NEUROPROTECTIVE EFFECTS OF DITERPENOIDS-ENRICHED FRACTION OF *ANDROGRAPHIS PANICULATA* AND ANDROGRAPHOLIDE IN ISCHAEMIA-INDUCED NEURONAL DEATH AND COGNITIVE IMPAIRMENT: *IN VITRO* AND *IN VIVO* MODELS

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by

ROZ AZINUR BINTI CHE LAMIN

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LIST OF SYMBOLS

A	Ampere
α	Alpha
β	Beta
γ	Gamma
δ	Delta
σ	Lower sigma, slope of regression line
%	Percentage
°C	Degree Celsius
=	Equal to
±	Plus minus
E	degree of freedom
n	Number of animals
x	Multiplication
>	Greater than
<	Lesser than
cm	Centimetre
CV	Coefficient of variation
et al.	And other
eV	Electron volt
g	Gram
g/day	Gram per day
g/kg	Gram per kilogram
g/L	Gram per litre
h	Hour (s)

Hz	Hertz
i.e	That is
i.p	Intraperitoneal injection
L	Litre
LOD	Limit of detection
LOQ	Limit of quantification
m	Metre
mA	Milliampere
mm	Millimetre
mL	Millilitre
ms	Milliseconds
min	Minute (s)
mg/day	Milligram per day
mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitre
mg/L	Milligram per litre
mL/kg	Millilitre per kilogram
mL/min	Millilitre per minute
mmol/L	Millimole per litre
p.o	Per os or per oram
R^2	Correlation coefficient
RSD	relative standard deviation
SD	Standard deviation
SEM	standard error of the mean
µl	Microlitre
µmol/L	Micromole per litre
µg/mL	Microgram per millilitre

U/L	Units per litre
ΔΨm	Mitochondrial membrane potential

LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
ADG	Andrographolide
AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMPAR	A-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
AP3	14 deoxy-11,12 didehydroandrographolide
APP	Amyloid-β precursor protein
ARASC	Animal Research and Service Centre
ARCD	Age-related cognitive decline
BACE1	B secretase 1
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CA	Cornu ammonis
CaMKII	Calcium/calmodulin-dependent protein kinase ii
CBF	Cerebral blood flow
CCH	Chronic cerebral hypoperfusion
CCI	Chronic cerebral ischaemia
CD4	Cluster of differentiation 4
ChEIs	Cholinesterase inhibitors
CMIs	Cortical cerebral microinfarcts
CUS	Chronic unpredictable stress
cNOS	Constitutive nitric oxide synthase
CNS	Central nervous system
CREB	cAMP-response element binding protein
CUS	Chronic unpredictable stress
CV	Precision
dCA1	Dorsal CA1
DCF	Dichlorofluorescein

DCFH	Dichloro-dihydro-fluorescein
DCFH-DA	Dichlorofluorescein diacetate
DEOXY	14-deoxyandrographolide
DG	Dentate gyrus
DMEM/F12	Dulbecco's modified eagle medium/nutrient mixture f-12
EC_{50}	Half maximal effective concentration
ELT	Escape latency time
E-LTP	Early long-term potentiation
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Federal and Drug Administration
fEPSP	Field excitatory postsynaptic potential
GSH	Glutathione
HCoV-OC43	Human coronavirus organ culture 43
HD	Huntington's disease
HIF	Hypoxia-induce factor
HIV	Human immunodeficiency viruses
HL-TBS	High-intensity long-theta-burst stimulation
HPLC-UV	High-performance liquid chromatographic-ultraviolet
HRE	Hypoxia-response element
IBI	Inter-burst intervals
ICH	Intracerebral haemorrhage
IC_{50}	Half-maximal inhibitory concentration
I/O	Input-output curves
LD_{50}	Lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
L-TBS	Long-theta-burst stimulation
LTD	Long-term depression
LTP	Long-term potentiation
MAGUK	Membrane-associated guanylate kinase family
MAPK	Mitogen-activated protein kinases
MDA-MB-231	MD Anderson - metastatic breast - 231

MetS	Metabolic syndrome
MID	Multi-infarct dementia
Mn-SOD	Manganese-superoxide dismutase
MPP^+	1-methyl-4-phenylpyridinium
MPT	Mitochondrial permeability transition
MS	Multiple sclerosis
NADH	Nicotinamide-adenine-dinucleotide hydrogen
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NE	North-east
NEO	Neoandrographolide
NeuN	Neuronal nuclei
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	Neurofibrillary tangles
NLEM	National List of Essential Medicines
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NO	Nitric oxide
NOS	Nitric oxide synthase
NRS	Nitrogen reactive species
NW	North-west
MCI	Mild cognitive impairment
MWM	Morris water maze
OD	Optical density, absorbance
OGD	Oxygen/glucose deprivation
PAT	Passive avoidance test
PBOCCA	Permanent bilateral occlusion of the common carotid arteries
PD	Parkinson's disease
PEG	Polyethylene glycol
PKA	Protein kinase A
PKC	Protein kinase C
PMS	Progressive multiple sclerosis
PPF	Paired-pulse facilitation
PS1	Presenilin 1
PSD-95	Post-synaptic density -95

PVDF	Polyvinylidene fluoride
PI3K	Phosphatidylinositol 3-kinase
RNS	Reactive nitrogen species
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	South-east
SRS	Sulfide reactive species
S-TBS	Short-theta-burst stimulation
SW	South-west
SYN	Synaptophysin
TBS	Theta-burst stimulation
TBST	Tris-buffered saline containing tween 20
TEMED	Tetramethylethylenediamine
TMRE	Tetramethylrhodamine ethyl ester
TrkB	Tropomyosin receptor kinase B
VCI	Vascular cognitive impairment
VD	Vascular dementia
VDCC	Voltage-dependent calcium channels
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
2VO	Common carotid arteries
IPS	Institut Pengajian Siswazah
URTI	Upper respiratory tract infection
USM	Universiti Sains Malaysia

LIST OF APPENDICES

KESAN PERLINDUNGAN NEURO OLEH FRAKSI *ANDROGRAPHIS PANICULATA* YANG DIPERKAYA DITERPENOID DAN ANDROGRAPHOLIDE TERHADAP KEMATIAN NEURON DAN GANGGUAN KOGNITIF ARUHAN ISKEMIA: MODEL *IN VITRO* DAN *IN VIVO*

ABSTRAK

Andrographis paniculata (Burm.f.) Wall. ex Nees, yang dikenali sebagai "hempedu bumi", telah dikaji untuk kesan peningkatan kognitif dan aktiviti perlindungan neuronya. Walau bagaimanapun, kajian tentang potensi fraksi *A. paniculata* yang diperkaya diterpenoid terhadap gangguan pembelajaran dan ingatan, adalah terhad terutamanya dalam demensia yang melibatkan patologi vaskular. Oleh itu, kajian ini bertujuan untuk mempertingkatkan ekstrak metanol *A. paniculata* dengan pengkayaan diterpenoid melalui proses pemfraksian dan menilai kesan peningkatan kognitif dan perlindungan neuro bersama-sama dengan andrographolide, iaitu sebatian utama dalam *A. paniculata*, menggunakan model *in vitro* dan *in vivo*. Kandungan diterpenoid tertinggi didapati pada fraksi 4 yang diperolehi menggunakan 80% metanol dengan hasil keseluruhan sebanyak 583.5 mg per 1 g ekstrak metanol. Model tikus iskemik serebrum kronik (CCI) yang dihasilkan melalui oklusi dua hala kekal arteri karotid biasa (PBOCCA) telah digunakan untuk menilai potensi peningkatan kognitif. Tikus teraruh CCI telah dirawat secara oral dengan fraksi 4 diperkaya diterpenoid pada dos 100 dan 200 mg/kg, sementara andrographolide diberikan pada dos 1, 5 dan 10 mg/kg selama 14 hari. Peningkatan signifikan diperhatikan dalam ujian pengelakkan pasif pada dos 200 mg/kg fraksi 4

diperkaya diterpenoid (p<0.01) dan andrographolide pada dos 5 mg/kg (p<0.05) dan 10 mg/kg (p<0.01). Kesan fraksi 4 diperkaya diterpenoid dan andrographolide disiasat selanjutnya dengan melakukan ujian pagar sesat air Morris, yang menunjukkan pemulihan memori spasial yang signifikan pada 5 dan 10 mg/kg andrographolide (p<0.05) dan 100 mg/kg fraksi 4 diperkaya diterpenoid (p<0.01). Kemudian, keplastikan sinaptik *in vivo* di hipokampus otak dinilai menggunakan rakaman elektrofisiologi potensiasi jangka panjang (LTP). LTP menunjukkan peningkatan signifikan ke atas tikus teraruh CCI pada semua dos rawatan fraksi 4 diperkaya diterpenoid (p<0.05) dan andrographolide pada 5 mg/kg (p<0.01) dan 10 mg/kg (p<0.001). Walau bagaimanapun, pengekspresan faktor neurotropik berasal dari otak (BDNF), dan protein sinaptik, iaitu protein kinase II alfa bersandar kalsium/kalmodulin (CaMKIIα), possinaptik ketumpatan-95 (PSD-95) dan sinaptofisin telah meningkat dengan signifikan (p<0.05) pada dos 10 mg/kg andrographolide tetapi tidak pada tikus yang dirawat dengan fraksi 4 diperkaya diterpenoid. Sebaliknya, kesan perlindungan neuro telah ditunjukkan dengan menggunakan hipoksia aruhan $CoCl_2$ dalam titisan sel neuroblastoma SH-SY5Y . Kesan perlindungan neuro ke atas sel SH-SY5Y teraruh hipoksia ditunjukkan dalam sel yang diberikan prarawatan dengan 2.5 dan 5 μg/mL fraksi 4 diperkaya diterpenoid, dan 7.5 dan 15 μg/mL andrographolide. Penemuan ini disokong oleh pengurangan penghasilan spesies oksigen reaktif (p<0.0001) sementara potensi membran mitokondria ($\Delta\Psi m$) meningkat dengan signifikan (p<0.05) pada semua kepekatan rawatan berbanding dengan sel SH-SY5Y teraruh hipoksia kawalan yang dirawat dengan bahan pembawa. Fraksi 4 diperkaya diterpenoid dan andrographolide didapati dapat memulihkan kemerosotan pembelajaran dan ingatan dalam tikus teraruh CCI, dan melindungi daripada neurotoksisiti aruhan hipoksia dalam sel SH-

SY5Y, dengan itu menonjolkan potensi peningkatan kognitif dan penawar perlindungan saraf mereka, terutamanya dalam demensia yang berkaitan dengan patologi vaskular.

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ABSTRACT

Andrographis paniculata (Burm.f.) Wall. ex Nees, known as "*hempedu bumi*", has been studied for its cognitive-enhancing and neuroprotective effects. However, the study on the potential of *A. paniculata* diterpenoids-enriched fraction against learning and memory impairment is limited, particularly in vascular-pathology-related dementia. Thus, this study aimed to enhance the methanolic extract of *A. paniculata* via the enrichment of diterpenoids through a fractionation process and evaluate its cognitive-enhancing and neuroprotective effects along with andrographolide, the major diterpenoid of *A. paniculata,* using *in vitro* and *in vivo* models. The highest diterpenoid content was found in fraction 4, which was obtained using 80% methanol with a total yield of 583.5 mg per 1 g of methanol extract. The chronic cerebral ischaemic (CCI) rat model, developed by a permanent bilateral occlusion of the common carotid arteries (PBOCCA), was used to evaluate the cognitive-enhancing potential. The CCI-induced rats were treated orally with diterpenoids-enriched fraction 4 at doses of 100 and 200 mg/kg, while andrographolide was administered at doses of 1, 5 and 10 mg/kg for 14 days. A significant improvement was observed in the passive avoidance test at 200 mg/kg diterpenoids-enriched fraction 4 (p<0.01), and andrographolide at doses of 5 mg/kg (p<0.05) and 10 mg/kg (p<0.01). The effects of diterpenoids-enriched fraction 4 and

andrographolide were further investigated by performing the Morris water maze test, which resulted in significant spatial memory restoration at 5 and 10 mg/kg of andrographolide (p<0.05) and 100 mg/kg of diterpenoids-enriched fraction 4 (p<0.01). Then, the *in vivo* synaptic plasticity of the brain hippocampus was assessed using long-term potentiation (LTP) electrophysiological recording. LTP showed significant enhancement in the CCI-induced rats treated with all doses of diterpenoids-enriched fraction 4 (p<0.05) and andrographolide at 5 mg/kg (p<0.01) and 10 mg/kg (p<0.001). However, the expression of the brain-derived neurotrophic factor (BDNF) and the synaptic proteins, namely the calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα), post-synaptic density-95 (PSD-95) and synaptophysin were increased significantly (p<0.05) at 10 mg/kg of andrographolide but not in the diterpenoids-enriched fraction 4-treated rats. On the other hand, the neuroprotective effect was explored by using $CoCl_2$-induced hypoxia in neuroblastoma SH-SY5Y cell lines. The neuroprotective effect on the hypoxia-induced SH-SY5Y was demonstrated in the cells pre-treated with 2.5 and 5 µg/mL diterpenoids-enriched fraction 4, and 7.5 and 15 µg/mL andrographolide. The findings were supported by suppression of reactive oxygen species production (p<0.0001), while mitochondria membrane potential ($\Delta\Psi m$) increased significantly (p<0.05) at all concentrations of treatment compared to hypoxia-induced SH-SY5Y control cells treated with the vehicle. Diterpenoids-enriched fraction 4 and andrographolide were found to restore learning and memory deficits in CCI-induced rats and protect against hypoxia-induced neurotoxicity in SH-SY5Y cells, thus highlighting their potential as cognitive-enhancing and neuroprotection remedies, especially in vascular pathology-related dementia.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Neurodegeneration has been identified as a pathophysiological change, especially neuronal loss-related, leading to neurodegenerative or brain-related disorders (Lamptey et al., 2022). Neurodegenerative diseases are categorised as heterogeneous groups of disorders with a broad spectrum of aetiology. Based on the diversity of the emphasis on neuronal cluster degeneration and pathophysiology, it could result in Alzheimer's disease (AD), which is related to neuronal loss in the hippocampus and cortical regions. Parkinson's disease (PD) is a neurodegenerative disease that affects the brain, particularly in the striatal regions. Different disease involves different primary neuronal losses; for instance, amyotrophic lateral sclerosis (ALS) is due to the loss of spinal motor neurons and cortical regions, and Huntington's disease (HD) is related to the neuronal loss at striatal and cortical regions (Muddapu et al., 2020). The characteristics of these conditions are related to the inexorable ageing process, typically dementia, which is associated with memory and cognitive deficits and affects the patient's routine activities, such as communicating with other people, body movement and breathing (Gitler et al., 2017).

The World Health Organization (WHO) projects that by the year 2040, neurodegenerative diseases will surpass cancer as the second leading cause of death (Esmaeili et al., 2022). Approximately, around 50 million individuals across the globe suffer from AD. This staggering figure is expected to double every 5 years and is anticipated to rise to more than 150 million cases by the year 2050 (WHO, 2017). Data published by the Department of Statistics, Malaysia, indicates that the

proportion of people aged 65 and above rose from 6.7% in 2019 to 7.0% in 2020. According to Alzheimer's disease International, the anticipated prevalence of dementia in Malaysia was estimated at 123,000 individuals in 2015, increasing to 204,00 by 2020, 261,000 by 2030 and potentially reaching 590,000 by 2050 (Arumugam, 2022). This illustrates a consistent increase in the elderly population in Malaysia each year. Furthermore, it is projected that by 2030, the elderly population will make up 15% of the total population, signifying that Malaysia will become an aged nation (Lai et al., 2022). The burden of AD impacts people individually as well as their families, with an estimated $1 trillion in annual global cost (Breijyeh & Karaman, 2020). These facts predict that this disorder will represent a huge burden in the future, including socio-economic and healthcare systems (Drzezga, 2009).

Although the exact aetiology of neurodegenerative diseases largely remains unknown, ageing is the primary risk factor, and some of the main mechanisms underlying neurodegenerative diseases include amyloid-beta (amyloid-β) and tau proteins aggregation (Breijyeh & Karaman, 2020), oxidative stress (Cho et al., 2012), autophagy (Esmaeili et al., 2022), lysosomal dysfunction, mitochondrial dysfunction, and neuroinflammation (Zeydan & Kantarci, 2023). It has also been shown that vascular, environmental, and physical factors contribute to the development of AD and dementia (Bhushan et al., 2018). Likewise, vascular-related pathology is the common aetiology for vascular cognitive impairment (VCI), which is characterised by dementia. The most common VCI, vascular dementia (VD), is a neurological condition with clinically substantial cognitive impairment associated with cerebrovascular injury, ischaemic stroke, neuron loss and impact on the connectivity of neural pathways of the brain (Bir et al., 2021; Morgan & Mc Auley, 2024).

The highlight of this thesis is more focused on the issue of cognitive impairment resulting from ischaemia-induced neuronal death. Hence, the methodology encompasses different settings, *in vivo* study (animal model) and *in vitro* (human neuronal cell culture) experimental techniques. Some animal models have been established to induce ischaemic injuries in the rat brain with different levels of severity, enabling thorough investigations into the cellular metabolic processes and behavioural impacts of interrupted cerebral blood flow. Therefore, the suitable model for this experiment is permanent bilateral occlusion of the common carotid artery (PBOCCA). In this model, a ligation procedure of particular major arteries that provide blood to the brain was executed to induce cerebral hypoperfusion and initiate pathophysiological alterations in the brain, resulting in neuronal damage (Damodaran et al., 2014). To ascertain the metabolic mechanism of ischaemia, which leads to hypoxia-induced response, an *in vitro* investigation employing human neuronal cell culture methodologies was established. The SH-SY5Y cell line is a widely used neuronal cell-like model that is valuable for evaluating the neuroprotective or neurotoxic properties of various substances (Merino et al., 2015). The SH-SY5Y cells have been extensively utilised to mimic neurons and investigate the pathogenic molecular mechanisms related to neurodegenerative diseases and pain disorders (Gangras et al., 2022). The SH-SY5Y cells are treated with damage or stress inducers, frequently employing chemical mimics of hypoxia, like cobalt chloride (Muñoz-Sánchez & Chánez-Cárdenas, 2019).

The current focus of clinical treatments is on controlling clinical symptoms rather than addressing the root pathological cause. The Food and Drug Administration (FDA) has permitted several drugs to alleviate neurodegenerative progression and symptom control, such as donepezil and rivastigmine to treat AD,

levodopa to treat PD and tetrabenazine drug for HD (Lamptey et al., 2022). However, these medications have adverse effects that include severe headache, diarrhoea, bradycardia, insomnia, seizures, tremors, and disruption of gait (Adlimoghaddam et al., 2018). This demonstrates that while existing drugs can lessen symptoms, there are no long-term fixes to protect the remaining neuronal population (Muddapu et al., 2020).

Exploring neuroprotective agents from natural products is an interesting and promising alternative treatment. Therefore, numerous studies have focused on natural products, including extracts and phytochemicals, as bioactive molecules for combating neurodegenerative diseases. These include medicinal plants such as olive (Angeloni et al., 2017), *Schisandra chinensis* (Liu et al., 2019a), *Polygonum tinctorium* (Shin et al., 2022), *Clitoria ternatea* (Ahad et al., 2023), *Scutellaria incarnata Vent*. (Vásquez-Londoño et al., 2023), and *Angelica sinensis* (Choi et al., 2024). Among local medicinal herbs, *Andrographis paniculata* and its bioactive constituent have the potential to address the complex nature of the neurodegenerative disease, which is supported by a few pharmacological studies (Guccione et al., 2017; Prasertsuksri et al., 2023).

1.2 Problem Statement

Vascular dementia (VD) is the second most frequent cause of dementia, with a progressive deterioration in cognitive function as its hallmark. In neurological illnesses like VD, cerebral ischaemic stroke or chronic cerebral ischaemia (CCI) is thought to be a significant adverse outcome that leads to neurodegeneration and memory impairment. As VD is a heterogeneous disease, it is challenging to discover effective treatment for this disease. Various efforts are being made to improve the

VD symptoms, but none of the currently available drugs can stop the disease progression. With the limitation of available drugs in the market, herbal medicines, which consist of multicomponent and multitarget approaches, could provide viable therapies for VD events for other neurodegenerative diseases. Medicinal herb, *A. paniculata* and its extract have been reported to ameliorate neurological disorders. However, very few studies have yet to show the potential protection of *A. paniculata*, especially its diterpenoids-enriched fraction, against ischaemia-induced neurotoxicity and animal behaviour of learning and memory. This has sparked an interest in investigating the protective role of *A. paniculata* diterpenoid-enriched fraction and its major compound, andrographolide, against ischaemia-induced neuropathology. A previous study revealed the effectiveness of andrographolide in ameliorating cognitive impairments, reducing amyloid-β aggregation, suppressing microglial activation, and diminishing the release of proinflammatory inducer in the amyloid precursor protein/presenilin (APP/PS1) mice models (Zhang et al., 2021). This thesis presents a constructive exploration of the potential benefits of fractioned *A. paniculata* plant extracts in enhancing cognitive function and learning capability in the chronic cerebral ischaemia (CCI) rat model and alleviating the effect of hypoxia-inducible factor in human neuroblastoma cells. Hence, for a clear understanding of the methodologies and experimental process involved, Figure 1.1 provides a summary and illustration of this thesis framework.

1.3 Hypothesis

Diterpenoids-enriched fraction of *A. paniculata* and andrographolide is capable of restoring learning and memory deficits in chronic cerebral ischaemia (CCI)-induced rat models and showing a protective response in hypoxia-induced

neuronal cells. In addition, the treatment could restore the molecular synaptic signalling proteins that are differentially expressed in the brains of CCI rats. Collectively, the effect of diterpenoids-enriched *A. paniculata* fraction and andrographolide would offer a comprehensible explanation of behavioural reactions and neuroplasticity responses in the CCI-induced rat model and molecular protection mechanism in the hypoxia-induced neurotoxicity cell model.

1.3.1 Objectives

The general objective of this research is to investigate the neuroprotective effects of diterpenoids-enriched fraction of *Andrographis paniculata* and andrographolide in ischaemia-induced neuronal death and cognitive impairment. The specific objectives are as follows:

1) To prepare a diterpenoid-enriched fraction of *A. paniculata*, obtain the chemical fingerprint and standardise the fraction using the selected diterpenoids as marker compounds.

2) To evaluate the cognitive-enhancing effect of the diterpenoids-enriched fraction of *A. paniculata* and andrographolide in a chronic cerebral ischaemia-induced rat model.

3) To determine the expression of synaptic signalling proteins that mediate synaptic plasticity in the hippocampus of the CCI rat model following diterpenoids-enriched fraction of *A. paniculata* and andrographolide administration using western blot analysis.

4) To determine the range of non-toxic concentrations and the cytotoxicity behaviour of diterpenoids-enriched fraction of *A. paniculata* and andrographolide on human neuroblastoma cells.

5) To evaluate the neuroprotective effect of the diterpenoids-enriched fraction of *A. paniculata* and andrographolide at the non-toxic concentrations in the hypoxia-induced neurotoxicity cells model.

6) To elucidate the molecular mechanisms of neuroprotection of the diterpenoids-enriched fraction of *A. paniculata* and andrographolide in the hypoxia-induced neurotoxicity cells model.

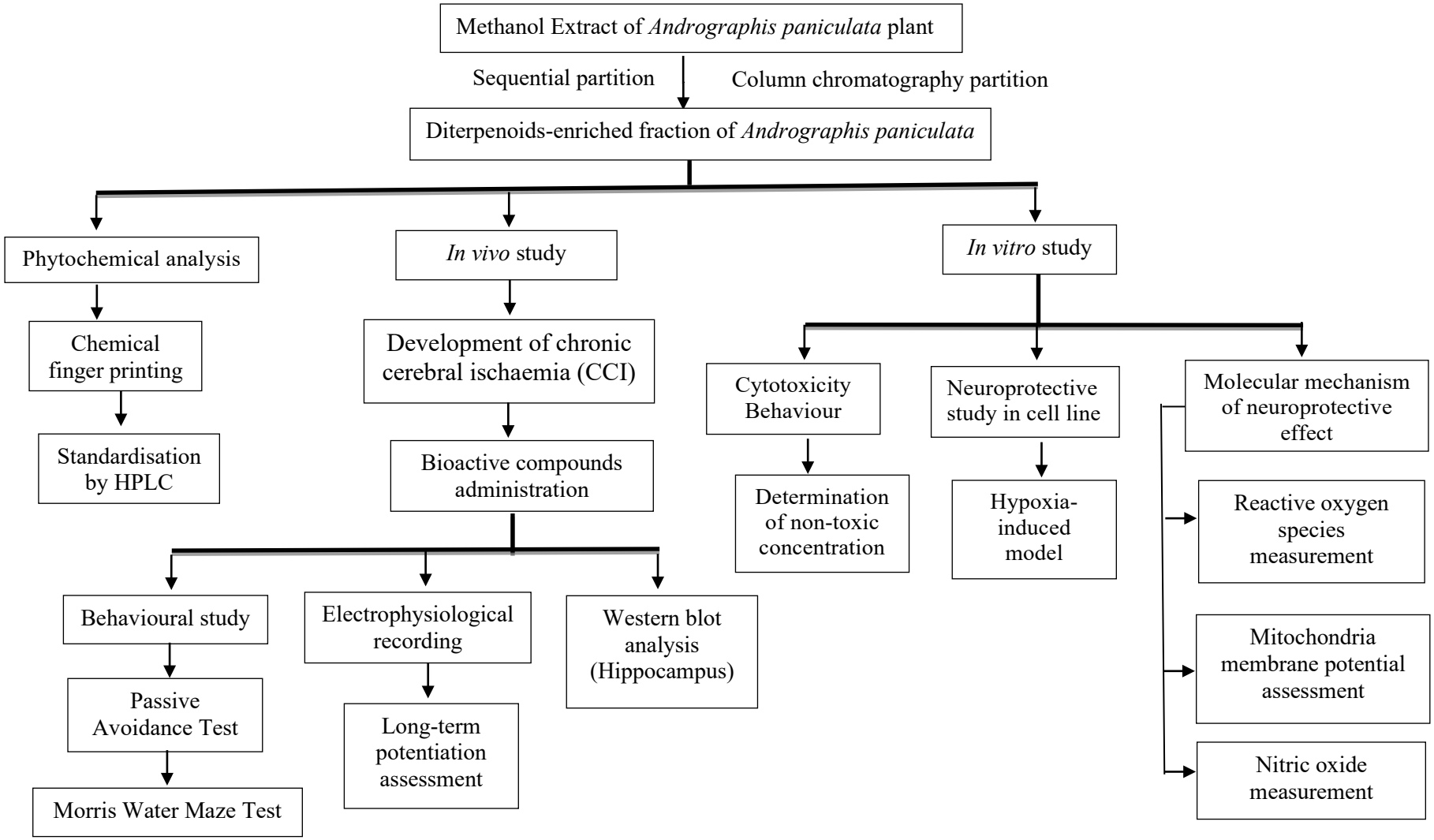

Figure 1.1 Thesis framework

CHAPTER 2

LITERATURE REVIEW

2.1 Dementia

Dementia encompasses a broad spectrum of neurocognitive disorders characterised by a progressive decline in cognitive function (WHO, 2017). This decline primarily affects memory but also involves other cognitive abilities and behavioural regulation. Such impairments significantly hinder an individual's capacity to perform daily living activities, ultimately affecting their overall quality of life (Alhazmi & Albratty, 2022). The risk of developing dementia escalates significantly among elderly folk (Yoon et al., 2022). Epidemiological estimates that approximately 6% of individuals between the ages of 75 and 79 years old are affected, while the prevalence rises to 18.3% among those aged 85 to 89 years old. Notably, the incidence further surges to 41.1% for individuals exceeding 95 years old (Morgan & Mc Auley, 2024).

Alzheimer's disease represents the most prevalent form of dementia, comprising approximately 60 to 70% of reported cases, followed by VD and mixed dementias, which can present concurrently with AD's pathology (Lai et al., 2022). The hypothesis that altered vascular and lifestyle factors were linked to the onset of dementia and predementia symptoms in later life was validated by epidemiological research. According to epidemiological evidence, the complicated relationship in age-related cognitive decline disorder may be intertwined with metabolic syndrome, such as glucose intolerance, obesity, hypertension and hypertriglyceridemia (Frisardi et al., 2010). These metabolic disease connections are essential to promote awareness of cognitive health in ageing populations.

2.1.1 Alzheimer’s disease

Alzheimer's disease (AD) is a complex neurological disorder and is the primary cause of dementia in the elderly. AD is prevalent among individuals aged above 65 years old and is the primary cause of brain dysfunction in people over the age of 85 (Apostolova, 2016). Studies have indicated that chronic cerebral hypoperfusion or ischaemia is one of the most common pathologies that co-occur in VCI and AD (Chen et al., 2022; Ma & Ji, 2022). Patients with AD will lose healthy nerve cell numbers and connections, and the size of the patient’s brain will shrink (Bhushan et al., 2018). When AD progresses, individuals experience a subtle cognitive loss that eventually becomes irreversible (Silva et al., 2014). The primary neuropathological features of AD include the aggregation of amyloid-β plaques, hyperphosphorylation of tau protein, and reduction of cholinergic, which subsequently triggers multiple undesired metabolic reactions in the neuronal brain. The accumulation of plaque first began in the medial temporal lobe, particularly prior to the onset of behavioural symptoms. This phenomenon is succeeded by the deterioration of neuronal and synaptic functions, along with a decline in learning and memory capabilities (Sajad et al., 2022).

There are several phases of Alzheimer's disease: pre-dementia, mild, moderate, and severe. The pre-dementia stage is frequently challenging to differentiate from normal ageing or stress-related issues (Bhushan et al., 2018). At the mild stages of AD, cognitive symptoms such as increased memory loss may be apparent, but the deterioration does not affect their daily living activities. However, about 15–30% of these patients will experience aggravated symptoms over time (Drzezga, 2009). The moderate AD stage occurs when it begins to affect the area of the cerebral cortex. At this point, patients can still handle their routine life, but

assistance is needed for certain tasks like reading and writing. The patient will also experience significant memory loss, such as trouble recognising family and friends and a loss of impulse control (Breijyeh & Karaman, 2020). In cases of late-stage or severe AD, the entire cortical area has deteriorated due to a massive accumulation of neurotic plaques and neurofibrillary tangles, resulting in a major loss of function and cognition. Patients may become dependent, bedridden, and experience many health management issues, including death complications, which may ultimately cause death (Apostolova, 2016; Breijyeh & Karaman, 2020).

2.1.2 Vascular cognitive impairment

Vascular cognitive impairment (VCI) is currently the preferred terminology in the research classification systems. The term "VCI" refers to a range of cognitive severity syndromes, including subclinical vascular brain injury and executive dysfunction, as well as other brain vascular diseases that may cause cognitive symptoms (Gorelick et al., 2009). Classifying cognitive impairment associated with stroke has traditionally involved the use of terminologies like vascular dementia (VD) and multi-infarct dementia (MID) (Gorelick et al., 2009). Moreover, VCI is a recognised disease category that includes a range of cognitive impairments, from mild cognitive impairment to dementia, that are predominantly brought on by cerebrovascular diseases. Cognitive deterioration is a typical complication of stroke and has significant consequences for post-stroke functioning (Bir et al., 2021).

2.1.3 Vascular dementia

The second most prevalent type of dementia after AD is vascular dementia (VD), including dementia brought on by any vascular disease. Memory, cognitive function and learning skills may gradually deteriorate as a result of VD, which can happen when the brain's blood supply is blocked or when the affected blood vessel

system is compromised (Zhang et al., 2019). Numerous terminologies have evolved to acknowledge the various mechanisms of VD, implicating both large and small vessels. The root of VD pathogenesis can be caused by primary central nervous system vascular impairment, such as *in situ* thrombosis related to atherosclerosis, fibrinoid necrosis, or lipohyalinosis. This pathogenesis can coincide with certain mechanisms associated with hypertensive intracerebral haemorrhage (ICH) (Bir et al., 2021). In addition, ischaemic stroke, which has a high correlation with VD, can be caused by atherosclerosis. Arteriosclerosis and atherosclerosis affect various mechanisms that could lead to the development of cortical cerebral microinfarcts (CMIs) (Huang et al., 2024). These CMIs may result from cerebral hypoperfusion, a condition brought about by the mismanagement of blood flow in the brain, itself a consequence of atherosclerosis (Morgan & Mc Auley, 2024).

According to Kang et al. (2023), clinical conditions of inadequate cerebral blood flow are recognised as chronic cerebral hypoperfusion (CCH). This condition is highly prevalent among patients with VD and AD and is frequently observed in old folks. In CCH models, white matter lesions were particularly susceptible to pathological alterations, and hypoperfusion was discovered early in the leukoaraiosis region. Therefore, the injury in the white matter was a critical pathological change in VD, and the degree of leukoaraiosis, which is known as an abnormal change in the appearance of white matter near the brain lateral ventricles, was associated with neurodegenerative development in the elderly (Chen et al., 2022).

There exists a distinct intersection between VD and AD, as well as another neurodegenerative disease that contributes to cognitive decline during ageing. This cognitive impairment may affect the capacity to work and quality of life, including

patients facing problems maintaining routine activities, social relationships and living independently, especially difficulties in learning and storing memory (El Husseini et al., 2023).

2.2 Learning and Memory

Learning is the process of gaining new knowledge, behavioural patterns, or skills demonstrated by changes in behaviour due to training, education, or life experience. Memory is the ability to store and retrieve information in the brain and maintain behavioural patterns over time (Proctor & Niemeyer, 2020). Learning and memory progression are mediated by electrochemical signalling integration within the brain's neuronal networks to function. The essential mechanism of the intelligent network is the electrical-chemical signal transductions at the synaptic junctions of neurons, which construct numerous neural connections and circuits that form multimodal brain processes (Wu et al., 2023). This chemical signalling can also be mediated through peptides, amino acids, biogenic monoamines, acetylcholine and gasotransmitters. Notably, the most extensive and diverse category within these signalling molecules is constituted by neuropeptides, which are organised by more than 20 different gene families (Gøtzsche & Woldbye, 2016).

2.2.1 Types of memory

Memory is defined as the ability to acquire and retain knowledge from new experiences, and consolidation is the process of reinforcing newly acquired information for storage. Memory can also be categorised based on the area of interest, such as temporal (short- or long-term memory), function (working or reference memory), nature (associative or non-associative) and content (explicit or implicit memory) (Gøtzsche & Woldbye, 2016). Short-term memory, or primary

memory, indicates memory systems that hold bits of information for up to 30 seconds of short-term storage (Cascella & Khalili, 2025). Whereas, a long-term memory serves as an extension storage of information and records of past experiences, and it is acknowledged across all theoretical perspectives (Cowan, 2008).

Among the differences between short- and long-term memory stores, two important factors involved are duration and capacity. A difference in duration indicates that items stored in short-term memory deteriorate over time. Meanwhile, a difference in capacity refers to the limitation in the number of items that can be held in short-term memory compared to long-term memory (Cowan, 2008). Meanwhile, working memory is a distinctive and temporary active reservoir and can manipulate information for various complex cognitive activities, such as language processing, reasoning and decision-making (Sridhar et al., 2023).

Explicit memory (or declarative) and implicit memory (non-declarative) are both grouped as long-term potentiation or memory, but are distinct in terms of physiology. Explicit/declarative memory is associated with a conscious condition. The origin of memory can often be accessed alongside details of the memory itself, such as a life event, and it reflects the things that can be explained to others. Implicit/non-declarative memory resulted from direct involvement or experience, and a proactive process to retrieve it may not be necessary. Therefore, it is also recognised as conceptual memory (Ghafarimoghadam et al., 2022).

Another important memory is spatial memory, which refers to the ability to get input of the layout information and recall the orientation of the location. The brain part that is responsible for spatial memory is the entorhinal-perirhinal cortex,

which is located at the hippocampus projection to the neocortex. Experimental assessments such as the Morris water maze (Neumann et al., 2013) and 8-arm radial maze (Stevens & Brown, 2015) are used to measure spatial memory in animal models. Memory impairments can occur due to the neuronal fundamental structure destruction which hinders memory storage and may fail to recall memories. The spectrum of memory issues varies either temporary term or fast progressive impairment (Apostolova, 2016). Other disorders that can affect memory and competency in learning include head trauma and amnesia (Ghafarimoghadam et al., 2022).

2.2.2 Hippocampal long-term potentiation (LTP)

The vital part of the brain that is essential in forming memories is the hippocampus, as it integrates the cognitive experience with different sensory and emotional feelings related to various contexts of memory (Knierim, 2015). It is located at the cornu ammonis (CA) and consists of CA1, CA2, CA3, and dentate gyrus sub-regions (DG) (Figure 2.1). However, experience-driven synaptic changes in the CA1 sub-region output are believed to be crucial for most hippocampal-dependent memories (Wang et al., 2021). Notably, the formation of memories enhances the particular Schaffer collateral-to-dorsal CA1 area (dCA1) synapses and CA3 schematic strengthening. This enhancement is facilitated through synaptic plasticity that relies on N-methyl-D-aspartate receptor (NMDAR), associated with the growth and formation of new dendritic spines, which contain glutamatergic synapses (Clark et al., 2015; Ziółkowska et al., 2023). Accordingly, NMDAR-dependent plasticity of dCA1 synapses is commonly believed to be a primary cellular learning mechanism.

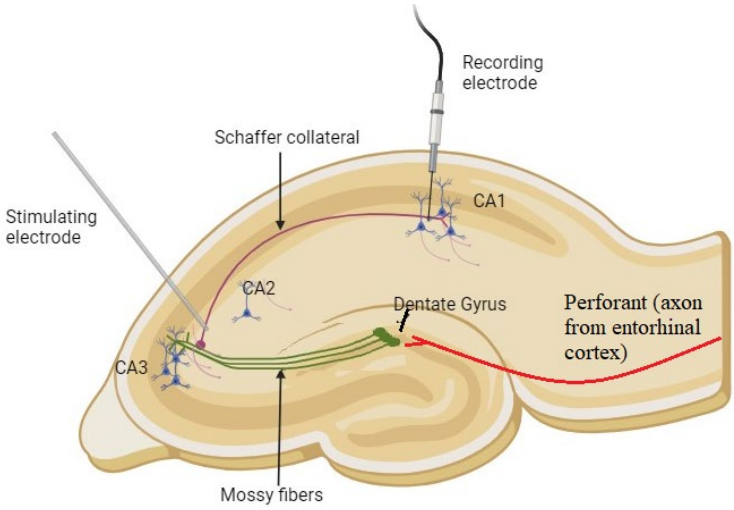

Figure 2.1 The hippocampus structure, which comprises the cornu ammonis 1 (CA1), CA2, CA3, and dentate gyrus sub-regions. This figure was created by using Biorender (www.biorender.com)

The primary gateway cortical input to the hippocampus is via the entorhinal cortex, and its projection is primarily through the perforant track to the dentate gyrus (DG) region or Synapse 1. Next in the synapse 2, the mossy fibre provides a projection pathway from the DG to the CA3. Then, in the event of synapse 3, CA3 projects stimulation to CA1 by passing the Schaffer Collateral pathway. Ultimately, CA1 returns the projections to the entorhinal cortex to close the synaptic circuit. In the classic tri-synaptic circuitry mechanism, it is determined that, besides CA3 projection to CA1, it also delivers collaterals to produce synapses onto other CA3 neurons. The persistence of the collateral pathway led to the development of several important theories about CA3 as an auto-associative memory system, exhibiting essential attractor dynamics for facilitating memory dissemination (Knierim, 2015).

In the neurology study of the ischaemic effect, the hippocampus is the most interesting region to focus on CA1 neuron reactions that are generally based on the ischaemic level or phases. In the early phase, the onset of oxygen/glucose deprivation (OGD) is associated with minor neuronal depolarisation and increased excitability. The prolonging of this phase can spread to the surrounding neurons and

then stimulate a massive glutamate release (Neumann et al., 2013). This excessive release of glutamate can also induce ischaemic injury and lead to neuronal cell death, thus reducing the density of CA1 hippocampal neurons and synapses (Neumann et al., 2013). In another study, the CA1 region was observed to have less neuronal population compared to the CA2 and CA3 regions of the hippocampus among AD patients (Padurariu et al., 2012).

2.2.3 Long-term potentiation (LTP) induction protocol

The long-term potentiation mechanism is one of the reliable assessments that underlie learning and memory studies. The advancement of the LTP phenomenon has been explored up to animal models, human tissues and extensive investigation at the molecular level (Rygvold et al., 2022). LTP represent an essential mechanism underlying the rapid establishment of new memory formation. LTP mechanism involves Schaffer's collaterals and mossy fibres of the hippocampus. This process enhances synaptic efficiency in response to the elevated frequency of activity at the presynaptic terminal. This effect can last for numerous days, resulting in increased activity of the post-synaptic neurons. The increased rate of activity contributes to the buildup of calcium ions within these post-synaptic neurons, which initiates the LTP. Despite the cessation of the initial external stimulus, the signals continue to be transmitted consistently from the synapses in the hippocampus during the process of LTP formation (Chauhan et al., 2021).

An established technique for inducing LTP involves employing theta-burst stimulation (TBS) protocols. These protocols have been shown to effectively facilitate synaptic strengthening, which is critical for understanding the underlying mechanism of learning and memory (Rivera et al., 2016; Damodaran et al., 2018; Goussakov et al., 2021). Based on Figure 2.2, TBS is categorised into three

terminologies: short-TBS (S-TBS), long-TBS (L-TBS), and high-intensity long-TBS (HL-TBS).

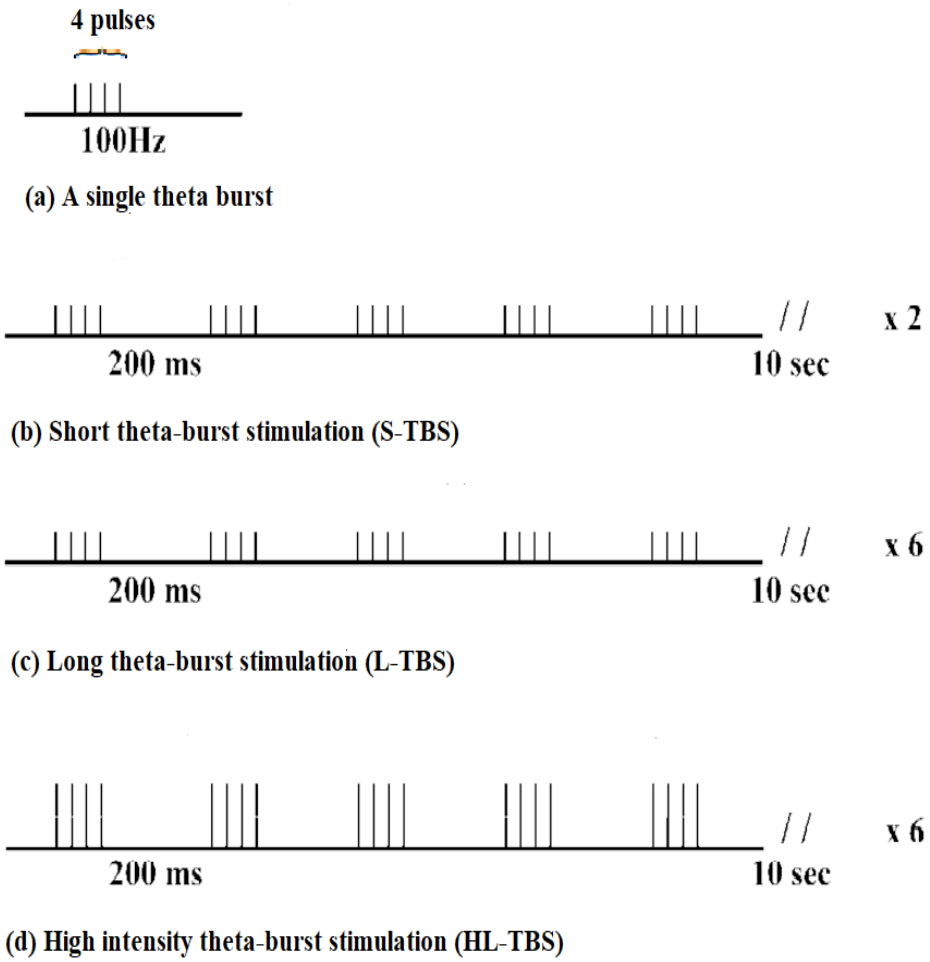

Figure 2.2 Various theta-burst patterns of schematic illustration. (a) Each of the tetanus patterns is composed of single theta bursts consisting of 4 pulses at 100 Hz. (b) S-TBS stimulation consisted of 5 bursts separated by 200 ms repeated 2 times with 10 s between the 2 trains. (c) L-TBS is the same as S-TBS but repeated 6 times with an interval of 10 s between each train. (d) High-intensity long–TBS (HL-TBS) is the same as L-TBS, but the intensity of the stimulation was increased. This figure is reused from Morgan & Teyler (2001)

TBS was performed following a baseline of at least 30 minutes. S-TBS was composed of five trains of four pulses delivered at a frequency of 100 Hz with 200 ms gaps and repeated two times at 10-second inter-burst intervals (IBI). L-TBS utilises the same stimulation approaches as S-TBS, but it is administered six times

with 10 second of IBI interval between each stimulation. The S-TBS and the L-TBS trains had enough amplitude to cause a 0.75 to 1 mV population spike in the layer of cell bodies. Similar to L-TBS, the stimulus intensity in HL-TBS was heightened to produce a population spike of 2.5–3 mV as recorded in the cell body layer (Morgan & Teyler, 2001). An established technique for inducing LTP involves employing theta-burst stimulation (TBS) protocols. These protocols have been shown to effectively facilitate synaptic strengthening, which is critical for understanding the underlying mechanism of learning and memory (Rivera et al., 2016; Damodaran et al., 2018; Goussakov et al., 2021).

2.2.4 Mechanisms underlying LTP

LTP is conceptualised as a fundamental part of information storage in the brain. It considers the synaptic plasticity capacity and strength to facilitate cognitive processes such as learning and memory processes (Clark et al., 2015). LTP induction and expression are mediated by the transient elevations in intracellular calcium concentration and followed by the subsequent activation of a calcium-dependent mechanism. This calcium signalling is related to learning and memory and is linked to the cellular processes that contribute to LTP of synaptic strength (Clark et al., 2015). Meanwhile, at hippocampal CA3-CA1 synapses, there are at least two distinct mechanisms of LTP identified to coexist: N-methyl-D-aspartate receptor-dependent LTP (NMDAR LTP) (Bliss & Collingridge, 1993) and a non-NMDA receptor-dependent LTP (non-NMDAR LTP), which is facilitated through the activation of L-type voltage-dependent calcium channels (VDCC) (Clark et al., 2015). Furthermore, pharmacological evidence also supports that both forms of LTP participate in the generation of spatial memory (Clark et al., 2015).

2.2.5 Phases of LTP

LTP can be categorised into three phases: short-term potentiation, early LTP (E-LTP), and late LTP (L-LTP). Short-term and E-LTP function without depending on gene transcription and *de novo* protein synthesis. Conversely, L-LTP requires gene transcription and *de novo* protein synthesis for their induction and maintenance (Balua & Coyle, 2011). E-LTP begin right after the stimulus that induces LTP, lasts for several hours, and relies mostly on the activity of short-term kinases. A single train of high-frequency stimuli is sufficient to induce E-LTP. In contrast, L-LTP commences a few hours after the initial stimulus, persists for a minimum of eight hours, and relies on the activation of gene transcription. To recruit L-LTP, stimulation intensity must be higher than in E-LTP protocols, requiring three to four-spaced delivery trains of stimuli (Huang, 1998).

E-LTP is recognised as a protein synthesis-independent, while L-LTP is protein-dependent and/or transcription-dependent (Abbas et al., 2015). E-LTP functions as the activation of CaMKII to initiate metabolic reactions and protein trafficking, both of which eventually increase synaptic transmission. On the other hand, L-LTP depends on the gene expression and local protein synthesis controlled by TrkB receptor and functional prions CPEB2-3-mediated translation. Furthermore, structural changes in the gene expression, along with the synthesis of de *novo* protein and mRNA become apparent, and new synapses are formed in young animals during L-LTP (Baltaci et al., 2019). BDNF-TrkB signal also plays an important role in regulating adult hippocampal in both E-LTP and L-LTP phases (Balua & Coyle, 2011).

2.2.6 Synaptic plasticity

Synaptic plasticity is the ability of neurons to regulate the structure and strength of excitatory synapses, and it is crucial for the development of adult learning and memory as well as the refinement of neural circuits in response to sensory input (Wang et al., 2021). Excitatory synaptic transmission occurs when the receptors on the postsynaptic neuron are activated by glutamate, the excitatory neurotransmitter. In the postsynaptic compartment, the pattern of calcium influx through activated glutamate receptors can affect the removal of receptors and shrinkage, or new receptors and synapse enlargement. The removal of certain receptors and the enlargement of the synapse resulted in long-term depression and long-term potentiation, respectively. Small GTPases and enzymes regulated by calcium/calmodulin work together to control this highly tuned mechanism (Kennedy, 2016).

The most common method for studying synaptic plasticity is to record electrical responses from the Schaffer-collateral pathway synapses (Figure 2.1), which link two distinct groups of hippocampal excitatory neurons. When this pathway and its spine synapses are repeatedly stimulated at a frequency ranging from 10 to 100 Hz for a few seconds, a phenomenon known as long-term potentiation (LTP) is typically triggered. During this process, the activated synapses enlarge and more successfully depolarise the postsynaptic membrane (Kennedy, 2016). In contrast, activation of these several minutes typically commences a process known as long-term synapses at a lower frequency, between 1 and 5 Hz, for long-term depression (LTD), in which the activated synapses reduce in size, and the depolarisation at the postsynaptic membrane becomes less effective. The change in synaptic strength can endure for hours or even for a lifetime, depending on the

stimulation's repetition frequency (Kennedy, 2016). LDP can also be NMDA receptor-dependent and independent (Balua & Coyle, 2011).

2.2.7 Synaptic plasticity protein expression

Synapses consist of proteins, some of which are directly involved in the transmission of signals, while others either regulate synaptic function or act as structural scaffolds. Since proteins, including those at the synapse, have a limited lifespan, they must be continuously replaced with newly synthesised proteins. Additionally, alterations in the function of the specific synapses necessitate changes in their protein contents regarding quantity, distribution, and post-translational modifications (Rosenberg et al., 2014). Instances of synaptic plasticity that are closely associated with memory formation encompass critical factors such as brain-derived neurotrophic factor (BDNF) (Abdelhamid et al., 2024), calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) (Lu et al., 2024), post-synaptic density-95 (PSD-95) (Levy et al., 2022), synaptophysin (Kokotos et al., 2019), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) (Dore et al., 2021), and N-methyl-D-aspartate receptors (NMDARs) (Goussakov et al., 2021).

Brain-derived neurotrophic factor (BDNF) is a small protein composed of two identical subunits that bind with high affinity to tropomyosin-related kinase B (TrkB) receptors. The ligand BDNF activates the receptor tyrosine kinase TrkB, which is a strong modulator of many kinds of LTP (Pre et al., 2022). BDNF influences the growth and survival of neurons, and it is associated with the formation of memories, LTP and LDP (Ye et al., 2015). Endogenous BDNF pro-peptide is released from neuronal cells and is thought to multiply the post-translational process, hence amplifying the biological effect of BDNF (Joshi et al., 2019). BDNF plays a crucial role in various neurological conditions. Individuals suffering from psychiatric

and neurodegenerative disorders have shown lower levels of BDNF in their blood and brain (Giacobbo et al., 2019). Specifically, BDNF is associated with the decline of neurogenesis linked to AD, and levels of nerve growth factor also decrease as the disease progresses (Ye et al., 2015). Researchers have additionally validated the reduced expression of BDNF mRNA in the hippocampus and temporal cortex of individuals with AD, indicating that the lack of BDNF may contribute to the continuous deterioration of neurons in the context of the disease (Wang et al., 2024).

Calcium/Calmodulin-Dependent Protein Kinase II (CaMKII) is a complex serine/threonine holoenzyme that has intriguing implications for the dynamics of calcium/calmodulin activation. CaMKII interacts with various proteins found in the post-synaptic density (PSD), such as NMDA receptor, synapsin 1, F-actin, and calcium channels (Zalcman et al., 2018). CaMKII is crucial for specific types of synaptic plasticity and the consolidation of memory, achieved through the binding to substrates and the phosphorylation within the molecules of the holoenzyme's subunits (Liu & Murray, 2012). CaMKII is mostly found in the cerebrum, making up 0.7% of striatal protein, 1.3% of cortical protein, and roughly 2% of total hippocampus protein. It is less abundant in the lower brain areas, with a range of 0.1% in the medulla to roughly 0.3% in the hypothalamus (Erondu & Kennedy, 1985). The activity of CaMKII is crucial for the initiation of LTP in the CA1 region of the hippocampus. LTP reflects the enhancements in synaptic strength that may occur with high-frequency or paired stimulation and is claimed to be the cellular basis of memory. The kinase in mammals is produced by four genes, α, β, γ, and δ, (Griffith, 2004). CaMKII γ and CaMKII δ have been identified within multiple tissues beyond the central nervous system. Meanwhile, CaMKIIα and CaMKIIβ are commonly in the brain (Liu & Murray, 2012). The α isoform calcium/calmodulin-

dependent kinase II or CaMKIIα is well recognised for its dual role in regulating synaptic plasticity (Ren et al., 2016).

Impairments in spatial learning are frequently associated with LTP deficiency, and animals lacking the CaMKIIα isozyme do not normally learn in a task (Griffith, 2004). CaMKIIα is one of the primary proteins in the postsynaptic density and localises in presynapses and dendrites in neurons. Its placement inside postsynaptic density (PSD) is calcium-dependent and regulated by CaMKIIβ F-actin binding. It has been demonstrated that N-methyl-D-aspartic acid receptors (NMDAR) stimulation induces this process, whereas α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPAR) or metabotropic glutamatergic receptor stimulation does not. Moreover, the structure is almost entirely composed of synapses that receive inputs from glutamatergic terminals (Zalcman et al., 2018).

Postsynaptic density protein-95 (PSD-95) is categorised as a part of the membrane-associated guanylate kinase family (MAGUK) and serves as a key scaffolding protein found within the postsynaptic density of excitatory synapses. PSD-95 is vital for the development of excitatory synapses as it interacts with and transports NMDAR and AMPAR to the postsynaptic membrane (Coley & Gao, 2019). PSD-95 interaction with NMDAR and AMPAR is essential in the synaptic plasticity of glutamatergic synapses due to its important functional roles (Béïque et al., 2006). PSD-95 stands as a highly stable protein found in PSDs at excitatory synapses. Additionally, PSD-95 effectively regulates synaptic strength by primarily controlling the quantity of AMPA receptors at synapses (Chen et al., 2011).

In the hippocampal region, AMPAR-mediated current is significantly reduced in the presence of PSD-95 loss, indicating the establishment of silent synapses