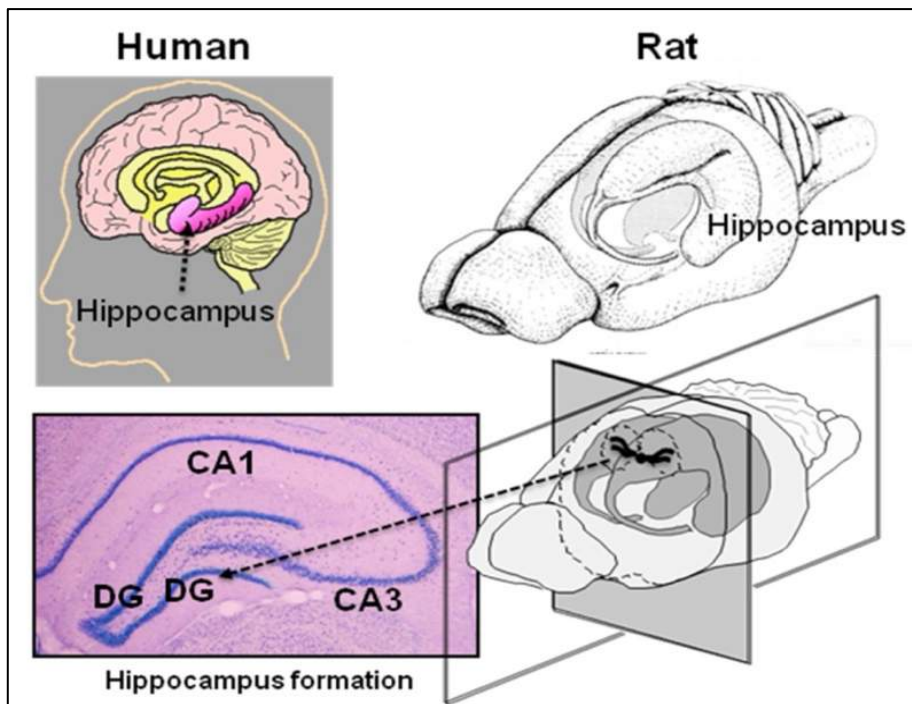


Figure 2.20 Anatomical Process of Hippocampus Showing the Ammon's Horn (CA1-CA3) and the Dentate Gyrus (DG) (Soya et al., 2011)

The trisynaptic pathway in the hippocampus is a neuronal circuit that connects three major structures; the entorhinal cortex, DG and CA3 region, ultimately influencing information flowing to CA1 region (entorhinal cortex → DG → CA3 → CA2 → CA1). This pathway requires the interconnection between three main synaptic connections; perforant path (entorhinal cortex → DG, mossy fibers (DG → CA3) and Schaffer collaterals (CA3 → CA1). Perforant pathway is mostly an inflow tract for the hippocampal formation that receives fibers from the entorhinal cortex of the parahippocampal gyrus and other parts of the temporal lobe that will be transmitted to the DG. This process contributes to memory, autonomic, hormonal regulation, motivation and the central regulation of emotional behavior. Therefore, if there are minor disturbances in these mechanisms then it will lead to inability to form long-term memories. Perforant mechanism starts with the initiation of the trisynaptic circuit that underlies the critical hippocampal memory processing.

This consists of progression from DG granule cells (which support pattern separation) to CA3 pyramidal neurons via mossy fibers, and crucially moving to CA2 and CA1 regions via Schaffer collaterals at which pattern completion and memory consolidation occur (Yassa & Stark, 2021; Denny et al., 2021). Meanwhile in fornix pathway, fornix is a major white matter tract that connects the hippocampus specifically CA1 and subiculum to mammillary bodies and anterior thalamic nuclei enabling memory retrieval and contextual association (Christiansen et al., 2020). Physiologically, alveus fibers converge forming fimbria near the medial border of the hippocampus. This fimbria leaves the hippocampus as the crus of the fornix, a structure that shapes curving posteriorly and superiorly beneath the splenium of the corpus callosum and around the posterior surface of the thalamus. Hence, degeneration of these perforant and fornix would lead to diminished cortical input to the hippocampus and weakening synaptic plasticity.

Aligned with AD progression, fornix atrophy correlates with early episodic memory impairment and hippocampal volume loss (Grothe et al., 2020; Liu et al., 2021). CA1 is the first region from which a significant output pathway goes into the layers of entorhinal cortex and its main output is to the subiculum. This region is the primary output node of the hippocampus, particularly by integrating these signals through dendritic compartmentalization allowing for coincidence detection and the formation of long-term potentiation (LTP) important for learning processes (Bittner et

al., 2019; Grienberger & Mage, 2020). Neurons in this region are highly responsive to signal strength changes and cellular activities especially those related in episodic and declarative memory. Recent studies suggest that hippocampus plays a role in temporal coding as well as which enabling the regions to not only store where events have occurred but also when were the occurrences (Lux et al., 2023). In early stage of AD, the marked episodic memory deficits and cognitive decline in CA1 have been relevant as its dysfunctional leads to dendritic spine loss, impaired long-term potentiation of neurons and reduced excitatory-inhibitory balance (Dumitrescu et al., 2020; Yang et al., 2021). Hence, maintenance of CA1 will not only crucially integrating transmit of hippocampal outputs but enhancing the precision and organization of learning and memory processes.

Meanwhile, CA2 region is situated between CA1 and CA3 and the morphology of the pyramidal cells in CA2 are distinguished looking similarly to those in CA3 region rather than cells in CA1. However, due to its small size and location is in between the other two main bigger regions, it is often mislook in neurological studies. Despite that, CA2 plays a significant role in receiving excitatory inputs and making sure its path are extended to the right region for continuous firing of the neurons to support long-term potentiation (LTP), essential for synaptic plasticity (Sheffield & Dombeck, 2019). CA2 has emerged as a unique subfield possessing strong resistance to synaptic plasticity compared to other subfields. CA2 receives input signals from entorhinal cortex and hypothalamus and sends projections directly to CA1. In AD, this is crucial as these would play their part in retrieving social memory and contextual encoding such as recognition of familiar individuals (Hitti & Siegelbaum, 2020). There are interferences in CA2 functional properties have been linked to psychiatric and neurodegenerative disorders, including schizophrenia and AD. As these diseases progress over time in patients, CA1 and CA2 showed to be one of the areas to be earlier compared to the rest and would affect the correlation in memory deficits and hippocampal atrophy (Sanfilippo et al., 2021).

For CA3, a key physiological functional of this region is encoding heteroassociative memories using its recurrent circuitry. This was supported by a recent hypothesis that postulates CA3 sequences are built pair by pair during memory encoding depending on the precisely timed input from the entorhinal cortex. This occurs when the synapses of the CA3 recurrent axon collaterals on the dendrites of the CA3 population to form a complete matrix of connections. Physiologically, CA3 receives

input from mossy fibers of the granule cells in the DG and also from cells in entorhinal cortex via perforant pathway. Pyramidal cells in CA3 region have unique type of dendritic spine called a thorny excrescence or thorn only found in this area and hilar mossy cells. These pyramidal cells will send some axons back to the DG hilus, however, mostly are projected towards CA3, CA2 and CA1 regions via schaffer collaterals. There are some recurrent connections are terminated in CA3 instead, but both recurrent connections and Schaeffer collaterals terminate preferentially in the septal area. With that, CA3 also responsible in sending a small set of output fibers to the lateral septum. CA3 overall has been considered to be the “pacemaker” of the hippocampus as much of the synchronous bursting activity associated with interictal epileptiform activity appears to be generated in CA3. In that case, CA3 excitatory collateral connectivity might be potent for this. When there is a signal transduction, the pyramidal cell axon collaterals will ramify extensively with the local regions around it and create excitatory connections with it.

As for CA4 region, or also known as the hilus of the dentate gyrus is a transitional zone situated between CA3 and DG. Neurons in CA4 also contains a mix of mossy cells and interneurons that modulate the excitability of DG and CA3 maintaining the excitatory-inhibitory balance necessary for synaptic plasticity and learning. The mossy cells would create excitatory projections which are in a feedback loop that regulates neuronal excitability, pattern separation and sparse coding essential for memory formation (Bui et al., 2018; Myers et al., 2022). The neuronal signalling in CA4 is predominantly glutamatergic allowing propagation and modulation of excitatory inputs contributes amplification and precision of memory encoding (Synder & Cameron, 2020). Furthermore, structural or functional disruptions among the mossy cells has been linked to the vulnerability in AD contributing to hyperexcitability and cognitive declines (Leal et al., 2020; Provenzano et al., 2022). Hence, other upstream areas will be disrupted functionally including those originating from CA4. Despite major critical functions of the other regions, CA4 still serves as a critical integrator within the entorhinal-hippocampal circuit that needs to be maintained its integrity and performance alongside with the other essential components of cognitive processing.

In DG, a tooth-like band of gray matter that lies in between fimbria and the parahippocampal gyrus is labelled as a primitive three-layered cortical structure. It receives afferent input from enthorhinal cortex (EC) via perforant pathway which synapse onto granule cells in DG. Granule cells generate sparse, highly selective

electrosignal firings to distinguish inputs (Yassa & Stark, 2011; Hainmueller & Bartos, 2020). Granule cells project their axons in a pattern of mossy fibers which made up of mossy cells forming feedforward circuit regulated by local inhibitory interneurons and excitatory mossy cells within the hilus (CA4 region) to modulate granule cell excitability and temporal precision (Senzai, 2019). Furthermore, neurons are depending on DG as it is a region that undergoes neurogenesis and exhibit enhanced synaptic plasticity particularly involved in encoding novelty, contextual fear memory and flexible learning (Anacker & Hen, 2017; Berdugo-Vega et. al., 2020). Any interferences through reduced neurogenesis, oxidative stress or amyloid pathology due to failure of cellular functions of granule cells will affect accurate memory formation and retrieval, even worser in already-diagnosed AD individuals as neurodegenerative effects might jeopardize the retain, recall and apply information essential for daily functioning, independence and quality of life.

2.15 Learning and Memory Circuit in the Brain

2.15.1 Memory Consolidation, Retention and Formation

In memory transmission, perforant pathway is a component of the trisynaptic pathway of memory transmission. It is the process which consists of signals from the entorhinal cortex reaching DG, a subregion important for pattern separation; the ability to distinguish between similar inputs in the brain (Hainmueller & Bartos, 2020). From DG, signals are transmitted via mossy fibers to the CA3 region, known for its recurrent excitatory circuits facilitating pattern completion that allows the occurrence of retrieving complete memories from partial cues (Rolls, 2016). This means that this mechanistic transfer of information will travel through all CA regions respectively until it reaches CA1 through Schaffer collateral pathways completing the trisynaptic pathway at which long-term potentiation (LTP), a primary cellular mechanism of memory encoding that strengthens synaptic connections in response to activity (Grienberger & Magee, 2020). Reaching CA1 region, the outputs are sent back to the entorhinal cortex and from there, there will be a chain of transmission and distribution to the prefrontal cortex and other cortical regions for memory consolidation. Figure 2.21 shows the flow of learning and memory circuit in the brain via perforant pathway, mossy fibers and Schaffer Collaterals from cortex to CA1 region.

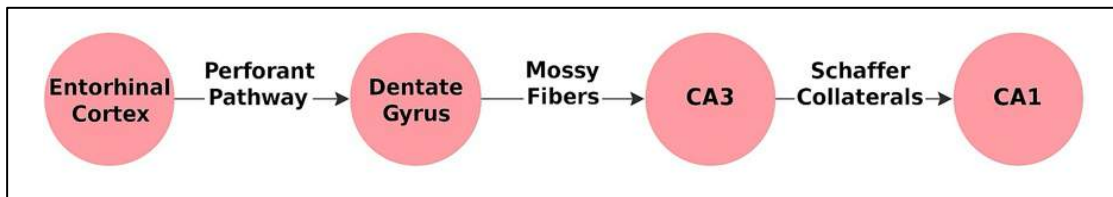


Figure 2.21 Neural Circuit of Learning and Memory (Nakazawa et al., 2016; Palop & Mucke, 2016; Eichenbaum, 2017)

Creation of memories is through complex pathway involving changes in the strength of connections between synapses allowing the brain to adapt and learn new information. In the process of creating new memories and retaining the memories in the brain requires a string of processes. The brain acts as a hub for encoding, consolidating and retrieving episodic and spatial memories. Specifically, hippocampus is crucial for forming new declarative memories especially those related to episodic memory (facts and events) and for spatial navigation. It is required in converting short-term memories into long-term memories through consolidation in neocortex. In the hippocampus, there are two main processes vital for memories; memory consolidation and memory retention. Memory consolidation is a process transforming short memories into long memories requiring strengthening and stabilizing newly formed memories, increasing its resistance to interference and integrating into existing memory networks. Memory consolidation is defined as the stabilization of memories after initial acquisition (Dudai et al., 2015). This process is enhanced by hippocampal-neocortical by transforming the memories from short-term into long-term memories especially for spatial and episodic memories. The strength of synaptic connections within the trisynaptic pathway can be modified and enhanced through learning and experiences, a process called synaptic plasticity.

This process needs the brain to reactivate and reorganize traces of memory, normally with the aid of proper sleep. This aims to possess stable memories that can be retrieved later and without consolidation, memories would easily be lost or undergo distortions. In AD, memory consolidation is significantly impaired due to the pathological disruptions in these memory networks (Busche & Hyman, 2020; Palop & Mucke, 2016). Furthermore, sleep disturbances, a common thing among AD patients prevent effective memory replay and further affecting the downstream processes by hindering the formation and stabilization of memory traces which lead to learning and

memory deficits. Even though, memories are consolidated through time via hippocampus in forming memories but neocortex gradually will take over its function as becoming the storage of memories. Notably, memory consolidation is influenced and modulated by neuromodulation involving dopamine and noradrenaline. Memory formation begins with the processing of sensory and contextual information by the association cortices of the brain. The information will be relayed to the entorhinal cortex which serves the main gateway into the hippocampal formation.

In the brain, there are three types of memory consolidation namely synaptic consolidation, systems consolidation and reconsolidation. Synaptic consolidation occurs within hours of learning and involves strengthening of specific synaptic connections through long-term potentiation (LTP) and one more, systems consolidation which unfolds over days to weeks. This process requires memory traces from the hippocampus to transfer gradually among the areas in the cortex for long-term storage (Wang & Morris, 2010; Dudai et al., 2015). Based on their mechanism, synaptic consolidation as the name suggest it occurs at the synaptic level in order to increase its efficiency. This memory consolidation requires short-term memories are slowly transformed into long-term stable representations. During the early-phase, long-term potentiation (LTP) strengthen specific synaptic connections primarily involving the synergy of NMDA receptor activation and calcium influx that will trigger intracellular cascades (Takeuchi et al., 2014; Bliss & Collingridge, 2013).

Next stage is the systems consolidation which involves gradual transfer of memory from the hippocampus to the cortex. Over time, these hippocampus-dependent memories will gradually transfer and integrate themselves into neocortex for storage specifically in the prefrontal, temporal and parietal cortices. Interestingly, this process is facilitated during offline brain states such as during slow-wave sleep (SWS) at which this is the time that memory traces are reactivated and replayed from the hippocampus to the cortex (Diekelmann & Born, 2015; Klinzing et al., 2019). Finally, the last stage is reconsolidation referring to the process at which the existing memories might be vulnerable to change or modification when retrieved. Thus, entorhinal cortex acts as an important gateway for communication between hippocampus and cortex. Via this route, information from neocortex enters via perforant pathway passing through all regions in CA and DG, respectively. Finally, the information will exit via the subiculum and fornix (Suh et al., 2011; Bartos & Vida, 2018).

As for memory retention, it is defined as the ability of the brain to maintain and retrieve these consolidated memory traces over time. This involves in the maintenance of brain integrity of previous strengthened synaptic pathways along with the reactivation and recognition during recall. There are factors affecting memory retention such as sleep, emotional salience, repetition and neurochemical modulation (Sekeres et al., 2018; Klinzing et al., 2019). Different from consolidation where it is an active process of needing the brain to reorganize memory representations, retention is more to relying on the stability and accessibility of the memories made or representations over extended periods. Both memory consolidation and retention rely on synaptic plasticity mechanisms such as LTP and neurotransmitter systems via receptors especially for glutamate, acetylcholine and dopamine (Lisman et al., 2018). However, in AD, soluble A β oligomers will disrupt memory consolidation through impairing synaptic LTP, affects functional NMDA receptors and reduce spine density (Mucke, 2016; Busche & Hyman, 2020). Meanwhile, tau pathology disturbs axonal transport and microtubule stability and interfering with the neuronal connectivity necessary for memory consolidation and recalling (Hoover et al., 2010). These efforts in memory recalling might be a challenge towards AD brains as individuals often experience sleep disruptions that interfere with memory replay and coordinated hippocampal-neocortical dialogue necessary for retention (Mander et al., 2017). This leads to destabilization and deterioration of neuronal synapses and structures.

Aside from CA regions, granule cells in DG also play a pivotal role in memory retention, particularly for episodic and spatial memory through adult neurogenesis at which new neurons are generated. The newly formed cells are more plastic compared to the old ones, these may integrate among the existing cells into the neuronal circuit allowing memory renewal and long-term storage (Anacker & Hen, 2017). In the hippocampus, CA3 region acts a buffer in ensuring partial retrieval inputs can reactivate entire memory traces via short-term memory retention and pattern completion (Rolls, 2016). This is very important for memorial storage and any disturbances disrupt this hippocampal-cortical coupling mechanism may lead to deficits in learning and memory, as expressed in AD individuals. In AD pathology, synaptic losses in CA and DG regions along with the disruption in neurogenesis results in weakened or memory traces loss, hence, contributing to more deteriorations as the disease progresses (Zhao et al., 2019; Moreno-Jiménez et al., 2019). Figure 2.22 shows the trisynaptic pathway related to learning and memory from entorhinal cortex to the dentate gyrus.

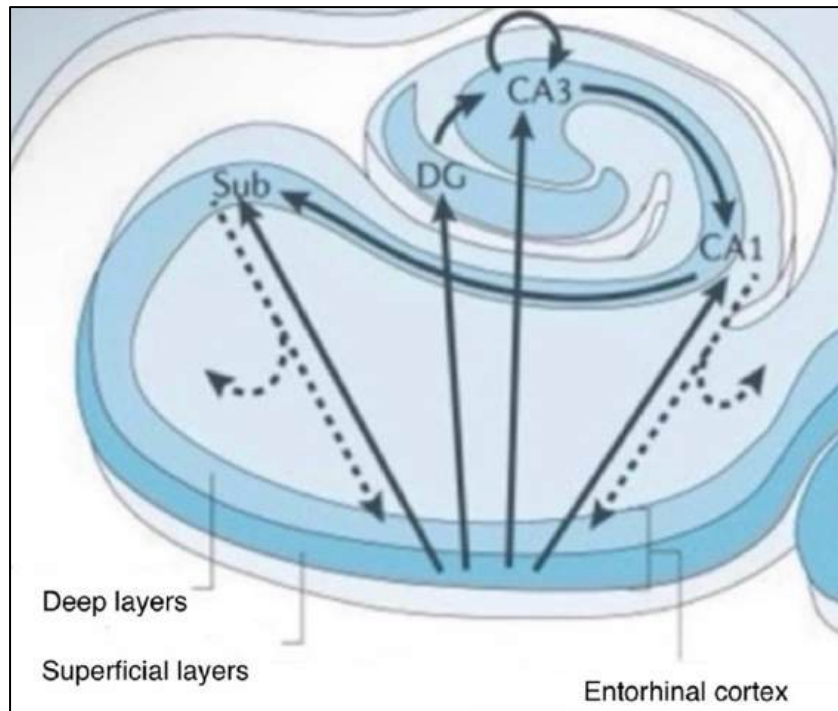


Figure 2.22 Trisynaptic Pathway Related to Learning and Memory Involving Information Flowing from Entorhinal Cortex to the Dentate Gyrus (DG), CA3, CA2 and CA1 Regions Travelling through Subiculum and Other Brain Areas (Scott et al., 2011).

2.15.2 Neural Plasticity and Metaplasticity

Neural plasticity or known as neuroplasticity is defined as the ability of the brain to reorganize its structure, function and connections in response to intrinsic and extrinsic stimuli. In the hippocampus, a region critical for creating declarative memory and spatial navigation; neural plasticity encompasses several dynamic processes that alters its mechanistic mediation such as synaptic plasticity (changes at the synapse level), structural plasticity (growth or retraction of dendrites and spines), homeostatic plasticity (changes according to stimulation), non-synaptic plasticity (due to the changes in functions or expression of its ion channels) and neurogenesis (generation of new neurons, primarily in the CA and DG regions). These forms of plasticity are able to modify and enhance the regulation of forming new memories and adapt to new learning environments (Gonçalves et al., 2016; Mateos-Aparicio & Rodríguez-Moreno, 2019).

As for synaptic plasticity, it is the mostly known form of neuroplasticity as it occurs almost all the time. It involves changes at the synapse at which acts as the junction between neurons where they communicate between each other. Also, synaptic plasticity is important for the brain in learning new skills, acquiring information and forming memories. Speaking of the brain, it plays a vital role in the development of the brain as well as its reorganization responding to stimuli such as neuronal injury or damage. The key mechanism of synaptic plasticity is long-term potentiation (LTP) at which it is a sustained strengthening of synapses after high-frequency stimulation. There are factors that need to be considered as the process can be affected by stress, trauma and substance use.

Synaptic plasticity is mostly consist of either long-term potentiation (LTP) and long-term depression (LTD) at which the synaptic connections is either increased or decreased respectively. The occurrences are primarily at the hippocampus and cortex mediated by N-methyl-D-aspartate receptor (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors via cAMP response element-binding protein (CREB) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) pathways to enable experience-dependent learning, memory consolidation and contextual memory recall (Nicoll, 2017; Park & Poo, 2013). Aside from synaptic plasticity, there is another type of communication that plays a significant role between neurons. Structural plasticity complements the rest by modifying the architecture of neural circuits by targeting in enhancing the dendritic spine density and synaptic remodelling (Chidambaram et al., 2019). These key processes in neural plasticity are crucial as the brain is always developing and it is in the most need for enhancement in learning and memory, especially for brain recovery after injuries or diseases like Alzheimer's disease. This type of plasticity is defined as the physical remodelling of neuronal connections such as dendritic spine growth, axonal sprouting and synaptogenesis (Koleske, 2013). So, it plays a huge role in neural network adaptability and memory storage in regions exquisite for learning and memory like hippocampus, cortex and dentate gyrus.

There are researches that have highlighted structural modifications support long-term memory persistence and adaptability throughout life (Mostany et al., 2018). For example, the process of brain changes after injury such as AD, stroke and traumatic brain injury (TBI) definitely need restoration of functional neurons to accommodate the neurodegeneration that have affected along the disease progression. Structural changes

will accompany these functional deficits by ameliorating dendritic spine loss and reduced synaptic density that highly correlates with memory impairments (Terry et al., 2020). Lastly, homeostatic plasticity as the name suggests this process aims to maintain overall neural stability by adjusting synaptic strengths or excitability in response to chronic neuronal changes, especially in neurodegenerative diseases like AD. This differs with synaptic plasticity that enhances or limits the synaptic strengths, homeostatic plasticity aids in maintaining balance in neural networks and avoiding hyperexcitability silencing. This is important because as AD progresses, synapses degenerate, both short-term and long-term potentiation mechanisms deteriorate and consequently disrupting the stability of hippocampal circuit to encode, store and retrieve information.

One of the main reasons there is a need in maintaining or re-enhancing neural networks is because neural silencing might occur, if there is no prompt to regulate it. Neural silencing refers to inhibition or suppression of the activities of neurons. This includes intersecting the transmission by preventing firings signals through methods like optogenetics, chemogenetics or even manipulation of the related cellular mechanisms in order to inhibit action potentials and its corresponding synaptic transmissions between neurons. So, if the homeostatic plasticity is kept at optimum at all times, overall neural stability in synaptic strengths or excitability to changes will be maintained. However, neurodegenerative diseases have higher chances of possessing excitatory/inhibitory imbalances. This describes the major differences between synaptic plasticity (strengthens or weakens specific synapses) and homeostatic plasticity (ensures neural balance and avoids hyperexcitability). These can be achieved through synaptic scaling and modulation of intrinsic excitabilities among synapses (Turrigiano, 2017).

Other than synaptic plasticity and homeostatic plasticity, there is another aspect of neuroplasticity that needs attention upon it as it involves modulation of functions among neurons. It is termed as intrinsic plasticity or non-synaptic neuroplasticity in a more general term, this process occurs when neuronal excitability is due to the changes in functions or expression of its ion channels. The changes might also include alterations in its resting membrane potential or action potential threshold that affects how neurons respond to incoming signals and can influence on its overall contribution to neural circuits (Debanne et al., 2019). Example of intrinsic plasticity includes individuals learning a new skill causing changes in the excitability of neurons in the motor cortex

allowing for more precise movements (Shim et al., 2018). As for non-specific neuroplasticity, the situation might be post-stroke at which the brain may need to reorganize its structure and function to compensate for the damaged area. This mechanism depends on the changes in synaptic connections and changes in the intrinsic properties of surrounding neurons.

Aside from neuroplasticity, there is another more complex process which is called metaplasticity or may be described as plasticity of synaptic plasticity. This refers to a higher degree of regulation at which prior synaptic activities can modulate the threshold for future LTP or LTD. According to Abraham et al., (2019), metaplasticity is able to regulate hippocampal response to receive repetitive inputs and prevents saturation of memory capacity. However, even if metaplasticity does not occur, both short (STP) and long term potentiation (LTP) are highly efficient to trigger the hippocampus for neuronal potentiation. STP is a transient increase in synaptic strength lasting from milliseconds to a few minutes. This occurs as there is an increased calcium influx and enhancement of neurotransmitter release that arises from presynaptic mechanistic processes. STP involves a sequent of reactions such as facilitation, augmentation and post-tetanic potentiation at which all of those are relying on calcium influx at the presynaptic terminals (Zucker & Regehr, 2002; Jackman & Regehr, 2017). During the neuronal potentiation, it is primarily postsynaptic and involves the activation of NMDA receptors, calcium influx and downstream signalling cascades including CaMKII, CREB and brain-derived neurotrophic factor (BDNF) to aid gene expression and dendritic spine growth (Nicoll, 2017; Nabavi et al., 2014). Even though STP is essential for signal transmission and memory encoding, but the duration of transmission is considered as rapid and non-long lasting. Unlike LTP, the potentiation is widely considered as long-term and its consolidation of learning experiences will steadily change into stable memories. However, STP and LTP is closely related to each other as STP is able to serve as a precursor to LTP due to repeated stimulation. With that, it has been proved that STP and LTP are interdependent to each other in order to contribute to synaptic plasticity and co-act together maintaining neurotransmitter dynamics.

In AD brains, cognitive declines significantly impair the functionality of neurons as oligomers of A β have been shown to affect NMDA receptor function, block the stimulation of long term potentiation and facilitate aberrant long-term depression (LTD). This causes imbalance in synaptic maintenance leading to weakened synapses (Palop & Mucke, 2016; Li et al., 2018). This further aiding in AD progression as it

couples with tau pathology at the same time disrupting intracellular signalling and impairs synaptic scaffolding proteins, degrading LTP even further. According to Wang et al., (2017), it has been proved that early A β accumulation impairs STP as presynaptic vesicle cycling and calcium homeostasis is disrupted. Thus, this creates failure in the rapid synaptic signalling. To prove this even further, according to *in-vivo* studies either through environmental enrichment or learning tasks, there were positive results in studies that used naturalistic stimuli has successfully induced potentiation in specific hippocampal subfields highlighting the effects in responsive of the circuit (Sakai et al., 2021). Furthermore, presynaptic potentiation also has been observed at mossy fibres of CA3 synapses proved by an increased probability of neurotransmitter release at which non-NMDA receptor mechanisms contribute to spatial memory and rapid encoding (Nicoll & Schmitz, 2005; Rebola et al., 2017).

2.15.3 Memory Retention in Neuronal Circuits

Memory retention in the hippocampus relies on stabilization and maintenance of neural circuits formed during encoding. Once memory is encoded via long-term potentiation (LTP), the activity must be consolidated and maintained throughout the ongoing molecular and structural modifications. LTP in CA1 region starts with the induction of synaptic tagging and capture, a process at which occurs due to activated synapses are “tagged” in need of plasticity-related proteins (PRPs) such as BDNF and CaMKII, which are essential for sustaining long-term synaptic changes (Redondo & Morris, 2011; Lisman et al., 2018). Furthermore, these proteins aid in supporting the maintenance of dendritic spine morphology, important for memory persistence over time. According to Hebb (1949), the neurophysiological changes underlying learning and memory processes would rise through three stages; synaptic changes, formation of cell assembly and formation of phase sequence, at which stimulates cognitive abilities in the brain. This mechanism occurs when synaptic efficacy increases triggering the coordinated firing of the pre- and post-synaptic cell (Hebb, 1949). In synaptic plasticity, Hebbian and non-Hebbian potentiation are two distinct forms of synaptic plasticity that affect directly the strength of the neuronal communications. Hence, it is termed as Hebbian plasticity, or known as associative plasticity that synapses are strengthened when pre- and postsynaptic neurons fire simultaneously. Non-Hebbian plasticity postulated that strengthen or weaken synapses is based on the postsynaptic activity

alone, independent from the neurons firing from the presynaptic input. This may occur due to mechanisms such as synaptic scaling.

Synaptic scaling is a phenomena that occurs when neurons might increase in the number of AMPA receptors on its postsynaptic membrane to increase the overall strength of its synapses. There are few types of neuronal plasticity that are important for the brain including intrinsic plasticity, heterosynaptic plasticity and homeostatic plasticity. Intrinsic plasticity is when a neuron might change due to its own intrinsic excitability of action potentials, while heterosynaptic plasticity is a potentiation at one synapse that can lead to changes in the strength of the other synapses on the same neuron. As for homeostatic plasticity, it can occur through non-Hebbian plasticity where the brain adjusts synaptic strengths to maintain stable network activity. These non-Hebbian forms of potentiation such as synaptic tagging and capture (STC) occurs by allowing stronger potentiated neurons to spread and activate the weakly activated synapses. This will positively create memory linkages at which memories formed are close in time through the overlapped communications (Redondo & Morris, 2011). From this, there is one more type of synapse that emerged, called as homeostatic synaptic scaling that functions in making adjustments in the synaptic strength to maintain stability in response to prolonged changes in the network activity, especially in neuronal circuits at the DG and CA regions (Turrigiano, 2017).

2.15.4 Memories Affected in AD

Learning and memory is a complex cognitive function that involves a whole circuit to encode, store and retrieve information. It consists specialized of brain regions and neural circuits to make a whole complete neural flow. In general, memory is divided into sensory memory, short-term memory (STM) and long-term memory (LTM) and the flow of converting STM into LTM is done by perforant pathway. STM is can be maintained or enhanced through attentional control and if reinforced through rehearsal or emotional salience, STM can be encoded into long-term memory (D'Esposito & Postle, 2015). On the other hand, LTM is a memory process over extended periods which can be further divided into explicit (declarative) and implicit (non-declarative) memory systems. However in AD, long-term memory is the critical part of learning process as it occurs in the hippocampus and cortex and has been affected throughout AD progression. The neurodegeneration results in impairments in the trisynaptic circuit

responsive for encoding, retrieval and storage affecting the distinct subdivisions of memory such as episodic memory, working memory, semantic memory and procedural memory.

Episodic memory is the autobiographical recall of personal experiences embedded in specific spatial and temporal contexts mostly relying on CA1 and CA3 subregions of the hippocampus and the medial temporal lobe structures (e.g., entorhinal cortex). This type of memory allows the brain to mental time travel and have the capacity to re-experience the past and imaging the future. While in AD patients, individuals have difficulty in forming new episodic memories and struggle to retrieve recent personal events. Due to amyloid-beta plaques and tau tangles tend to accumulate in the hippocampus, functional magnetic resonance imaging (MRI) studies have showed decreased connectivity in the default mode network (DMN). At norm, this brain network becomes more active during rest and while the brain is not focused on external tasks. Yet it undergoes self-referential thinking specifically causing a reduced functional connectivity in the cortex and hippocampus. Comparing between mild and moderate AD, more extensive decreased connectivity can be seen in moderate AD rather than mild AD. Disturbances to these areas would cause diseases like AD resulting profound episodic memory loss (Zhao et al., 2023) making episodic memories categorized as the mostly severely impaired in early AD.

The next type of memory affected is working memory. In the brain's system, this functions to temporarily hold and manipulate information. Essential for reasoning, learning and goal-directed behaviour that requires a network of brain regions include prefrontal cortex, parietal cortex and anterior cingulate cortex specifically. Differ from long-term memory, working memory operates on a short timescale (from seconds to minutes), however, its neuronal deficits are becoming more apparent as the disease progresses even further. Individuals that suffer from this would find themselves struggling to hold multiple tasks in mind especially when it needs updating or manipulating information. Furthermore, since working memory is heavy depending on the frontal lobes, AD neurodegenerative mechanism will suffer an executive dysfunction that affects frontal lobes would impair ability to organize and control attention along processing memories. Evidently, functional MRI and electroencephalogram (EEG) studies have showed reduced activity and disrupted connectivity in the frontoparietal networks that are important in maintenance of working memory.

Other than the ability in making reasonings on information and processing memories, there is also semantic memory that encompasses general world knowledge including meanings of words, concepts and factual information that is greatly affected in AD. Unlike the other types, semantic memory tends to involve in specific personal experiences and usually the first to undergo degradation. However, it deteriorates gradually over time, often begins in the early to moderate stages of AD. Individuals may exhibit difficulty in word-processing activities; word-finding difficulties (anomia), loss of category fluency, confusion between semantically related concepts (e.g., misinterpreting a tiger as a lion). However, if it is not treated and the disease keeps on progressing hence there would be effects in core conceptual knowledge at which this will disturb one's understanding regarding objects, tools or emotions, and even worse high chances can lead to memory loss as semantic knowledge serves as a foundation for encoding, organizing and integrating new information.

Semantic networking in the brain, particularly in the anterior and lateral temporal lobes including anterior temporal pole acts key hubs for storing and retrieving semantic information (Wang et al., 2020; Pini et al., 2023). This is the reason why AD patients have difficulty in understanding new concepts, categorizing new stimuli or applying prior knowledge to learning tasks. This leads to consequent increased difficulties or inefficiency in both explicit learning (e.g., learning new facts) and implicit learning (e.g., associating objects or words according to their functions). In terms of pathophysiology of AD, disruptions in semantic memory is due to neuronal degeneration in the temporal lobes particularly inferior and lateral anterior temporal cortices, which are the areas highly vulnerable towards tau accumulation and atrophy in AD. Hence, creating disturbances in the integral parts of the default mode network (DMN) in creating internal thought, memory retrieval and semantic processing. In recent studies, tau pathology have been successfully proved to affect functional connectivity between memory systems exacerbating further cognitive declines (Jefferies et al., 2021; Pini et al., 2023). These memory systems were proved to link to atrophy in the left inferior frontal gyrus and related frontotemporal circuits (Jefferies et al., 2021).

Another interesting standpoint, semantic memory is not primarily related or let alone depending on hippocampal neurogenesis, however there is an indirect link between neurogenesis, neuroplasticity and semantic learning. Impaired hippocampal neurogenesis in AD patients have shown reduced adaptive capacity, affecting the

integration of new conceptual knowledge with existing ones (Mu & Gage et al., 2020). As neurogenesis is primarily occurring in DG of the hippocampus, the decline in synaptic plasticity and glial support in neocortical areas have limit the potential for semantic memory recovery or compensation. This contributes in disruption of overall cognitive rigidity and diminished learning flexibility. However, there is no rigid evidence suggesting semantic memory degradation is directly connected due to reduced neurogenesis, but the loss of plasticity across the brain networks significantly contributing towards semantic learning deficits. Thus, understanding these mechanisms is essential for developing interventions aim to preserve semantic function and support compensatory learning in individuals with AD.

Lastly, memory can be categorized into procedural memory, a stage of memory formation often preserved until the late stage, physiologically linked to basal ganglia, cerebellum and premotor cortex. Procedural memory refers to the implicit memory system responsible for learning and performing motor skills and habits, such as riding a bicycle, typing on computer or even brushing teeth. Interestingly, its pattern of neurodegeneration differs with episodic and semantic memory in AD, procedural memory is often preserved during early and moderate stages. This is because procedural memory primarily depends on brain areas that are relatively less affected by early AD pathology (Chau et al., 2019; Schwarb et al., 2021). The regions include those important in facilitating motor learning and routine behaviors allowing individuals to retain their familiar tasks or daily activities stimulating their ability to recall personal experiences or factual knowledge. However as AD advances, procedural memory also begins to deteriorate especially when the progression spreads to frontal and subcortical areas involved in motor planning and control.

The basic motor routines may still be functional, but a more higher degree of AD would result in compromised recently acquired motor skills. Additionally, declined procedural memory will affect implicit learning system leading to complications in rehabilitation strategies for AD. These therapies focus on formation of habits and repetition routines so when procedural memory is greatly affected, the chances in developing and maintaining new motor routines are greatly reduced making it harder for rehabilitation efforts. In AD, accumulation of tau and amyloid plaques will disrupt neural circuits specifically frontostriatal circuits and causing patients to exhibit increased motor and cognitive inflexibility. These affects not only existing neural circuits in procedural memory but impairs the ability in forming new habits and learning

new skills, which are critically needed for AD brains (Becker et al., 2023). In terms of its relation with hippocampus, the procedural memory is less reliant on neurogenesis in hippocampus, whereas it relies on synaptic plasticity and neural connectivity in motor-associated cortical and subcortical regions. This sums up the dependence of procedural learning onto neuroplastic mechanisms to increase the brain's adaptability.

Comparing the effectiveness of compensatory pathways between procedural and episodic memory, procedural is not as strong as the latter but both result in overall decrease in neuroplasticity and glial support across the brain as AD progresses becoming worse. Procedural learning actually take benefits from intact white matter tracts and dopaminergic systems. In later stages of AD, these two systems would deteriorate and disrupt the whole processes undermining motor learning and habit formation (Mu & Gage, 2020; Bettio et al., 2019). Although, physiologically the motor system is more resilient but the cumulative effects of neuronal death, reduced neurotransmitter availability and circuit disintegration lead to declining path in procedural capabilities. On another degree, this degeneration further impacts both daily functioning and effectiveness of therapeutic interventions based on routine and habit. Even though the interdependence relationship between procedural learning and neurogenesis is not as enhanced compared to declarative memory systems, it still suffers from the downstream effects in AD such as neuronal death and diminished neuroplasticity pathways across cortical-subcortical regions (Kanishka & Jha, 2022).

2.16 Other Affected Brain Regions in AD

2.16.1 Neocortex

Maintaining the structural integrity of the brain is crucial for optimal memory and learning functions due to its central role in encoding, consolidating and retrieving information. The hippocampus, particularly the CA1, CA3 and DG subregions is responsible for mechanisms underlie learning. However, there are a few other regions that are related that comprises cognitive functions in the brain; neocortex, amygdala, basal ganglia, cerebellum and cerebrum. Neocortex is the outermost layer of the cerebral hemispheres that plays a vital role in higher-order cognitive functions including attention, language, decision-making and long-term memory storage. The process begins when sensory input is received and transmit the processed information to

associative cortical areas (Yamins & DiCarlo, 2021). In these regions of the neocortex, the brain integrates multisensory information with prior knowledge and context. This is an area that are heavily involved in memory and attention as the brain temporarily hold and manipulate information essential for decision-making as well as understanding new concepts, depending on how the information is interpreted and stored (D'Esposito & Postle, 2023).

As for long-term learning and memory formation, neocortex plays a vital role in strengthening connections between neurons via long-term potentiation (LTP) through reinforcing specific neural pathways that encode stable memory representations over time (Takeuchi et al., 2023). With this, memories are gradually transferred and reorganized by reactivating of memory traces and reinforcing neural cortical circuits (Ghosh et al., 2022). Prefrontal cortical areas are able to coordinate memory recall in AD brains, however the retrieval is dynamic as memories formed can be updated or reconsolidated based on experiences and flexible adaptation. This will ameliorate the advancements in AD pathology especially the accumulated amyloid plaques and tau tangles in the neocortical layers that responsible in impairing synaptic transmission, reducing plasticity and ultimately inhibiting cortical neurogenesis causing the brain to lose its abilities in thinking and recalling (Pini et al., 2023; Badhwar et al., 2017).

2.16.2 Amygdala and Basal Ganglia

Amygdala is a medial temporal lobe structure that has significant role involving emotional learning and memory through integrating sensory stimuli with emotional significance, helping prioritizing certain experiences to be encoded into long-term memories. The amygdala communicates closely with hippocampus, prefrontal cortex and thalamus in modulating attention, memory consolidation and behavioural responses. Memory and learning functions act through activation and enhancement of memory storage via stimulation noradrenergic and glucocorticoid systems. Importantly, this causes an increase in synaptic plasticity of the amygdala and hippocampus (Phelps & Hofmann, 2020). In AD, amygdala shows early atrophy and tau pathology that leads to a loss of emotional salience in memory and reduced affective regulation (Piulin et al., 2019). On an interesting note, this degradation contributes to impairments in learning processes such as emotional blunting, impaired social cognitive and dampened

response to emotionally significant cues which will be hard for individuals that are affected by AD. Compared to other vital regions for learning, amygdala is not known as a site of adult neurogenesis, however, its regulation in terms of hippocampal plasticity can indirectly affect neurogenesis mechanistic pathway in DG.

Basal ganglia, a group of subcortical nuclei including the caudate nucleus, putamen and globus pallidus are primarily known for their role in motor control, but also important for procedural learning, habit formation and reward-based memory. The basal ganglia receive inputs from the cortex and relay processed information back via the thalamus forming loops that support decision-making, skill acquisition and cognitive flexibility (Yin & Knowlton, 2021). In the early AD stage, basal ganglia are less severely affected than the hippocampus or cortex yet changes in the neurons in basal ganglia may cause changes that impair memory functions such as dopaminergic deficits, synaptic loss and disruption of cortico-striatal connectivity (Guerreiro et al., 2022). Consequently, these altered effects will impair procedural memory and the ability to learn from reinforcement that lead to deficits in tasks requiring attention shifting, motor learning and adaptive behavior (Schwarb et al., 2021). Furthermore, the relationship with AD is proven to be even stronger when amyloid and tau pathology have been successfully observed in basal ganglia contributing to circuit dysfunction and executive impairments seen in advanced stages of the disease. Dopaminergic signalling is also shown to be disrupted among the basal ganglia degenerating the process for reinforcement of learning, specifically the brain's ability to learn from feedback and reinforcement. Although, neurogenesis is not functionally prominent to be involved among basal ganglia but the loss of striatal plasticity and white matter integrity affects the cellular communication between neuronal cells in the memory-related regions.

2.16.3 Cerebellum

Cerebellum is a region that communicates with the cerebral cortex, basal ganglia and limbic system in the brain through dense afferent and efferent connections, thereby signifying its role in broader cognitive networks involved in attention, working memory, motor coordination and language processing (Koziol et al., 2022). Traditionally, cerebellum is associated with motor coordination and balance which are vital in cognitive timing, prediction and procedural memory. Recent evidences suggests

that there are connections between cerebellum with implicit learning and attention modulation. In normal condition, learning and memory pathway in cerebellum primarily involves its Purkinje cells, which are the main output neurons of the cerebellar cortex. These cells receive excitatory input from parallel fibers (originating from granule cells) and climbing fibers. Recent research has shown that cerebellum was functionally and structurally affected especially in moderate to advanced stages of the disease; neurodegenerated structures of Purkinje cells, granule cells and deep cerebellar nuclei. Thus, previous studies have strongly showed that disturbed functions due to A β accumulation has been linked to reduced cognitive and affective processing (Jacobs et al., 2021). Other than that, the capacity of the cerebellum in supporting motor learning and procedural memory would be impaired as well. This actually modulates cortical and subcortical activity patterns and may influence plasticity indirectly through these pathways.

In another prospective, AD-related disruption of cerebello-cortical loops, especially the connections between cerebellum and prefrontal cortex has been associated with decreased cognitive efficiency and coordination of thought processes. These were proved further based on the reduced connectivity and hypoactivation in the cerebellum during cognitive tasks in AD patients, suggesting the cerebellar impairments leads to cognitive decline, not just only to motor symptoms (Guell et al., 2022). Cerebello-cortical loops, particularly those connecting the cerebellum to the prefrontal cortex are increasingly recognized as essential networks for higher-order cognitive processing including attention, working memory, executive function and coordination of thought. In healthy individuals, cerebellum helps in predicting, modulating and optimizing the flow of cognitive networks and these pathways are related reciprocally. Neural signals from prefrontal cortex will be sent to cerebellum via pontine nuclei and processed through cerebellar cortex and deep nuclei which will be relayed back to the prefrontal cortex via thalamus. This occurs in a continuous feedback manner as to allow cerebellum to refine and regulate prefrontal activity in real-time (Schmahmann et al., 2019).

As AD progresses, recent neuroimaging and postmortem studies have resulted with progressive disruption of cerebello-cortical loops that associates with cognitive inefficiency at which the ability to manage brain activities reduced. Inefficiency in these scopes would lead further difficulties in attention shifting, planning and decision-making. In AD patients, MRI studies have proved reduced connectivity and

synchronization between cerebellum and prefrontal cortex in AD patients correlating with impaired executive function and cognitive flexibility (Guell et al., 2022). These impairments were caused by AD most-known pathological basis including all of which compromising the integrity of cerebello-thalamo-cortical pathways due to A β and tau deposition, Purkinje cell loss, white matter degeneration and thalamic dysfunction. As a result, the cerebellum can no longer involve in modulations of prefrontal processes in the brain causing disorganized or slowed thinking, impaired judgement and decreased cognitive adaptability signifying hallmarks of advanced cognitive decline in AD (Jacobs et al., 2021). Loss of cerebellar modulation further affects the brain capacity for error prediction and correction, processes necessary for learning, behavior regulation and fluid thought. Thus, cerebellum being one of the factors of AD have shown its ability in offering a crucial perspective on the multidimensional nature of the disease.

2.16.4 Cerebrum

Cerebrum is the largest part of the brain comprising about 80% of its weight, responsible for higher-level cognitive functions. The area can be divided into two hemispheres, with each hemisphere controlling movement and sensation on the opposite side of the body. It encompasses the neocortex limbic system and subcortical structures including hippocampus and entorhinal cortex which function as the primary regions for conscious cognition, memory, language and voluntary motor function. In normal condition, these regions work together in an integrated network to process sensory information, encode and consolidate experiences and guide decision-making in the brain. The prefrontal cortex is essential for executive functions; attention control, working memory and planning while the hippocampus acts on formation of new declarative memories. Thus, intercommunication between these allows the brain to coordinate thoughts, shift attention flexibly and retain relevant information for immediate or long-term use (D'Esposito & Postle, 2023).

Interestingly, in AD, cerebellar involvement is relatively late compared to other regions during the progression but it is still considered to be as significant as it leads to tau pathology spreads beyond the limbic and cortical areas. When cerebrum is affected such as one of its downstream effects; cerebellar atrophy can exacerbate difficulties in motor learning and cognitive timing affecting the coordination between the mind and

movements (Guo et al., 2018). The interplay between the affected mind and movements include initiation and coordination of muscle contractions for walking, writing or playing an instrument. This supports that cerebrum indeed plays a role in enhancement and maintenance of higher mental functions including sensory inputs especially for processing, storing memories as well as receiving and interpreting sensory information from the sensory organs of the body. This would indirectly affects the downstream processes such as learning, thinking, reasoning and problem-solving too.

In AD and other neurodegenerative conditions, cerebrum is one of the first regions to be affected showing structural and functional decline contributing to reduced cognitive efficiency. These effects would lead to disorganization of neural activity and impaired connectivity across brain networks especially deterioration of synaptic integrity. This was supported based on functional MRI studies in the early stages of AD at which functional connectivity within default mode network (DMN) that includes prefrontal cortex and posterior cingulate cortex, has resulted a measurable drop in its readings (Buckley et al., 2021). DMN involves both cortical and hippocampal nodes correlated with deficits in memory performance, attention and executive functioning (Jones et al., 2022). Thus, when neural connectivity is disrupted, eventually this will result in interferences in the flow of information between hippocampus and cortex during systems-level consolidation, a process necessary for converting short-term memories into stable long-term representations (Takeuchi et al., 2023). When these processes are degraded as AD progresses, individuals affected would slow but increasingly face in struggle to form new memories, retrieve back past experiences or maintain coherent of thoughts. This failed cognitive integration in creating memories further highlights the importance of cerebrum in the brain.

Another possible factor that supports cerebrum being related to AD is when decreased white matter integrity was found to impair speed and coordination through the slowing down of neural signalling processes. Disrupted coordination may occur as behaviourally-affected outcomes such as mental sluggishness, distractibility and an inability to adapt to new information, all of which severely hinder learning and memory. From a structural perspective, the cerebrum function is disrupted as seen in aging and AD due to cortical thinning, synaptic loss and hippocampal atrophy. Not only these deteriorations happen to affect neural processes but these impair the abilities in managing cognitive demands such as orchestrating thoughts and goal-directed behaviour. Thus, diseases related to these factors, in this case specifically AD, the

downregulated neural processes may lead to widespread disruption of cerebrum's central role in sustaining the coherence, speed and adaptability of human thought processed by the brain. Even worse when there is no counteraction to reverse back or reduce the effects. This weakens the critical communication pathways between hippocampus and cortex especially those necessary for systems-level memory consolidation needed long-term memories conversion (Deng et al., 2023). The damage in cortical and hippocampal areas will result in disruptions in broader brain ability to integrate, prioritize and act on complex information memory processes. If there is no intervention, these would spread across the brain areas over time and enhance more brain deficits.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Materials and Method

Seventy healthy male Albino Wistar rats (n=70), 7-8 weeks old weighing 200-250g were used in this study. The rats were purchased from a local supplier, Takrif Bestari Enterprise, Seri Kembangan, Selangor, Malaysia. The rats were housed 2-3 rats per cage under same laboratory conditions (temperature $22 \pm 25^{\circ}\text{C}$, 12h light: 12h dark cycle) and were acclimatized for one week before starting the experiment. They were fed with standard commercially available rats chow (autoclavable pellet) and water *ad libitum*. All experimental protocols were carried out during the light cycle and performed at Animal Behavioural Laboratory, Faculty of Medicine and Health Sciences, University of Putra Malaysia with ethic approval from Animal Ethic Committee of University of Putra Malaysia (UPM/IACUC/AUP-R094/2019). AlCl_3 , D-gal and Donepezil were purchased from Sigma-Aldrich, St. Louis, MO, USA, meanwhile $\Delta^9\text{THC}$ was purchased from Lipomed, Switzerland. D-gal and Donepezil were dissolved in distilled water for intraperitoneal injection (i.p). Meanwhile, AlCl_3 was dissolved in normal saline for oral administration (Chiroma et al., 2018). All the drugs and chemicals were freshly prepared prior to the experimental work.

3.2 Animal Experimental Design

All animals were screened for health prior to experiments, and only clinically healthy rats with normal activity and feeding behaviour were included. Inclusion criteria required the absence of infection, motor impairment, or weight loss exceeding 10% during the induction period. Predefined exclusion criteria consisted of failure of AD induction, post-procedural complications, severe illness unrelated to treatment, or inability to perform behavioural testing. After delegating, no animals met exclusion criteria thus none were removed prior to analysis. A total of 70 rats was included in the experimental design and categorized into seven groups (n=10). To minimise selection

bias, animals were allocated to experimental groups using computer-generated block randomisation, stratified by baseline body weight to ensure comparable distribution across groups. Group identity was concealed using numerical or alphanumeric codes during behavioural testing, histological scoring, immunofluorescence quantification, and western blot densitometry. The investigators responsible for data collection and image or band analysis were blinded to treatment allocation until completion of statistical analysis.

Table 3.1 shows the animal groups and treatments they received. Group 1 served as Control group (A) treated with distilled water + 0.9% normal saline, Group 2 is the AD Model group (B) received 60 mg/kg D-gal + 200 mg/kg AlCl₃ and Group 3 acts as the AD group (C) which did not receive any treatment to prove the capability of AD and its potential effects in reducing neurodegenerative pathology without being prompted by pharmacological intervention. This aims to see if non-treated alternative can give more benefits than the treated ones. As for Group 4, it acts as a positive control group (D) that received 60 mg/kg D-gal + 200 mg/kg AlCl₃ together with Donepezil treatment at 1.0 mg/kg meanwhile Group 5, Group 6 and Group 7 received 60 mg/kg D-gal + 200 mg/kg AlCl₃ together with Δ^9 THC treatment at 0.75 mg/kg (E), 1.5 mg/kg (F) and 3.0 mg/kg (G) respectively. The rats were given a combination administration of 200 mg/kg of AlCl₃ and 60 mg/kg of D-gal to induce AD in the rat models (Chiroma et al., 2018) as seen in Table 3.1.

The total induction duration was 10 weeks. After the completion of animal induction, the rats went through neurobehavioural assessments specifically Morris Water Maze (MWM). For Group 2 (B) only, the rats were decapitated meanwhile the other animal groups proceeded to undergo further treatments. Three different concentrations at which 0.75 mg/kg (E), 1.5 mg/kg (F) and 3.0 mg/kg of Δ^9 THC (G) were administered respectively to investigate the potential therapeutic effect. The concentration was according to the previous study done by Suliman et al (2018). The dosage of Donepezil at the concentration of 1.0 mg/kg was given in the rats intraperitoneally. All the drug solutions were prepared immediately prior to the experiment and administered daily via intraperitoneal injection (i.p) except for AlCl₃ (orally administered). The volume of i.p injection is 0.1 ml/100 g body weight. Upon completion of treatment period for 28 days, the rats were undergone MWM before decapitated using guillotine. Brain samples were collected for histological findings and molecular studies.

Table 3.1
Animal Groups and Treatments

Group	Treatment	Dosage
(A) Control	Distilled Water + 0.9% Normal Saline	0.1 ml/100 g b.wt.
(B) AD Model*	D-gal + AlCl ₃	60 mg/kg D-gal + 200 mg/kg AlCl ₃
(C) AD Without Treatment	D-gal + AlCl ₃	60 mg/kg D-gal + 200 mg/kg AlCl ₃
(E) Positive Control	D-gal + AlCl ₃ + Donepezil	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 1.0 mg/kg donepezil
(F) AD Model + Δ^9 THC Low Dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 0.75 mg/kg Δ^9 THC
(G) AD Model + Δ^9 THC Medium Dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 1.5 mg/kg Δ^9 THC
(H) AD Model + Δ^9 THC High Dose	D-gal + AlCl ₃ + Δ^9 THC	60 mg/kg D-gal + 200 mg/kg AlCl ₃ + 3.0 mg/kg Δ^9 THC

*Animals in the Group 2 were decapitated upon completion of induction process (10 weeks)

3.3 Neurobehavioral Assessment

The neurobehavioural test carried out was Morris Water Maze (MWM). MWM was designed to study AD-like cognitive deficits in rats in terms of hippocampal-dependent spatial memory. This test allowed rodents to depend on distal cues to navigate from start locations to locate the submerged of escape platform. The parameters such as swimming trajectories, escape latency and residence times in escape platform quadrant were recorded by a video camera linked to computer with ANYmaze™ software for analysis. MWM was conducted as a well-established assay for hippocampal dependent spatial memory in which the rats learned to navigate the spatial location of a platform submerged 2 cm in a circular pool of water using distal cues. The MWM tank was filled with opaque water to a depth of 40 cm and kept at the consistent temperature of 24 ± 2 °C (Zhang et al., 2016; Li et al., 2016). MWM tank was equipped with a video tracking camera that was positioned above it. This camera was then connected to a laptop with software ANYmaze™, Stoelting Co., Chicago, USA. With the aid of the software, the tank was divided into four equal quadrants (Figure 3.1). A platform with a diameter of 10 cm was positioned at the centre of a designated quadrant, referred to as the target quadrant. On the first day, the rats were given trainings

in order to locate a visible escape platform that was positioned 2 cm above water surface.

The following consecutive four days (Day 2 until Day 5), the animals were trained to find the hidden escape platform submerged 2 cm below water surface. The location of the platform remained in the same quadrant throughout the whole five days testing. During the place navigation task, each rat has completed three trainings per day and in each of the trial, the rats were randomly introduced from one of the three quadrants, excluding the one containing the platform. They were given a maximum time of 120 seconds to locate the hidden platform. If the rats successfully locate the platform, they were allowed to stay on the platform for 20 seconds to aid in spatial recognition and memory consolidation. However, for the rats that failed to locate the platform within the allocated time, the rats were gently guided to it and allowed to remain there for 20 seconds (Chiroma et al., 2018). This spatial probe test was conducted for 24 hours after has completed the final place navigation trial. The rats were released into the quadrant that is located opposite from the former location of the platform. The rats were allowed to search for the removed platform for a duration of 30 seconds (Chiroma et al., 2018). A video tracking system connected to ANY-maze software was used to monitor and record the behavioural parameters.

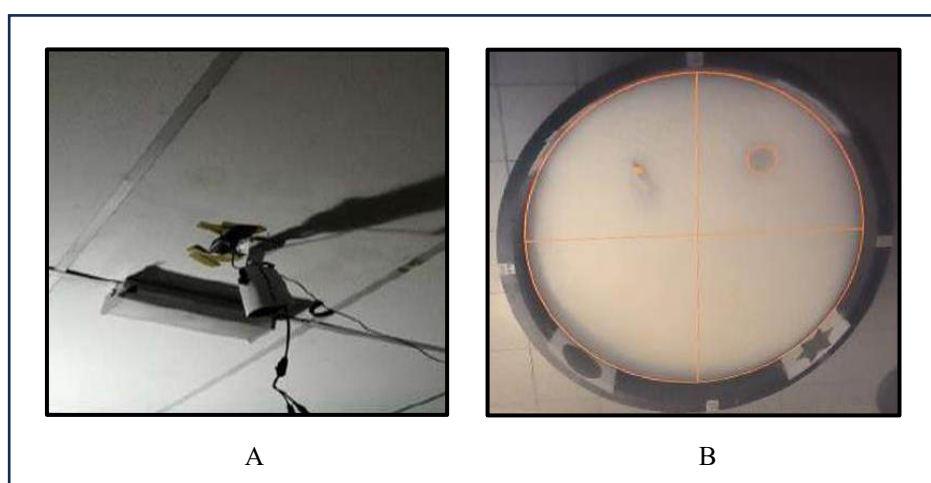


Figure 3.1 Experimental Setup during Morris Water Maze (MWM) Test. (A) Logitech Camera Fixed on the Ceiling Directly Facing the MWM Tank to Capture the Movement, (B) ANYmaze Video Tracking Software Connected with Logitech Camera for Recording of Rat Movement such as Swimming Trajectories, Escape Latency, Time Spent in the Target Quadrant.

3.4 Brain Retrieval and Preparations

Following the completion of neurobehavioural assessments, the rats were euthanized by decapitation to allow rapid brain retrieval while minimizing post-mortem tissue degradation. Immediate decapitation is commonly employed in neurohistological and molecular studies to preserve neuronal morphology and protein integrity by limiting ischemia-induced artefacts. Brain retrieval was performed on an ice-containing tray to maintain low tissue temperature, thereby reducing enzymatic activity and autolytic processes that could compromise histological and molecular outcomes. The excised brains were promptly collected and prepared for downstream histological and molecular analyses. Tissue processing was conducted using an automated tissue processor (Leica TP1020), which ensures standardized and reproducible processing conditions across all samples. The automated processing protocol included sequential dehydration, clearing, and paraffin infiltration steps, following established histological procedures as previously described by Woods and Ellis (1994). The use of an automated processor minimizes operator-dependent variability and improves consistency in tissue preservation and embedding quality. Brain retrieval was carried out inside an ice-containing tray and the samples were placed in appropriately labelled tissue cassettes at which the samples were oriented in an upright position to maintain its anatomical integrity.

Tissue thickness was standardized at approximately 5 mm to facilitate efficient fixative penetration. The samples were immersed in 10% neutral buffered formalin for a fixation period of seven days, a duration sufficient to achieve complete cross-linking of tissue proteins while preserving cellular morphology. Adequate fixation is critical for maintaining structural fidelity of neuronal and glial components and preventing post-processing artefacts. Following fixation, the cassettes were arranged in the tissue basket and processed automatically for 22 hours. Prior to embedding, paraffin wax was melted in the wax reservoir, and metal embedding moulds were pre-warmed to prevent premature solidification. The infiltrated brain tissues were carefully removed from the cassettes, oriented correctly within the moulds, and embedded in molten paraffin wax at an approximate thickness of 3 mm. The moulds were subsequently transferred to the cooling platform of the embedding station to allow controlled solidification of the paraffin. The resulting paraffin blocks were labelled and stored at 4 °C to facilitate final hardening prior to sectioning.

3.5 Histology Study

3.5.1 Hematoxylin and Eosin Staining (H&E)

After completion of the embedding process, paraffin blocks were sectioned using a rotary microtome (SLEE MAINZ CUT 5062). Initial trimming was performed at a section thickness of 10 μm to expose the tissue surface uniformly. Subsequently, the microtome thickness was adjusted to 5 μm to obtain thin, continuous paraffin ribbons suitable for histological staining. The use of 5 μm sections provides optimal resolution for visualizing neuronal architecture and identifying subtle morphological changes associated with apoptosis. Next, Hematoxylin and Eosin (H&E) staining was conducted using a standardized regressive staining protocol comprising nine sequential steps: Deparaffinisation, Rehydration, Hematoxylin Staining, Differentiation, Bluing, Eosin Staining, Dehydration, Clearing, and Coverslipping. This protocol was meticulously followed to ensure optimal preservation and visualization of tissue architecture, which is essential for accurate histopathological evaluation. The procedure commenced with deparaffinisation, involving two sequential immersions in xylene (2 minutes each) to completely dissolve paraffin wax. Effective removal of paraffin is critical, as residual wax can impede reagent penetration and compromise staining uniformity. Following deparaffinisation, tissue sections were rehydrated through a graded ethanol series, including two immersions in 100% ethanol, followed by 95% and 70% ethanol. This gradual reintroduction of water prevents tissue distortion and restores the hydrated state required for aqueous staining.

The slides were then rinsed thoroughly with distilled water to remove residual alcohol and prepare the tissue for nuclear staining. Hematoxylin staining was performed for 3 minutes, allowing selective binding to basophilic structures such as nuclear chromatin and nucleic acids, thereby providing clear visualization of nuclear morphology. Excess stain was removed by rinsing in wash buffer, followed by a differentiation step to selectively eliminate nonspecific hematoxylin staining, enhancing nuclear contrast. Bluing was subsequently performed using a mildly alkaline solution to convert the hematoxylin to a stable blue coloration, further improving nuclear clarity and contrast. Following nuclear staining, brief dehydration in 95% ethanol was carried out to prepare the tissue for eosin staining. Eosin was applied for 45 seconds to stain cytoplasmic and extracellular components in varying shades of pink,

providing contrast to the hematoxylin-stained nuclei and enabling comprehensive visualization of tissue architecture. The sections were then progressively dehydrated through increasing concentrations of ethanol, cleared in xylene to render the tissue transparent, and mounted with a coverslip. Mounting preserves the stained sections, protects them from degradation, and provides an optically suitable surface for microscopic analysis. This carefully controlled staining procedure ensured consistent, high-quality histological sections suitable for quantitative and qualitative assessment of neuronal morphology. A detailed summary of the H&E staining protocol is presented in Table 3.2.

Table 3.2
Hematoxylin and Eosin (H&E) Staining Processes

Process	Reagent	Duration
Deparaffinisation	Xylene	2 minutes
Deparaffinisation	Xylene	2 minutes
Rehydration	100% Ethanol	2 minutes
Rehydration	100% Ethanol	2 minutes
Rehydration	95% Ethanol	2 minutes
Rehydration	70% Ethanol	2 minutes
Rinsing	Distilled Water	Tap for 2-3 times
Rinsing	Distilled Water	Tap for 2-3 times
Staining	Hematoxylin	3 minutes
Rinsing	Wash Buffer	1 minute
Staining	Differentiator	1 minute
Rinsing	Wash Buffer	1 minute
Staining	Bluing	1 minute
Rinsing	Wash Buffer	1 minute
Dehydration	95% Ethanol	1 minute
Staining	Eosin	45 seconds
Dehydration	95% Ethanol	1 minute
Dehydration	100% Ethanol	1 minute
Dehydration	100% Ethanol	1 minute
Clearing	Xylene	2 minutes
Clearing	Xylene	2 minutes
Mounting	Cover Slip	Few minutes

3.5.2 Determination of Morphological Changes

Histological evaluation was performed using a compound research microscope equipped with a digital image acquisition system (Olympus BX51 TRF, Japan). Stained brain sections were examined to assess neuronal morphology and identify apoptotic cells within the hippocampal CA1 and CA3 subfields, as well as granule cells in the dentate gyrus (DG). Quantification of apoptotic neurons was conducted under the guidance of a trained pathologist to ensure accurate identification and minimize observer bias. Apoptotic neurons were identified based on characteristic morphological features observed in H&E-stained sections, including cellular shrinkage, cytoplasmic condensation, and circular or pyknotic nuclei. These features are widely accepted histological indicators of neuronal apoptosis. For each experimental group, five coronal brain sections encompassing both the hippocampus and dentate gyrus were randomly selected from each animal, with a total of five rats per group ($n = 5$). Within each section, apoptotic cells were counted in at least five randomly selected fields located in the mid-regions of the CA1 and CA3 subfields, using a magnification of $200\times$. The same counting strategy was applied to the dentate gyrus to ensure methodological consistency across regions. This random sampling approach reduces selection bias and improves the representativeness of the quantified data (Mahdi et al., 2021). The total number of apoptotic cells from all selected fields was summed, and the mean value was calculated for each group. This mean apoptotic cell count was expressed as the apoptosis index and used as a quantitative measure of neuronal degeneration across experimental conditions.

3.6 Immunofluorescence

Immunofluorescence assay was employed to evaluate neurogenesis-related cellular changes within the hippocampus. In this study, an indirect immunofluorescence approach was used, involving a primary antibody specific to the target protein and a fluorophore-conjugated secondary antibody. The primary antibody selectively binds to the antigen of interest, while the secondary antibody binds to the primary antibody, enabling signal amplification. The resulting fluorescence intensity was quantified as an indirect indicator of neurogenesis-associated protein expression in hippocampal cells. Paraffin-embedded tissue blocks

were retrieved from cold storage and maintained under chilled conditions throughout trimming to preserve tissue integrity. Sections were initially trimmed at a thickness of 10 μm using a rotary microtome (SLEE MAINZ CUT 5062) until the tissue surface was exposed. Subsequently, the microtome thickness was adjusted to 5 μm to generate uniform paraffin ribbons. The ribbons were floated on a water bath and carefully mounted onto silane-coated slides at 40 °C to enhance tissue adhesion. Slides were then placed on a hotplate and allowed to dry until the paraffin wax had completely melted.

Deparaffinisation was performed by immersing the slides in xylene for three consecutive changes, each lasting 10 minutes, followed by rehydration through two changes of 100% ethanol (5 minutes each). The sections were then sequentially immersed in 95% ethanol for 3 minutes and 70% ethanol for 3 minutes before being rinsed in distilled water to complete the rehydration process. Antigen retrieval was conducted by heating the slides for 30 minutes at approximately 97 °C in either low-pH retrieval buffer (for GFAP) or high-pH retrieval buffer (for Calbindin), using a water bath or laboratory oven. This step facilitates the unmasking of antigenic epitopes that may have been altered during fixation and embedding. The slides were subsequently allowed to cool for 15 minutes at room temperature in retrieval buffer and then placed in wash buffer to maintain tissue hydration. To prevent cross-contamination of reagents, the tissue sections were circumscribed using a hydrophobic barrier pen. Endogenous peroxidase activity was quenched by applying 1–2 drops of hydrogen peroxide (H_2O_2) to each marked area and incubating for 30 minutes at room temperature. This step minimizes background signal and reduces the likelihood of false-positive fluorescence.

Slides were then washed three times with wash buffer and briefly rested. Non-specific antibody binding was blocked by applying protein blocking solution to each section and incubating for 30 minutes at room temperature. Following blocking, the slides were washed three times and maintained in wash buffer for an additional resting period. The sections were then incubated with 100 μL of the respective primary antibodies (GFAP or calbindin) for 30 minutes. After primary antibody incubation, the slides were washed three times, with wash buffer retained on the tissue for 5 minutes during the final wash. Excess buffer was gently removed by tapping the slide edge against absorbent tissue. The sections were then incubated with the appropriate fluorophore-conjugated secondary antibody for 30 minutes,

followed by washing with wash buffer for 10 minutes. Nuclear counterstaining was performed by incubating the slides overnight with DAPI at a dilution of 1:500. Following incubation, slides were washed with wash buffer, air-dried, and mounted using a fluorescent mounting medium before being cover-slipped. The mounting medium preserves fluorescence and eliminates the need for dehydration steps. Prepared slides were stored in a light-protected container at 4 °C until imaging.

Fluorescence imaging was conducted at the Cell Imaging Centre, Collaborative Drug Discovery Research (CDDR), Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Puncak Alam campus. Slides were examined in a dark room using an inverted fluorescence microscope (Nikon Eclipse Ti) equipped with NIS-Elements imaging software. Photomicrographs were captured for each fluorescent marker in combination with DAPI staining. Quantitative analysis of fluorescence intensity was performed using the open-source image processing software ImageJ (Fiji; version 2.14.0/1.54f). The mean fluorescent area was calculated for each image, and the average values were tabulated for subsequent graphical representation. A detailed step-by-step summary of the immunofluorescence protocol is presented in Table 3.3.

Table 3.3
Immunofluorescence (IF) Processes

Process	Reagent	Duration
Light Phase		
Deparaffinisation	Xylene	10 minutes
Deparaffinisation	Xylene	10 minutes
Deparaffinisation	Xylene	10 minutes
Rehydration	100% Ethanol	5 minutes
Rehydration	100% Ethanol	5 minutes
Rehydration	95% Ethanol	3 minutes
Rehydration	70% Ethanol	3 minutes
Resting phase	Distilled Water	Few minutes
Baking	Low pH Buffer (GFAP)/High pH Buffer (Calbindin)	30 mins at 97°C
Dark Phase (No Light)		
Unmasking	Retrieval Buffer	15 minutes
Resting	Wash Buffer	Few minutes
Blocking	1-2 Drops	30 minutes
Resting	Wash Buffer	3x wash
Blocking	1 Drop of Protein Block	30 minutes
Resting	Wash Buffer	3x wash
Resting	Resting Phase	30 minutes
Resting	Wash Buffer	3x wash
Staining	100 µl of Primary Antibody (GFAP or Calbindin)	30 minutes
Resting	Wash Buffer	5 minutes
Staining	Secondary Conjugated Antibody	30 minutes
Resting	Wash Buffer	10 minutes
Staining	1:500 DAPI Solution	Overnight
Resting	Wash Buffer	10 minutes
Mounting	Fluorescent Mounting Solution	Few minutes

3.7 Western Blotting

The brain specifically cortex was collected immediately after decapitation and stored at -80°C until further analysis. The analysis of total expression of p-Tau Thr 231 protein and Beta-secretase 1 (BACE 1) was done.

3.7.1 Preparation of Brain Lysate

The frozen cortex tissue was thawed for 30 mins at room temperature before washing with distilled water to eliminate any clotted blood. Then, it was weighed at 0.01g before mixed with 400 μ l RIPA lysis buffer together with 15 μ l phosphatase inhibitor and 15 μ l protease inhibitor. The cortex was homogenized using a homogenizer (D130, Wigggen Hauser, USA) with sucrose lysis buffer, protease inhibitor and phosphatase inhibitor for 10 minutes on ice. The lysate was centrifuged at 13,000 rpm for 10 minutes at 4°C. Resulting supernatant and pellet were aliquoted and stored at -20 °C. The protein content of brain lysate was determined using the Bradford Protein Assay kit. Microplate reader was used to read its absorbance at 562 nm. The concentration of protein in brain lysate samples were calculated with a standard curve of reference protein.

3.7.2 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Electro Transfer

For the gel preparation, 10% resolving gel and 4% stacking gel were applied. 20 μ l of brain lysate containing 20 μ g of protein sample was added to 20 μ g of Laemmli sample buffer supplemented with 1:19 dilution of β -mercaptoethanol and was heated at 95°C for 5 minutes. The samples were then vortexed, centrifuged at 1000 rpm for 1 minute followed by loading into the gel 20 μ l per well. Known molecular weight marker (Precision Plus Standard Kaleidoscope Marker) was loaded into the gel as well. After that, SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was conducted. The separated protein was then transferred onto a nitrocellulose membrane and was stained with Ponceau S dye to observe any presence of protein bands.

3.7.3 Blocking and Antibody Incubation

Blocking of the non-specific binding was conducted by incubating the membrane with 5 % w/v skimmed milk powder in Tween 20 solution for 2 hours at 4°C. The membrane was incubated with antibodies from Invitrogen (Thermo Fischer Scientific); primary anti-BACE 1 (1:1000) and anti-p-Tau Thr231 (1:1000) overnight in Abcam protein blocking solution (ab64226) at 4°C. The next day, the membrane was washed for three times with Tris-buffered saline Tween 20 (TBST 20) for 5 minutes each and

incubated with secondary horseradish peroxidase (HRP) antibody (1:5000) in protein blocking solution for 2 hours at 4°C. The membrane again washed for three times at 10 minutes each.

3.7.4 Detection and Visualization

Following antibody incubation, protein bands were detected using an enhanced chemiluminescence (ECL) method. The membrane was incubated with a horseradish peroxidase (HRP)-based chemiluminescent substrate prepared by mixing WesternBright™ ECL and WesternBright™ Peroxidase at a 1:1 ratio for 5–10 minutes. This substrate reacts with HRP conjugated to the secondary antibody, producing a light-emitting signal proportional to the abundance of the target protein. Chemiluminescent signals were captured using a gel documentation system (Infinity System, Vilber Lourmat, Germany) under optimized exposure conditions to prevent signal saturation. Digital images corresponding to the protein bands of interest were acquired and subsequently subjected to densitometric analysis. The intensity of each band was quantified and used for downstream statistical analysis to determine relative protein expression levels.

3.7.5 Quantification of Protein of Interest

The quantification of proteins was done using ImageJ system protocol (Davarinejad, 2013). Data were normalised by dividing the mean intensity of protein interest with housekeeping protein (β -actin) for each reading. The protein level was presented as percentage changes compared with control group, designated as 100%.

Table 3.4
Western Blot Procedure

Process	Reagent
Homogenization	Add 400 μ l RIPA lysis buffer mixed with 15 μ l phosphatase inhibitor + 15 μ l protease inhibitor and leave it for 10 minutes
Determination of Standard Protein Concentration	Pierce BCA Protein Assay kit (Thermo Fischer Scientific, USA)
Gel preparation	Add 20 μ l Laemli sample buffer and β -mercaptoethanol for 5 minutes
SDS-PAGE	10% resolving gel and 4% stacking gel. Run at 100 V for 60 minutes followed by 150 V for another 30 minutes
Transfer	Transfer onto 0.45 μ M nitrocellulose membrane. Stain with Ponceau S dye
Blocking	Block with a duration of 2 hours in 5% skimmed milk powder in TBST Tween 20 (for BACE1 and APP) Leave it overnight in 5% BSA in TBST Tween 20 (p-tau Thr231)
Incubation 1$\square$ Antibody	Incubate the membrane protein with primary anti-BACE1 (1:1000) and anti p-tau Thr231 (1:1000) overnight
Washing	3 times for 5 minutes each
Incubation 2$\square$ Antibody	Incubate with blocking solution horseradish peroxidase (HRP) antibody (1:5000) for 2 hours
Washing	3 times for 10 minutes each
Detection	Incubate with chemiluminescence HRP substrate (1:1 of WesternBright™ Peroxidase
Viewing	Using gel documentation system

3.8 Statistical Analysis

In order to achieve optimum results, few statistical analyses specific to each methodology of this research were conducted. An overall experimental design was portrayed in a flowchart below to develop a deeper understanding and coherence of knowledge on research niche (Figure 3.2). Statistical analyses were selected according to the specific experimental endpoints of this study to ensure rigorous and appropriate data interpretation. A schematic overview of the experimental workflow is provided to enhance clarity and conceptual coherence of the research framework (Figure 3.2). Neurobehavioural performance assessed using the Morris Water Maze (MWM) was analyzed by One-way Analysis of Variance (ANOVA), followed by Tukey's multiple comparison post hoc test using SPSS software version 25.0. Data were expressed as mean $\pm$ standard error of the mean (SEM), and statistical significance was defined as $p < 0.05$.

Quantitative data obtained from histological assessment using Hematoxylin and Eosin (H&E) staining were analyzed using one-way ANOVA followed by Tukey's post hoc test with SPSS version 25.0. Immunofluorescence analysis was performed to evaluate the effects of Δ^9 THC on the expression of GFAP (Glial Fibrillary Acidic Protein) and Calbindin as neurogenesis-related protein markers in an AD-induced Wistar rat model. Immunoreactivity was quantified as the percentage of immunoreactive area using ImageJ software, and the resulting data were subjected to one-way ANOVA followed by Tukey's multiple comparison test using SPSS version 25.0.

Finally, quantification via western blot analysis was conducted in assessing the effects of Δ^9 THC on the relative protein band intensity of Beta-site Amyloid Precursor Protein Cleaving Enzyme 1 (BACE1) and Phosphorylated Tau (Thr231) proteins in the cortex of the brain specifically. The means quantified were normalized by dividing the mean band intensity of each target protein by that of the housekeeping protein β -actin for each sample. Normalized values were expressed as percentage changes relative to the control group, which was designated as 100%. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. All data are presented as mean $\pm$ SEM, and statistical significance was established at $p < 0.05$.

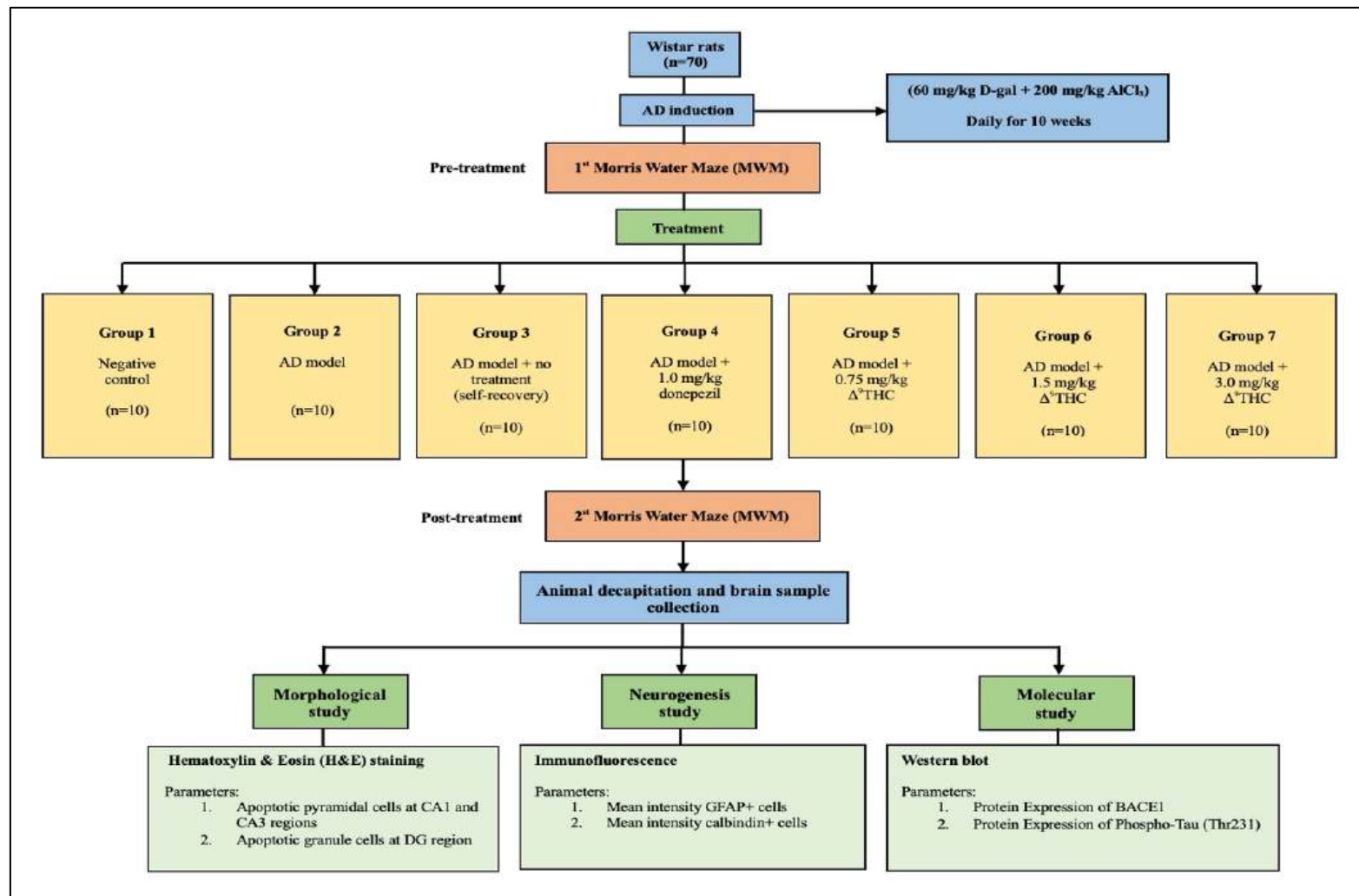


Figure 3.2 Flowchart of Research Methodology

CHAPTER 4

RESULTS

4.1 Δ^9 THC Improves Spatial Learning and Memory in Rats Induced by D-gal and AlCl_3 through Morris Water Maze (MWM)

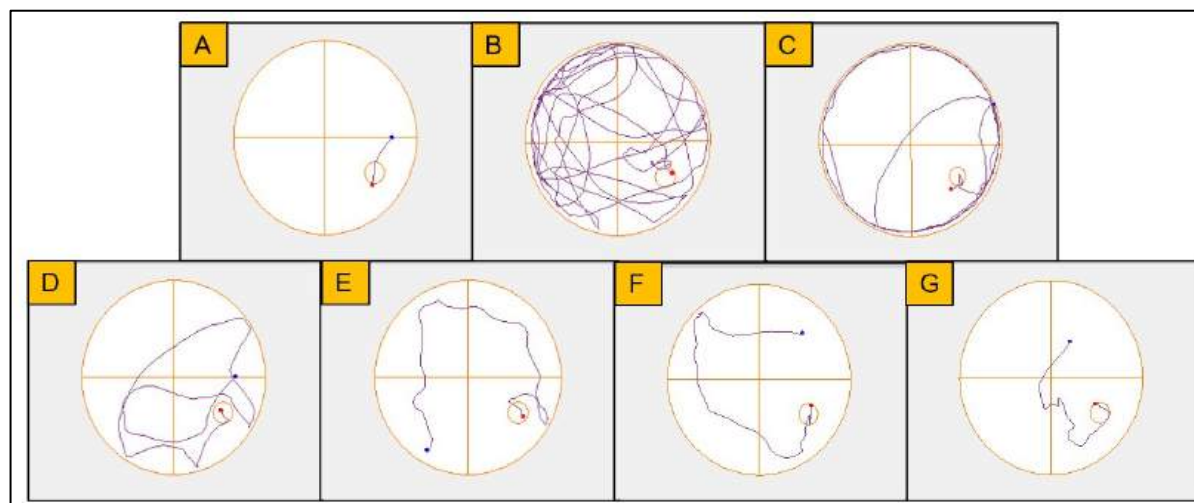


Figure 4.1 Representative Swimming Path on the Final Day (Day 5) of the Place Navigation Test via Spatial Learning and Memory through MWM among the Rats. Control Rats (A), AD Model (B), AD Without Treatment (C), Treated Group with 1 mg/kg Donepezil (D), 0.75 mg/kg Δ^9 THC (E), 1.5 mg/kg Δ^9 THC (F) and 3.0 mg/kg Δ^9 THC (G) respectively. Blue Dots – Starting Point During Place Navigation Test; Red Dots – Hidden Escape Platform Point during Place Navigation Test.

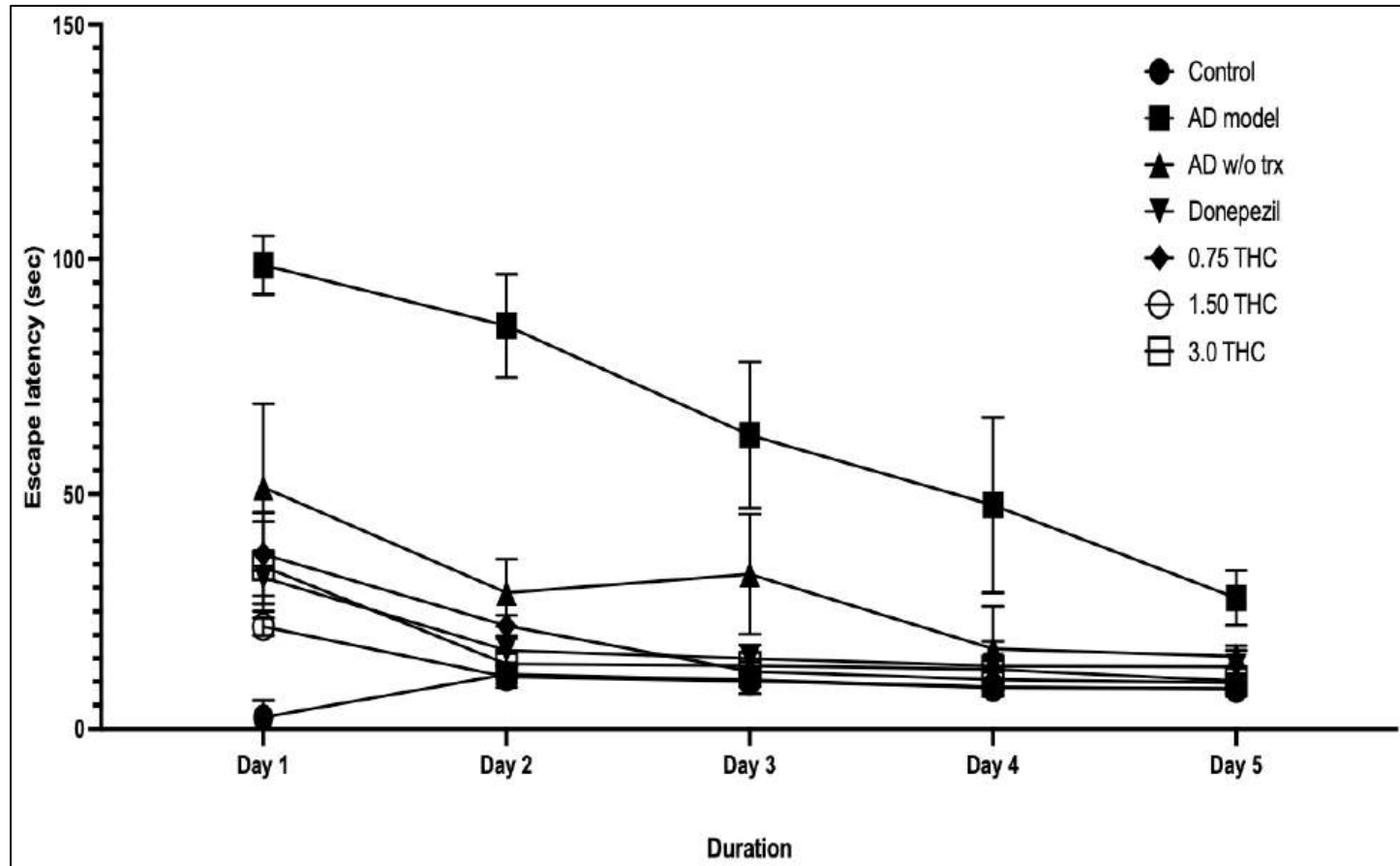


Figure 4.2 Spatial Learning and Memory via MWM (Mean Escape Latency in 5 Days to Find the Escape Platform in Place Navigation Test).

Data Were Expressed as Mean $\pm$ SEM. $^*p < 0.05$ vs AD model, $^{\#}p < 0.05$ vs AD Without Treatment

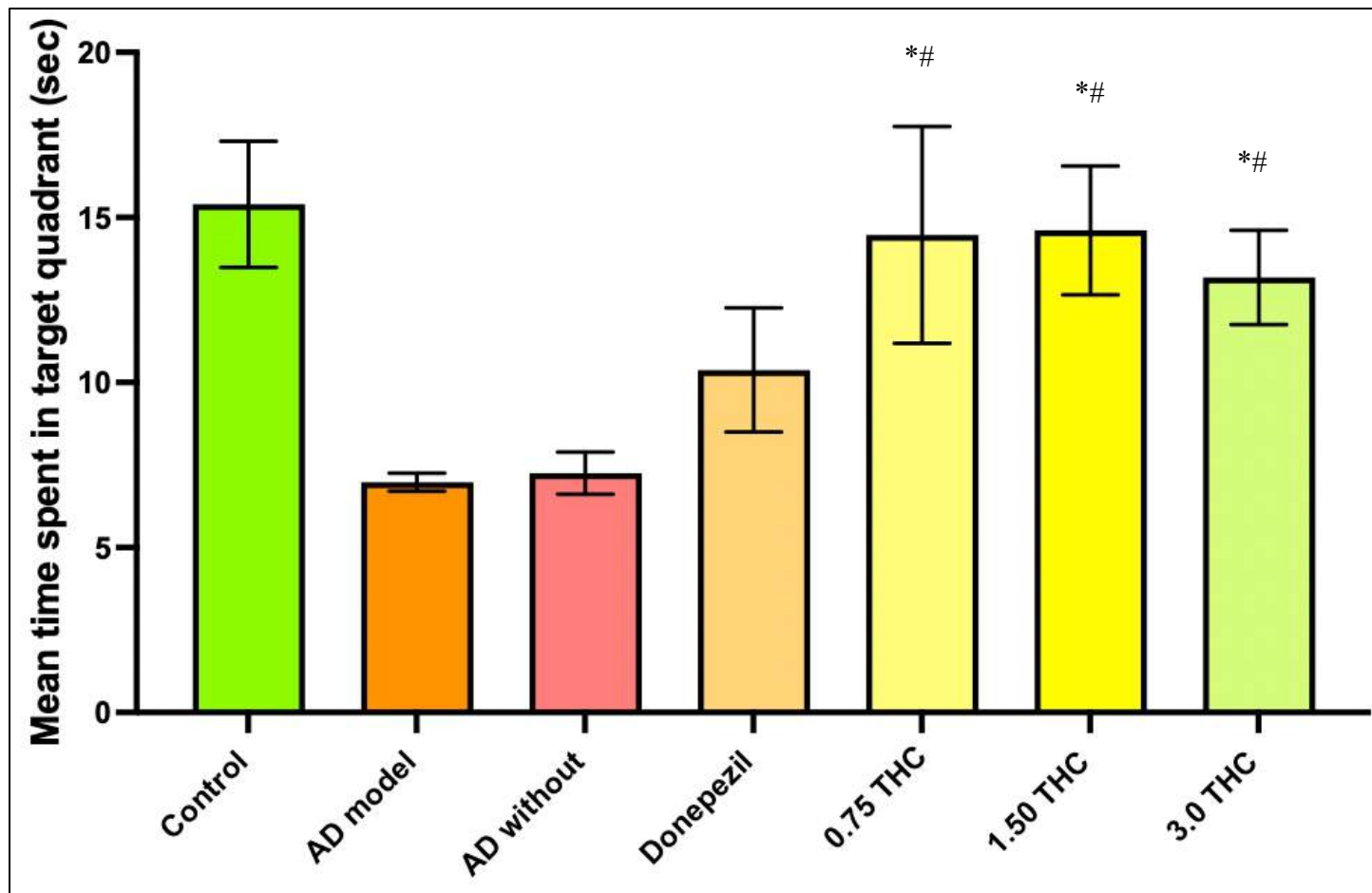


Figure 4.3 Spatial Learning and Memory via MWM (Mean Time Spent in Target Quadrant in Probe Test). Data Were Expressed as Mean $\pm$ SEM. * $p < 0.05$ vs AD model, # $p < 0.05$ vs AD Without Treatment

Figure 4.1 shows the representative swimming path on the Final Day (Day 5) of the Place Navigation Test via Spatial Learning and Memory through MWM among the Rats. By comparing the results of AD model (B), it can be seen that the swimming trajectories were randomly scattered throughout all four quadrants. However, in the group of AD Without Treatment (C), there was a slight improvement observed in the swimming trajectories although the movements were focused more towards the perimeter of the tank. As for Figure 4.2, the graph shows the mean escape latency in 5 days for the rats to find the escape platform in Place Navigation Test. Interestingly, AD Model (B) and AD Without Treatment (C) were seen to have a reduced mean escape latency however, the reduction was progressively slow per day as compared to those treated with 0.75 mg/kg (E), 1.5 mg/kg (F) and 3.0 mg/kg Δ^9 THC (G).

As for Figure 4.3, it shows the mean time spent in target quadrant in probe test. Based on the results, a statistically significant interaction was defined between the efficacy of the treatment and its duration at which the treatment was given. Tukey's post hoc test revealed that AD Model group differed significantly from the Control Group and all treated groups ($p < 0.05$). One way ANOVA [$F(6,33) = 3.123, p = 0.016$] showed a statistically significant difference in the time spent by the rats (seconds) in the targeted quadrant during probe trial. This was proved when AD Model (6.98 ± 0.27) and AD Without Treatment (7.25 ± 0.64) spent statistically significant less time in the targeted quadrant when compared to the Control (15.40 ± 1.92). As for all treated groups, there were statistically differences seen among the mean differences; 0.75 mg/kg Δ^9 THC (14.47 ± 3.28), 1.5 mg/kg Δ^9 THC (14.61 ± 1.95) and 3.0 mg/kg Δ^9 THC (13.18 ± 1.43), as revealed by Tukey's post hoc (Figure 4.3).

4.2 Δ^9 THC Remediates Neuronal Apoptotic at CA1, CA3 and DG Regions of the Hippocampus in a Rat Model Induced by D-gal and AlCl_3 through Histological Study

The hippocampus consists of 4 regions altogether; CA1, CA2, CA3 and CA4. In this study, the effects of Δ^9 THC on neurodegeneration induced by D-gal and AlCl_3 at CA1 and CA3 regions of the hippocampus were examined. Figure 4.4, 4.6 and 4.8 consists the histological results showing CA1, CA3 and DG regions for all groups. The histological findings depict the mean number of apoptotic pyramidal cells at CA1, CA3 region and granule cells at DG region respectively. To evaluate its significance, one-way between groups Analysis of Variance (ANOVA) and Tukey's post hoc comparisons were conducted to investigate the effect of Δ^9 THC in remediating neuronal apoptosis in an Alzheimer-induced rat model brains. In comparison with the control group of rats, the group that depicted AD model which was exposed to chronic exposure of D-gal and AlCl_3 , and the group that serves as AD Without Treatment showed an increase in neuronal apoptosis among the cells, contributing to the progression of the disease. The presence of numerous apoptotic cells were determined by comparing their morphological differences with viable cells as the viable cells have the presence of light cytoplasm and dark nucleus with well-arranged intact layers of pyramidal cells meanwhile apoptotic cells observed in the AD group of rats have hyperchromic dark cells, easily differentiating between viable and apoptotic cells.

4.2.1 Histological Results Showing Δ^9 THC Reduces the Number of Apoptotic Pyramidal Cells at CA1 Region in Rats Induced by D-gal and $AlCl_3$

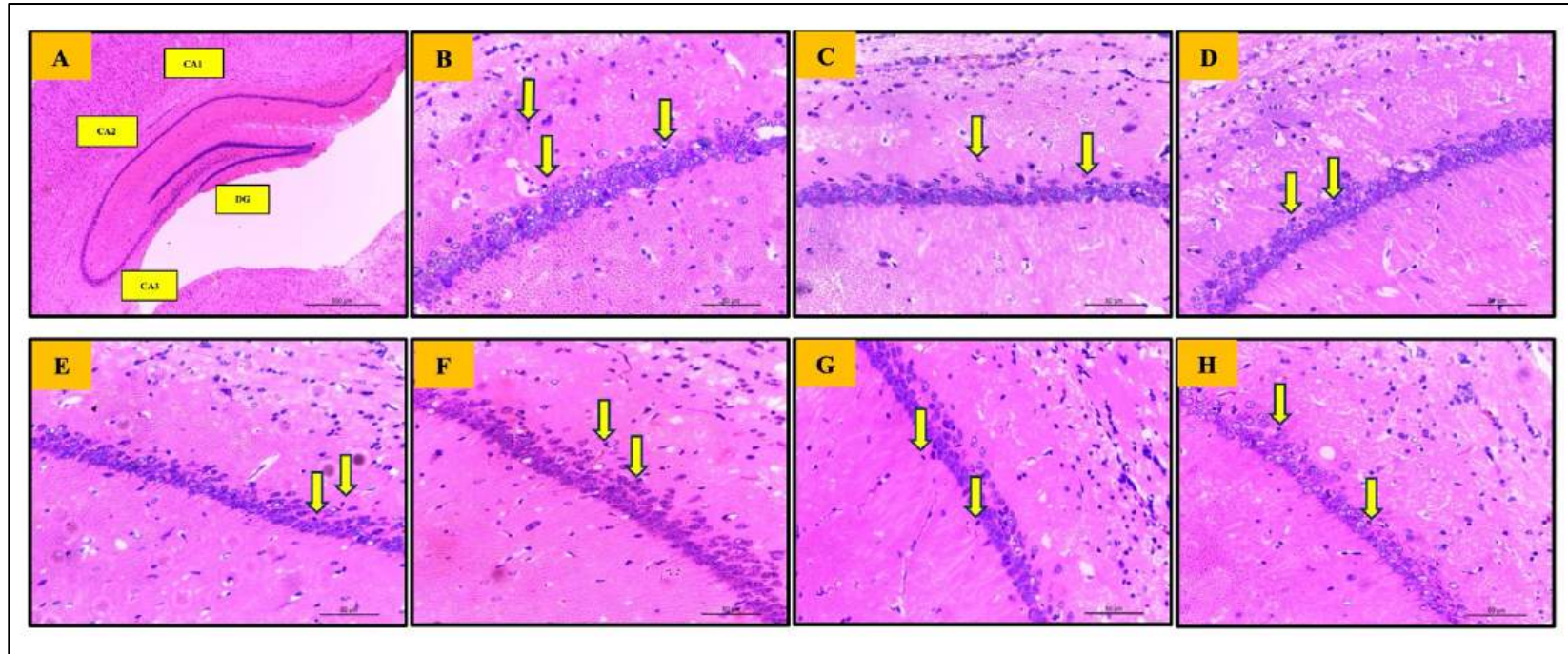


Figure 4.4 Hematoxylin and Eosin-Stained Photomicrographs of Brain Sections, specifically Cornu Ammonis 1 (CA1) Region of the Hippocampus. (A) The Structure of Whole Hippocampus Showing All Parts at Magnification 50x; CA1, CA2, CA3 and DG, Control Rats (B), AD Model (C) Demonstrates an Increase of Apoptotic Cells, AD Model Without Any Treatment (D), Treated with 1mg/kg Donepezil (E), (F-H) Treated with 0.75mg/kg Δ^9 THC, 1.5mg/kg Δ^9 THC and 3.0mg/kg Δ^9 THC Respectively, Has Showed a Decrease in the Number of Apoptotic Cells at CA1 Region. Magnification of 400x for (B-H), Scale bar 50 μ m. CA1- Cornu Ammonis 1; CA2 – Cornu Ammonis 2; CA3 – Cornu Ammonis 3; Δ^9 THC – Delta-9-Tetrahydrocannabinol.

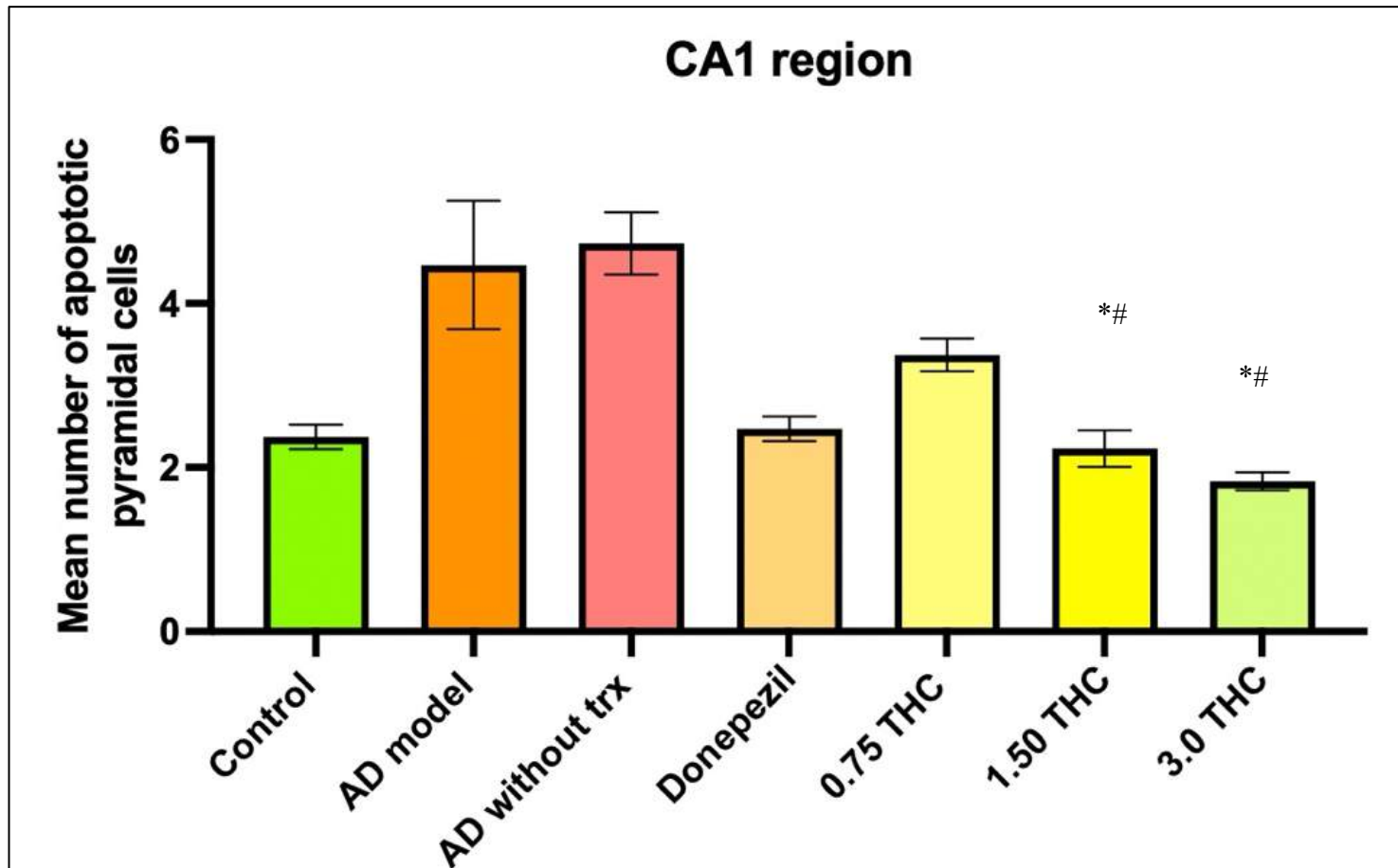


Figure 4.5 Mean Number of Apoptotic Pyramidal Cells at CA1 Region for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test Among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). * $p < 0.05$ vs AD model, # $p < 0.05$ vs AD Without Treatment. CA – Cornu Ammonis

One-way ANOVA revealed statistically differences in the number of apoptotic pyramidal cells in CA1 region [$F(6,35) = 10.227, p = 0.001$]. Tukey's post hoc test revealed that there was a statistically increase ($p < 0.05$) in the number of neuronal apoptotic cells within the subfields of AD Model group of rats; CA1 (4.47 ± 0.78) as compared to their respective Control Group; CA1 (2.37 ± 0.15) (Figure 4.5). Based on the morphology of the neuronal cells observed, the administration of varying doses of Δ^9 THC alleviates neuronal apoptosis across the treated groups. Tukey's post hoc analysis confirmed the administration of Δ^9 THC has resulted in significantly reduced neuronal apoptosis of pyramidal cells in the CA1 subfield; 1.50 mg/kg (2.23 ± 0.22) and 3.0 mg/kg (1.83 ± 0.11) treatment groups, compared to the AD Model (4.47 ± 0.78) and AD Without Treatment (4.73 ± 0.38). By comparing statistically, it was proven that our results were aligned with Donepezil as shown in the results at which the treated groups has significantly reduced number of neuronal apoptotic cells (2.47 ± 0.15) among the groups.

4.2.2 Histological Results Showing Δ^9 THC Reduces the Number of Apoptotic Pyramidal Cells at CA3 Region in Rats Induced by D-gal and AlCl_3

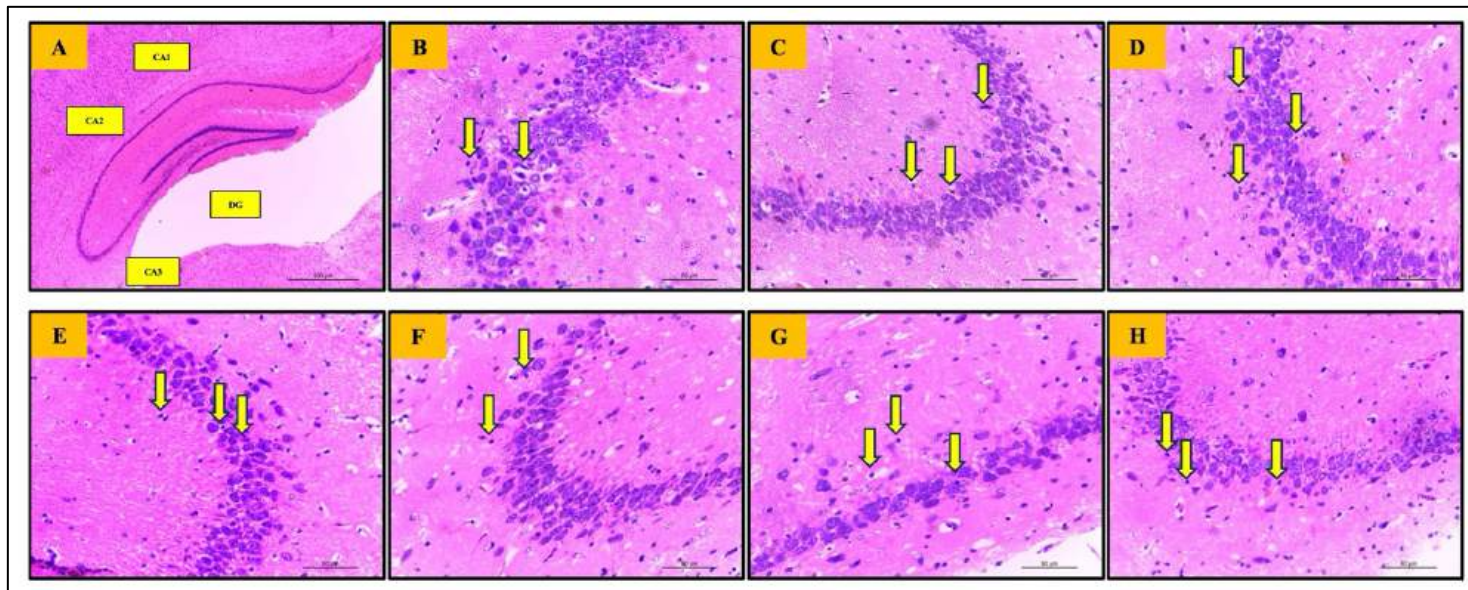


Figure 4.6 Hematoxylin and Eosin-Stained Photomicrographs of Brain Sections, specifically Cornu Ammonis 3 (CA3) Region. (A) The Structure of Whole Hippocampus Showing All Parts at Magnification 50x; CA1, CA2, CA3 and DG, (B) Control Rats, (C) AD Model Demonstrates An Increase of Apoptotic Cells, (D) AD Model Without Any Treatment, (E) Treated with 1mg/kg Donepezil, (F-H) Treated with 0.75mg/kg Δ^9 THC, 1.5mg/kg Δ^9 THC and 3.0mg/kg Δ^9 THC Respectively, Has Showed a Decrease in the Number of Apoptotic Cells at CA3 Region. Magnification of 400x for (B-H), Scale Bar 50 μ m. CA1- Cornu Ammonis 1; CA2 – Cornu Ammonis 2; CA3 – Cornu Ammonis 3; Δ^9 THC – Delta-9-Tetrahydrocannabinol.

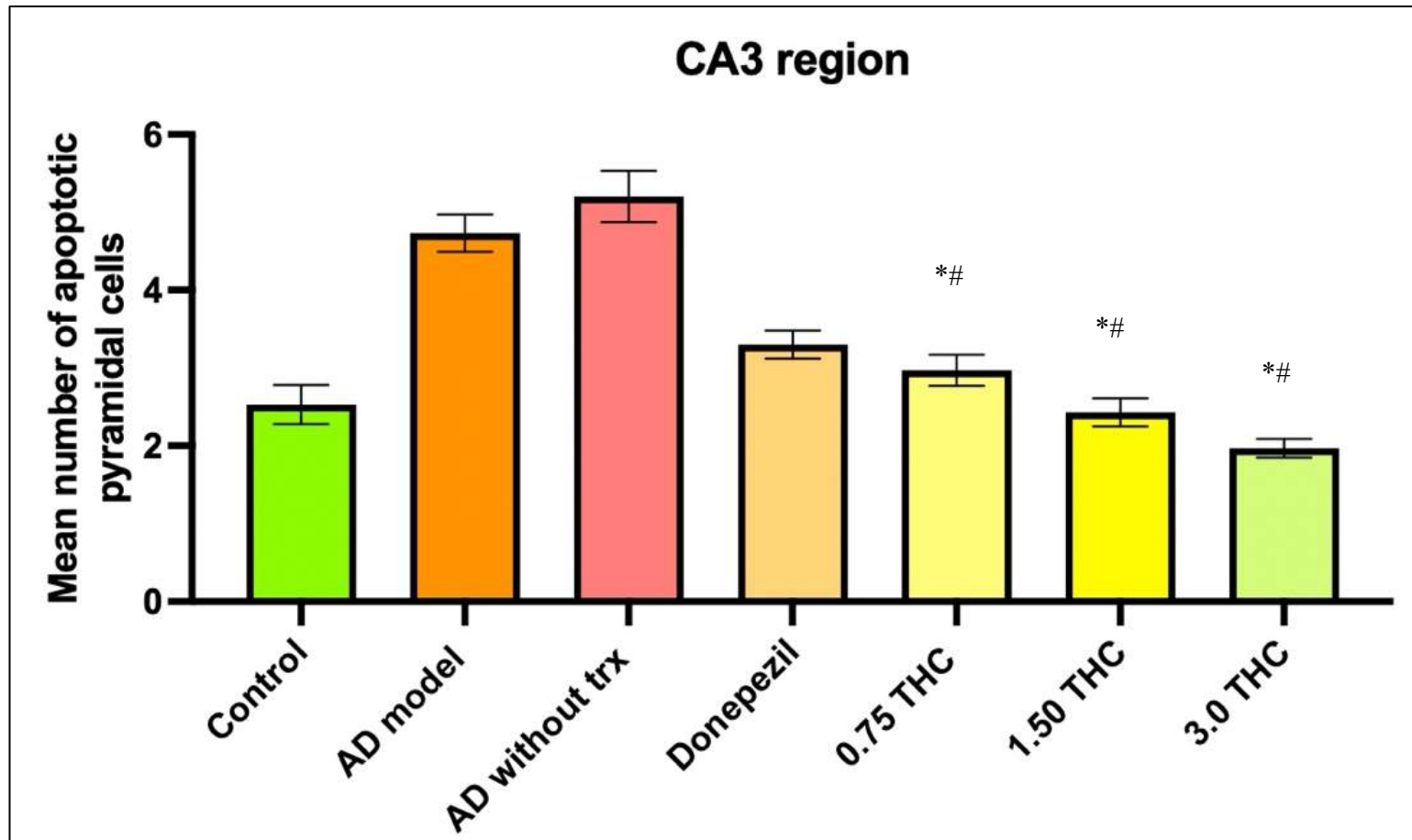


Figure 4.7 Mean Number of Apoptotic Pyramidal Cells at CA3 Region for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test Among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). * p < 0.05 vs AD model, # p < 0.05 vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.6 depicts the subfields of CA3 region of the rat hippocampus in Control Group and the treatment groups of Δ^9 THC with varying doses; 0.75mg/kg, 1.50mg/kg and 3.0mg/kg respectively. The presence of viable cells is distinguished by the observed light cytoplasm, dark nucleus and well-arranged intact layers of pyramidal cells in the control group of rats. In the AD Model, some hyperchromic cells were seen with altered morphology of pyramidal cells that signified cellular deterioration has occurred. Statistically, One-way ANOVA revealed there is a significance difference in the number of apoptotic pyramidal cells in CA3 region [$F(6,35) = 29.398$, $p = 0.001$] of the hippocampus (Figure 4.7). This was proved with the reduction in the number of apoptotic pyramidal cells at CA3 region compared to AD Model (4.73 ± 0.25) and AD Without Treatment (5.20 ± 0.33); 0.75 mg/kg (2.97 ± 0.20), 1.50 mg/kg (2.43 ± 0.18) and 3.0 mg/kg (1.97 ± 0.12) respectively. Interestingly, there is only a slight difference between the results of Δ^9 THC and Donepezil (3.30 ± 0.18), one of the approved drugs used to treat AD. The mean number of apoptotic cells of concentration of Δ^9 THC at 1.50 mg/kg and 3.0 mg/kg are found to be even lower than Donepezil proving a better chance in remediating AD. In addition, between the three doses of Δ^9 THC, the difference in means of neuronal apoptosis showed that 1.50 mg/kg and 3.0 mg/kg have a much significantly lower mean than 0.75 mg/kg. All in all, Tukey's post hoc analysis confirmed that Δ^9 THC treatment has resulted in a decrease in the number of apoptotic cells caused by the declining progression of AD.

4.2.3 Histological Results Showing Δ^9 THC Reduces the Number of Apoptotic Granule Cells at Dentate Gyrus (DG) Region in Rats Induced by D-gal and AlCl_3

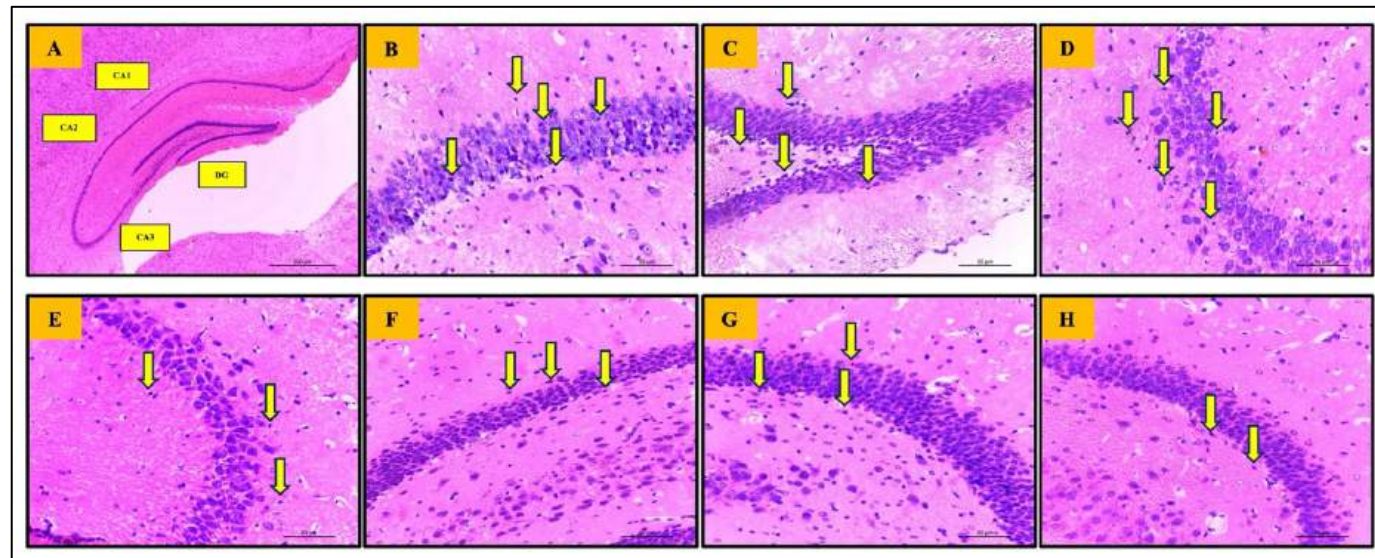


Figure 4.8 Hematoxylin and Eosin-Stained Photomicrographs of Brain Sections, specifically Dentate Gyrus (DG) Region. (A) The Structure of Whole Hippocampus Showing All Parts at Magnification 50x; CA1, CA2, CA3 and DG, (B) Control Rats, (C) AD Model Group Demonstrates An Increase of Apoptotic Cells, (D) AD Model Without Any Treatment, (E) Treated with 1mg/kg Donepezil, (F-H) Treated with 0.75mg/kg Δ^9 THC, 1.5mg/kg Δ^9 THC and 3.0mg/kg Δ^9 THC Respectively, Has Showed A Decrease in the Number of Apoptotic Cells at DG Region. Magnification of 400x for (B-H), Scale Bar 50 μ m. CA1- Cornu Ammonis 1; CA2 – Cornu Ammonis 2; CA3 – Cornu Ammonis 3; Δ^9 THC – Delta-9-Tetrahydrocannabinol.

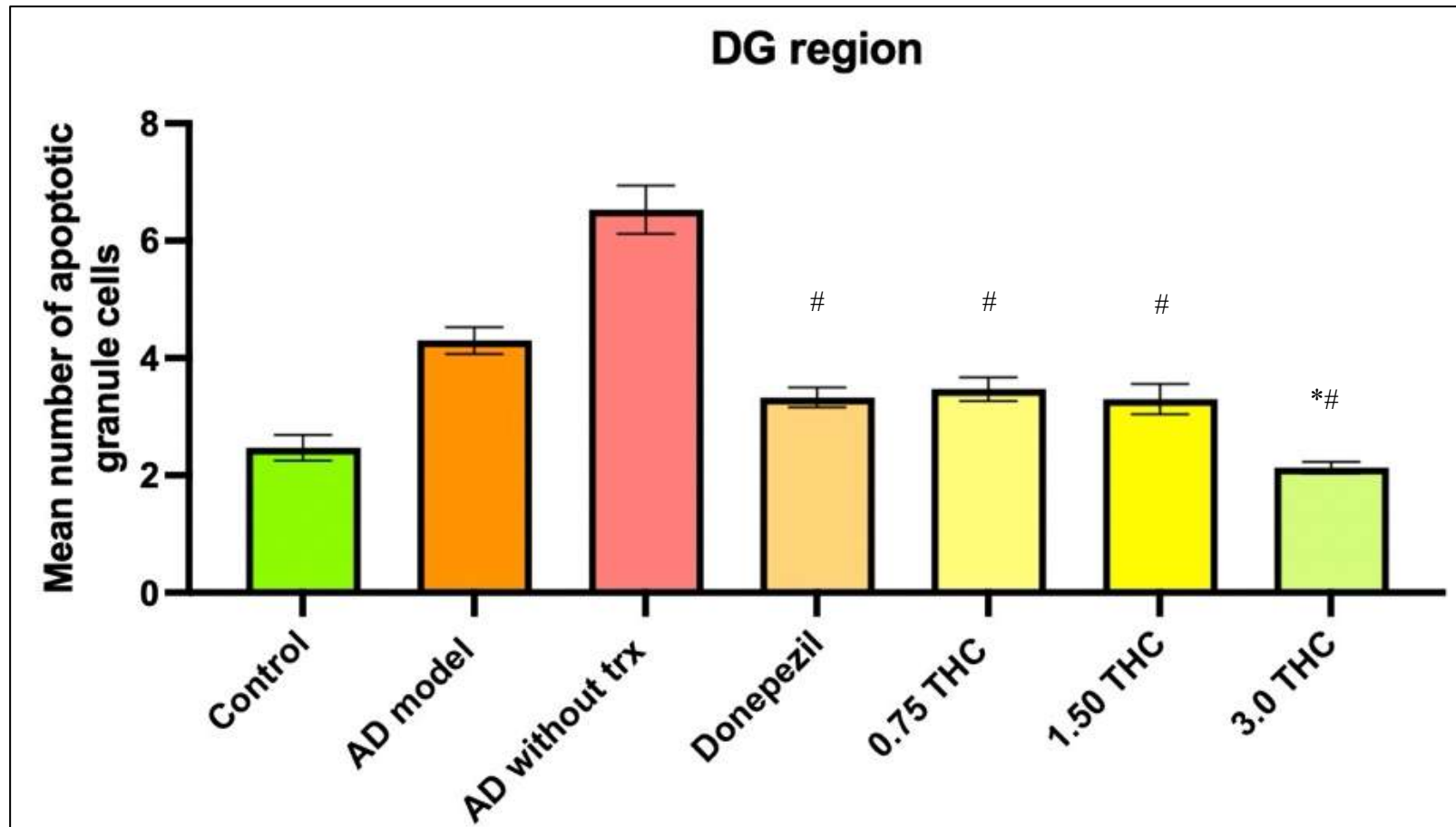


Figure 4.9 Mean Number of Apoptotic Granule Cells at DG Region for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test Among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). * p < 0.05 vs AD model, # p < 0.05 vs AD Without Treatment. CA – Cornu Ammonis

The therapeutic potential of Δ^9 THC in alleviating neurodegeneration in D-gal + AlCl_3 induced AD model were also studied on its impacts in the dentate gyrus region (both upper and lower blade). Figure 4.8 depicts the histology of the dentate gyrus sections for all groups. Compared to the AD Model, the histology of the dentate gyrus in the control group showed a normal cytoarchitecture with a dense layer of granule cells, differentiating between the dark nuclei in the centre and its surrounding. However, in the AD-induced model of rats, apoptotic granule cells were seen with its morphological features that include cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and the formation of membrane-bound apoptotic bodies. This confirms the apoptotic cells is due to the chronic exposure of rats to D-gal and AlCl_3 resulting dentate gyrus degeneration. One-way ANOVA showed statistically differences in the number of apoptotic granule cells [$F(6,35) = 36.335$, $p = 0.001$] in the dentate gyrus. Tukey's post hoc test revealed that there was a statistically increase ($p < 0.05$) in the number of apoptotic granule cells in the dentate gyrus of the AD Model (4.30 ± 0.23) as compared to the Control Group (2.47 ± 0.22) (Figure 4.9). Furthermore, a decrease in the number of apoptotic granule cells was observed when the rats were treated with Donepezil (3.33 ± 0.17) and all doses of Δ^9 THC; 0.75 mg/kg (3.47 ± 0.20), 1.5 mg/kg (3.30 ± 0.26), 3.0 mg/kg (2.13 ± 0.10) when compared to the AD Model of Rats (4.30 ± 0.23). From the post-hoc Tukey's analysis, the dosage of Δ^9 THC at 3.0 mg/kg was observed to have a significantly higher value of mean neuronal apoptosis as compared to the other dosages.

4.3 Δ^9 THC Intensifies the Expression of Calbindin and GFAP as Neurogenesis Protein Markers in an AD Rat Model Induced by D-gal and AlCl_3 through Immunofluorescence Technique

4.3.1 Quantification of Mean Fluorescence Intensity of GFAP Expression

4.3.1.1 Expression of GFAP as Hippocampal Neurogenesis Marker at CA1 Region

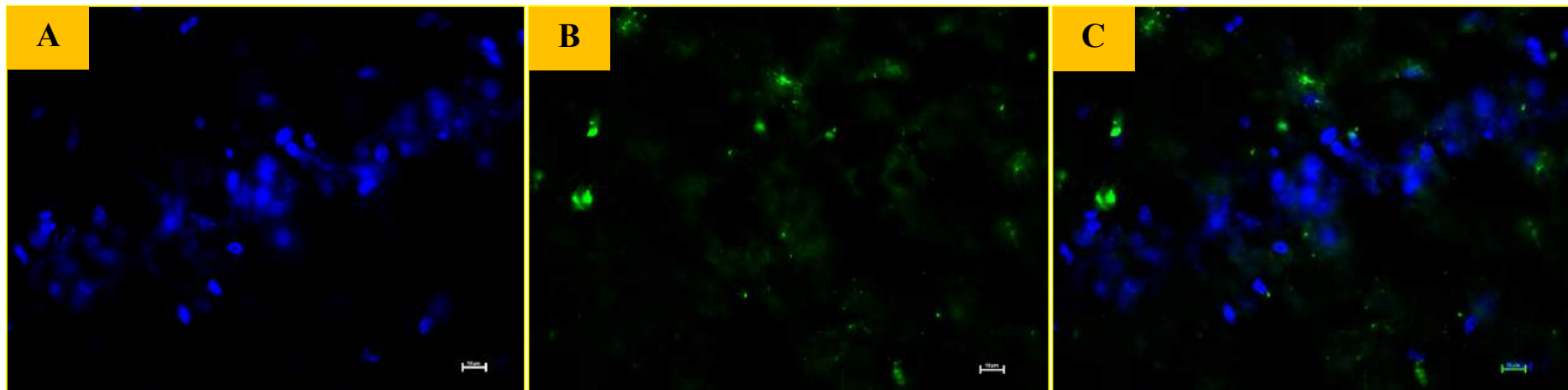


Figure 4.10 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in Control Group, specifically at the CA1 Region of the Hippocampus. The Results Showed Normal Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μm . Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

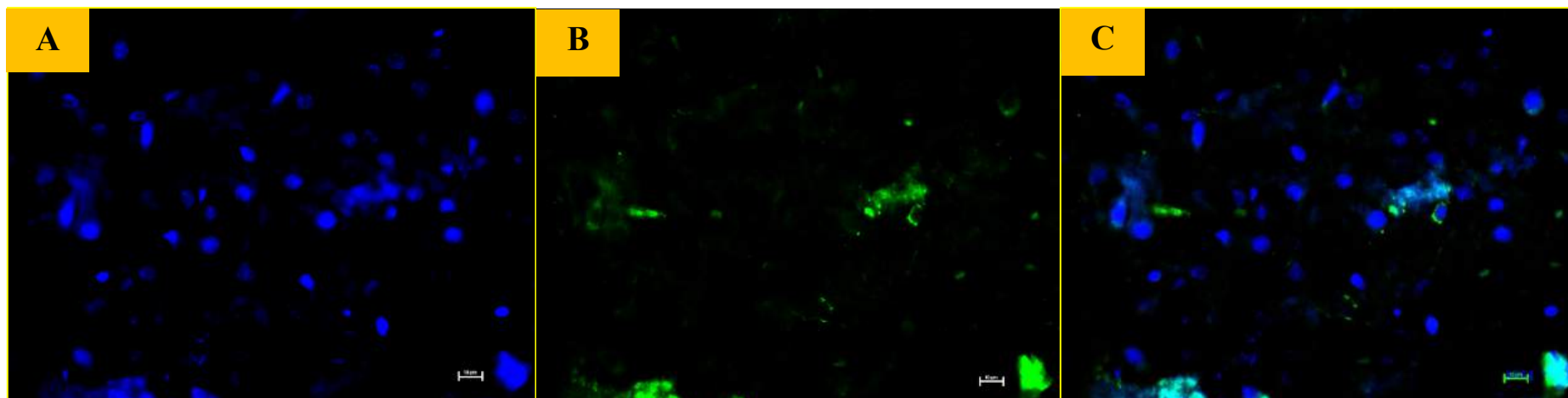


Figure 4.11 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model, specifically at the CA1 Region of the Hippocampus. The Results Showed Less Intense of Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Reduced Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

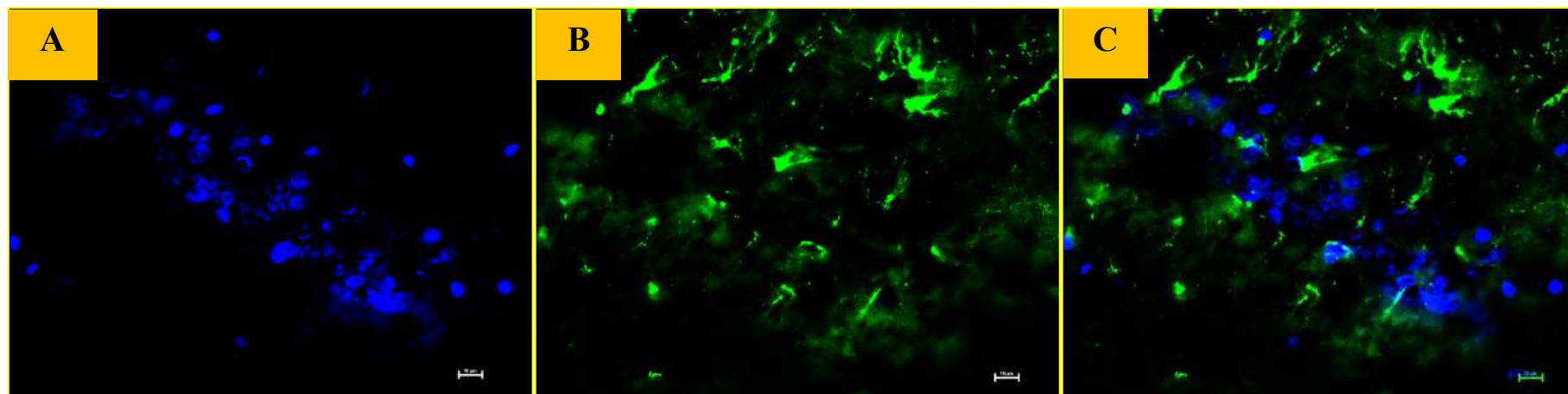


Figure 4.12 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model Without Treatment, specifically at the CA1 Region of the Hippocampus. The Results Showed Less Intense Co-Staining of DAPI And GFAP surrounding the Pyramidal Cells, Indicating Reduced Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates The Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

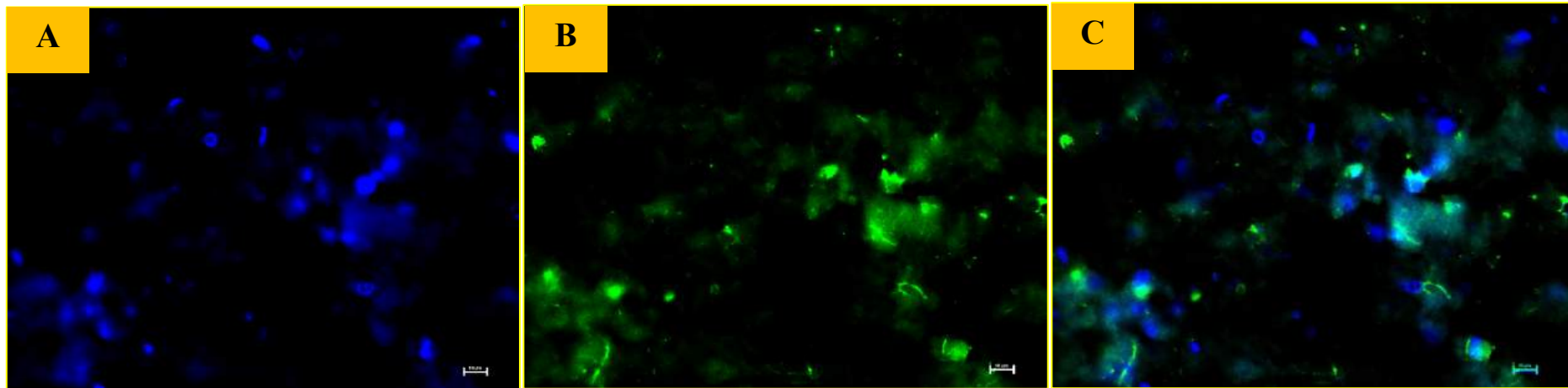


Figure 4.13 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil, specifically at the CA1 Region of the Hippocampus. The Results Showed Intensive Co-Staining Of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

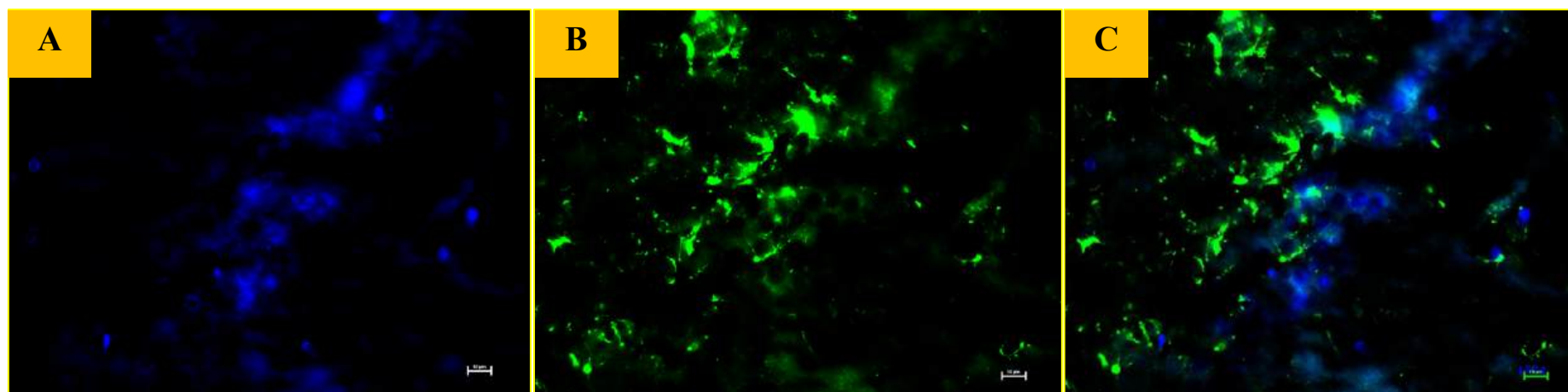


Figure 4.14 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 0.75 mg/kg Δ^9 THC, specifically at the CA1 Region of the Hippocampus. The Results Showed High Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

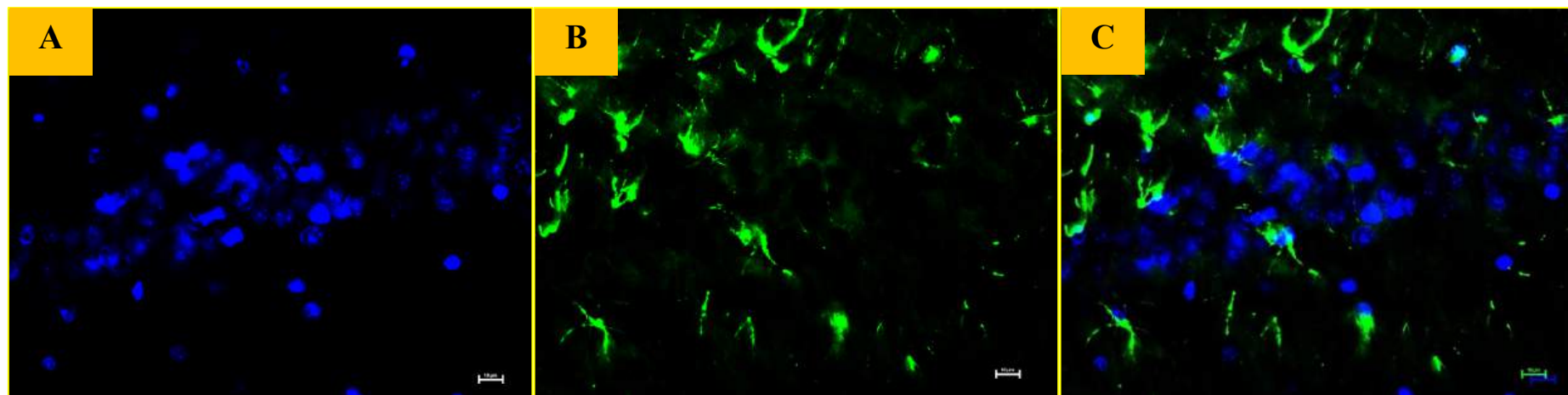


Figure 4.15 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.5 mg/kg Δ^9 THC, specifically at the CA1 Region of the Hippocampus. The Results Showed High Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates The Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

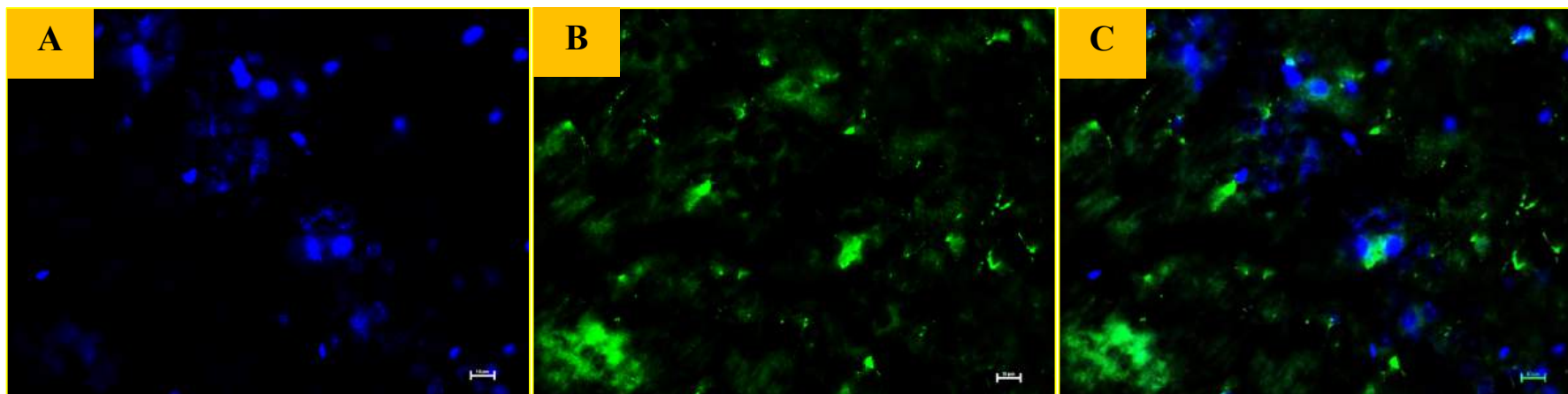


Figure 4.16 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 3.0 mg/kg Δ^9 THC, specifically at the CA1 Region of the Hippocampus. The Results Showed Highest Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

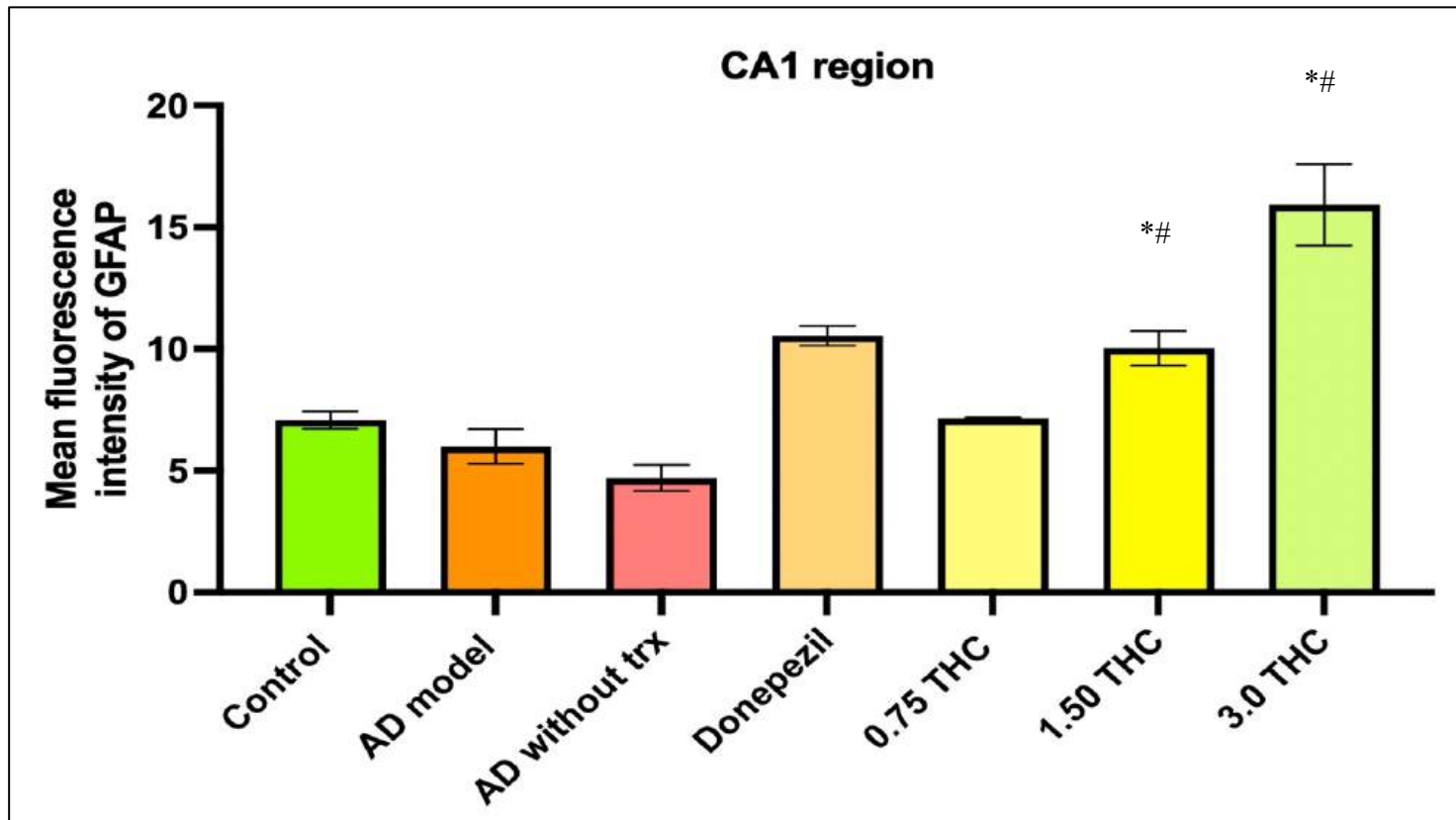


Figure 4.17 Mean Area of Immunofluorescent-Stained Pyramidal Cells of GFAP at CA1 Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test Among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). # p < 0.05 vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.10 until Figure 4.16 depict the photomicrographs of immunofluorescent-stained images of GFAP in the subfields of CA1 region of the rat hippocampus in the Control Group and the treatment groups of Donepezil and Δ^9 THC with varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. The analysis of the immunofluorescence is determined by its levels of fluorescent intensity among the cells indicating neurogenesis has occurred. The mean of immunofluorescent-stained neuronal results showed an increase in the intensity of GFAP+ cells among the pyramidal cells. Results show that mean intensities of GFAP+ cells are higher in the treatment groups (0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg) as compared to the AD Model (5.99 ± 0.71) and AD Without Treatment (4.70 ± 0.53). Morphologically, it can be observed that the treated groups specifically at 1.50 mg/kg and 3.0 mg/kg, showed more glow-stained cells compared to AD Model and AD Without Treatment among the pyramidal cells. This shows that neurogenesis was enhanced parallel with the increased mean fluorescence intensity of neurogenesis markers in the rats treated with Δ^9 THC. Statistically, One-way ANOVA revealed an increased neurogenesis in the CA1 region [$F(6,49) = 4.43$, $p = 0.001$] when compared to both AD Model and AD Without Treatment (Figure 4.17). Furthermore, compared with the Donepezil-treated group (10.54 ± 0.40), Δ^9 THC specifically at the concentration of 1.50 mg/kg (10.03 ± 0.72) and 3.0 mg/kg (15.92 ± 1.67) have successfully resulted either on par with the FDA-approved drug, Donepezil or even to our interesting note, successfully scored much higher mean intensities of GFAP as seen in the above result of the highest concentration of Δ^9 THC, 3.0 mg/kg (15.92 ± 1.67). The results were drastic as AD Model (5.99 ± 0.71) and AD Without Treatment (4.70 ± 0.53) showed apparent minimized neurogenesis with low intense fluorescence.

4.3.1.2 Expression of GFAP as Hippocampal Neurogenesis Marker at CA3 Region

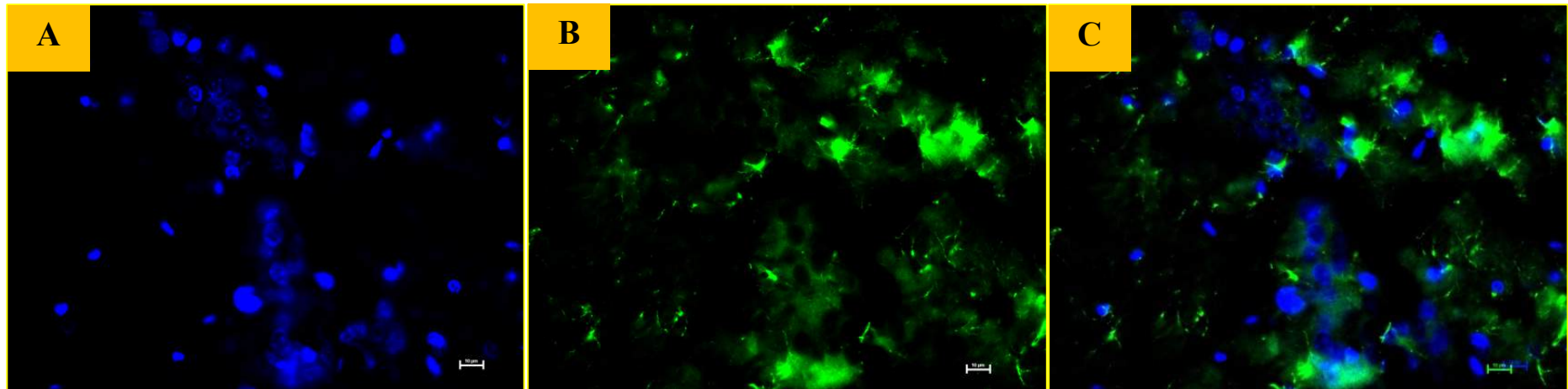


Figure 4.18 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in the Control Group, specifically at the CA3 Region of The Hippocampus. The Results Showed Normal Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among The Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

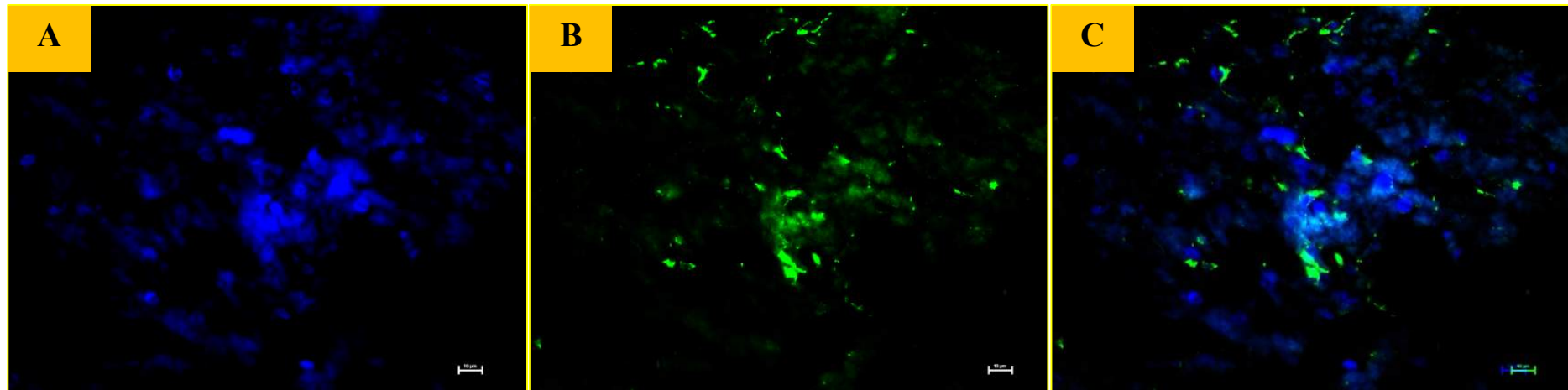


Figure 4.19 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model, specifically at the CA3 Region of the Hippocampus. The Results Showed Less Intense Co-Staining of DAPI And GFAP surrounding the Pyramidal Cells, Indicating Reduced Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

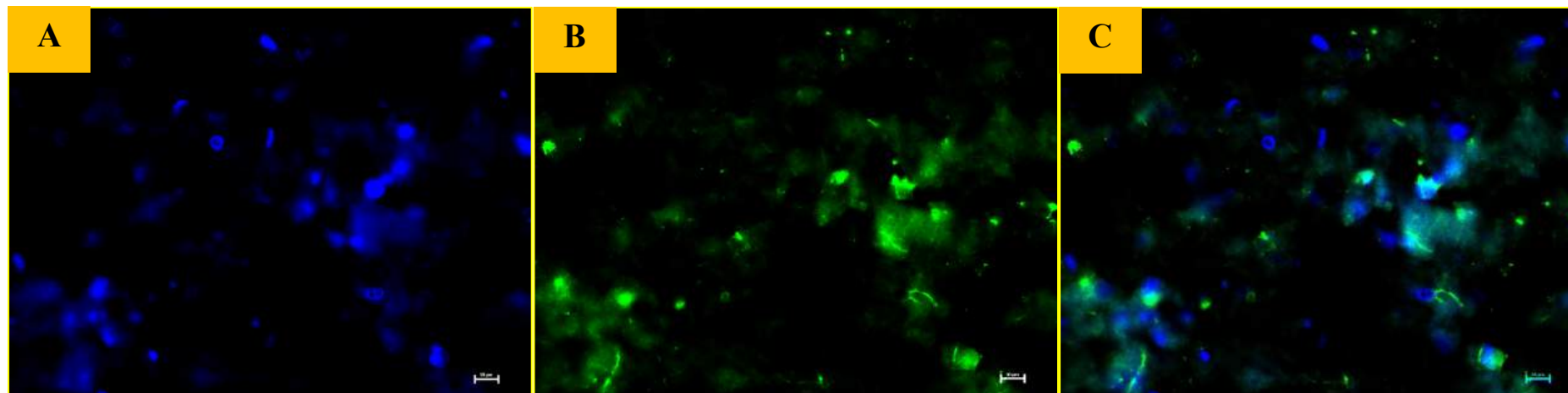


Figure 4.20 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model Without Treatment, specifically at the CA3 Region of the Hippocampus. The Results Showed Less Intense Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Reduced Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

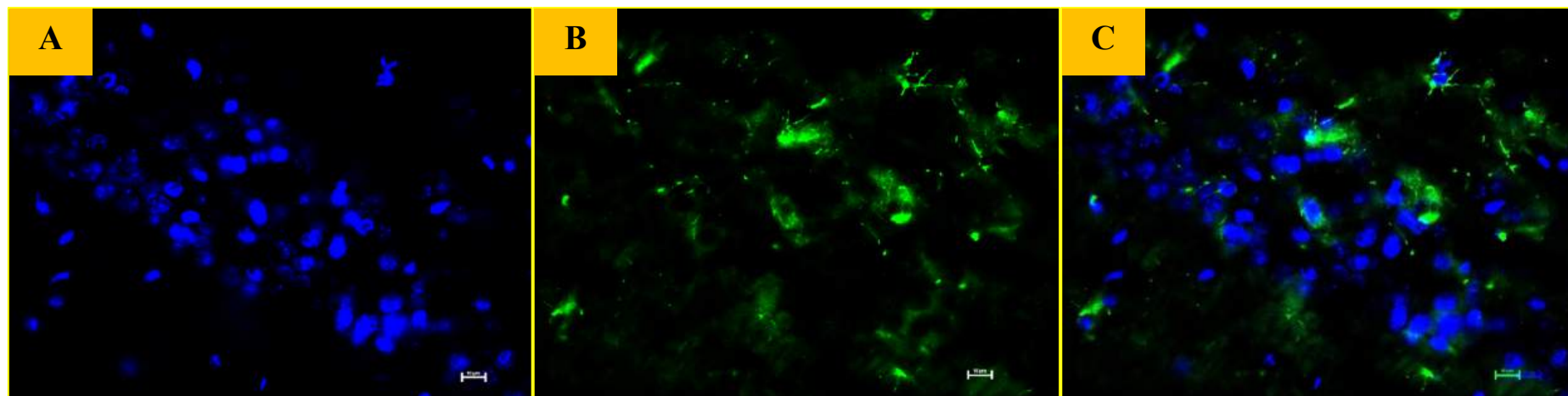


Figure 4.21 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil, specifically at the CA3 Region of the Hippocampus. The Results Showed Slightly Higher Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

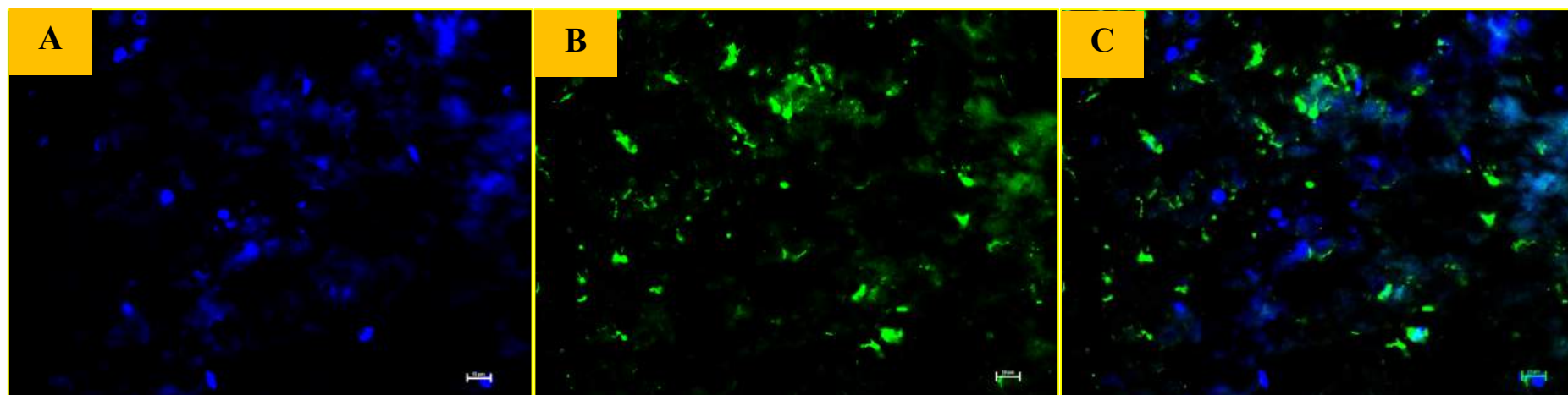


Figure 4.22 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 0.75 mg/kg Δ^9 THC, specifically at the CA3 Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

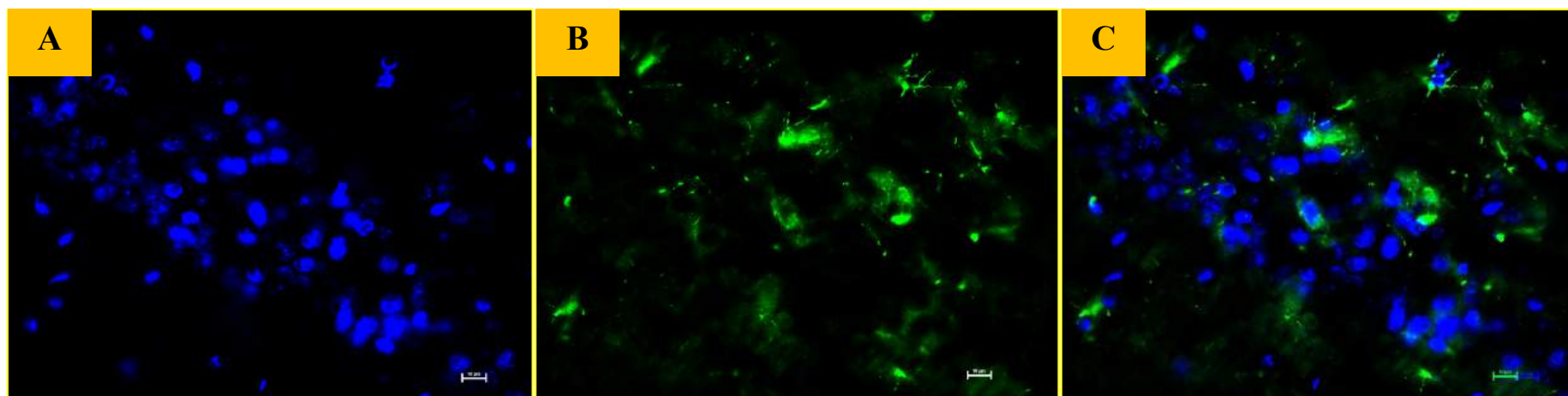


Figure 4.23 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.50 mg/kg Δ^9 THC, specifically at the CA3 Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

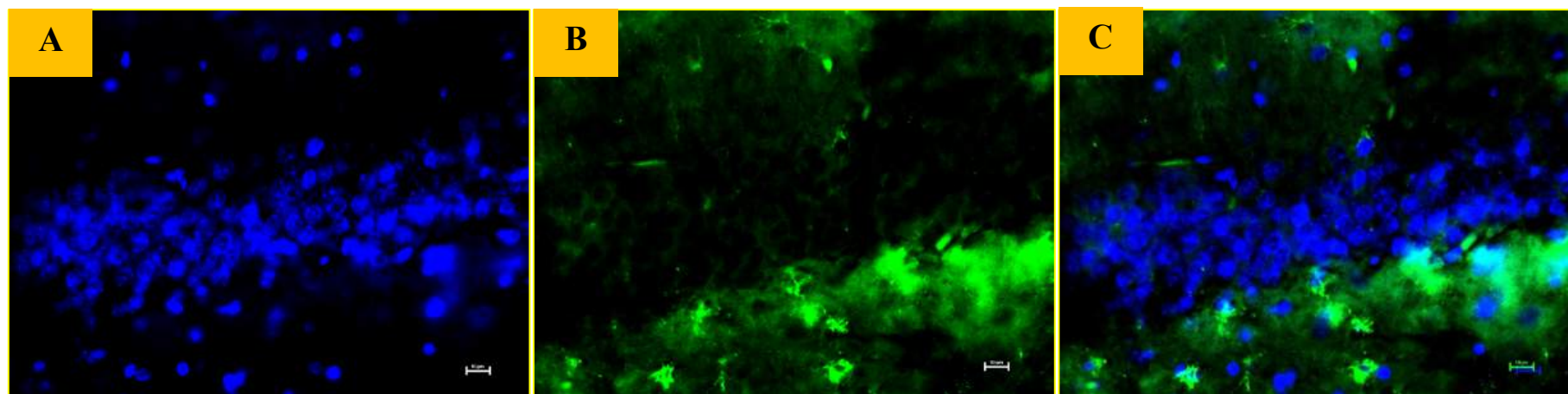


Figure 4.24 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated With 3.0 mg/kg Δ^9 THC, specifically at the CA3 Region of the Hippocampus. The Results Showed Highest Intensive Co-Staining of DAPI and GFAP surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

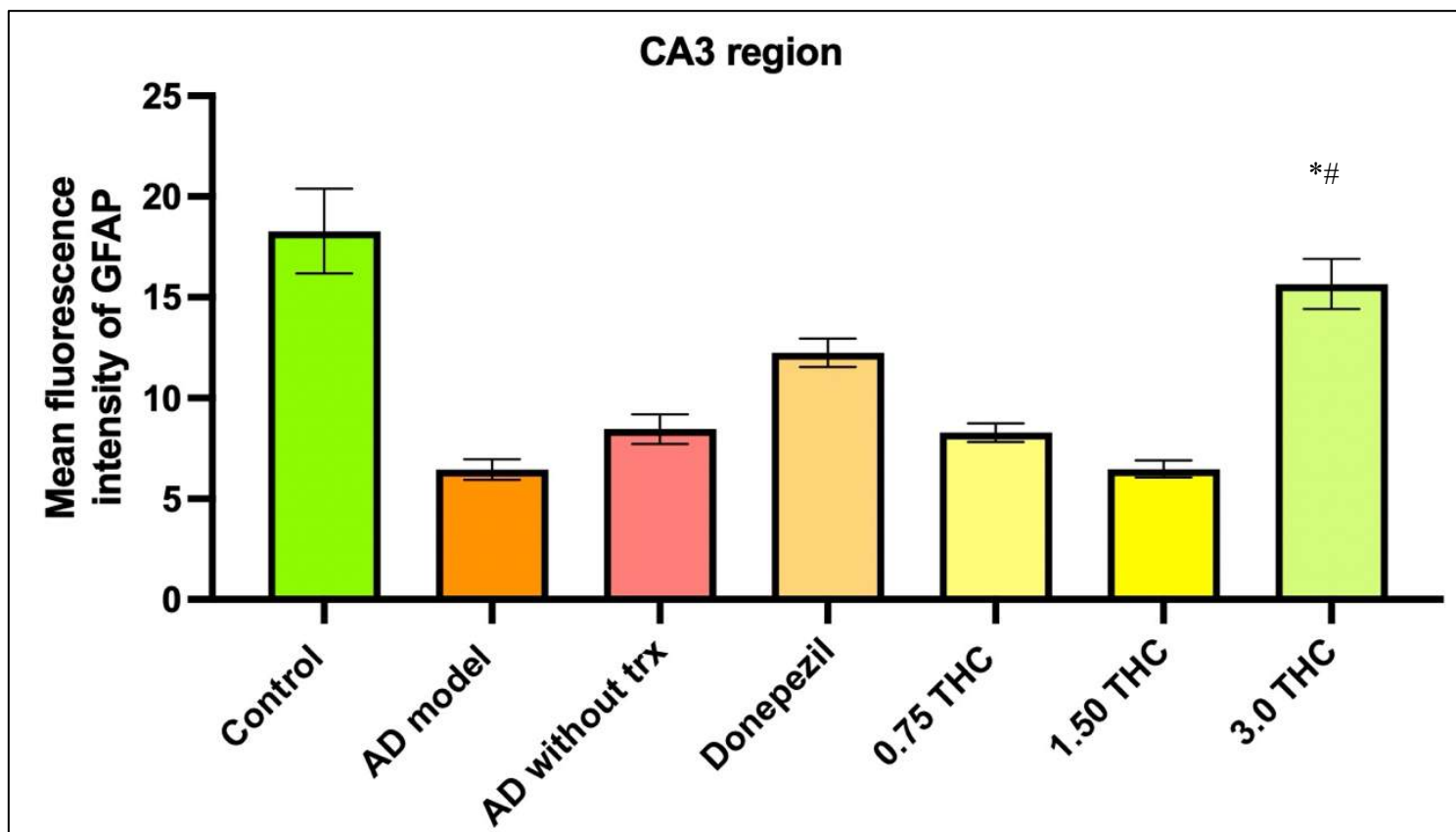


Figure 4.25 Mean Area of Immunofluorescent-Stained Pyramidal Cells of GFAP at CA3 Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). * p < 0.05 vs AD Model, # p < 0.05 vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.18 until Figure 4.25 depict the photomicrographs of immunofluorescent-stained images of GFAP in the subfields of CA3 region of the rat hippocampus in the Control Group and the treatment groups of Donepezil and Δ^9 THC with varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. The analysis of the immunofluorescence is determined by its levels of fluorescent intensity among the cells indicating neurogenesis has occurred. Interestingly, the mean of immunofluorescent-stained neuronal results showed a definite increase in intensity of GFAP especially among the treated groups. However, based on the results shown, morphologically it can be seen that the intensity is the highest at 3.0 mg/kg as compared to the AD Model (6.45 ± 0.50) and AD Without Treatment Group (8.46 ± 0.74) as the glow-stained cells increased. In comparison with the photomicrographs in the AD Model and AD Without Treatment groups, there is a lesser glow of GFAP among the pyramidal cells. Hence, lesser neurogenesis occurred in that area as compared to the rats treated with Δ^9 THC. When the treatments are being compared to Donepezil, an approved drug for AD (12.25 ± 0.71), the Δ^9 THC-treated group specifically 3.0 mg/kg (15.41 ± 1.20) has resulted an obvious higher value and this represents promising positive results in mean intensities of GFAP respectively. Statistically, the results of One-way ANOVA justified that Δ^9 THC has a potential to stimulate GFAP+ cells in the CA3 region [$F(6,49) = 19.97$, $p = 0.001$] when compared with both AD Model and AD Without Treatment (Figure 4.25).

4.3.1.3 Expression of GFAP as Hippocampal Neurogenesis Marker at DG Region

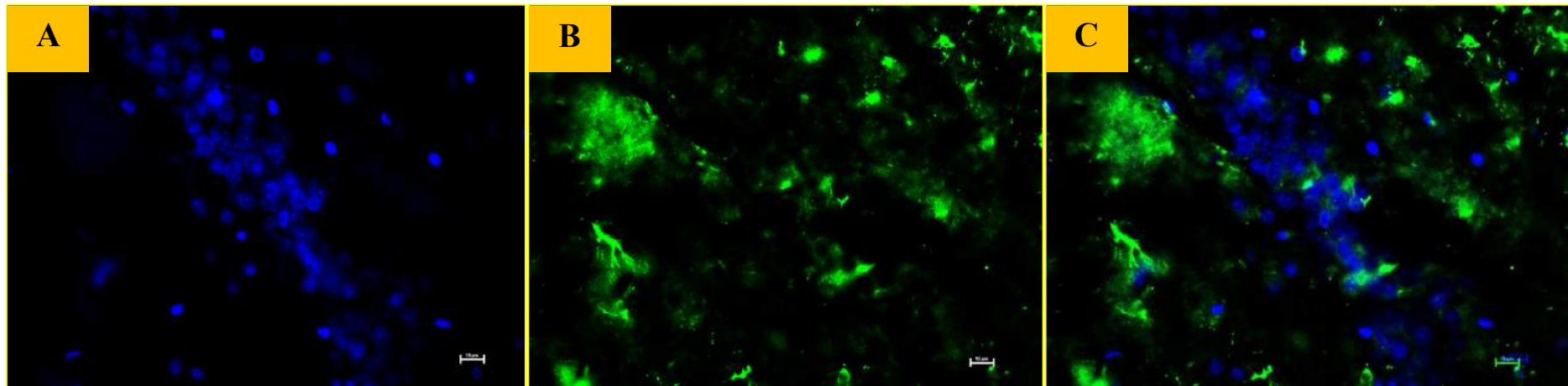


Figure 4.26 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in Control Group, specifically at the DG Region of the Hippocampus. The Results Showed Normal Intensive Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

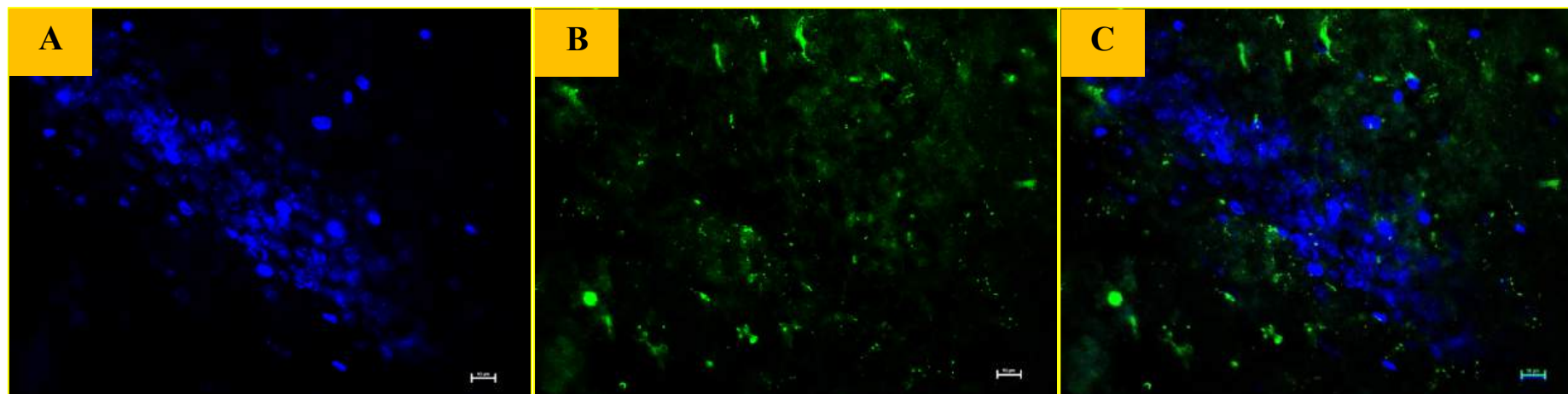


Figure 4.27 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model, specifically at the DG Region of the Hippocampus. The Results Showed Less Intense Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Reduced Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

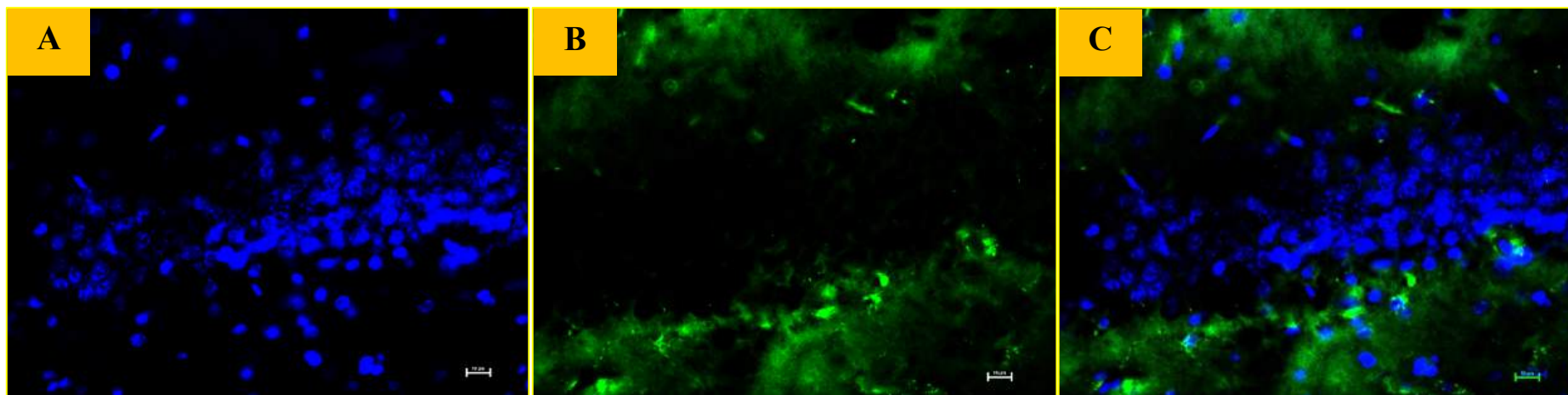


Figure 4.28 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model Without Treatment, specifically at the DG Region of the Hippocampus. The Results Showed Less Intense Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

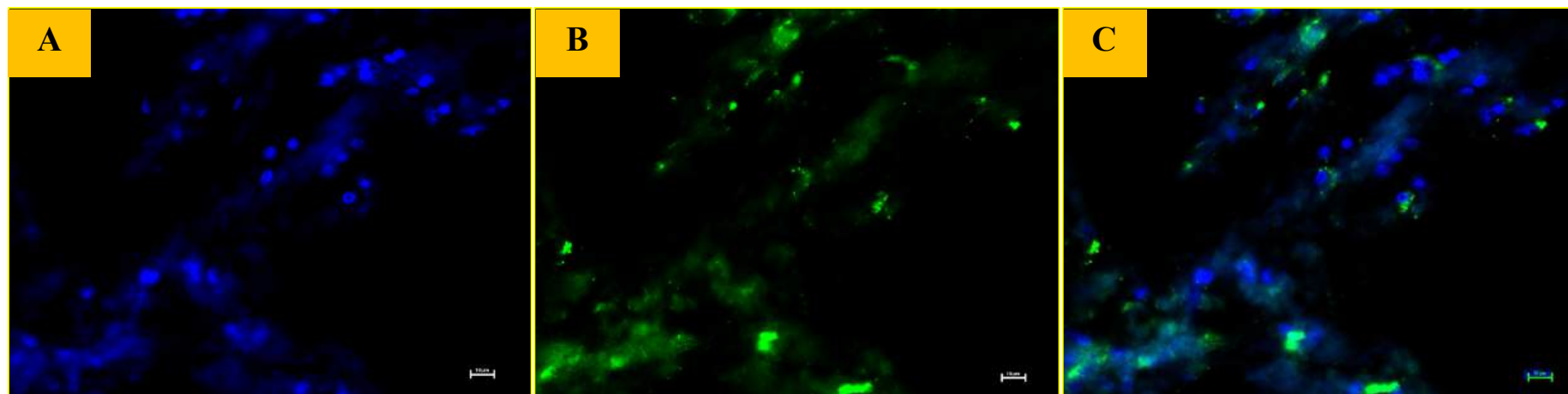


Figure 4.29 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil, specifically at the DG Region of the Hippocampus. The Results Showed Slightly Higher Intensive Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

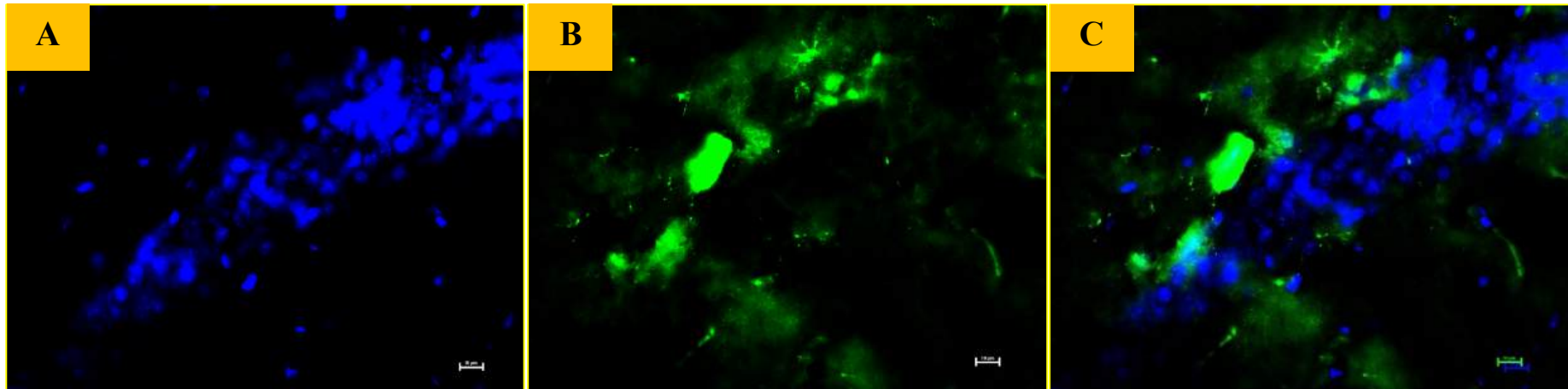


Figure 4.30 Representative Immunofluorescent-Stained Photomicrographs of The Brain Sections Treated with 0.75 mg/kg Δ^9 THC, specifically at the DG Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

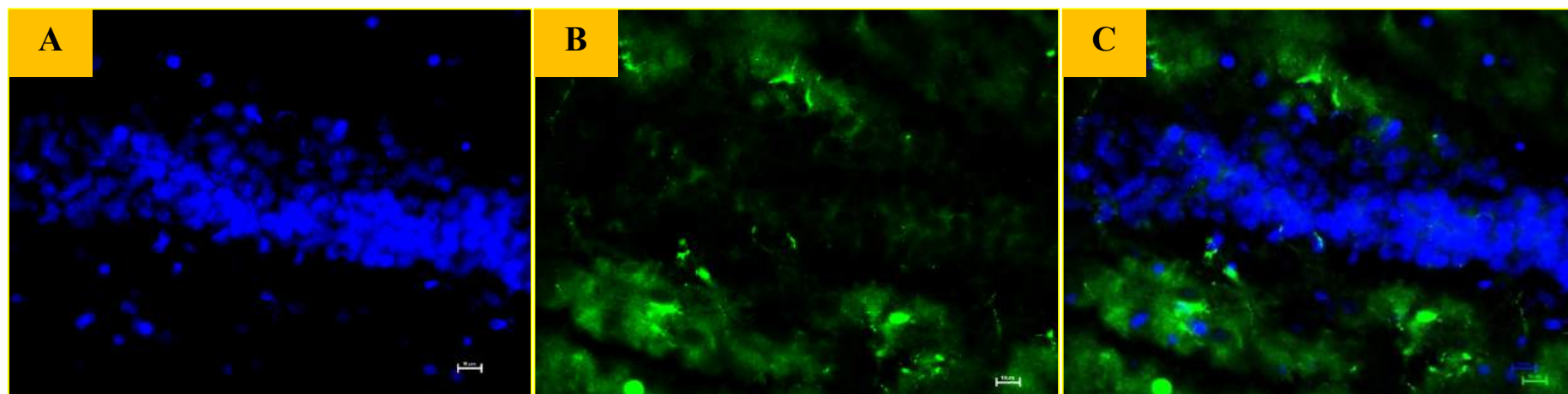


Figure 4.31 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.5 mg/kg Δ^9 THC, specifically at the DG Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

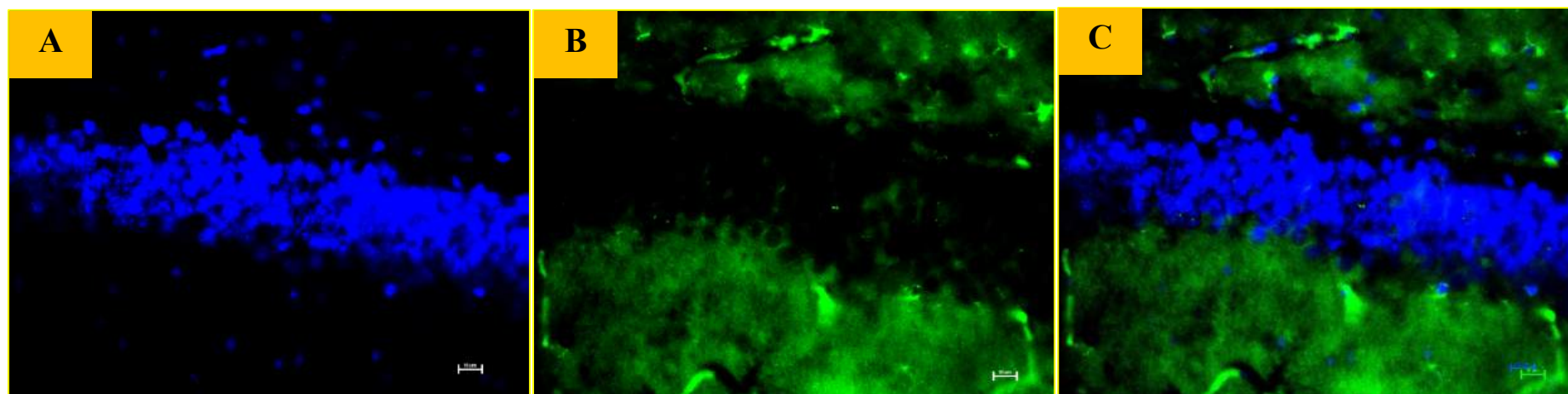


Figure 4.32 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 3.0 mg/kg Δ^9 THC, specifically at the DG Region of the Hippocampus. The Results Showed Highest Intensive Co-Staining of DAPI and GFAP surrounding the Granule Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Granule Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker GFAP among Granule Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and GFAP respectively.

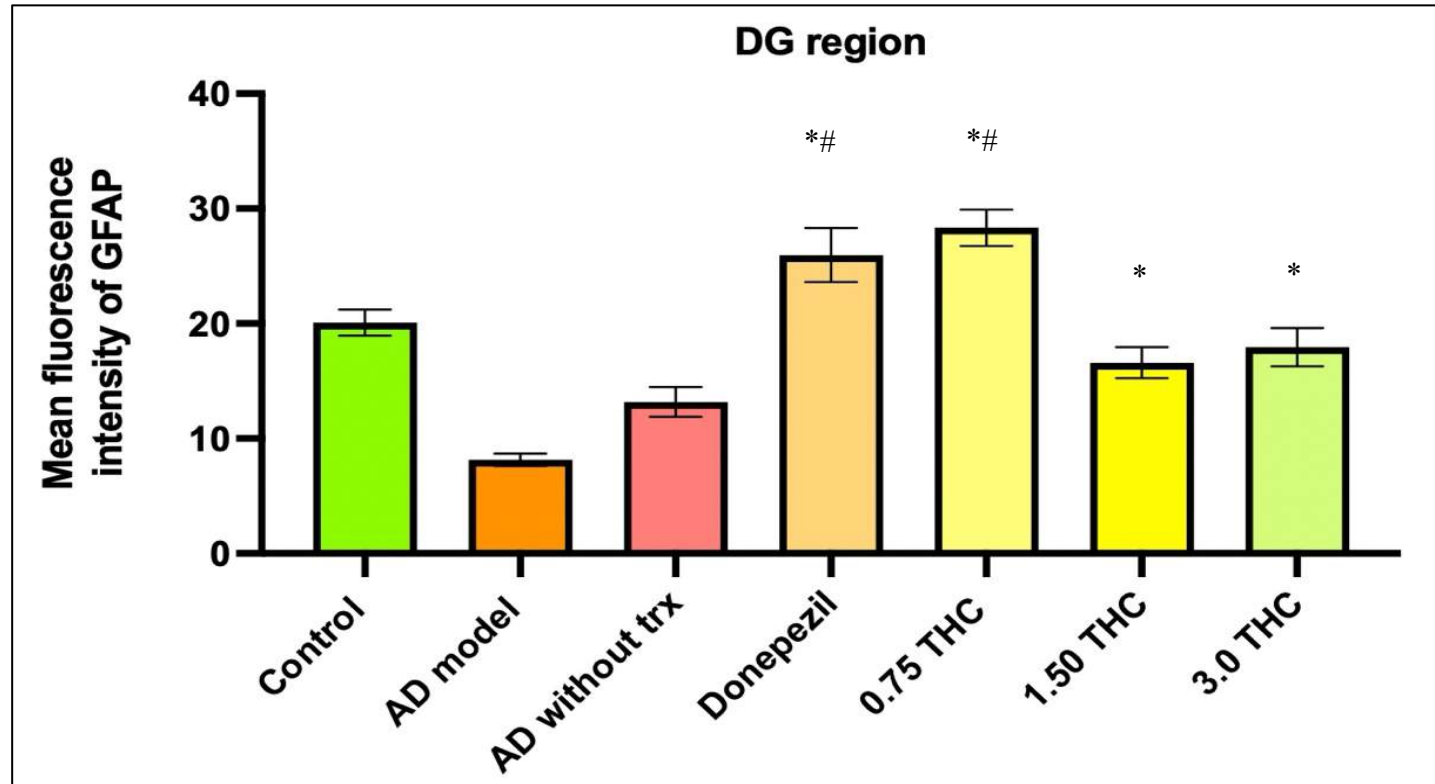


Figure 4.33 Mean Area of Immunofluorescent-Stained Granule Cells of GFAP at DG Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM

(n = 7). * p < 0.05 vs AD Model, # p < 0.05 vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.26 until Figure 4.32 depict the photomicrographs of immunofluorescent-stained images of GFAP in the subfields of DG region of the rat hippocampus in the control group and the treatment groups of Donepezil and Δ^9 THC with varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. The analysis of the immunofluorescence is determined by its levels of fluorescent intensity among the cells indicating neurogenesis has occurred. The mean of immunofluorescent-stained neuronal results showed an increase in intensity of GFAP especially among the treated groups (0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg). Results indicated that the intensity of GFAP is higher in the treatment groups as compared to the AD Model (8.14 ± 0.54) and AD Without Treatment (13.19 ± 1.29). Based on the results showed, it is apparent that the mean intensity of GFAP increased among granule cells in the treatment groups as the glow-stained cells increased. Compared with the photomicrographs in the AD model and AD Without Treatment, there is a lesser glow of GFAP among the granule cells. Hence, lesser neurogenesis has occurred in that area as compared to the rats treated with Δ^9 THC. When the treatments are being compared to Donepezil, an approved drug for AD (25.95 ± 2.35), only the 0.75 mg/kg Δ^9 THC-treated group (28.34 ± 1.58) has resulted slightly higher mean intensities of GFAP justifying the effectiveness of the treatment. Despite the other two concentrations of Δ^9 THC-treated resulted lower mean intensities; 1.50 mg/kg (16.59 ± 1.35) and 3.0 mg/kg (17.95 ± 1.67), this still shows potential therapeutic values as it has progressively increased compared to those of AD Model (8.14 ± 0.54) and AD Without Treatment (13.19 ± 1.29). Statistically, the results of One-way ANOVA justified that Δ^9 THC has a potential to stimulate GFAP in the DG region [$F(6,49) = 21.59$, $p = 0.001$] when compared with both AD Model and AD Without Treatment (Figure 4.33).

4.3.2 Quantification of Mean Fluorescence Intensity of Calbindin Expression

4.3.2.1 Expression of Calbindin as Hippocampal Neurogenesis Marker at CA1 Region

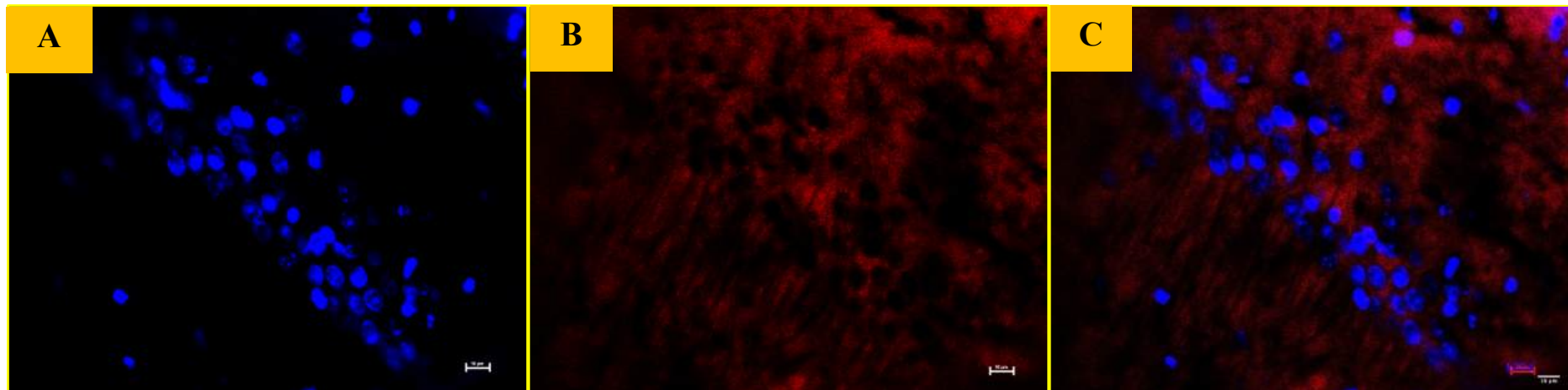


Figure 4.34 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in Control Group, specifically at the CA1 Region of the Hippocampus. The Results Showed Normal Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Intensive Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

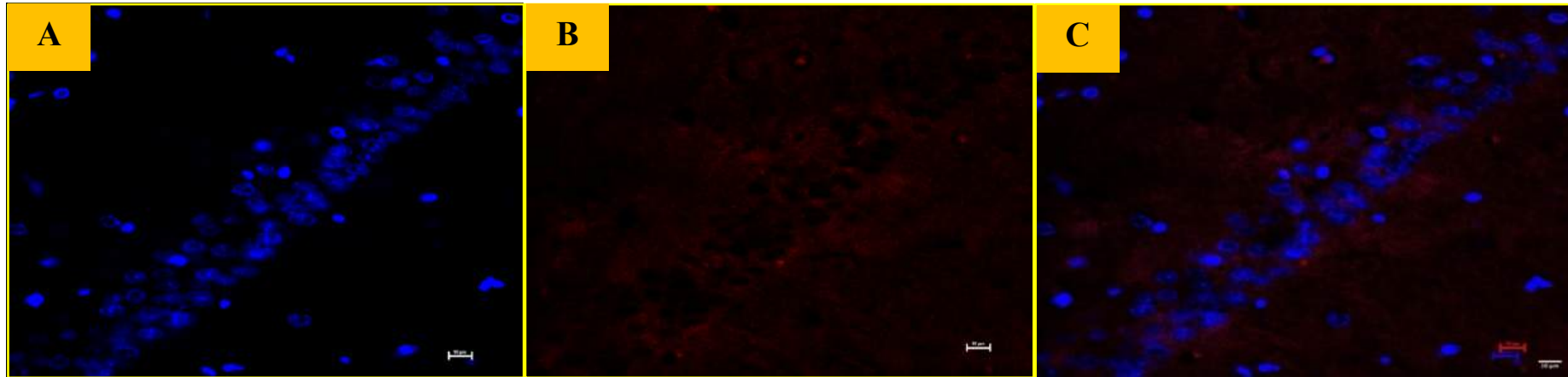


Figure 4.35 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model, specifically at the CA1 Region of the Hippocampus. The Results Showed Less Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Decreased Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

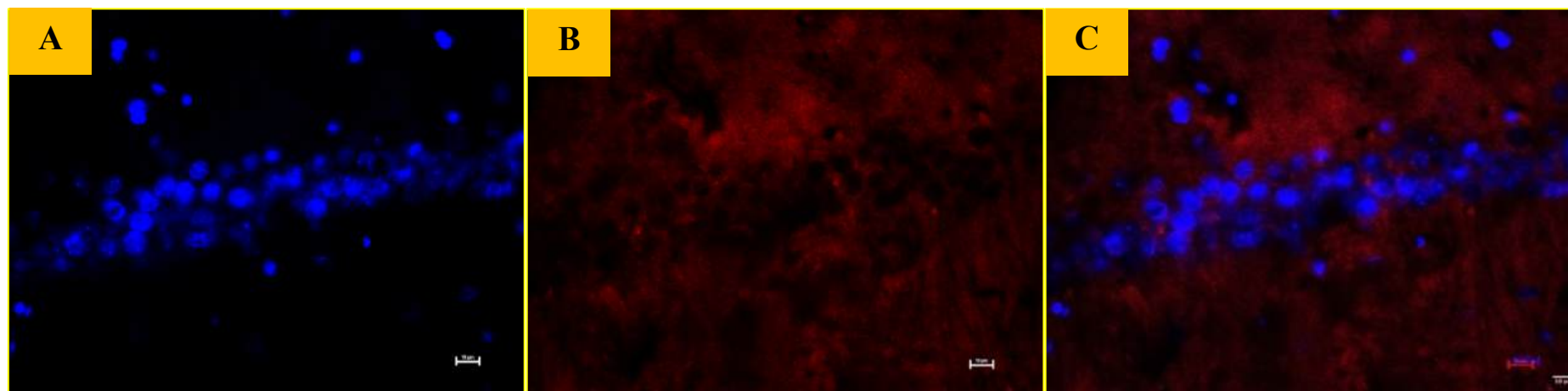


Figure 4.36 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model Without Treatment, specifically at the CA1 Region of the Hippocampus. The Results Showed Less Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Decreased Neurogenesis among the Cells Has Occurred. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

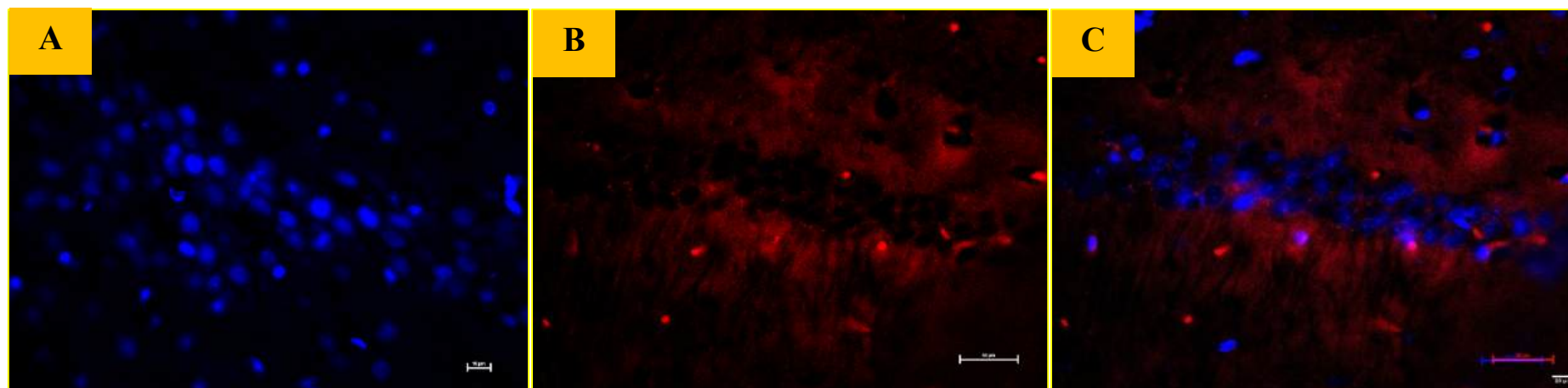


Figure 4.37 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil at CA1 Region of the Hippocampus. The Results Showed Slightly Higher Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Increased Neurogenesis. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

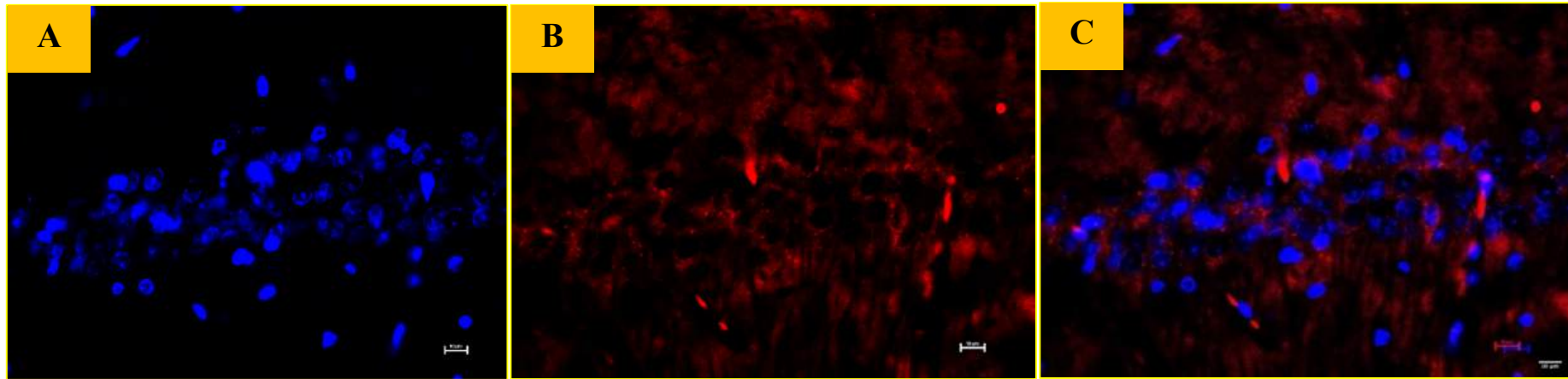


Figure 4.38 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 0.75mg/kg Δ^9 THC at CA1 Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Increased Neurogenesis. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

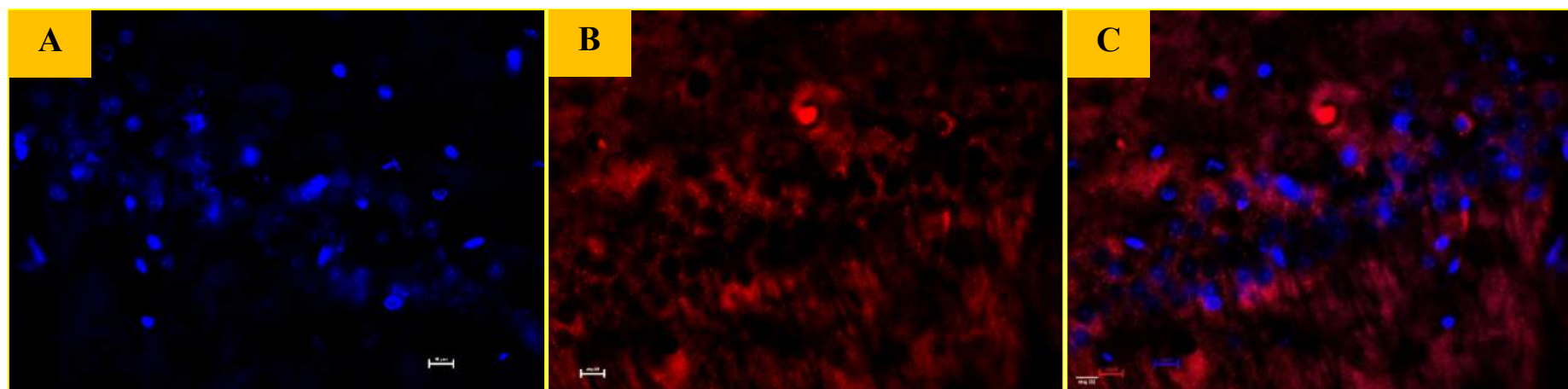


Figure 4.39 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.5mg/kg Δ^9 THC at CA1 Region of the Hippocampus. The Results Showed Higher Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells, Indicating Increased Neurogenesis. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

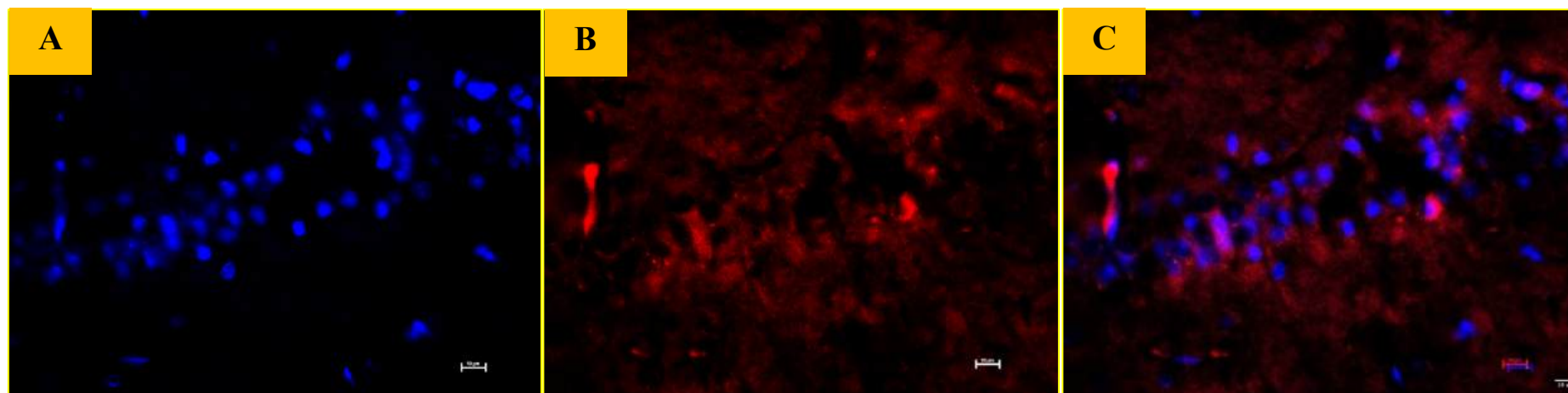


Figure 4.40 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 3.0mg/kg Δ^9 THC at CA1 Region of the Hippocampus. The Results Showed Higher Intensity of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

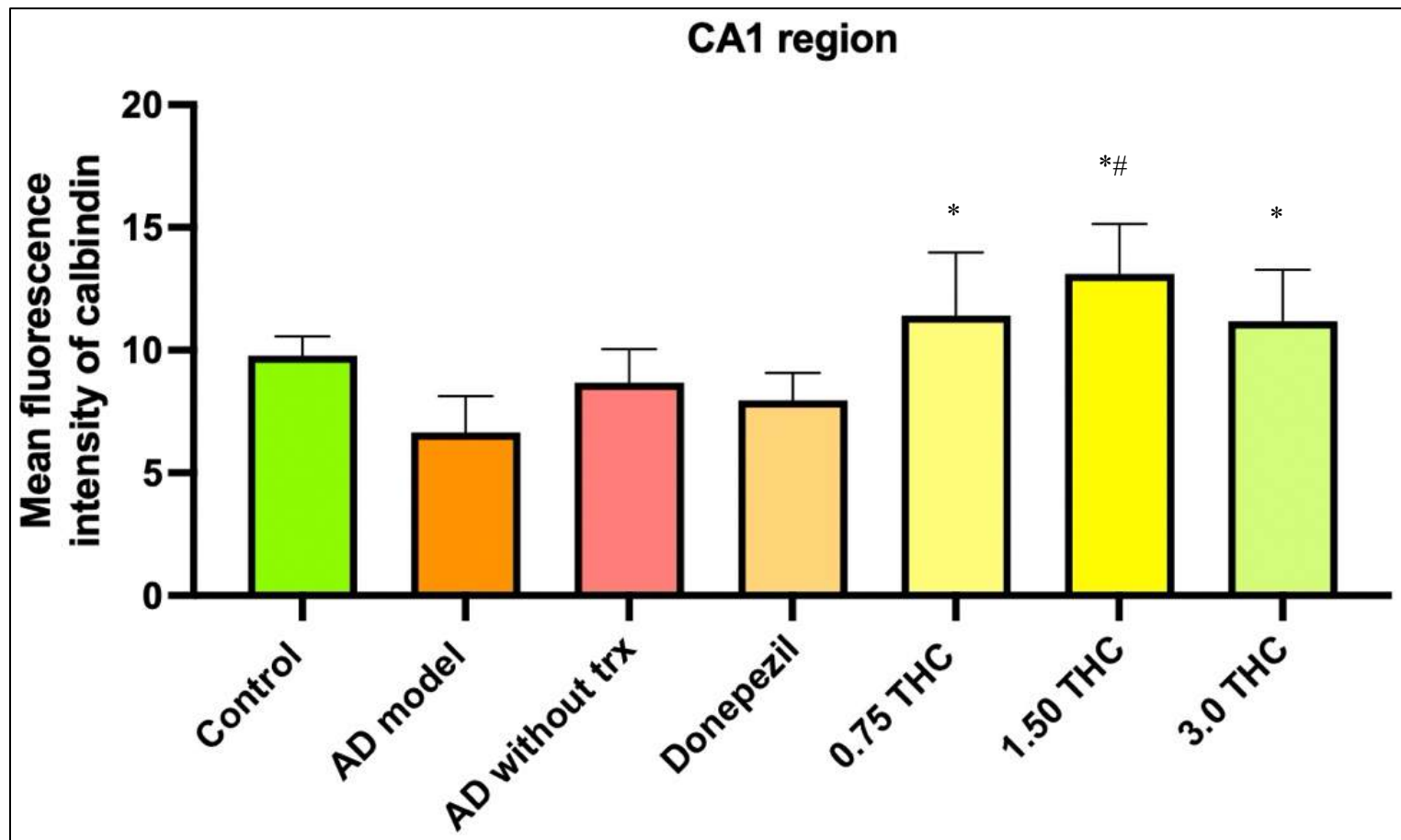


Figure 4.41 Mean Area of Immunofluorescent-Stained Pyramidal Cells of Calbindin at CA1 Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM (n = 7). * $p < 0.05$ vs AD Model, # $p < 0.05$ vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.34 until Figure 4.40 depict the photomicrographs of immunofluorescent-stained images of Calbindin in the subfields of CA1 region of the rat hippocampus in the Control and treated groups of Donepezil and Δ^9 THC with varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. Figure 4.41 shows the mean area of immunofluorescent-stained pyramidal cells of Calbindin for all groups. The analysis of the immunofluorescence is determined by its levels of fluorescent intensity among the cells indicating neurogenesis has occurred. The mean of immunofluorescent-stained neuronal results showed an increase in the intensity of Calbindin especially among the treated groups (0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg). Comparing with the photomicrographs in the AD and AD Without Treatment group, there is little to no intense glow among the pyramidal cells. This indicates that there is less neurogenesis occurring, in contrast to the marked increase intensity of the neurogenesis marker when the rats were treated with Δ^9 THC.

To determine the efficacy of Δ^9 THC, the results were compared to the Donepezil-treated group. Results showed that all of the treated groups have higher mean intensities of Calbindin than Donepezil (7.96 ± 0.37). Statistically, among the treatment groups, One-way ANOVA showed that all three groups have increased neurogenesis in the CA1 region [$F(6,50) = 11.316$, $p = 0.001$] when compared with both AD Model and AD Without Treatment. This was proved when the treated groups had higher mean intensities compared to AD Model (6.64 ± 0.53) and AD Without Treatment (8.68 ± 0.48); 0.75 mg/kg (11.41 ± 0.91), 1.50 mg/kg (13.10 ± 0.72) and 3.0 mg/kg (11.17 ± 0.75) respectively. Among these three doses, 1.50 mg/kg of Δ^9 THC resulted the highest mean of Calbindin intensity (13.10 ± 0.72). Meanwhile, 0.75 mg/kg and 3.0 mg/kg of Δ^9 THC only has a slight difference in mean intensities, still proved its efficacy far better than both AD Model and AD Without Treatment. Donepezil (7.96 ± 0.37) was proved successfully to increase neurogenesis, however, the treatment groups have much higher values of mean intensities. The distinct gap between the mean fluorescence intensity in the results of Δ^9 THC resembles positive insights in the vital potential of Δ^9 THC in remediating AD. All in all, Tukey's post hoc analysis has confirmed Δ^9 THC is potent in alleviating the neurogenesis among pyramidal cells in CA1 region which enlightens the progression of the disease.

4.3.2.2 *Expression of Calbindin as Hippocampal Neurogenesis Marker at CA3 Region*

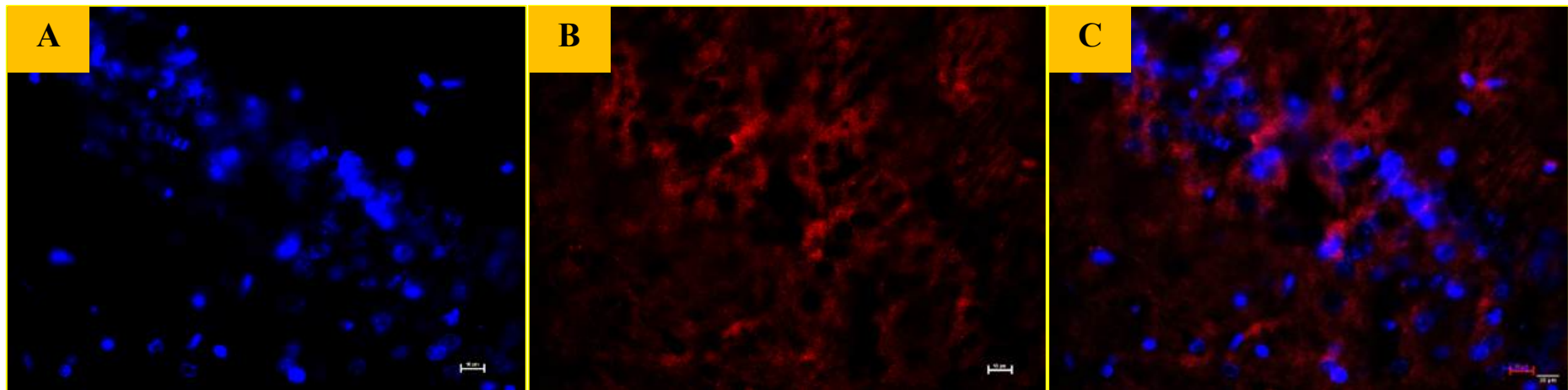


Figure 4.42 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in Control Group at CA3 Region of the Hippocampus. The Results Showed Normal Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

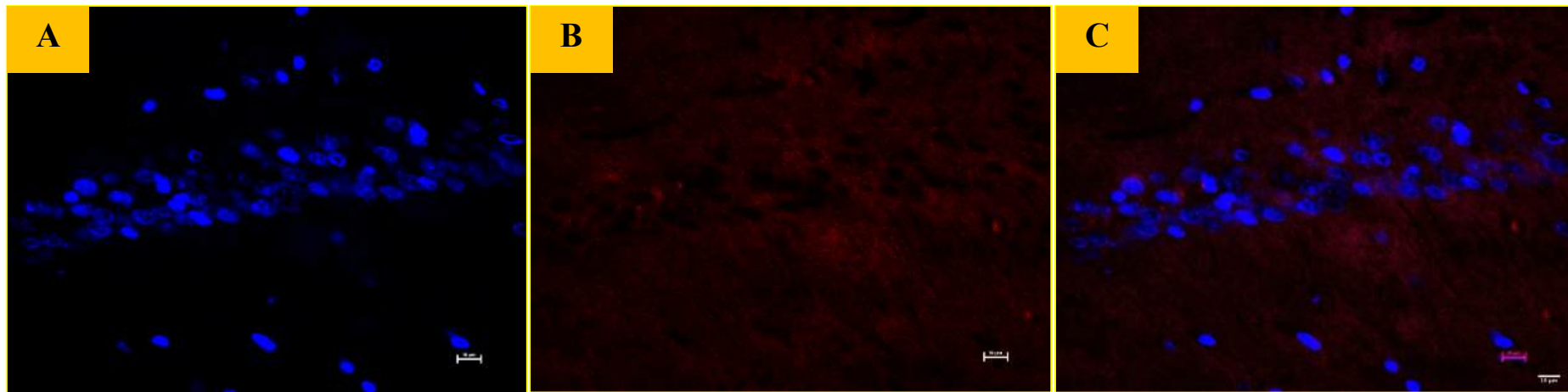


Figure 4.43 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model at CA3 Region of the Hippocampus. The Results Showed Less Intensive Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

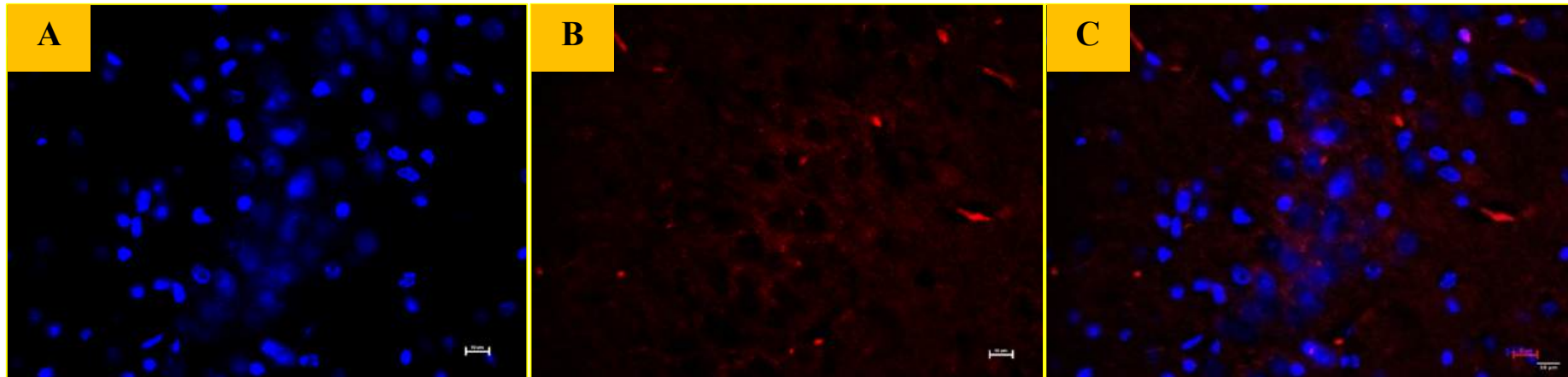


Figure 4.44 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Without Treatment at CA3 Region of the Hippocampus. The Results Showed the Less Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respective.

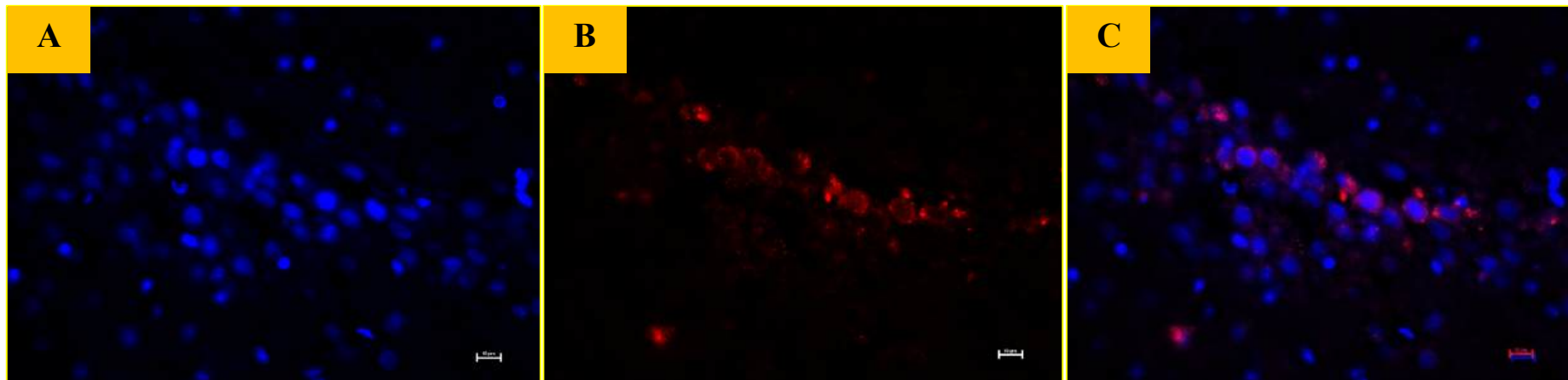


Figure 4.45 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil at CA3 Region of the Hippocampus. The Results Showed Slightly Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respective.

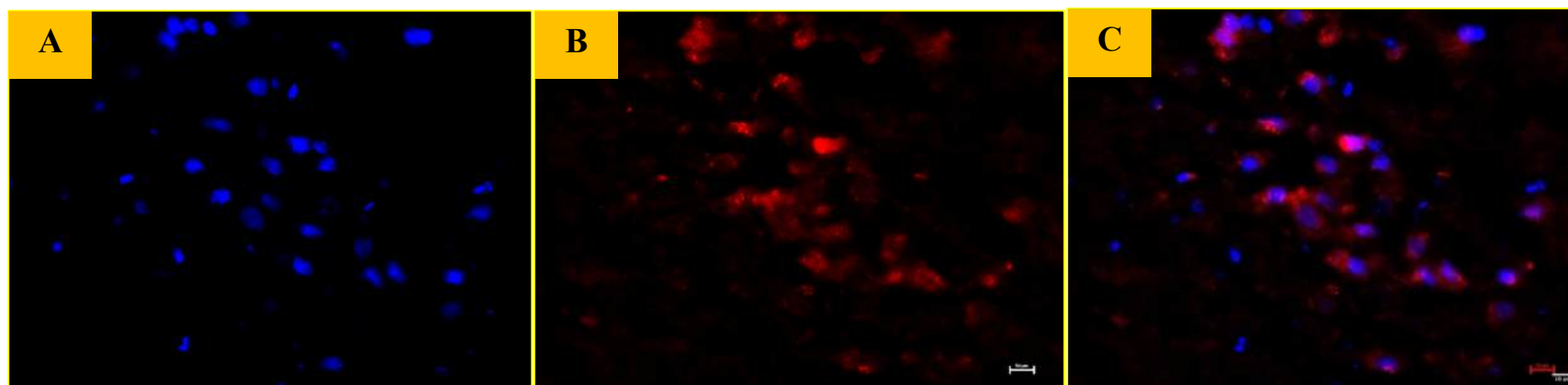


Figure 4.46 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 0.75mg/kg Δ^9 THC at CA3 Region of the Hippocampus. The Results Showed Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

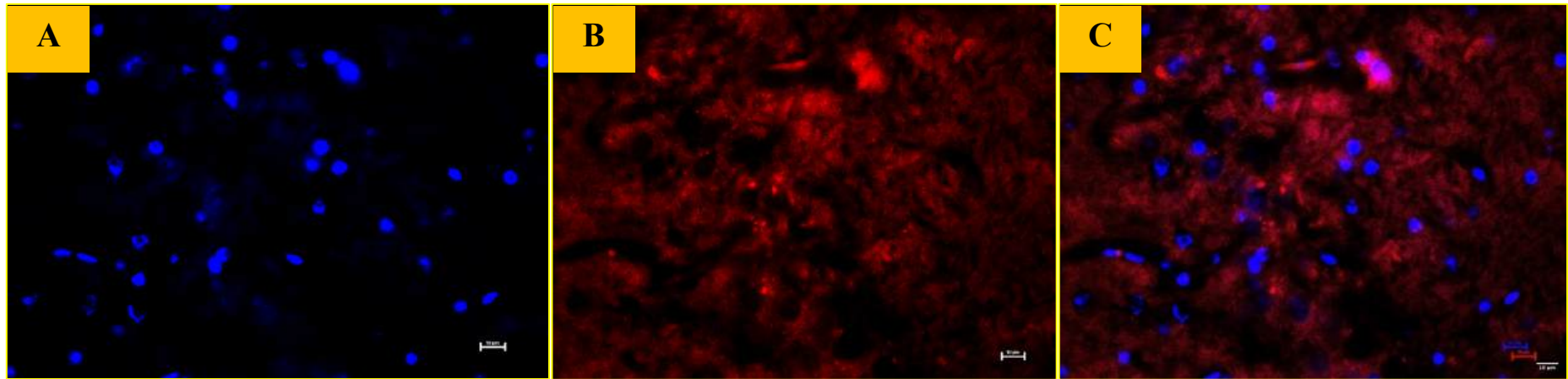


Figure 4.47 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.50mg/kg Δ^9 THC at CA3 Region of the Hippocampus. The Results Showed Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

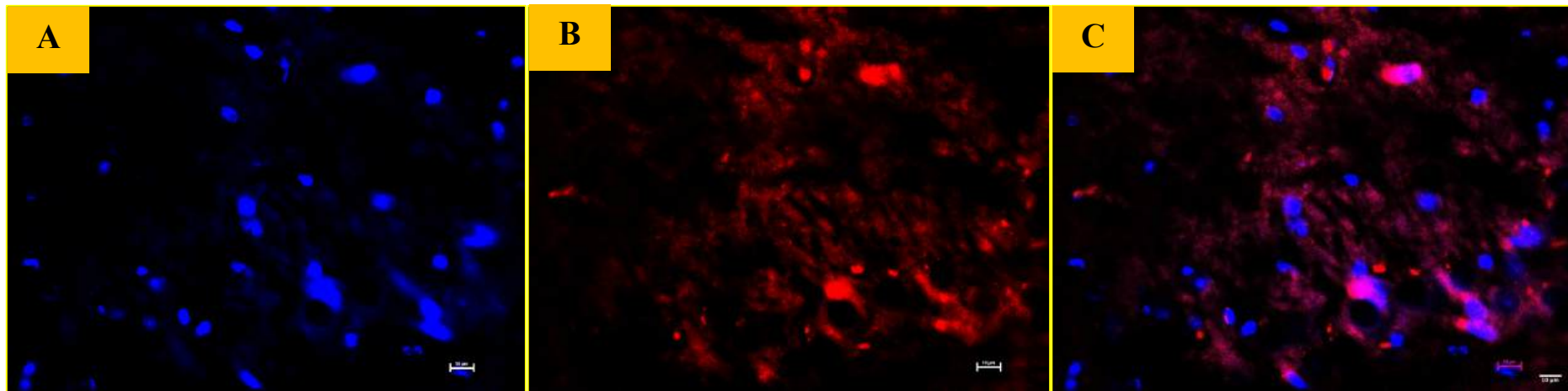


Figure 4.48 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 3.0mg/kg Δ^9 THC at CA3 Region of the Hippocampus. The Results Showed the Highest Intensive of Co-Staining of DAPI and Calbindin surrounding the Pyramidal Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

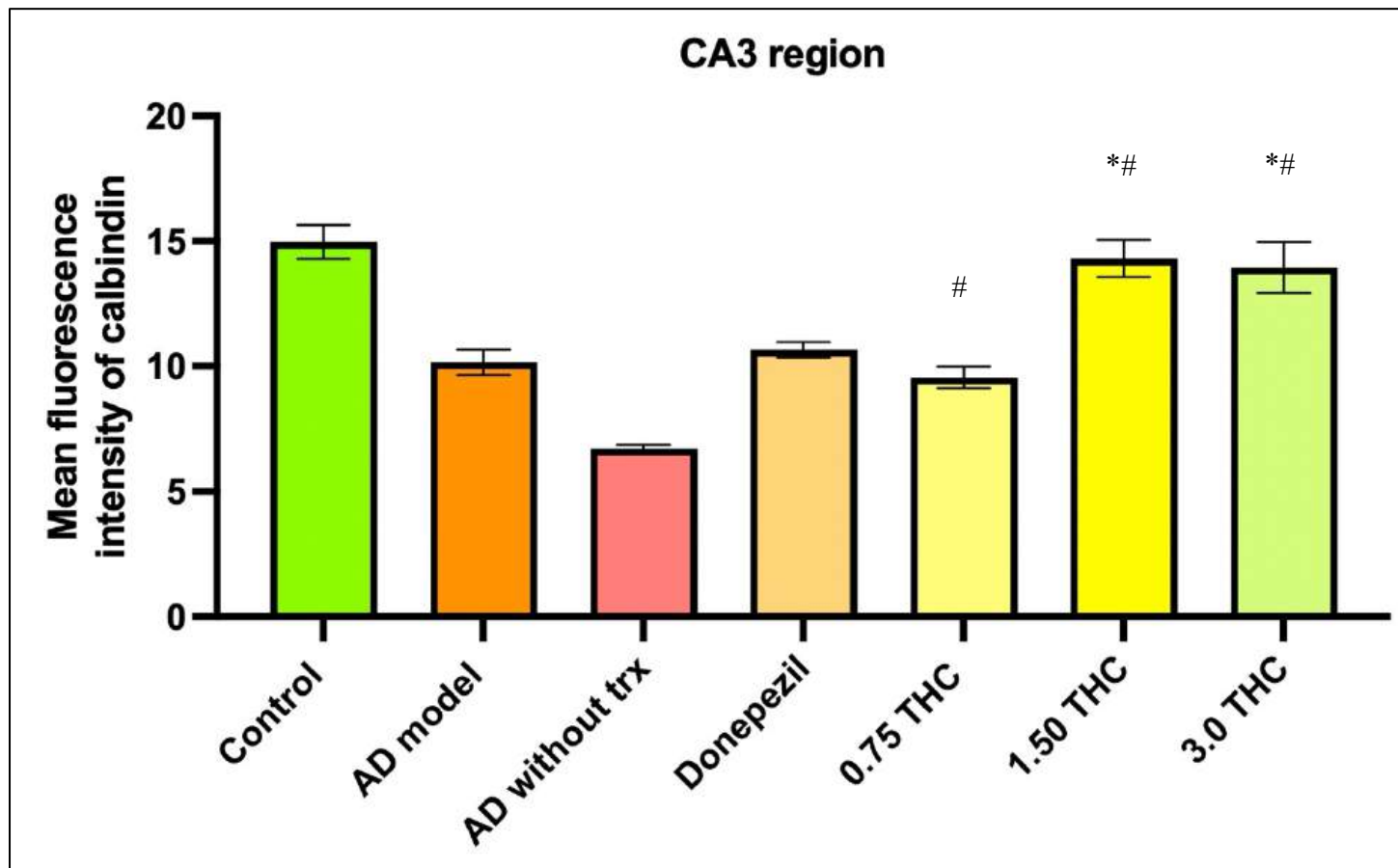


Figure 4.49 Mean Area of Immunofluorescent-Stained Pyramidal Cells of Calbindin at CA3 Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test among the Groups of Rats. Data Were Expressed As Mean $\pm$ SEM (n = 7). * $p < 0.05$ vs AD Model, # $p < 0.05$ vs AD Without Treatment. CA – Cornu Ammonis

Figure 4.42 until Figure 4.48 depict the photomicrographs of immunofluorescent-stained images of Calbindin in the subfields of CA3 region of the rat hippocampus in the control group and the treatment groups of Donepezil along with Δ^9 THC with varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. Figure 4.49 shows the mean area of immunofluorescent-stained pyramidal cells of Calbindin for all groups. The analysis of the immunofluorescence is determined by its levels of fluorescent intensity among the cells indicating neurogenesis has occurred. Observing the treatment groups, the mean immunofluorescent-stained neuronal results showed a drastic increase in the results showing Calbindin-stained neurons especially the obvious difference among the treated groups (1.50 mg/kg and 3.0 mg/kg). Comparing with the photomicrographs in the AD Model and AD Without Treatment, there is a lesser intense of fluorescent apparent in the mean intensity of Calbindin among the pyramidal cells. This indicates less neurogenesis has occurred compared to the treatment groups at which the rats were treated with Δ^9 THC.

This can be seen as there are more fluorescent-stained areas signifying marked intensity of neurogenesis is higher when treated with Δ^9 THC among the pyramidal cells in the treatment groups as to the AD Model and AD Without Treatment. Statistically, among the treatment groups, One-way ANOVA showed increased neurogenesis in the CA3 region [$F(6,28) = 24.34$, $p = 0.001$] when compared to both the AD Model (10.17 ± 0.51) and AD Without Treatment (6.72 ± 0.17). This was proved when the treated groups had slightly higher mean intensity; 0.75 mg/kg (9.56 ± 0.96), 1.50 mg/kg (14.31 ± 0.74) and 3.0 mg/kg (13.95 ± 1.03) respectively. When being compared to an approved-drug for AD, Donepezil (10.67 ± 0.31) was seen to increase neurogenesis, but for the groups treated with Δ^9 THC, specifically at both 1.50 mg/kg and 3.0 mg/kg, the statistics has resulted even higher mean intensities; (14.31 ± 0.74) and (13.95 ± 1.03) respectively. To conclude, Tukey's post hoc analysis has proved that Δ^9 THC has great potential in improving neurogenesis to remediate AD progression at specific concentrations.

4.3.2.3 *Expression of Calbindin as Hippocampal Neurogenesis Marker at DG Region*

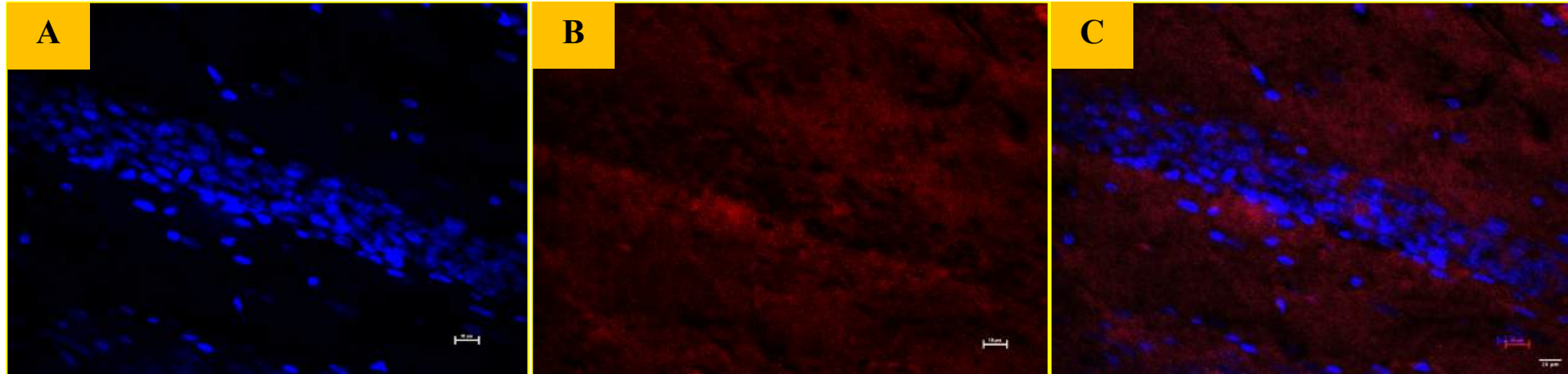


Figure 4.50 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in Control at DG Region of the Hippocampus. The Results Showed Normal Intensive of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

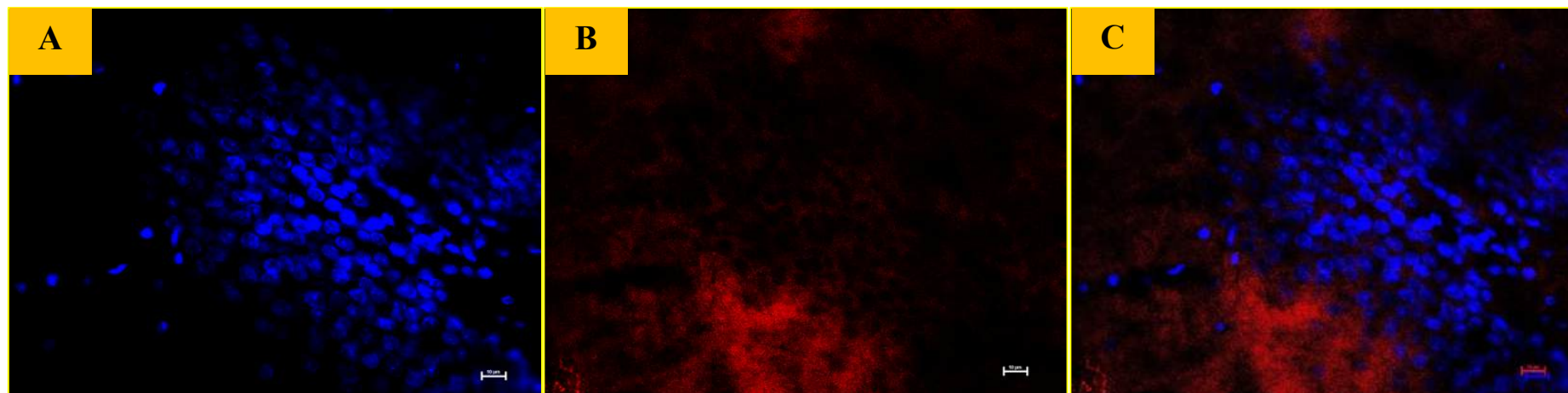


Figure 4.51 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model at DG Region of the Hippocampus. The Results Showed the Less Intense of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

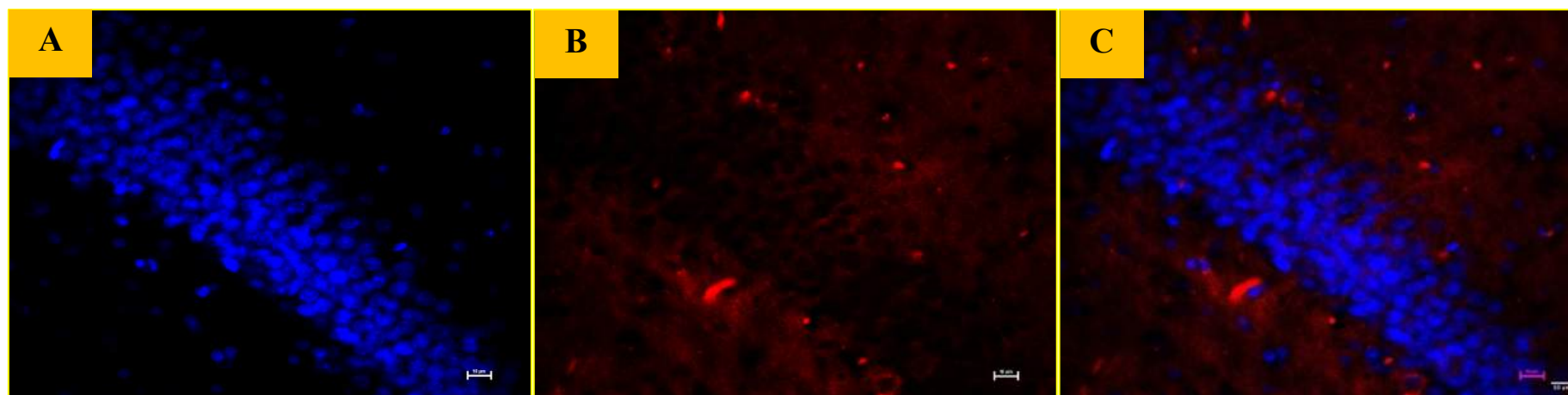


Figure 4.52 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections in AD Model Without Treatment at DG Region of the Hippocampus. The Results Showed Less Intense of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

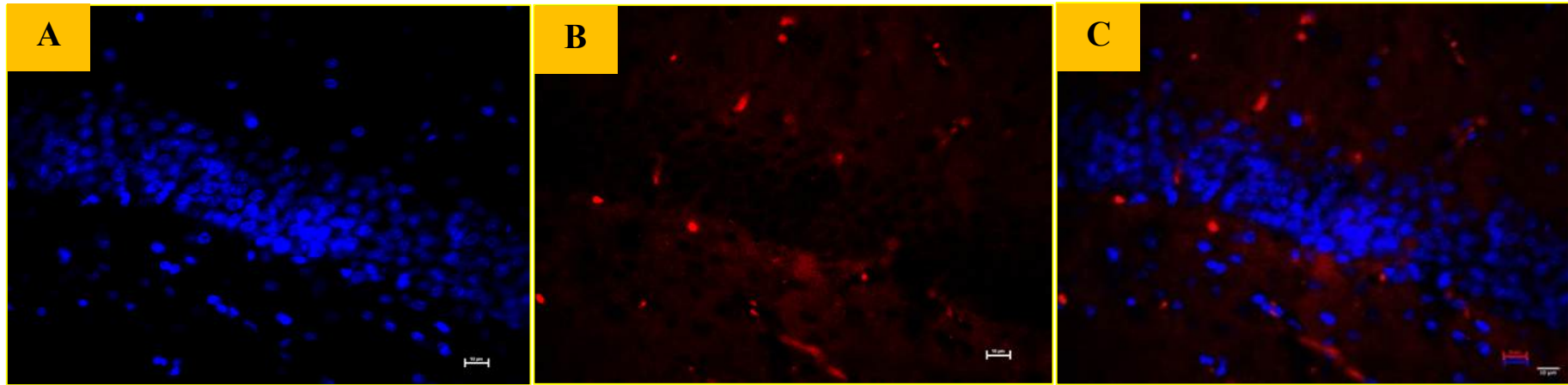


Figure 4.53 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with Donepezil at DG Region of the Hippocampus. The Results Showed Slightly Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

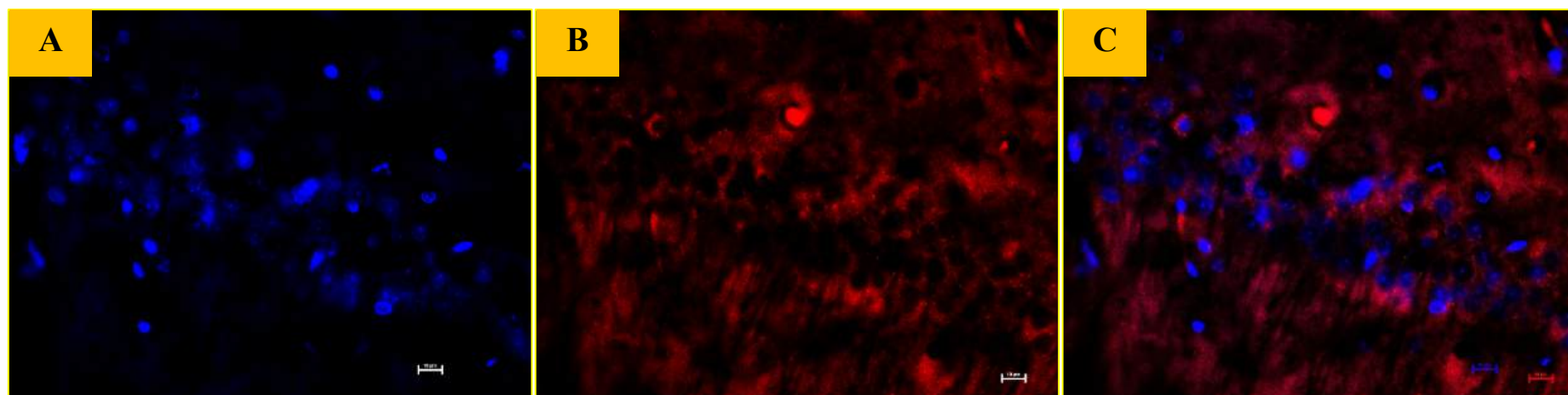


Figure 4.54 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 0.75 mg/kg Δ^9 THC at DG Region of the Hippocampus. The Results Showed the Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

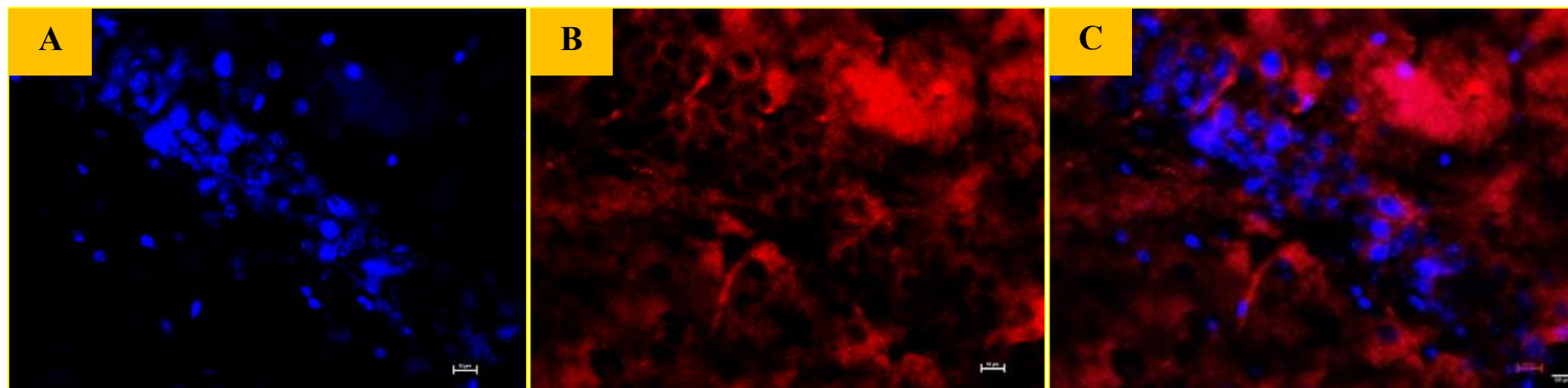


Figure 4.55 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 1.5 mg/kg Δ^9 THC at DG Region of the Hippocampus. The Results Showed the Higher Intensive of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

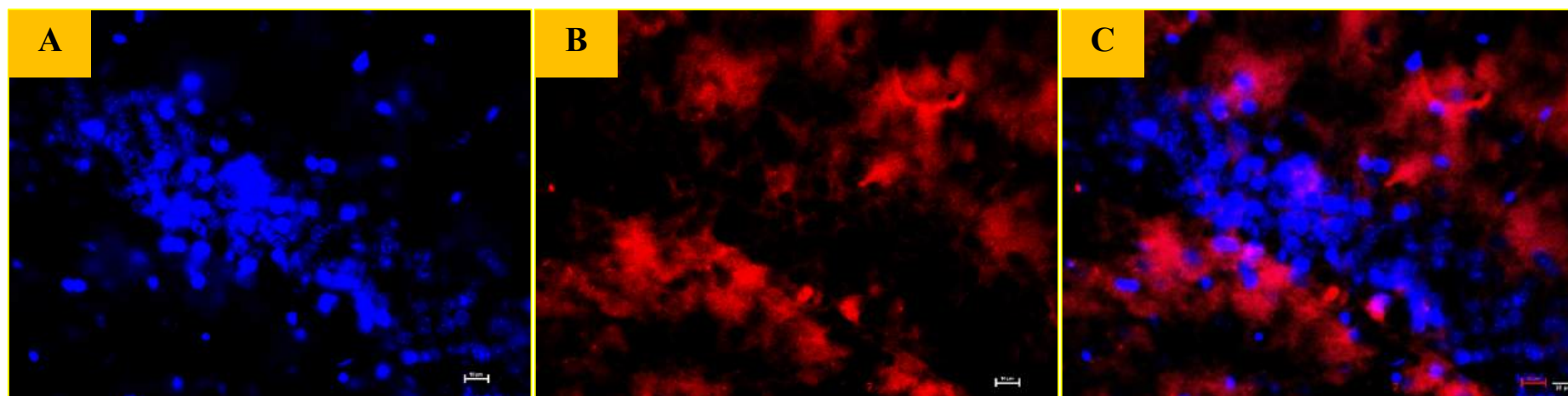


Figure 4.56 Representative Immunofluorescent-Stained Photomicrographs of the Brain Sections Treated with 3.0 mg/kg Δ^9 THC at DG Region of the Hippocampus. The Results Showed the Highest Intensive of Co-Staining of DAPI and Calbindin surrounding the Granule Cells. Scale Bar: 10 μ m. Magnification of 400x. (A) The Positive Area of Immunostained DAPI Expression among Pyramidal Cells. (B) The Positive Area of Fluorescence Indicates the Expression of Immunostained Neurogenesis Marker Calbindin among Pyramidal Cells. (C) The Merged Photomicrographs of Fluorescence-Positive Areas of Immunostained DAPI and Calbindin respectively.

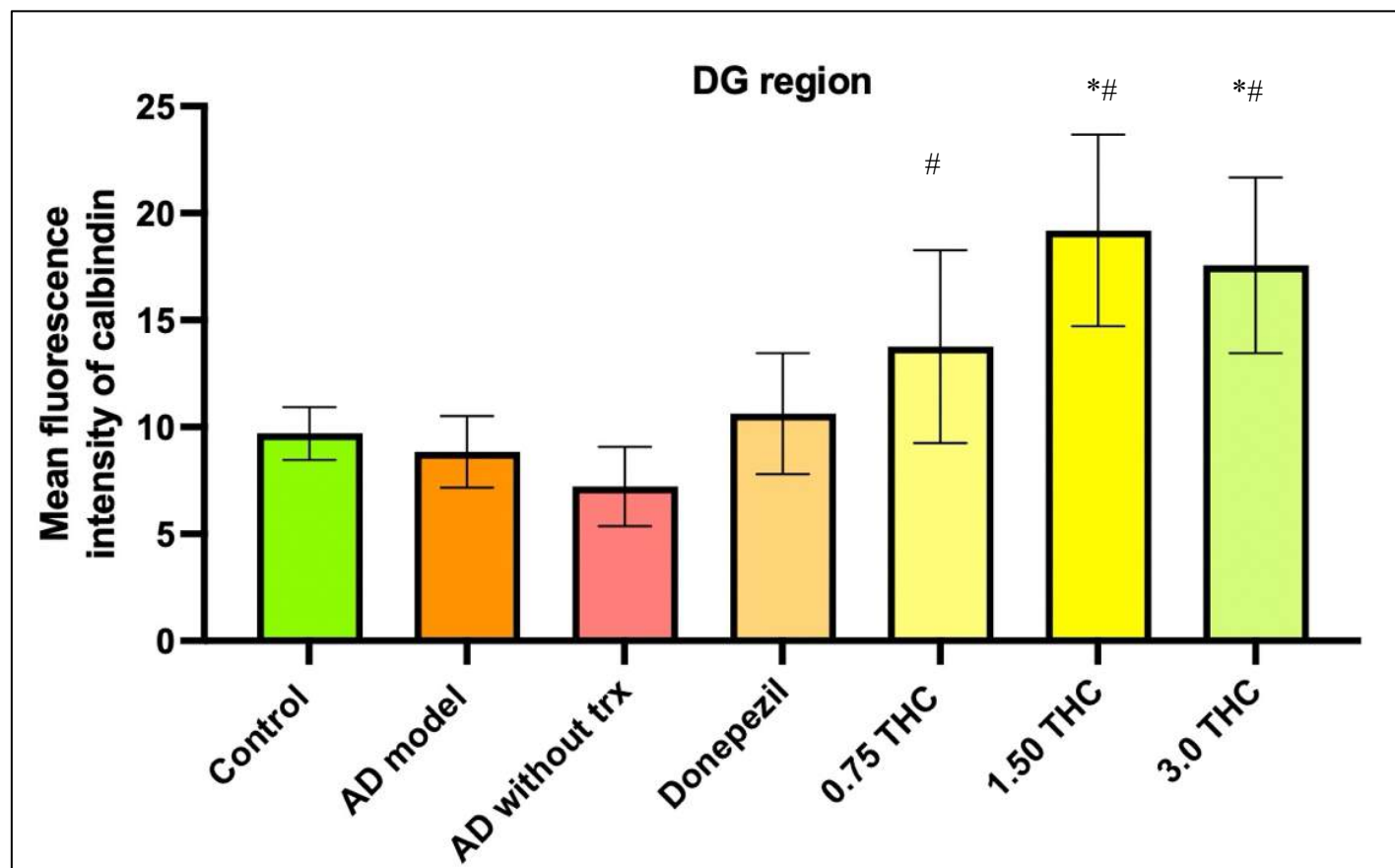


Figure 4.57 Mean Area of Immunofluorescent-Stained Granule Cells of Calbindin at DG Region of the Hippocampus for All Groups. Data Were Analyzed using One-way ANOVA Followed by Tukey's Post Hoc Test among the Groups of Rats. Data Were Expressed as Mean $\pm$ SEM

(n = 7). * p < 0.05 vs AD Model, # p < 0.05 vs AD Without Treatment. DG – Dentate Gyrus

Figure 4.50 until Figure 4.56 depict the photomicrographs of immunofluorescent-stained images of Calbindin in the subfields of DG region of the rat hippocampus in the Control group and the treatment groups of Donepezil along with Δ^9 THC at varying doses; 0.75 mg/kg, 1.50 mg/kg and 3.0 mg/kg respectively. Figure 4.57 shows the mean area of immunofluorescent-stained granule cells of Calbindin for all groups. The mean area of the fluorescence was analyzed using One-way ANOVA followed by Tukey's post hoc test among the group of rats. The levels of fluorescent intensity was analysed among the cells to compare the levels of neurogenesis with and without treatment. From the results, the mean of immunofluorescent-stained neuronal results showed increase Calbindin intensity in the treatment groups. Compared to the groups of AD group and AD without treatment group, the Δ^9 THC treatment groups have resulted major differences as there was an increase in fluorescent seen among the granule cells.

Based on the photomicrographs, the marked increase in the fluorescent glow among the granule cells was more obvious and apparent in the treated groups, especially 1.50 mg/kg and 3.0 mg/kg. Statistically, One-way ANOVA showed that all treatment groups are potent to increase neurogenesis [$F(6,49) = 13.908$, $p = 0.001$] when compared to the AD Model and AD Without Treatment. This was justified as the mean intensities of Calbindin increased along with the concentration of Δ^9 THC; 0.75 mg/kg (13.76 ± 1.59), 1.50 mg/kg (19.19 ± 1.58) and 3.0 mg/kg (17.56 ± 1.45) respectively. To further prove the effectiveness of Δ^9 THC, the results were compared to Donepezil, an approved-drug for AD. Donepezil (10.62 ± 1.0) was seen to successfully increase neurogenesis. Interestingly, all of the concentration of Δ^9 THC specifically at 0.75 mg/kg (13.76 ± 1.59), 1.50 mg/kg (19.19 ± 1.58) and 3.0 mg/kg (17.56 ± 1.45) have resulted a much higher mean intensities of Calbindin respectively. The observed gap of differences between the mean fluorescence intensity in the results of Δ^9 THC signifies potential positive therapeutic values in improving neurogenesis in AD. To conclude, Tukey's post hoc analysis has proved that Δ^9 THC has great therapeutic potential in improving neurogenesis by upregulating its neurogenesis markers.

4.4 Δ^9 THC Reduces Beta-site APP Cleaving Enzyme 1 (BACE1) and Phospho-Tau (Thr231) Protein Band Intensity in a Rat Model through Western Blot Technique

4.4.1 Effects of Δ^9 THC on the BACE1 Protein Band Intensity in the Cortex of D-gal and AlCl_3 -Induced Rats

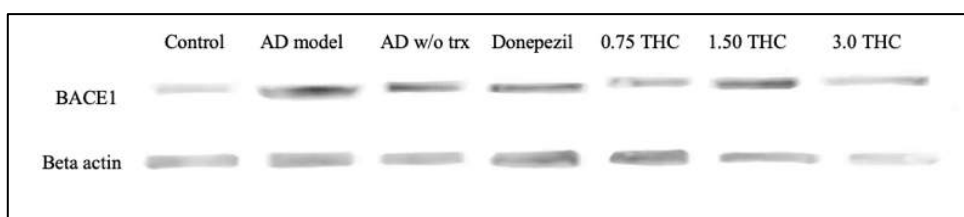


Figure 4.58 Therapeutic Effects at Different Doses of Δ^9 THC in D-gal and AlCl_3 -induced Rats on BACE1 Protein Band Intensity in the Cortex. Representative Immunoblots of BACE1 and Beta Actin (Loading Control) Proteins Measured by Western Blot Analysis in the Cortex.

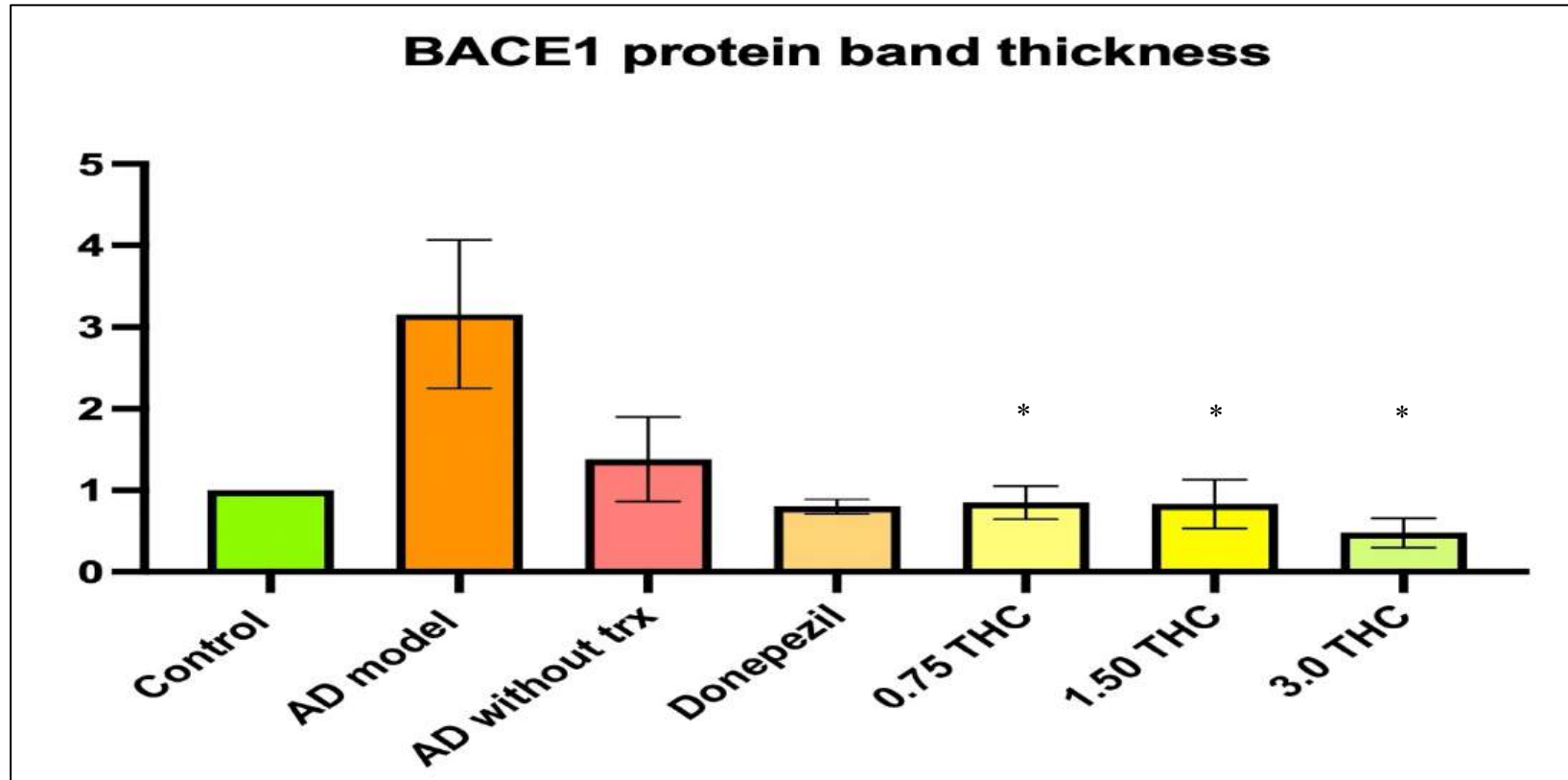


Figure 4.59 Relative Protein Band Intensity of BACE1 in the Cortex Analysed by Image J Software. The Data Were Normalised by Dividing the Mean Intensity of BACE1 with the Housekeeping Protein, Beta Actin for Each Reading. Data Shown Were Presented as Mean $\pm$ SEM, n = 5.

* $p < 0.05$ vs AD Model.

BACE1 is the primary β -secretase responsible for initiating the amyloidogenic pathway by cleaving Amyloid Precursor Protein (APP) to produce A β peptides. Elevated BACE1 levels have been observed in its protein band intensity in the brains of AD patients and animal models, correlating with increased A β production and cognitive decline. To assess the improvements in the disease progression, a gold-standard technique for quantifying specific analysis was conducted to evaluate the changes at high sensitivity and specificity, as an early indicator of AD. Thus, the relative protein band intensity of BACE1 was determined and analysed as shown in Figure 4.58 to provide insights into the enzyme's role in disease progression and understanding its distribution and regulation in AD. Given its pivotal role in A β production, BACE1 serves as a prime target for therapeutic strategies aimed at reducing A β levels. Monitoring BACE1 relative quantitative values through Western blotting enables researchers to evaluate the efficacy of potential modulators in AD models. The altered levels will provide insights closely-related to APP processing and A β production which contribute greatly to the neurodegeneration in AD.

Observing the decrease across the treated groups, there was an obvious marked reduction in the values as seen in the Cortex respectively (Figure 4.58), especially in the treated groups compared to the AD Model (3.16 ± 0.91); $0.75 \Delta^9$ THC (0.85 ± 0.20), $1.50 \Delta^9$ THC (0.83 ± 0.30) and $3.0 \Delta^9$ THC (0.48 ± 0.18). Even among the three concentrations, they have resulted such great results especially $3.0 \Delta^9$ THC which gave the highest decrease in the BACE1 protein band intensity compared to those of AD Model and AD Without Treatment. To further analyse its effectiveness, One-way ANOVA was conducted [$F(6,14) = 4.47$, $p = 0.001$] (Figure 4.59). The Tukey's post hoc test has revealed a significant reduction among the treated groups of rats ($p < 0.05$); Donepezil (0.80 ± 0.91), $0.75 \Delta^9$ THC (0.85 ± 0.20), $1.50 \Delta^9$ THC (0.83 ± 0.30), $3.0 \Delta^9$ THC (0.48 ± 0.18) when compared to AD Model group of rats (3.16 ± 0.91) and AD Without Treatment (1.38 ± 0.52). This signifies such gaps in differences especially those treated with the highest concentration of $3.0 \Delta^9$ THC. Thus, this proves that it is a core factor in understanding the involvement of BACE1 protein throughout the whole AD progression crucial for future development of specific-targeted therapeutic strategies.

4.4.2 Effects of Δ^9 THC on the Phospho-Tau (Thr231) Protein Band Intensity in the Cortex of D-gal and AlCl_3 -Induced Rats

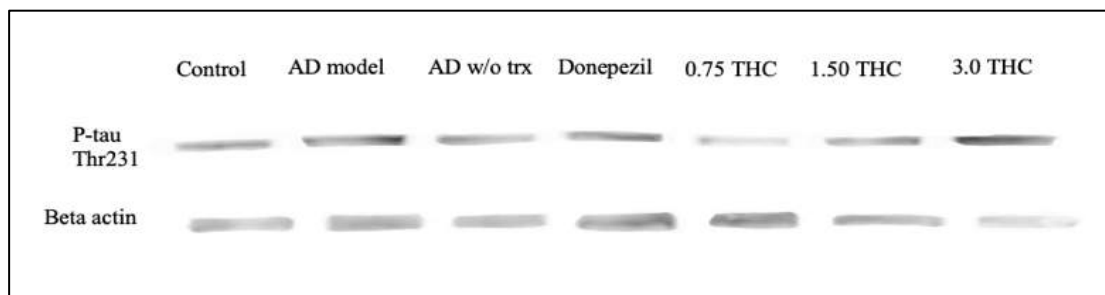


Figure 4.60 Therapeutic Effects at Different Doses of Δ^9 THC in D-gal and AlCl_3 -induced Rats on p-tau Thr231 Protein Band Intensity in the Cortex.
Representative Immunoblots of p-tau Thr231 and Beta Actin (Loading Control)
Proteins Measured by Western Blot Analysis in the Cortex.

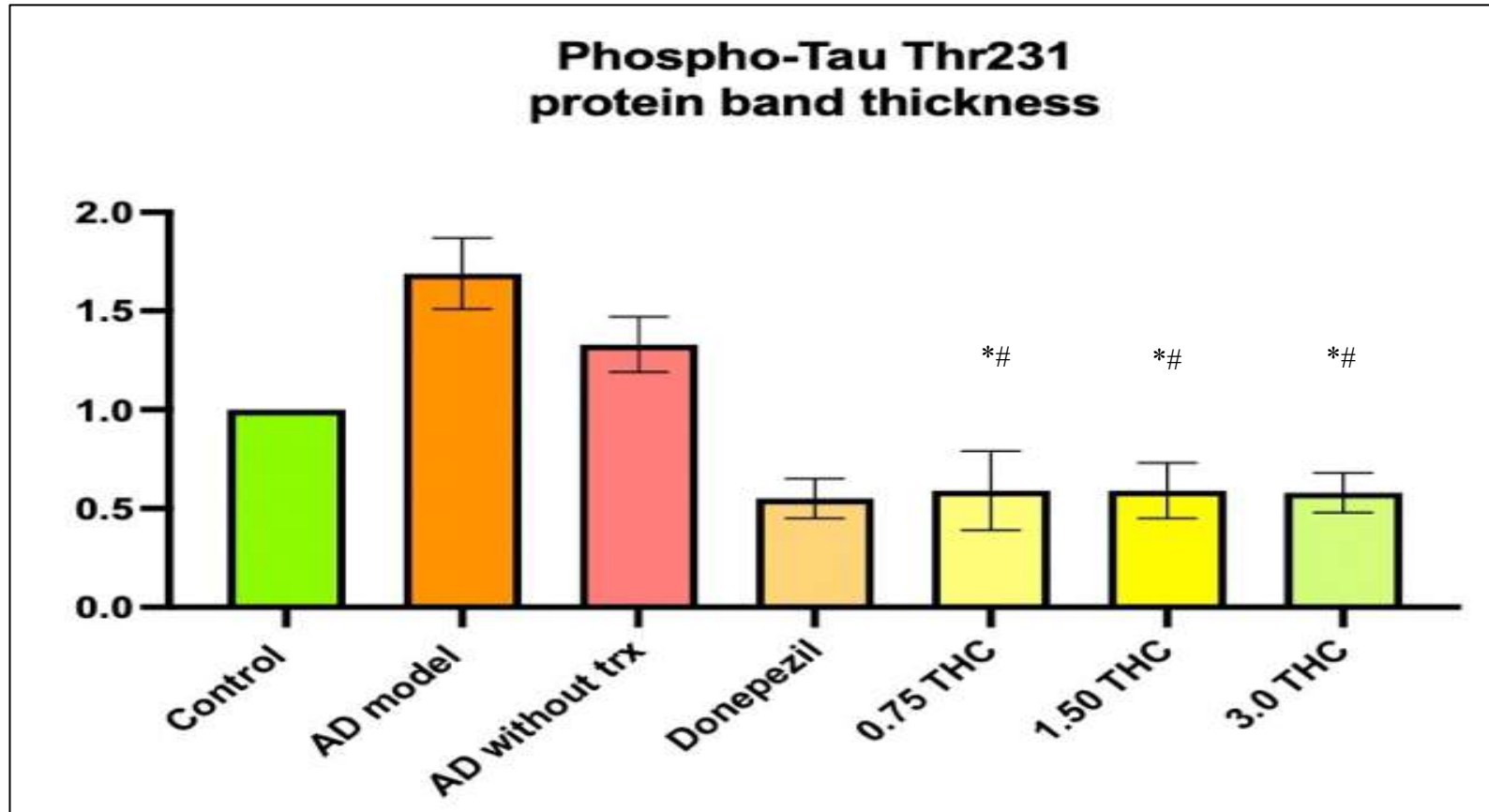


Figure 4.61 Relative Protein Band Intensity of p-tau Thr231 in the Cortex Analysed by Image J Software. The Data Were Normalised by Dividing the Mean Intensity of p-tau Thr231 with the Housekeeping Protein, Beta Actin for Each Reading. Data Shown Were Presented as Mean $\pm$ SEM, $n = 5$. * $p < 0.05$ vs AD model, # $p < 0.05$ vs AD Without Treatment

Aside from BACE1 as the distinguishable protein marker for AD, another vital marker that may aid in detection of disease progression is through analysis on tau proteins. In AD, dysregulation of tau will lead to formation of neurofibrillary tangles (NFT), one of its major hallmarks. In normal conditions, this phosphorylation regulate interactions between tau and microtubules, but in AD, excessive phosphorylation would lead to misfolded tau and formation of aggregates. Thus, phosphorylated tau proteins may serve as one of the signals in disease progression and biological targets to remediate AD. Thr231 proteins among the treated groups would be useful in detecting the effectiveness of the. In this research, p-tau Thr231 is included as the phosphorylation at Thr231 is particularly when the phosphorylation leads to a cis conformation has been linked to toxic activity and tau-dependent neurodegeneration as it is highly phosphorylated and aggregated. Hence, this research focuses on analysing the specific effects of Δ^9 THC on the protein band intensity of p-tau as conducted via western blot. The representative figures of the protein bands were shown in Figure 4.60. From the changes, this would give valuable insights to identify the possible reduction in the p-tau Thr231 levels post-treatment by comparing with other treated groups.

According to the results from ANOVA, the relative protein band intensity was seen to successfully create a large gap for p-tau Thr231; 0.75 Δ^9 THC (0.59 ± 0.20), 1.50 Δ^9 THC (0.59 ± 0.14) and 3.0 Δ^9 THC (0.58 ± 0.10). The positive changes in mean intensity have showed that Δ^9 THC was indeed able to reduce p-tau Thr231 and this was justified further when it is being compared to the AD Model (1.69 ± 0.18) and AD Without Treatment (1.33 ± 0.14). Interestingly, when analysing results of the animal group that was being treated with an-approved drug for AD, Donepezil (0.55 ± 0.10), the results were at par with slight differences so this shows there is a brighter future in incorporating Δ^9 THC as an intervention. This proves Δ^9 THC has the chance to subsequently create an initial reduction affecting corresponding plaques formation processes later as the disease progresses. Besides, this research aimed to discover if there is any self-renewing potential, as targeted earlier by portraying AD Without Treatment group. Based on the results, statistically One-way ANOVA [$F(6,21) = 0.001$] have justified its changes through further evaluation via Tukey's post hoc test that proved the significance of the treatments on p-tau Thr231 was aligned with the results and positively reflected in all three treated groups of Δ^9 THC ($p < 0.05$) (Figure 4.61).

CHAPTER 5

DISCUSSION

Alzheimer's disease (AD) is neuropathologically characterized by two principal hallmarks: extracellular amyloid-beta ($A\beta$) plaques and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. These pathological features collectively disrupt synaptic homeostasis and impair regulatory mechanisms responsible for maintaining the balance between $A\beta$ production and clearance, as well as tau phosphorylation dynamics, ultimately contributing to progressive cognitive decline (Li et al., 2016). As the disease advances, widespread synaptic dysfunction, neuronal loss, and impaired neuroplasticity become increasingly evident, highlighting the necessity for therapeutic strategies that target multiple interconnected pathways involved in AD pathogenesis. Thus, any effort to meddle in the disease progression will act as a therapeutic strategy to halt the disease from becoming worse.

In this research, the experimental design aimed to evaluate the efficacy of a new potential intervention, delta-9-tetrahydrocannabinol (Δ^9 THC) as one of the future AD treatments. From a physicochemical standpoint, Δ^9 THC is categorized as lipophilic compound that is susceptible to degradation and exhibits variable bioavailability when it is maintained in solution over time (Reisdorph et al., 2024). To mitigate these limitations and ensure dosing consistency, all drug solutions used in the experimental setup were prepared immediately prior to administration preserving its chemical values. Furthermore, fresh prepared chemicals tend to minimize degradation by ensuring each animal receives the intended active compound at the specified concentration, contributing to the reduction in variabilities. Daily administration of Δ^9 THC was conducted via intraperitoneal injection (i.p) except only for aluminium chloride ($AlCl_3$) which was through oral administration. The selected route of administration was a well-established method in rodent-based cannabinoid analysis to facilitate rapid and reliable absorption into the systemic circulation in the rats, which is particularly important when evaluating central nervous system effects of cannabinoids (Reisdorph et al., 2024; Nguyen et al., 2023). Additionally, comparative pharmacokinetic studies have demonstrated that the route of administration significantly affects the absorption,

central accumulation, and time course of Δ^9 THC and its metabolites, supporting the use of i.p. injection for mechanistic studies (Nguyen et al., 2023; Ruiz et al., 2021).

As for the volume of intraperitoneal injection, the benchmark was standardized at 0.1 mL per 100 g body weight in accordance with the accepted rodent welfare guidelines to minimize any physiological stress and reduce the risk of peritoneal irritation along any potential adverse effects that could confound both behavioural and biochemical outcomes (Reisdorph et al., 2024; Nguyen et al., 2023). This is crucial as excessive volumes of the drugs will alter the peritoneal absorption dynamics and impact further pharmacokinetic profiles in the brain resulting toxic exposures. In contrast, aluminium chloride (AlCl_3) was administered orally as this experiment aimed to better simulate environmental and dietary exposure routes commonly associated with aluminium toxicity, thereby enhancing the translational relevance of the AD induction models. This is due to the fact that orally-administered AlCl_3 proved to induce elevated oxidative stress, cholinergic dysfunction, and cognitive impairments which are categorized as surrogate features of AD-like pathology in preclinical studies (Khan et al., 2024; Rahman & Banu, 2025). Thus, this route of administration would enhance the translational relevance of the animal models by aligning the symptoms or behaviours that are more closely towards the primary pathways of human exposure towards aluminium compounds.

Moreover, Δ^9 THC and its therapeutic effects were examined via AD-induced rats to explore not only one aspect but multiple targets in ameliorating disease mechanism. This includes aiming improvements in cognitive functions assessed through Morris Water Maze (MWM), a widely validated behavioural paradigm for evaluating spatial learning and hippocampal-dependent memory. Back in the days, this behavioural test was initiated by Richard G. Morris in 1981 to test the rodents' spatial learning ability and long-term memory consisting of trials to differentiate between spatial (hidden platform) and non-spatial (visible platform) conditions relying on their distal cues to find the submerged platform (Bromley-Brits et al., 2011; Wang et al., 2018). To justify, MWM is used extensively in the study of neurobiology and neuropharmacology of spatial learning and memory due to its capabilities in allowing its environment to reduce odour trail interference when the experiment is being conducted.

The experimental setup was performed in a large tank filled with opaque water and there was an escape platform hidden beneath the water. In order to specifically

locate the platform, the rodents needed to depend on their distal cues travelling from their initial positions. The rodents would swim and travel along the open swimming arena targeting to reach the location of the submerged escape platform (Wang et al., 2018). The initial trial of MWM was characterized by the tendency of the rodents to spend most of their time near the walls (thigmotaxis behaviour) or typically searching around the pool randomly. Interestingly, in the training trials, the rodents demonstrated gradual changes in their behaviour which can be distinguished by the progressive active search for the platform. Based on the results, the findings revealed that the administration of D-gal and AlCl₃ has successfully declined cognitive abilities exposed by the similar neurotoxin. In the behavioural test of MWM, the parameter conducted was based on the time taken to escape in reaching the submerged platform. The escape latency was evaluated by observing the decrease in time spent at the target quadrant after the submerged platform was removed. The results from the neurobehavioral changes would give in-depth insights as the rats would rely solely on the capabilities of the hippocampus in recalling and activating learning processes.

Analysing the results, Δ^9 THC attenuated the cognitive deficits induced by D-gal and AlCl₃ based on the decreased escape latency aimed to find the location of submerged platform and the reduction in the number of platform quadrant entries and platform zone entries during the probe trial. Rats treated with Δ^9 THC has spent longer time in target quadrant searching for the removed escape platform during the probe test of MWM. Interestingly, findings from this study were supported and aligned with Nitzan et al., (2021) at which a single injection of ultra-low dose Δ^9 THC (0.002 mg/kg) is able to improve cognitive performances via MWM in a 5xFAD tg-mice AD model. There is another study that supported Δ^9 THC as a pharmacotherapy for AD which resulted in Δ^9 THC improved spatial learning and memory in aged mice in MWM (Nidadavolu et al., 2021). The findings is consistent with the observed results, in which rats treated with Δ^9 THC spent a significantly longer duration in the target quadrant during the probe trial of the Morris Water Maze (MWM), actively searching for the location of the previously removed escape platform. This behavioural pattern is indicative of enhanced spatial memory retention, as it reflects the ability of recalling the learned spatial location using distal environmental cues rather than relying on random search strategies.

In MWM paradigm, spatial learning is typically assessed during the acquisition phase through repeated training trials, where a progressive reduction in escape latency

reflects the ability to encode and consolidate spatial information. This process is largely dependent on hippocampal synaptic plasticity, particularly long-term potentiation (LTP), which strengthens neural circuits involved spatial navigation and memory formation. In contrast, reference memory is evaluated during the probe trial, where the platform is removed, and the preference for the target quadrant serves as a measure of memory retention and recall accuracy.

Apparently, the degenerative memory recalling was observed among the rats in AD model and AD without treatment as the swimming trajectories showed random movements at the edge of the pool. Unlike healthy rats, their swimming paths remain inefficient and disorganized, characterized by random navigation patterns, frequent changes in direction and an inability to adopt goal-directed search strategies. One of the hallmark behaviours observed was thigmotaxis, defined as tendency to swim along the walls of the pool rather than exploring the area reaching the submerged hidden platform. This shows that the rats experienced heightened anxiety and impaired spatial processing, as the animal fails to utilize distal visual cues and may display reduced swimming activity which can indicate decreased motivation or altered neurological function. As predicted, most of the animal trials failed to locate the submerged platform in the target quadrant as compared to the control and Δ^9 THC-treated groups. However, there was an increased in the search observed in their active progressive search near the platform area and the ability to reach the platform in much lesser time. From this, it can be deduced that the delayed time and increasing difficulty in its behaviour to search the platform during the trials confirmed impaired learning and memory in the brains of the rats. This aligned with the quantitative data obtained which further confirms that the cognitive deficits have definitely occurred similar with the characteristics of AD proven before (Zhang et al., 2016; Wei et al., 2017).

However, Δ^9 THC-treated groups have demonstrated marked improvements quantitatively apparent in its reduced time taken to reach the submerged platform compared to AD rats. Specifically, treated rats exhibited reduced escape latency, indicating enhanced spatial learning and increased time spent in the target quadrant during probe trials, reflecting improved memory retention. These observations were consistent with prior findings reporting that low-dose Δ^9 THC administration enhances cognitive function in transgenic AD models and aged rodents (Nitzan et al., 2021; Nidadavolu et al., 2021). The combination successfully decreased escape latency for the rats in locating the submerged escape platform. Moreover, dose dependent Δ^9 THC-

treated rats have demonstrated more directed search strategies and efficient navigation toward the platform location, indicating improved cognitive processing and spatial memory encoding over time.

As for the control rats, there would be lesser time to learn to swim reaching the behavioural asymptote after multiple trials. These trials would be as a kind of training for the rats to regulate their learning and memory processes based on their experiences via failed attempts (Goodrich-Hunsaker et al., 2010; Daugherty et al., 2015). This was proven further when the same outcome was achieved prior by Nitzan et al., (2021), who reported that a single injection of ultra-low dose of Δ^9 THC (0.002 mg/kg) was potent to initiate improved cognitive performances via MWM in a 5xFAD tg-mice AD model. Meanwhile, comparing with a study aimed in enhancing spatial learning in aged mice via MWM at 1 mg/kg Δ^9 THC, the results successfully were on par with positive outcomes with no statistically significant differences between the two treatments. In addition, when there were no statistically significant differences observed between Δ^9 THC-treated and Donepezil, this shows that Δ^9 THC has a promising therapeutic capability with the corresponding FDA-approved drug.

Interestingly, the apparent structural changes in the treated rats were observed to be in relation to the concentration of the drug itself. The higher the dosage, the lesser neuronal damage were seen among the cells. Thus, beyond the effects on behavioural, Δ^9 THC was aimed to create an impact on neurodegeneration occurring via cellular death. Apoptosis is a vital process to maintain tissues homeostasis by elimination of damaged or unwanted cells and aid in organ developments. However, the process needs tight regulation maintaining the correct ratio of newly-developed cells and death of cells, particularly the cells are crucial being involved in memory and cognition. Preventing the cells from experiencing unregulated and accelerated apoptosis will directly contribute to the brain atrophy characteristic of the disease which refers to the loss of tissues including neurons and their connections resulting a decrease in brain volume. Our findings indicated that the positive insights among its gradual reduction in hippocampal apoptosis had successfully provide a greater impact counteracting the cognitive deficits affected by AD. Based on thorough observation, there were more cells with a normal shape of the nuclei with apparent cytoplasm in the Δ^9 THC-treated groups compared to the AD model with its apoptotic cells that were easily distinguished by observing its irregular shape and dark-coloured cells with no nuclei visible. On an

interesting note, the outcomes proved a strong interdependency in the relationship between Δ^9 THC and neuronal survivability.

Based on the results, the mean number of apoptotic cells were dependent on the Δ^9 THC concentration. This was proved drastically both in the brain morphology and statistical results as the higher the concentration of Δ^9 THC, the lesser rate of apoptosis was observed. Significant decreases were seen based on the number of neuronal apoptotic cells at CA1, CA3 and DG regions, especially in the higher concentrations of Δ^9 THC, 1.50 mg/kg and 3.0 mg/kg respectively. Despite that, results from the lowest concentration of Δ^9 THC at 0.75 mg/kg still proved its reduction in number even though it was significant only in CA3 and DG regions only. In CA1, there were reduced apoptotic cells, but it was not significant statistically. This aligned with the microscopic observation as well as there were abundant apoptotic cells distinguished in 1.5 mg/kg and 3.0 mg/kg dose of Δ^9 THC as compared to the ones treated with 0.75 mg/kg Δ^9 THC. Based on the statistical results, great number of apoptotic cells were reduced despite neuronal cells were susceptible to apoptotic deaths triggered by oxidative stress, mitochondrial dysfunction, amyloid-beta toxicity and neuroinflammation (Moreno-Jiménez et al., 2019). Triggering positive outcomes, findings from this study further prove that Δ^9 THC is able to trigger CB1 and CB2 receptors especially at higher concentrations of Δ^9 THC (1.50 mg/kg and 3.0 mg/kg). Reduced apoptotic cells were apparently distinguished according to their cellular morphology as lesser dark-coloured cells with irregular shape were seen by comparing the photomicrographs between AD model, AD without treatment and all the treated groups at CA1, CA3 and DG regions.

This was supported by several preclinical studies that provided converging evidences on Δ^9 THC and its capability to exert improvements through modulation of apoptotic signalling pathways in CA1, CA3 and DG regions thereby potentially preserving hippocampal-dependent learning and memory. Moreover, the reduction seen in apoptosis was correlated with improved spatial memory performance suggesting a functional consequence of reduced apoptosis (Sarne et al., 2018; Marchalant et al., 2020; Moreno-Jiménez et al., 2020; Khodaverdi et al., 2021). The morphological results proved statistical values measured in this study were aligned with the previous findings focusing Δ^9 THC as the pharmacotherapy via rodent models treated with chronic low-dose Δ^9 THC. The outcomes include significantly reduced markers of apoptosis

particularly in CA1 and CA3 subfields (Marchalant et al., 2020). Furthermore, ultra-low doses of Δ^9 THC had justified its capabilities especially in the DG region shown among the observed significantly lesser apoptosis in a study done by Sarne et al., (2018). Enhanced neurogenesis were seen especially in the aged mice models of acute neuronal injury. These are critical to ensure that a maintained equilibrium exists between neurogenesis (programmed cell birth) and apoptosis (programmed cell death) to further enhanced its maintenance in regulating brain plasticity and functional integrity (Yousef et al., 2019). Based on their findings, it can be concluded that the reductions in apoptosis was on par as targeted similarly in the initial part of this research. Importantly, between these regions, statistical results have showed that there were definite significant reductions in apoptotic cells with a steady reducing manner in DG regions across all three concentrations of Δ^9 THC. Interestingly, the effects were stated to be less pronounced in CA1 and CA3 highlighting potential region-specific sensitivity. Despite that, this study proved that Δ^9 THC was able to significantly reduce the mean number of apoptotic cells in CA1 and CA3 regions as impactful in DG. This might be only due to the use of higher concentrations of Δ^9 THC across all regions. According to the graphs among all regions, 0.75 mg/kg of Δ^9 THC was not sufficient to cause great reductions in apoptosis. It was seen at its best rate of reduction only in DG and slowly lost its significance in the other two regions.

According to Navarrete et al., (2020), Δ^9 THC affects the CB2 receptors primarily found on microglia in DG by reducing the production of inflammatory cytokines (e.g., IL-1 β , TNF- α) and oxidative stress mediators such as reactive oxygen species (ROS). Hence, by dampening the neuroinflammation, Δ^9 THC indirectly protects DG regions from apoptotic cascades triggered by microglial overactivation. Comparing the findings of both CA and DG regions on reducing apoptosis via Δ^9 THC-mediated processes, the underlying mechanisms and magnitude may differ but overall analysis particularly shows that Δ^9 THC is potent to limit apoptosis simultaneously having complimentary effects in cell survivability that could together underlie the observed improvements in hippocampal functions following Δ^9 THC treatment. Collectively, these findings point toward its potential however, dosage, receptor specificity along with the balance between apoptosis and neurogenesis may be differentially modulate its effects across

different subfields especially in human or translational AD models to fully characterize these regional differences and optimize therapeutic strategies.

Neurogenesis is a complex, in-depth process consisting six developmental stages altogether (Kempermann et al., 2015). The cycle begins at Stage 1 which is the proliferation phase which occurs 1-3 days after birth. After approximately 1 week, stages 2-4 occur and are defined as the differentiation phase at which neuronal progenitors finish their cell cycle and continue into their neuronal lineage entering stage 5, called the migration phase. Lastly, approximately 4 weeks after birth, stage 6 (synaptic integration) occurs at which newly generated neurons start to enter the pre-existing hippocampal circuits (Goncalves et al., 2016). Based on these mechanistic processes, there were six stages in neurogenesis based on the processes specifically proliferation (nestin), differentiation (GFAP, nestin, Pax6), migration (NeuroD, DCX, PSA-NCAM), axonal and dendritic targeting (PSA-NCAM, DCX, TUC-4, Calretinin), and synaptic integration (NeuN, TuJ-1, Calbindin) (Bohlen and Halbach, 2011). Hence, this study focused on examining initial improvements in neurogenic processes through analysing two important neurogenesis markers; GFAP and Calbindin.

GFAP plays a crucial role as it is one of the markers that involve earlier in adult hippocampal neurogenesis which is the differentiation stage. In contrast to GFAP, Calbindin involves in the synaptic integration, a stage much later in the neurogenesis process (Bohlen and Halbach, 2011). From the outcomes from this experimental design, the upregulation of markers could be the initial baseline or foundation for future researches in targeting ameliorating the rate of neurogenesis in AD brains. This is because effective molecular therapies are able to signify which learning and memory-dependent areas vital to emphasize at the same time enhance the possibility to incorporate more stage-specific markers in AD brains. However, if this regulation is disrupted or left untreated, this may jeopardize the consequent learning and memory processes due to a lack of or sudden fluctuation of these markers. The positive outcomes of this research prove that Δ^9 THC treatment successfully promotes neurogenesis activities in the hippocampus following prolonged exposure to D-gal and AlCl_3 were consistent with the findings of Suliman et al. (2018), who reported that acute and chronic administration of Δ^9 THC neurogenesis in the hippocampus of the rats in control groups. Thus, this portrays the interdependence play between the regulation of hippocampal neurogenesis and the administration levels of Δ^9 THC.

Based on the photomicrographs of both CA and DG regions, it was observed that there was a rise in the level of both stage-specific neurogenesis protein markers. The gradual restoration of neurogenesis was easily distinguished according to the intensity of the immunofluorescence glow via the fluorescent-stained cells. The higher the glow observed among the cells, the higher the intensity of the neurogenesis occurred. This research has proved that the mean area of immunofluorescent-stained cells is higher in the Δ^9 THC-treated groups compared to the AD model. Firstly, the photomicrographs of immunofluorescence showed a low intensity of GFAP-stained area in both AD model and AD without treatment compared to the control group. This is due to AD jeopardizing the neurogenesis process that affects the downstream decreasing cognitive and memory abilities seen in the rats. Restoration of GFAP was successfully observed when the Δ^9 THC-treated groups had a rapid increase of glow among the neuronal cells. As GFAP is important in neuronal differentiation, there is a strong correlation between Δ^9 THC and improvement of neurodegeneration.

Comparing the morphological differences between the findings among the treated groups in both CA and DG regions, the intensity of GFAP was obviously enhanced specifically at 1.50 mg/kg and 3.0 mg/kg as there were apparent glows among the cells. However, in CA1, the immunofluorescent glow was evenly dispersed indicating that a steady state of neurogenesis was sparked and initiated throughout the treatment process. As for the dose of 0.75 mg/kg Δ^9 THC in CA regions, the immunofluorescent glow observed among the cells was more focused on specific regions and not evenly scattered throughout the whole area. Interestingly, this was the opposite when being compared to 0.75 mg/kg Δ^9 THC that was treated in DG, as it is was recorded the highest GFAP mean fluorescent intensity among all the three regions. This may be due to the fact that in DG, the astrocytes that function in supporting neuronal activity, synaptic function and neurogenesis become reactive enough to increase GFAP expression supporting synaptic remodelling and growth factor release (Sarne et al., 2018). Thus, 0.75 mg/kg dose of Δ^9 THC proved to enhance neurotherapeutic glial activity but 1.50 and 3.0 mg/kg has resulted more evenly dispersed GFAP expression among cells.

To further justify the effectiveness of the treatment, results were compared to those of Donepezil-treated group and from the mean fluorescence intensity analysed, Δ^9 THC have successfully surpassed Donepezil in elevating fluorescence intensity of GFAP dose-dependently. This was crucial as Donepezil is a widely used acetylcholinesterase inhibitor in AD therapy that reduces reactive astrogliosis and

preserves radial glia-like progenitors. However, Donepezil-mediated signalling is typically less robust than combined CB1/CB2 modulation. The mechanism focuses on anti-inflammatory effects that targets to decrease microglial activation and lower pro-inflammatory cytokines (Kim et al., 2020). Based on their own mechanistic target pathways, there are few differences need to be noted. Δ^9 THC directly targets the neurogenic mice via CB1 activation and indirectly by CB2-mediated reduction in neuroinflammation, whereas Donepezil modulates cholinergic signalling that subsequently affects astrocyte activity. This might be the nature of Δ^9 THC that possesses a more targeted effect on astroglial signalling and allows Δ^9 THC-focused animal models to report stronger preservation of GFAP-positive progenitors treated with Δ^9 THC rather than Donepezil alone (Sarne et al., 2018; Marchalant et al., 2020). This was reflected in the steady increase following the dose-dependent concentrations among the Δ^9 THC-treated groups in this study. Based on the statistical findings above, 3.0 mg/kg Δ^9 THC has resulted the highest mean fluorescence intensity across CA1, CA3 and DG regions. From the graph, as the concentration of Δ^9 THC rises, the mean fluorescence intensity of GFAP increases accordingly in CA1 and CA3 regions.

However, mean fluorescence intensity of GFAP in DG region shows inconsistency yet still increasing across the treated groups following the concentrations of Δ^9 THC. The inconsistent rise of intensity might due to the differences in regulating rate and stages of neurogenesis in DG. This inherently create the temporal fluctuations in GFAP levels as observed in the results. Compared to granule cells in DG, CA regions primarily consist mature pyramidal neurons and supporting astrocytes with minimal neurogenic activity (Kempermann et al., 2015). GFAP in CA regions is relatively stable, largely reflecting homeostatic astrocytic support rather than active proliferation or lineage transition. Thus, the rate of neurogenesis among the cells depend on the processes including activation, proliferation, differentiation and gliogenesis. Hence, this proves that Δ^9 THC is potent to be considered as one of the agents in ameliorating AD progression. However, this study does not only aim to increase neurogenesis, it aims to discover the potential for self-recovery among the AD-induced hippocampal cells, in this context self-induced neurogenesis. This aims to address the central question in regenerative biology for a possibility whether endogenous neural stem/progenitor cells can be triggered to counteract the neurodegenerative pathway. This might be a potential pathway as adult hippocampal specifically DG region contains a population of radial glia-like neural stem cells that retain capacity for self-renewal and neurogenesis

throughout life (Goncalves et al., 2016; Kempermann et al., 2015). Thus, this research aims to discover the direct implications of Δ^9 THC in restoring neurogenesis and repair circuitry damage as relevant as traditional AD treatments largely focus on symptoms management (Mu & Gage, 2011).

By analysing the morphological results of AD without treatment group, it can be concluded that there is an increase in mean fluorescence intensity in GFAP, however, the differences were minimal. The differences were minor if compared to those treated with FDA-approved drug, Donepezil and Δ^9 THC. The self-potential in upregulating neurogenesis may not be sufficient in improving the neurodegeneration back to normal. Without therapeutic intervention, on-going A β deposition will trigger further synaptic dysfunction, excitotoxicity and mitochondrial impairment resulting in widespread apoptotic neuronal loss (Scheff et al., 2007). If progression of AD is left untreated, neurodegeneration will slowly but surely disrupts the connections between the connections among neurons in the trisynaptic hippocampal circuit essential for spatial learning and memory consolidation (Small et al., 2011). As AD progresses, chronic exposure to untreated A β oligomers and hyperphosphorylated tau will impair further neural proliferation and neurogenesis due to the prolonged inflammation and oxidative stress among the cells causing depleted pools of neural progenitors along its neurosurvivability and integration of newly born neurons (Mu & Gage, 2011). Untreated AD group then would lead to weakened hippocampal plasticity and contributing to even progressed cognitive decline among the hippocampal regions diminishing rate of neurogenesis (Hollands et al., 2016). Thus, this proves that AD is better treated rather than with no treatment at all.

Based on previous findings, their results aligned with the achieved outcomes in this study as untreated AD pathology is associated with sustained microglial activation and astrocytic gliosis which amplifies local neuroinflammation and exacerbate neuronal apoptosis (Heneka et al., 2015). Over time, these processes synergistically accelerate hippocampal atrophy, correlate with worsening clinical dementia conditions (Jack et al., 2010). Altogether, this sums up the evidences indicating without therapeutic interventions, CA regions undergone profound pyramidal loss and DG suffers depletion in generation of new cells, both culminating progressive learning and memory deficits in AD. This further supports the claim that AD is better being treated with treatment rather than no treatment at all. The possible reason may be that when there is no positive

prompt to initiate neurogenesis, the progression of deterioration in AD will degenerate the neuronal cells even further despite progression in a slow manner.

As for Calbindin, the neurogenesis marker involves in the later stage of neurogenesis known for its involvement in buffering calcium fluctuations, crucial for maintaining cellular calcium homeostasis and prevent cellular apoptosis. Specifically, it involves in the integration stage (the last stage of neurogenesis) of the synapses and it is apparent that AD model and AD without treatment group showed lesser intensity of Calbindin-stained area as there was little to no glow seen among the cells. From the differences observed in the immunofluorescent-glow between AD model, AD without treatment and Δ^9 THC-treated groups, it can be concluded that newly generated neurons in the treated groups have bypassed the initial phase of neurogenesis and have reached its matured state of stable neurogenesis as seen in increased Calbindin. Therefore, Δ^9 THC has proved its efficacy not only effective as observed in the early stages of neurogenesis, and prolonged its effectiveness until neurogenesis processes have completed.

Upregulating Calbindin is crucial as this indicates that a greater proportion of newly generated neurons have successfully progressed beyond early developmental stages and have undergone maturation and functional integration into existing hippocampal circuits. Increased fluorescence intensity therefore implies not only a higher number of mature neurons are present but also improved capacity for maintaining calcium homeostasis, which is crucial for preventing excitotoxicity and supporting sustained neuronal activity during learning and memory processes. In neurobiological perspective, calcium homeostasis suggests critical role in buffering intracellular calcium (Ca^{2+}) levels, thereby regulating calcium-dependent mechanisms. Analysing the mean fluorescence intensity of Calbindin across all regions, interestingly 1.50 mg/kg Δ^9 THC have resulted promising outcomes as it showed steady increases in its values (Figure 4.41, Figure 4.49 and Figure 4.57). Meanwhile, in CA3, only concentration of Δ^9 THC at 1.50 mg/kg and 3.0 mg/kg have resulted the highest mean fluorescence intensity of Calbindin. These might indicate a reversal of these pathological features and suggests a more uniform and widespread fluorescence pattern across the subregions indicating coordinated neuronal maturation and network integration, whereas localized or uneven intense fluorescence may reflect region-specific neurogenic responses.

In contrast to Δ^9 THC concentration at 0.75 mg/kg, it resulted lower mean intensity, yet almost as high as the mean intensities of Donepezil-treated group. With Donepezil being FDA-approved drug, this gives positive insights and a chance to be compared with the available drug interventions that are currently being used clinically. Taken together, the capability of inducing neurogenic and neurorestorative effects even at the minimal concentration of Δ^9 THC provides strong molecular evidence in supporting late-stage neurogenesis for future endeavours in clinical diagnostics and initial efforts prior involving human studies treated with Δ^9 THC. To measure its effectiveness, the results from enhanced Calbindin in this study were compared with other findings from previous studies focusing cannabinoid receptors, CB1 and CB2. According to the previous studies targeting CB1, it has been reported that there was a significant increase in Calbindin-positive granule cells in an acute neuronal injury mouse model and similarly in an aged mouse model that has been treated with Δ^9 THC (Sarne et al., 2018). Another rat model that mimics amyloid-induced neurodegeneration and apoptosis in AD has proved that Δ^9 THC successfully enhanced the activation of CB1 receptors and progressively decreased the pro-apoptotic factors while stabilized the mitochondrial integrity allowing promotion of survival among neural progenitor cells (Khodaverdi et al., 2021). This justifies the importance of CB1 receptors not only in upregulating neurogenesis in AD brains, but these receptors have important roles contributing to maintain cellular survivability through mitochondrial stability within the neuronal cells, especially those in learning and memory-sensitive areas.

Aside from CB1, Δ^9 THC also interacts with CB2 receptors to activate its functions in reducing neuroinflammation and oxidative stress (Navarrete et al., 2020). Unlike CB1, which is highly abundant in neurons, CB2 is mainly expressed in microglia, astrocytes and infiltrating immune cells in the brain regulating inflammation, glial reactivity and secondary neurotoxicity associating with neuroinflammatory and neurodegenerative conditions AD (Benito et al., 2018; Christino et al., 2020). In healthy brains, CB2 expression is relatively low but substantially elevated under neuroinflammatory conditions. In Δ^9 THC-treated AD brains, mechanistically activated CB2 promotes a shift of microglia from M1 pro-inflammatory phenotype to the M2 anti-inflammatory state. This switch reduces the release of pro-inflammatory mediators such as IL-10 and arginase-1 (Aso & Ferrer, 2014; Cristino et al., 2020). This was aligned with the findings from in-vivo AD models such as the APP/PS1 transgenic mouse and amyloid-beta injected rat models that has demonstrated CB2 activation via

decreased caspase-3 activation by Δ^9 THC through PI3K/Akt signalling allowing proper neuronal progenitor cells differentiate into mature neurons (Khodaverdi et al., 2021).

Furthermore, CB2 was crucially involved in modulating AD as CB2 is able to reduce oxidative stress and increase expression of antioxidant enzymes important for neuronal survival and through this engagement, CB2 helps protect neurons from amyloid-induced toxicity that can cause apoptosis through the enhanced clearance of A β via increased phagocytic activity of microglia to reduce accumulated plaques and local inflammation among the cells (Benito et al., 2018; Navarrete et al., 2020). The relationship was confirmed as there were complementary evidences from in-vivo and in-vitro models that proved chronic low-dose Δ^9 THC animal model in aged rodents resulted positive neurogenic effects on CB1/CB2 receptor-mediated involving cell-intrinsic and environment-modulating mechanisms (Marchalant et al., 2020). Despite in-vitro studies could not fully replicate the neurogenic niche in the brain, but results demonstrated that Δ^9 THC has successfully reduced A β -induced oxidative damage in cultured neuronal cells (Navarrete et al., 2020).

All in all, the findings from this study have aligned with the results in previous researches as the pro-apoptosis cells were reduced in the hippocampal regions yet the ratio of neurogenesis generating newly-developed cells were enhanced to replace the loss of neurons as the disease progresses. This was reflected in the mean fluorescence intensities of Calbindin-positive cells that were increased in the treated groups at all three regions. However, the results of enhanced neurogenesis were not in a dose-dependent manner. This can be observed as there was slightly obvious decrease in mean intensities of Calbindin at CA1 and DG regions when treated with the highest concentration of Δ^9 THC, 3.0 mg/kg as compared to the other two concentrations. Considering Calbindin is functional in the last stage of neurogenesis, the possible reason may be due to the factor of time-dependent across experimental groups. Specifically, the duration of exposure was insufficient to fully mature and integrate into existing hippocampal circuits. This was evidenced by the observation that increases in Calbindin fluorescence intensity, although significant, it was not reflected at the highest concentration of Δ^9 THC of 3.0 mg/kg. This neurogenic pattern indicates that a longer duration of exposure may be required.

However, out of all the three concentrations, it can be concluded that the concentration of 1.50 mg/kg of Δ^9 THC is the most stable concentration across all three regions in stimulating the intensity of Calbindin promoting neurogenesis altogether.

The temporal dynamics of neurogenesis are particularly relevant at which neural stem or progenitor cells continuously generate new neurons throughout adulthood. Thus, the observed variability in Calbindin expression across regions may therefore reflect differences in neurogenic timing rather than treatment efficacy alone. A longer duration of Δ^9 THC administration would allow for more enhanced of completing the neurogenic cycles, which in turn might result a more robust and consistent increases in late-stage markers, particularly Calbindin.

Another factor that portrays crucial effects in this treatment is the importance of exploring CB1- and CB2-mediated effects of Δ^9 THC in neurodegenerative contexts. The therapeutic effects of Δ^9 THC are primarily mediated through its interaction with the endocannabinoid system, specifically the dual engagement of CB1 and CB2 receptors which aids in maintaining the optimum function in learning and memory processes; CB1 (neuronal survival and maturation) and CB2 (anti-inflammatory and anti-oxidant effects). Enhanced fluorescence intensity of these two in the morphological results describes how overlapping roles of these receptor-mediated pathways is essential to suggest a synergistic mechanism of action. AD is characterized by a complex of interplay of synaptic dysfunction, neuroinflammation, oxidative stress, and impaired neurogenesis so convergence of functional improvements allow a more comprehensive target towards diverse pathology of AD.

Another objective of this study was to investigate the potential for intrinsic self-recovery in AD-induced neuronal cells over time. This was evaluated by analysing the outcomes observed in the untreated AD group. Notably, within the CA1 region, the AD model without treatment demonstrated a modest increase in the mean fluorescence intensity of Calbindin, suggesting a limited degree of endogenous neurogenesis in the absence of Δ^9 THC intervention. However, this increase was comparatively minimal when contrasted with both the Δ^9 THC-treated groups and the Donepezil-treated group, indicating that spontaneous neurogenic responses alone are insufficient to achieve substantial neuronal recovery. In healthy adult brain, self-renewal is a defining property of neural stem progenitor cells (NSPCs) that resides mainly in the subgranular zone (SGZ) of DG within the hippocampus and the subventricular zone (SVZ). This aligns statistical results with the morphological observations as there were significances observed for both AD model and AD without treatment in 1.5 and 3.0 mg/kg of Δ^9 THC in DG however, only presence of minimal Calbindin expression was observed in the untreated AD brains indicating endogenous self-renewal is not entirely abolished, it

remains insufficient to achieve meaningful functional recovery across regions. This underscores the necessity of therapeutic intervention, such as Δ^9 THC administration, to restore neurogenic capacity and counteract the inhibitory effects of AD pathology.

Under physiological conditions, adult hippocampal neurogenesis follows a well-orchestrated sequence originating primarily in the dentate gyrus (DG), with newly generated neurons progressively migrating and functionally integrating into hippocampal circuits, including the CA1 region. The graphical trends observed in this study suggest a degree of sustained cellular proliferation, consistent with the inherent capacity of neural stem/progenitor cells (NSPCs) to support lifelong neurogenesis. During this process, NSPCs undergo tightly regulated cell division, giving rise to progeny that differentiate into either neuronal or glial lineages, thereby maintaining the neurogenic pool throughout adulthood (Mu & Gage, 2011). However, the process of self-renewal is highly complex and governed by a network of regulatory mechanisms including transcription factors, cell cycle modulators, extracellular niche signals, and intracellular pathways such as PI3K/Akt and ERK/CREB. These pathways collectively ensure the survival of neural stem/progenitor cells, proliferation, and lineage specification.

Disruption of these regulatory systems can significantly impair the efficiency of neurogenesis. In this context, investigating the role of Δ^9 THC in modulating these pathways is critical, as emerging evidence suggests its potential to enhance neurogenic processes through cannabinoid receptor-mediated signalling. In the untreated AD group, the capacity for self-renewal appeared to be modestly diminished, as reflected by reduced immunofluorescence intensity associated with neurogenesis markers. A clear disparity was observed when compared to Δ^9 THC-treated groups, indicating that endogenous regenerative mechanisms alone are insufficient to sustain effective neurogenesis under pathological conditions. This observation is consistent with previous studies demonstrating that the accumulation of A β exerts direct inhibitory effects on NSPCs. Specifically, A β induces oxidative stress and activates pro-apoptotic signalling cascades, including the upregulation of Bax and caspase-3, thereby impairing NSPC survival and proliferation (Winner et al., 2011; Khodaverdi et al., 2021).

Evidence from in vitro AD models further supports these findings, where exposure of cultured NSPCs to A β results in reduced proliferation, increased apoptosis, and mitochondrial dysfunction (Navarrete et al., 2020). Importantly, these effects occur independently of systemic inflammatory influences, highlighting the direct neurotoxic

impact of A β on the neurogenic niche. Collectively, both in vivo and in vitro studies indicate that impaired neurogenesis in AD arises from a combination of intrinsic factors, such as oxidative stress and transcriptional dysregulation, and extrinsic factors, including chronic neuroinflammation and neuronal injury. Thus, understanding these mechanisms is essential to develop therapies to treat the neurogenesis niche in AD progression. Researchers aim to shift AD treatment from purely symptomatic relief towards interventions that are potent to repair and rebuild hippocampal circuits include stimulations among neurons specific in neuroplasticity and dendritic sprouting to compensate for progressive neuronal loss in the DG and CA regions (Winner & Winkler, 2015; Hollands et al., 2016). Deeper understanding on these dynamics would therefore help pave future strategies that address both amyloid and tau pathologies in AD progression.

Aside from the known downregulation of neurogenesis, progressive cognitive impairments of AD are strongly associated with the dysregulation of amyloid-beta metabolism and tau hyperphosphorylation. These pathological hallmarks lead to the accumulation of extracellular A β plaques and intracellular neurofibrillary tangles (NFT) which collectively disrupt synaptic integrity and promote neuronal loss (Li et al., 2016). Consequently, reducing the levels of these pathogenic proteins represents a critical therapeutic objective. In the present study, it was hypothesized that increasing concentrations of Δ^9 THC would correspond to reduced formation of neurotoxic A β peptides through modulation of the amyloidogenic pathway. The results demonstrated that Δ^9 THC treatment effectively reduced BACE1 protein expression across all treatment groups, indicating a suppression of amyloidogenic processing. Notably, the highest concentration of Δ^9 THC (3.0 mg/kg) produced the most pronounced reduction in BACE1 levels, suggesting a dose-responsive effect in mitigating AD pathology. Hence, targeting the key enzyme responsible for excitotoxicity will affect the downstream formation of small clusters of monomers, oligomers and fibrils associated with maintaining homeostatic balance between the cells (Gouras et al., 2015; Li et al., 2016).

Despite that, when compared to a drug being used in clinical settings particularly Donepezil-treated group, Δ^9 THC exhibited comparable, and in some instances slightly enhanced, efficacy in reducing BACE1 expression, further supporting its potential as a viable therapeutic agent. In contrast, the untreated AD model exhibited markedly elevated BACE1 levels, reflecting increased amyloidogenic activity and accelerated

plaque formation. Although a slight reduction was observed in the AD without treatment group, likely reflecting minimal endogenous compensatory mechanisms, this reduction was insufficient to counteract disease progression. Prolonged absence of intervention may further exacerbate neuronal damage, as the imbalance between A β production and clearance continues to promote neurotoxicity and synaptic dysfunction. To compare its effectiveness, results were compared with previous studies such as in-vitro studies that further clarify Δ^9 THC modifies established AD pathology at which has been demonstrated by Cao et al., (2014) using cell culture analysis. From the insights, it was proven that Δ^9 THC significantly inhibits BACE1 activity effectively reducing A β production, which underpins plaque accumulation. From this, Δ^9 THC has suggested its role in intervening the mechanism after amyloidogenic processes have begun, actively reducing existing amyloid load. In AD pathogenesis, BACE1 is crucial in generating A β peptides, which aggregate to form plaques, one of the hallmarks of the disease. Mechanistically, Δ^9 THC is proposed to interact by directly binding to the active site of BACE1 and reducing its enzymatic activity thus lowering cleavage of APP and subsequent attenuation of amyloidogenic cascades (Cao et al., 2014; Eubanks et al., 2006).

Further supporting evidence from transgenic AD models indicates that cannabinoid-based interventions reduce BACE1 expression through CB1 receptor-mediated pathways, potentially via modulation of oxidative stress and inflammatory signaling (Casarejos et al., 2013). Inhibition of BACE1 has also been associated with improved cognitive outcomes, as demonstrated by studies employing BACE1 inhibitors such as ML264, which enhanced spatial learning and memory performance in APP/PS1 mouse models (Singh et al., 2021). In addition to CB1-mediated effects, Δ^9 THC activation of CB2 receptors contributes to the reduction of neuroinflammation by suppressing microglial activation and decreasing the release of pro-inflammatory cytokines. This creates a neuroprotective environment that supports neuronal survival and reduces apoptosis (Fukumoto et al., 2002; Cai et al., 2011). The combined modulation of CB1 and CB2 receptors underscores the multifaceted therapeutic potential of Δ^9 THC in addressing the complex pathology of AD.

Parallel with the findings in this study, the improvement in BACE1 is likely due to the diminished accumulated A β and associated alleviation of neurotoxic effects. If no effort is done, increase of accumulated A β plaques due to the imbalance between production and clearance mechanisms will further lead to aggregations into A β

oligomers and fibrils. This forms the well-known plaques impairing functional synapses leading to loss of neurons, exacerbating cognitive decline in AD brains. The neuroinflammations were induced through activation of microglia, the resident immune cells in the brain. In the process of microglia attempting to clear A β , their prolonged activation can become neurotoxic as it releases pro-inflammatory cytokines and reactive oxygen species that further damage the neurons. This neuroinflammatory response accelerates neuronal dysfunction and death, creating a vicious cycle that exacerbates disease progression. This establishes a self-perpetuating cycle of neuronal damage and inflammation, ultimately accelerating disease progression. Collectively, these observations highlight the critical importance of targeting amyloid pathology in AD and provide strong evidence supporting the role of Δ^9 THC as a multi-factorial therapeutic agent capable of modulating both neurogenesis and amyloidogenic pathways.

Aside from BACE1, another pathological hallmark for AD is accumulation of neurofibrillary tangles (NFTs) caused by build-up of hyperphosphorylated tau proteins that can lead to dysfunctional neurons. In the present study, particular emphasis was placed on p-Tau Thr231, as phosphorylation at this residue is recognized as an early and mechanistically significant event that impairs tau–microtubule interactions and promotes the initiation of tangle formation. Consequently, p-Tau Thr231 serves as both a sensitive early biomarker and a relevant therapeutic target for intervening in AD progression. In the present study, analysis of relative protein band intensity revealed a marked reduction in p-Tau Thr231 levels across all Δ^9 THC-treated groups compared to both the AD model and untreated AD group. This consistent decrease across concentrations (0.75, 1.50, and 3.0 mg/kg) indicates that Δ^9 THC effectively attenuates early tau hyperphosphorylation.

When compared to the Donepezil-treated group, Δ^9 THC demonstrated comparable efficacy in reducing p-Tau Thr231 expression. Given that Donepezil is a well-established, clinically approved therapy for AD, achieving similar reductions in tau phosphorylation highlights the therapeutic potential of Δ^9 THC as an alternative or adjunct intervention. Supporting these findings, previous studies have demonstrated that chronic administration of cannabinoids reduces immunoreactivity of p-Tau Thr231 and improves cognitive performance in transgenic AD models (Aso et al., 2013). These effects have been attributed to the ability of cannabinoids to reduce neuroinflammation and oxidative stress which are two key drivers of kinase overactivation responsible for tau hyperphosphorylation. By attenuating these upstream pathological processes,

Δ^9 THC may indirectly suppress tau kinase activity and restore phosphorylation homeostasis (Ramírez et al., 2005; Eubanks et al., 2006). Collectively, findings from this study suggest that Δ^9 THC is capable of targeting early-stage tau pathology, which is critical for preventing the progression toward irreversible neurodegeneration.

Importantly, the multi-targeted nature of Δ^9 THC distinguishes it from conventional therapies and bring it to a higher spectrum in regulating the disease mechanism. These findings serve as the foundation needed before venturing into medical administration clinically aimed in reducing prominent pathogenic factors in AD to preserve neuronal integrity and brain functionalities (Eubanks et al., 2006; Wang et al., 2022). This drug has been in the centre of attention unlike many drugs giving it an advantageous prospect. These holistic effects will provide meaningful insights into how cannabinoid-based therapies might be developed to address each complexity of the disease progression. Thus, researchers need to recognize and maximize the therapeutic benefits while minimizing its psychoactive side effects to translate into pre-clinical treatments.

Other than that, blood-brain barrier (BBB) in the central nervous system presents a significant obstacle to effective drug delivery as it restricts the passage of many therapeutic agents. Notably, cannabinoids have been shown to modulate BBB permeability as CB1 and CB2 receptors are expressed in brain microvascular endothelial cells and astrocytes. By activation of these receptors, it has been associated with enhanced tight junction integrity and reduced endothelial permeability, thereby contributing to the maintenance of BBB stability under pathological conditions. The permeability of the BBB is a critical determinant of Δ^9 THC pharmacokinetics within the CNS. Due to its highly lipophilic nature, Δ^9 THC readily undergoes passive diffusion across the BBB via transcellular pathways, allowing it to traverse endothelial cell membranes without reliance on active transport mechanisms. This property facilitates its access to neuronal and glial targets implicated in AD pathogenesis.

Despite these contributing enhancement factors, this similar characteristic of lipophilicity also presents pharmacokinetic limitations as it may lead to extensive binding to plasma proteins and peripheral tissues, which reduces its free circulating fraction and may consequently limit its bioavailability in the bloodstream and eventually within the brain itself. This could attenuate its therapeutic efficacy at the neuronal level, thereby influencing its capacity to ameliorate AD-related neuropathology. All things considered, even with the given challenges associated with

Δ^9 THC, efforts in using it as a new neurotherapeutic approach would be an innovative strategy to enhance progressive modulation and be a potent agent for future multifaceted medical use to enhance the brain innate clearance systems in AD brains. Thus, this suggests Δ^9 THC as a potential component in future research endeavours that could integrate cannabinoid-based intervention into regenerative frameworks (Marchalant et al., 2009; Aso et al., 2015).

CHAPTER 6

CONCLUSION

6.1 Summary and Conclusion

Alzheimer's disease (AD) is the most prevalent form of dementia, differed from the rest through its progressive memory loss, cognitive decline and downstream behavioural effects. Throughout all these years, the focus of research has been to aim in discovering ways to treat its symptomatic symptoms and not focusing enough on relieving the core causes of the disease. In recent years, cannabinoids specifically Δ^9 THC, the primary psychoactive component of *Cannabis sativa*, have received a lot of attention as it can interact with the body's endocannabinoid system (ECS) that regulates numerous vital physiological processes such as neuroinflammation, neuroprotection and synaptic regulatory mechanisms. To further aim in presenting a complementary therapeutic strategy for Δ^9 THC and other cannabinoids, multi-modal targets and effects are much more effective compared to single-target interventions. Δ^9 THC has gained increased attention from the scientific community as early-phase clinical trials and animal studies have found its potential in improving common behavioural symptoms such as agitation, anxiety and sleep disturbances as seen in AD individuals especially in moderate to severe AD stages (Aso & Ferrer, 2014).

Importantly, beyond the effects on the symptomatic symptoms, there are potential benefits in improving quality of life of the individuals. Despite that, challenges and risks associated with the use of Δ^9 THC as pharmacotherapies need to be addressed properly. One significant challenge is the psychoactive effects that come along with its use as medication. This has raised concerns among researchers due to the vulnerability of elderly populations towards the cognitive side effects like disorientation, anxiety and psychosis. This might worsens if chronic use of Δ^9 THC give more negative effects than positive ones because there is a potential that leads to impairments of memory and executive functions, potentially exacerbating some symptoms it aims to alleviate. However, from the collective experimental results that have been examined, administration of Δ^9 THC is successfully proved to be beneficial as long as its

concentration is being controlled. The positive outcomes have reflected in neurobehavioural assessment, MWM at which it demonstrated significant improvements in spatial learning and memory indicating enhanced cognitive performance following treatment. Also, apoptotic neuronal cells were proven remarkably being downregulated suggesting that the intervention mitigates neuronal loss in AD-related pathology. Moreover, the increased mean intensities of neurogenic markers highlight the potential of the treatment to support neuronal regeneration and plasticity. Along with that, western blot analysis has further confirmed the molecular changes underlying these effects, showing altered expression of key proteins associated with AD progression. Taken together, these findings provide novel insights into therapeutic potential of Δ^9 THC for mitigating AD pathology.

6.2 Recommendation for Future Direction

The use of delta-9-tetrahydrocannabinol (Δ^9 THC) as a potential therapy for Alzheimer's disease (AD) has gained significant scientific attention as it possess multi-targeted neurotherapeutic properties. The future research needs to focus on aiming Δ^9 THC as a therapeutic agent for AD by unlocking its full potential by clarifying dose-response relationship in AD patients via large-scale clinical trials. Experimental animal models are able to discover its effectiveness only to a certain level, hence clinical trials using will be useful in identifying minimal effective dose that maximizes the positive effects while at the same time minimizing psychoactive side effects. These might depend on how it affects the mechanistic pathways of the drug intervention such factors as age-specific and disease-stage-specific because the drug is potent to affect differently based on age groups (young and elderly), stage of the disease (early, middle and late) or both concurrently. The in-depth molecular mechanisms underlying Δ^9 THC and its effects have to be known. This needs further exploration on how it modulates amyloid-beta processing, tau phosphorylation, microglial activation and synaptic plasticity through both cannabinoid signalling pathways via the respective receptors; CB1 and CB2. Unlike the available drugs that focuses on single-targeting have limited efficacy, these multi-targeted approach would act across interconnected pathways offering a more holistic neurotherapeutic strategy. However, the results from the findings still need further clarifications and in-depth comparison strategies to enhance its efficacy and safety prior being administered in clinical settings. This experimental design is still

considered as premature as it is not an actual representative of AD treatment. Thus, engaging with AD-affected individuals in future studies is vital to explore deeper regarding its dose-dependent relationship and mechanistic pathways in the human brain. Non-psychoactive cannabinoid derivatives and endocannabinoid system modulators need to be considered as alternatives to direct Δ^9 THC administration. This was seen possible as there are CB2-selective agonists could provide anti-inflammatory benefits without triggering the other CB1-mediated psychoactive pathways.

Novel drug delivery systems such as nanoparticle encapsulation, intranasal delivery and targeted liposomal Δ^9 THC formulations should be further explored other than dermally dose-injections to identify the best route of delivery to improve brain-specific delivery. This is helpful in enhancing bioavailability and reduce peripheral side effects. Future research should also extend the limits beyond cannabinoids in investigating combined ECS modulation and anti-amyloid or anti-tau pharmacotherapies in producing synergistic benefits to halt the disease progression. Within that prospect, exciting frontiers in exploring alternative methods for AD involves modulating the exogenous and endogenous cannabinoid systems at the molecular level. For endogenous cannabinoids, their potential roles in enhancing therapeutic properties in the brain should be more explored to increase preclinical evidences. Aside from animal models and human studies, gene therapy approaches need to be widen in targeting cannabinoid receptor expressions (eg., increasing CB1 and CB2 densities) due to its possible amplification of neurotherapeutic and anti-inflammatory effects without altering systemic cannabinoid administration in the body.

In summary, while current AD therapies mostly offer symptomatic relief or modest disease-modifying effects, challenges in exploring the scope of study remains being a concern. Psychoactive side effects, optimal dosing strategies and individual variability in treatment responses need to be considered. More refined and safer strategies for leveraging neurotherapies need to be discussed. In terms of clinical trials, more rigorous designs, larger sample sizes and long-term effects of Δ^9 THC in the learning and memory pathways need to be evaluated properly across all ages and society groups. In future, tailoring specific-based therapies to specific patient profiles could greatly improve while validating Δ^9 THC at mechanistic level. With cannabinoids being the centre of attention in AD strategies, early future directions should embrace precise approaches to fully exploit its potential especially preclinical and mild cognitive impairment (MCI) stages rather than the late stage of the disease.

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APPENDICES

APPENDIX 1

Accepted Original Article by Letters in Applied NanoBioScience (LIANBS) Entitled Delta-9-tetrahydrocannabinol (Δ^9 THC) Treatment Improves Neuronal Survivability by Increasing Calbindin Expression in the Pyramidal Cells of Alzheimer-induced Rat Hippocampus

Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article

Volume 13, Issue 2, 2024

<https://doi.org/10.33263/LIANBS120.000>

Delta-9-tetrahydrocannabinol (Δ^9 THC) treatment improves neuronal survivability by increasing Calbindin expression in the pyramidal cells of Alzheimer-induced rat hippocampus

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Received: date; Accepted: date; Published: date

Abstract: Alzheimer's disease (AD) is the most common form of dementia, an age-dependent neurodegenerative process that involves the gradual deterioration of neurons leading to a decline in cognitive abilities. Since the endocannabinoid system (ECS) is highly expressed in the hippocampus, Delta-9-tetrahydrocannabinol (Δ^9 THC) often been associated with learning and memory functions. This research is designed to evaluate the effect of Δ^9 THC on the cellular and physiological changes in the hippocampus induced by varying doses during the early stage of developmental AD focusing on neuronal survivability and possible alterations in upregulating the expression of neurogenesis protein marker. Male albino Wistar rats were firstly exposed to 60 mg/kg D-gal intraperitoneally and 200 mg/kg AlCl_3 orally, once daily for ten consecutive weeks to induce AD. Doses of Δ^9 THC at 0.75, 1.5, and 3.0 mg/kg were administered to all groups for 28 days and 1 mg/kg donepezil was used as a positive control measure. The results revealed Δ^9 THC significantly alleviated cognitive impairment by reducing neuronal apoptosis, increasing neuronal survivability and preventing morphological aberrations. The evaluation of immunoreactivity area of calbindin-positive-stained cells indicated Δ^9 THC promotes calbindin expression as one of the neurogenesis protein markers at corpus ammonis region. This suggests that Δ^9 THC might be a promising strategy that may ameliorate AD-associated cognitive deficits.

Keywords: Alzheimer's disease; cannabis; Δ^9 THC, hippocampus, cognitive impairment

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APPENDIX 2

Organized A Research Workshop Entitled “Mastering Protein Analysis: Western Blot” with Technical Talk and Showcase on Principle and Troubleshooting 27th May 2024 at Research Interest Group (RIG) Laboratory, Faculty of Health Sciences, UiTM Puncak Alam



APPENDIX 3

Group Representative Image of the Attended Participants and Research Committees for the Workshop on “Mastering Protein Analysis: Western Blot” with Technical Talk and Showcase on Principle and Troubleshooting on 27th May 2024 at the Postgraduate Lounge, Faculty of Health Sciences,UiTM Puncak Alam



APPENDIX 4

Organized a Scientific Discovery Research Programme Involving Primary and Secondary School Students Showcasing Scientific Basic Knowledge and Research Activities Involving Optometry, Nursing clinics along with Anatomy and Microbiology Laboratories on the 4th June 2024 at Faculty of Health Sciences, UiTM Puncak Alam



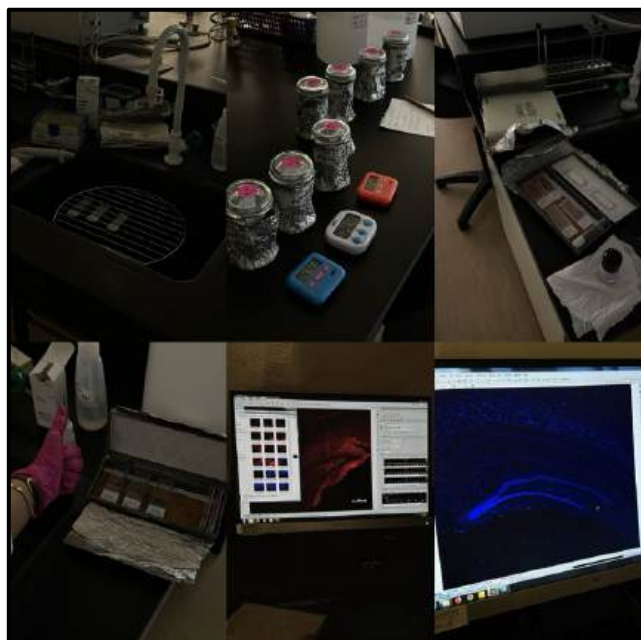
APPENDIX 5

**Experimental Procedures of H&E Staining to View the Apoptotic
Morphology of Neuronal Cells**



APPENDIX 6

Experimental Procedures of Immunofluorescence (IF) Staining to View the Neurogenesis among Neuronal Cells



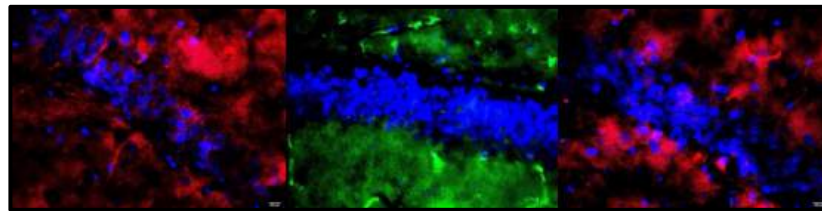
APPENDIX 7

Experimental Procedures of Western Blot (WB) Step-by-Step to Quantify Protein Band Intensities



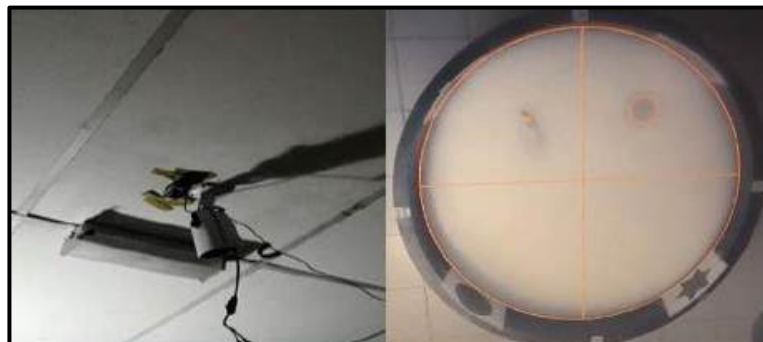
APPENDIX 8

Representative Photomicrographs of Granule Cells at Dentate Gyrus (DG) Region (x40 magnification) Submitted in the Postgraduate Research Image Competition 2025 Held on 24th June 2025 by UiTM Pulau Pinang Branch, Permatang Pauh Entitled Upregulated GFAP as a Neurogenesis Marker in an Alzheimer-induced Model by Delta-9-Tetrahydrocannabinol (Δ^9 THC), Major Constituent of *Cannabis sativa*



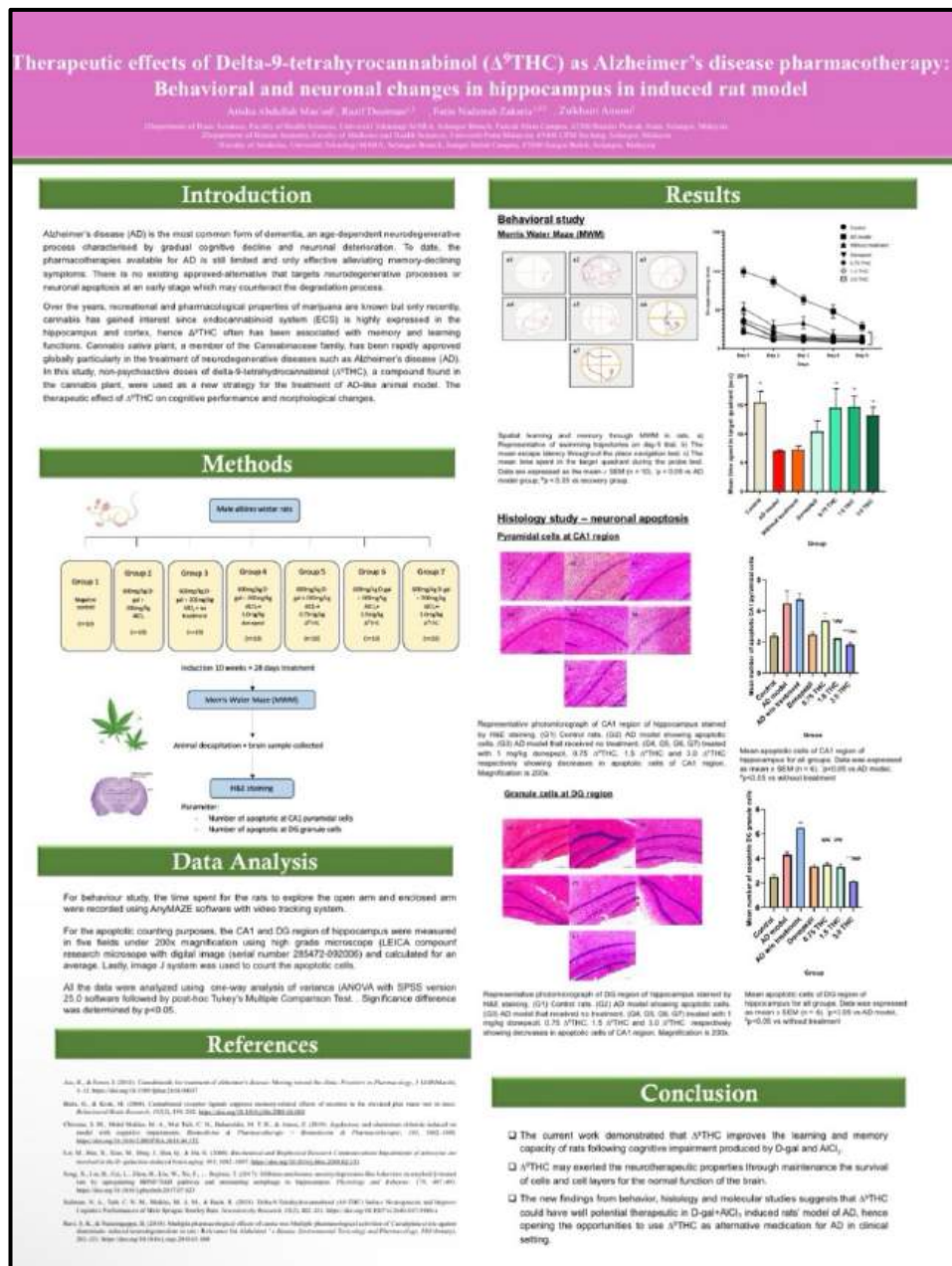
APPENDIX 9

Experimental Setup during Morris Water Maze (MWM) Analysing the Effects of Δ^9 THC on the Neurobehavioural Improvements in the Treatment Groups of AD-induced Wistar Rats via Video Tracking Software Connected to the Logitech Camera



APPENDIX 10

Received the Award for Young Investigator Travel Grant (YITG) in the 10th Federation of Asian and Oceanian Physiological Societies (FAOPS) Congress Held in Conjunction with the 75th Annual Meeting of the Korean Physiological Society in Daegu, South Korea from November 1-4 2023. Hosted at the Daegu Exhibition & Convention Center (EXCO)



APPENDIX 11

Poster Presentation and Award Ceremony during FAOPS Congress in Daegu, South Korea with Other Researchers and Established Professors from Multidisciplinary Fields of Expertise along with Sharing of Knowledge Sessions



APPENDIX 12

**Attended the Basic of Image Processing and Analysis with Image-J
Workshop Held on 15th March 2023 Organized by Imaging Center
(iMAGE), Faculty of Pharmacy, UiTM Puncak Alam**



APPENDIX 13

Attended the Basic Statistics using SPSS Workshop Held on 11-13th July 2023 Organized by Clinical Research Centre (CRC), Hospital Shah Alam

	HOSPITAL SHAH ALAM	
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	SEKSYEN 7, 40000 SHAH ALAM	
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Ruj. Kami : HSAS 600-22/1/4 (46)
Tarikh : 5 Julai 2023

SEPERTI SENARAI EDARAN

Tuan/ Puan,

PELEPASAN PESERTA BAGI MENGHADIRI KURSUS *BASIC STATISTICS USING SPSS 2023* DI HOSPITAL SHAH ALAM

Saya dengan segala hormatnya merujuk perkara tersebut di atas.

2. Sukacita dimaklumkan bahawa Pusat Penyelidikan Klinikal Hospital Shah Alam akan menganjurkan **Kursus "*Basic Statistics Using SPSS 2023*"** seperti ketetapan yang berikut:

Tarikh	: 11, 12 & 13 Julai 2023 (Selasa, Rabu & Khamis)
Masa	: 8.00 pagi - 5.00 petang
Tempat	: Bilik Latihan IT 1 & 2, Bahagian Teknologi Maklumat Hospital Shah Alam

3. Sehubungan dengan itu, pihak kami berbesar hati ingin menjemput dan memohon pelepasan pegawai dibawah seliaan tuan/ puan seperti di **Lampiran 1** bagi mengikuti kursus tersebut. Bersama-sama surat ini disertakan aturcara program untuk rujukan tuan/ puan **Lampiran 2**.

4. Peserta diminta untuk membawa bersama komputer riba bagi tujuan kursus. Sekiranya pihak tuan/ puan mempunyai sebarang pertanyaan, sila hubungi **Dr. Ili Najihah Muhammad Amin** di talian 03-55263000 no. sambungan 3304/3309/3316. Kerjasama dan sokongan daripada pihak tuan/ puan dalam menjayakan bengkel ini amatlah dihargai.

Sekian, terima kasih.

"MALAYSIA MADANI"

"BERKHIDMAT UNTUK NEGARA"

Saya yang menialankan amanah,

.....
(DATUK DR HJH NOR FARIZA BINTI NGAH MMC NO. 29577)
Ketua Unit CRC
Hospital Shah Alam

AUTHOR'S PROFILE



Arisha Abdullah Mas'od is a postgraduate neuroscience young researcher with a solid academic background in the life sciences and a growing expertise in the field of biological sciences. She began her academic journey with Foundation in Science from Universiti Malaya (UM), Malaysia, which provided her with a strong grounding in fundamental scientific principles. She then pursued a Bachelor of Science (Hons.) in Biology at Universiti Teknologi MARA (UiTM), Shah Alam, where she developed a keen interest in human physiology and neurodegenerative diseases. Currently, she is undertaking Master of Science by research in Physiology at UiTM Puncak Alam, with her thesis focusing on Alzheimer's disease, specifically exploring cannabinoid potential specifically Δ^9 THC along with its neurobiological mechanisms and therapeutic strategies for the disease modulation.

Her research interests lie in the interface of molecular neuroscience and experimental neurology, with a focus on brain neuroinflammation, amyloid-beta processing, and tau pathology in AD animal models. Arisha is actively involved in laboratory-based research using in vivo model to investigate potential interventions targeting key hallmarks in AD. She has contributed as the main author to a peer-reviewed journal and currently focusing on writing more manuscripts to be contribute to other scientific journals. She has presented her findings at academic conferences, receiving recognition for excellence in scientific communication, including an award for best oral presentation at an international neuroscience conference.

Arisha is known among her peers and academic mentors for her strong work ethic, meticulous attention to detail, and critical thinking skills. She demonstrates strong

research integrity, academic discipline, and adaptability in both collaborative and independent research settings. Her ability to manage complex experimental protocols and analyze data with precision reflects her competency in experimental design and scientific methodology. Furthermore, she shows a high level of self-motivation and a proactive approach to problem-solving, essential qualities in navigating the challenges in research. Arisha exhibits strong written and verbal communication skills, allowing her to effectively disseminate scientific findings to both specialized and broader audiences. She is committed to academic excellence and lifelong learning, with aspirations to contribute meaningfully to the field of neuroscience and the global effort to better understand and treat Alzheimer's disease.

LIST OF PUBLICATIONS:

- Mas'od, A. A., Zakaria, F. N., Dasiman, R., Chiroma, S. M., Liu, R., Moklas, M. A. M., M., & Amom, Z. (2024). Delta-9-tetrahydrocannabinol (Δ^9 THC) Treatment Improves Neuronal Survivability by Increasing Calbindin Expression in the Pyramidal Cells of Alzheimer-induced Rat Hippocampus. *Alzheimer's & Dementia*, 13(2). <https://doi.org/10.33263/LIANBS120.000> (Accepted).
- Mas'od, A. A., Zakaria, F. N., & Amom, Z. (2025). Delta-9-tetrahydrocannabinol (Δ^9 THC) Reduces Neuronal Apoptosis, Upregulates GFAP as Neurogenesis Protein Marker and Downregulates BACE1 Protein Expression at in an AD-induced Wistar Rat Model. *Basic and Clinical Neuroscience Journal (BCN)* (In Progress)
- Mas'od, A. A., Zakaria, F. N., & Amom, Z. (2025). Cannabinoid-Mediated Neurotherapy: Δ^9 -tetrahydrocannabinol (Δ^9 THC) Mitigates Apoptosis, Enhancing Neurogenesis and Reduces Phosphorylated-Tau (p-Thr231) in the Dentate Gyrus of an Alzheimer's Model. *Journal of Pharmacopuncture (JoP)* (In progress).