EFFECTS OF *SYZYGIUM POLYANTHUM* (WIGHT) WALP. LEAF EXTRACT WITH POTENTIAL CHOLINERGIC ACTIVITY ON COGNITIVE FUNCTION IN CHRONIC CEREBRAL HYPOPERFUSION RAT MODEL

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
&	And
<	Lesser than
=	Equals to
±	Plus or minus
×g	Gravitational force
°C	Degree celsius
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
2-VO	2-Vessel occlusion
4-VO	4-Vessel occlusion
Å	Angstrom
a.m.	Ante meridiem
a.m.u.	Atomic mass unit
Ca^{2+}	Calcium ion
cm	Centimeter
e.g.	For example
et al.	And others
g	Gram
g/kg	Gram per kilogram
h	Hour (s)
Hz	Hertz
K^{+}	Potassium ion
kDA	Kilodalton

kHz	Kilohertz
L	Liter
m/z	Mass to charge ratio
mA	Miliampere
mg/kg	Milligram per kilogram
mg/mL	Milligram per milliliter
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
min	Minute (s)
mL	Milliliter
mL/ min	Milliliter per minute
mL/kg	Milliliter per kilogram
mm	Millimeter
mM	Millimolar
ms	Milliseconds
n	Number of animals
Na^+	Sodium ion
Na_3OV_4	Sodium orthovanadate
nm	Nanometer
nmol	Nanomoles
NO	Nitric oxide
No.	Number
p	Probability
p.m.	Post meridiem
p.o.	Per os
pg/mL	Picograms per milliliter
psig	Pounds per square inch gauge

R^2	Correlation coefficient
s	Second (s)
U/g	Units per gram
v/v	Volume per volume
w/v	Weight per volume
x	Multiplication
α	Alpha
β	Beta
γ	Gamma
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	Analysis of variance
AOFT	Automated open field test
AP	Anteroposterior
ARASC	Animal Research and Service Centre
Arc	Activity-regulated cytoskeleton-associated
ATCI	Acethylthiocholine iodide
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCAS	Bilateral common carotid artery stenosis
BCTI	Butyrylthiocholine iodide
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
BuChE	Butrylcholinesterase
CA1	Cornu ammonis 1

CA3	Cornu ammonis 3
CADD	Computer aided drug designing
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	3′,5′-Cyclic adenosine monophosphate
CAS	Catalytic anionic site
CBF	Cerebral blood flow
CCH	Chronic cerebral hypoperfusion
CDR	Centre for Drug Research
ChAT	Choline acetyltransferase
ChEIs	Cholinesterase inhibitors
CHT1	Na^+-dependent choline transporter
CoA	Coenzyme A
CRE	cAMP-response element
CREB	cAMP-response element-binding protein
CSF	Cerebrospinal fluid
CT	Computerized tomography
CβG	Cystollic β-glucosidase
DB	Diagonal band of Broca
ddH_2O	Double distilled water
DG	Dentate gyrus
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dpf	Docking parameter file
DSA	Digital subtraction angiography
DTDP	4, 4-Dithiodipyridine
DTNB	5,5’-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol

EC	Entorhinal cortex
E-LTP	Early- long-term potentiation
ERK	Extracellular regulated kinase
FDA	Food and Drug Administration
fEPSP	Field excitatory postsynaptic potential
GA	Genetic algorithm
GC/MS	Gas chromatography–mass spectrometry
GluR1	Glutamate receptor 1
GLUT2	Glucose transporter 2
gpf	Grid parameter file
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HFS	High-frequency stimulation
HPTLC	High-performance thin-layer chromatography
HRP	Horseradish peroxidase
HTS	High throughput screening
I/O	Input/output
IL-18	Interleukin 18
IL-1ß	Interleukin 1 beta
IL-6	Interleukin 6
IR	Infrared spectroscopy
Iso-OMPA	Tetraisopropyl pyrophosphoramide
Ki	Constant of inhibition
LCMS-MS	Liquid Chromatography - Tandem Mass Spectrometry
LC-QTOF-MS	Liquid chromatography quadrupole time of flight mass spectrometry
LDT	Laterodorsal tegmental nucleus

L-LTP	Late-long-term potentiation
LPH	Lactase-phloridzin hydrolase
LTM	Long-term memory
LTP	Long-term potentiation
mAChRs	Muscarinic acetylcholine receptors
MAPK	Mitogen-activated protein kinases
ML	Mediolateral
MMSE	Mini-Mental State Examination
MoCA	Montreal Cognitive Assessment
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
MS	Medial septal nucleus
MTL	Medial temporal lobe
MWM	Morris water maze
nAChRs	Nicotinic acetylcholine receptors
NBM	Nucleus basalis meynert
NCBI	National Center for Biotechnology Information
NMDAR	N-methyl-D-aspartate receptor
NMR	Nuclear magnetic resonance
NOR	Novel object recognition
NR2B	N-Methyl D-Aspartate receptor subtype 2B
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PAS	Peripheral anionic subsite
PBS	Phosphate-buffered saline
PDB	Protein Data Bank
pH	Potential of hydrogen

PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phosphoinositide phospholipase C gamma
POBCCA	Permanent occlusion of bilateral common carotid arteries
PPT	Pedunculopontine tegmental nucleus
PSD-95	Postsynaptic density protein 95
RCSB	Research Collaboratory for Structural Bioinformatics
REM	Rapid eye movement
RI	Recognition Index
ROS	Reactive oxygen species
SD	Sprague-Dawley
SEM	Standard error mean
SFK	Src-family tyrosine kinase
SGLT1	Sodium-glucose-linked cotransporter 1
SK channels	Small conductance Ca^{2+}-activated K^{+} channels
SM	Sensory memory
STM	Short-term memory
TBS	Theta burst stimulation
TMB	3,3',5,5'-Tetramethylbenzidine
TNFα	Tumor necrosis factor α
TrkB	Tyrosine kinase B
UCCAO	Unilateral common carotid artery occlusion
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
VAChT	Vesicular acetylcholine transporter

VaD	Vascular dementia
WML	White matter lesion

LIST OF APPENDICES

KESAN EKSTRAK DAUN *SYZYGIUM POLYANTHUM* (WIGHT) WALP. DENGAN POTENSI AKTIVITI KOLINERGIK TERHADAP FUNGSI KOGNITIF DALAM MODEL TIKUS HIPOPERFUSI SEREBRUM KRONIK

ABSTRAK

Demensia vaskular (VaD) merupakan sejenis demensia yang paling biasa dalam populasi warga tua yang berpunca daripada hipoperfusi serebrum kronik (CCH). Disfungsi sistem kolinergik di sistem saraf pusat (CNS) telah dikenalpasti sebagai salah satu punca utama gangguan pembelajaran dan memori dalam pesakit VaD. Oleh itu, ubat-ubatan yang memulihkan tahap neurotransmiter asetilkolina (ACh) dengan merencat aktiviti kolinesterase telah dicadangkan sebagai calon yang berpotensi untuk mengubati pesakit VaD. Kajian ini bertujuan untuk menilai kesan empat ekstrak metanol tumbuhan dengan aktiviti anti-kolinesterase dalam tikus POBCCA. Ekstrak tumbuhan yang dipilih ialah *Coccoloba uvifera* (batang), *Mimusops elengi* (daun), *Syzygium aqueum* (daun) dan *Syzygium polyanthum* (daun). Kesan ekstrak tumbuhan terpilih (100 dan 200 mg/kg) terhadap pembelajaran dan memori telah dinilai menggunakan ujian pengenalan objek baharu (NOR) dan ujian berselirat air morris (MWM). Hasil penelitian daripada ujian tingkah laku menunjukkan ekstrak *S. polyanthum* (100 dan 200 mg/kg) dan *S. aqueum* (200 mg/kg) memperbaiki memori pengecaman jangka pendek ($p < 0.05$) dan panjang ($p < 0.05$) dalam ujian NOR. Selain itu, ekstrak *M. elengi* dan *S. aqueum* (200 mg/kg) serta *S. polyanthum* (100 dan 200 mg/kg) memperbaiki pembelajaran spatial (hari 4: $p < 0.05$; hari 5: $p < 0.05$) dan ekstrak *S. polyanthum* (100 dan 200 mg/kg) memperbaiki memori rujukan ($p < 0.05$) dalam ujian MWM. Ujian lapangan terbuka automatik

menunjukkan semua ekstrak tumbuhan tidak mengganggu fungsi motor dan eksplorasi tikus POBCCA. Ekstrak *S. Polyanthum* telah dipilih untuk mengkaji kesan bersandarkan dos (100, 200 and 300 mg/kg) terhadap tingkah laku (pembelajaran dan memori), potensiasi jangka panjang (LTP) dan sistem kolinergik tikus POBCCA. Ekstrak *S. polyanthum* memperbaiki memori bukan spasial (jangka pendek: $p < 0.0005$; jangka panjang: $p < 0.05$) dan spasial (latihan: hari 4, $p < 0.05$; hari 5, $p < 0.05$; percubaan probe: $p < 0.05$) dalam ujian NOR dan MWM, masing-masing. Ia juga mempromosikan peningkatan LTP berpanjangan pada sinaps kolateral Schaffer CA3-CA1 dalam hipokampus ($p < 0.05$) tikus POBCCA dan meningkatkan paras ACh, aktiviti kolina asetiltransferase (ChAT) dan paras faktor neurotropik yang berasal dari otak (BDNF) dalam korteks frontal ($p < 0.05$), hipokampus ($p < 0.05$) dan korteks serebrum ($p < 0.05$) dalam tisu otak tikus POBCCA. Menariknya, peningkatan aktiviti asetilkolinesterase (AChE) dalam hipokampus direncatkan secara signifikan ($p < 0.05$) selepas rawatan ekstrak ini. Tujuh sebatian terutamanya glikosida flavonoid dan asid fenolik dikenalpasti dalam ekstrak *S. polyanthum* daripada analisis LCMS-MS. Kajian pendokkan molekular *in siliko* dengan sebatian tersebut dan aglikon untuk glikosida flavonoid masing-masing terhadap struktur kristal enzim AChE dan butrilkolinesterase (BuChE) menunjukkan semua sebatian kecuali asid galik telah menunjukkan pengikatan yang sangat baik setanding dengan kawalan positif. Kesimpulannya, ekstrak daun *S. polyanthum* memperbaiki tahap pembelajaran dan memori serta keplastikan sinaptik *in vivo* melalui pemulihan sistem kolinergik dalam model tikus POBCCA. Hasil kajian ini menyokong potensi terapi ekstrak daun *S. Polyanthum* dalam rawatan VaD.

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ABSTRACT

Vascular dementia (VaD), is the most common type of dementia in the ageing population, initiated by chronic cerebral hypoperfusion (CCH). Cholinergic system dysfunction in the central nervous system (CNS) has been recognized as one of the main reasons for learning and memory impairment in VaD patients. Therefore, medications that restore the level of acetylcholine (ACh) neurotransmitter by inhibiting cholinesterase activity have been proposed as a potential candidate to treat VaD patients. Present study was conducted to evaluate the effect of four methanol plant extracts with anti-cholinesterase activity in POBCCA rats. The selected plant extracts were *Coccoloba uvifera* (stems), *Mimusops elengi* (leaves), *Syzygium aqueum* (leaves) and *Syzygium polyanthum* (leaves). The effects of these plants (100 and 200 mg/kg) on learning and memory were evaluated using novel object recognition (NOR) and morris water maze (MWM) tests. The behavioral tasks results revealed *S. polyanthum* (100 and 200 mg/kg) and *S. aqueum* (200 mg/kg) extracts improved short- ($p < 0.05$) and long-term ($p < 0.05$) recognition memories during NOR. Besides that, *M. elengi* and *S. aqueum* (200 mg/kg) along with *S. polyanthum* (100 and 200 mg/kg) extracts improved spatial learning (day 4: $p < 0.05$; day 5: $p < 0.05$) and *S. polyanthum* exract (100 and 200 mg/kg) improved reference memories ($p < 0.05$) in MWM test. Automated open field task showed all plant extracts did not impair motor and exploratory function in POBCCA rats. *S. polyanthum* extract was selected to further

study its dose dependent effect on behavioral (learning and memory), long-term potentiation (LTP) and cholinergic system of POBCCA rats. *S. polyanthum* extract (100, 200 and 300 mg/kg) improved both non-spatial (short: $p < 0.0005$; long: $p < 0.05$) and spatial memories (day 4, $p < 0.05$; day 5, $p < 0.05$; probe trial: $p < 0.05$) in NOR and MWM tests, respectively. It also promotes long-lasting LTP enhancement in Schaffer collateral CA3-CA1 synapses in the hippocampus ($p < 0.05$). It increases the ACh level, choline acetyltransferase (ChAT) activity and brain-derived neurotrophic factor (BDNF) level in frontal cortex ($p < 0.05$), hippocampus ($p < 0.05$) and cerebral cortex ($p < 0.05$) of POBCCA rat's brain tissue. Interestingly, the elevated acetylcholinesterase (AChE) activity in hippocampus was significantly inhibited ($p < 0.05$) after treatment with this extract. Seven compounds predominantly flavonoid glycosides and phenolic acids were identified in *S. polyanthum* extract from LCMS-MS analysis. *In silico* molecular docking of the identified compounds and their respective aglycones of flavonoid glycosides against AChE and BuChE enzyme crystal structures revealed all compounds except for gallic acid have shown excellent binding affinities (comparable to positive control). In conclusion, *S. polyanthum* leaves extract executes its learning and memory function and ameliorates *in vivo* synaptic plasticity via restoration of cholinergic system in POBCCA rat model. The findings of this study supports the therapeutic potential of *S. polyanthum* leaves extracts in the treatment of VaD.

CHAPTER 1

INTRODUCTION

Dementia is a neurodegenerative disorder characterized by progressive deterioration of cognitive functions which results in patients inability to carry out daily activities and live independently. The dramatic increase in the elderly population led to higher incidence of dementia worldwide. The number of dementia patient is estimated to reach 75.63 million in 2030 and approximately increase twofold to 135.46 million by 2050 (Ferretti *et al.*, 2018). In Malaysia, the incident of dementia was estimated to increase from 0.12 million in 2015 to 0.59 million by 2050 (Alzheimer's Disease International, 2014). The drastic increase of dementia cases is because most family members regard dementia symptoms as normal aging and hence, do not pursue for any medical treatments. Different forms of dementia exists such as Alzheimer's disease (AD), vascular dementia (VaD), dementia with Lewy bodies, fronto-temporal dementia, Parkinson's disease and Huntington's disease. Dementia remains as one of the greatest global public health challenges facing by society in terms of economic costs and social burdens, since most patients need long-term treatment at their home or nursing home. (Prince *et al.*, 2013).

VaD is the second most common type of dementia after AD (Lobo *et al.*, 2000). The incidence rate of VaD was 6 to 12 cases in every 1000 people over 70 years old annually (Van Der Flier & Scheltens, 2005). VaD is a progressive disease caused by reduced blood flow to the brain or also known as chronic cerebral hypoperfusion (CCH). Continuous decrease of cerebral blood flow (CBF) disrupts neural structures controlling memory and cognitive processes which ultimately contributes to the progressive decline of

memory and cognitive function (Snyder *et al.*, 2015). To date, there is no medication approved for VaD treatment. However, all approved drugs for AD such as cholinesterase inhibitors (ChEIs) and N-methyl-D-aspartate receptor (NMDAR) antagonist have been investigated in VaD patients in clinical trials (Wilcock *et al*., 2002; Black *et al*., 2003; Erkinjuntti, Roman & Gauthier., 2004; Nazish, Soomro & Alvi, 2015; Chen *et al*., 2016). There is growing evidence for cholinergic dysfunction in VaD and treatment with ChEIs have been reported to be effective and beneficial for VaD patients (Mcveigh & Passmore, 2006; Kavirajan & Schneider, 2007). ChEI are usually administrated empirically and continued when symptomatic improvements are noticed in VaD patients (Korczyn, Vakhapova & Grinberg, 2012).

Cholinergic signaling in the central nervous system (CNS) plays important role in cognitive processing such as attention, memory and motivation (Ballinger *et al.*, 2016). The cholinergic hypothesis of cognitive dysfunction was proposed by Bartus *et al.* (1982). Bartus and colleagues proposed that functional disruptions of cholinergic activity in the brain of healthy older adult leads to memory loss and cognitive problems. Meanwhile, restoring cholinergic function may decrease the severity of cognitive impairment. This hypothesis was supported by improvement of cognitive function in AD patients by the administration of ChEIs (Hansen *et al.* 2008). ChEIs act by inhibiting the cholinesterase enzyme activity and reducing the breakdown of acetylcholine to restore cholinergic function in the brain (Di Santo *et al.*, 2013; Anand & Singh, 2013; Andrieu *et al.*, 2015). Since the initial proposal, much clinical development research on ChEI agents has taken place, and although the overall clinical effects are limited, drugs that modulate

cholinergic function remain the most widely used medications to treat dementia (Dumas & Newhouse, 2011).

Permanent occlusion of bilateral common carotid arteries (POBCCA) in rats is a widely utilized CCH model that closely resemble human VaD conditions. POBCCA reduces the CBF and causes progressive neuronal degeneration, cholinergic dysfunction, learning and memory impairment or cognition deficits in rat (Kitamura *et al.*, 2012; Amenta, Di Tullio & Tomassoni, 2002). This model is useful to investigate the pathophysiology of CCH and discover drugs with potential therapeutic values for VaD (Institoris *et al.*, 2007).

1.1 Problem statement

Commercially available ChEIs such as donepezil, rivastigmine and galantamine have short half-life and often cause side effects such as nausea, vomiting, diarrhea, weight loss and muscle cramps (Sharma, 2019). Medicinal plants have attracted much attention in recent years as potential sources of new therapeutic agents for neurological disorders. Medicinal plants are rich in secondary metabolites and oils with therapeutic values. Hence, exploring this abundantly available natural resources may lead to discovery of potential agents with anti-cholinesterase activity which can efficiently improve congnitive function in dementia patients with less side effects. This study was conducted to determine the effect of 4 different plant extracts of *Coccoloba uvifera* (stem), *Mimusops elengi* (leaves), *Syzygium aqueum* (leaves) and *Syzygium polyanthum* (leaves) on cognitive function in POBCCA rats. The plants were selected based on previous *in vitro* study on screening of 177 plant extracts (Amir Rawa *et al.*, 2019) in which the selected 4 plant extracts possess

high anti-cholinesterase activity. Following *in vivo* behavioral studies, the most active plant extract was selected based on behavioral test performance to study its effect on cholinergic system and synaptic plasticity. Finally *in silico* study was performed to identify bio-compounds with potential anti-cholinesterase activity in the selected most active plant extract.

1.2 Objectives

1. To evaluate the effect of four plant extracts on motor, learning and memory function in POBCCA rats.
2. To determine the dose-dependent effect of the most active plant extract on motor, learning and memory function in POBCCA rats.
3. To study the effect of the most active plant extract on hippocampal long-term potentiation (LTP) in POBCCA rats using electrophysiology.
4. To determine the effect of the most active plant extract on cholinergic system in the brain of POBCCA rats (ex vivo).
5. To identify compounds present in selected most active plant extract using LCMS-MS and study the anti-cholinesterase activity of these compounds using *in silico* method.

CHAPTER 2

LITERATRE REVIEW

2.1 Vascular dementia (VaD)

VaD is associated with the loss of cognitive function to a degree that affects daily living activities due to reduced blood supply to the brain tissues (Roman, 2002). VaD patients display impairment in memories (short-term, long-term and working) and difficulty in making judgement, thinking, reasoning, planning and completing tasks (Venkat, Chopp & Chen, 2015). In addition, VaD patients often experiences confusion and mood swing such as depression and anxiety. Cognitive decline in VaD is generally associated with the widespread of vascular lesions involving subcortical brain areas such as basal ganglia and periventricular white matter. These lesions interrupt neuronal networks such as thalamo-cortical, striato-subfrontal, cortico-subcortical and limbic systems which involved in cognition, memory and behavior (Jellinger, 2013). The lifespan of VaD patients is shorter compared to AD patients with mean survival of 3 to 5 years after diagnosis due to the coexistence of other vascular diseases (Kua *et al.*, 2014).

Neurochemical studies have indicated abnormalities in key neurotransmitter systems, particularly in the central cholinergic system in VaD patients without any concomitant of AD pathology (Erkinjuntti, Román & Gauthier, 2004; Di Lazzaro *et al.*, 2008). Cholinergic dysfunction such as (i) decline of cholinergic neurons in the nucleus basalis of Meynert, (ii) lower concentrations of ACh in the cerebrospinal fluid and (iii) decrease of ChAT activity in the hippocampus and temporal cortex were found in VaD patients (Kalaria., 2002; Wang, Zhang & Tang, 2009). Based on these evidences,

cholinergic dysfunction plays a crucial role in deterioration of learning and memory in VaD patients.

2.1.1 Risk factors of VaD

The most common risk factor of VaD is ageing in which the occurrence of VaD exponentially increases with age above 65 years, as older people are more susceptible to cerebrovascular and cardiovascular diseases. VaD are more common in men as compared to women below 58 years old but there is no difference between the genders in age group above 85 years (Dong *et al.*, 2007). The reason behind men is more prone to VaD earlier than women may be associated with lifestyle indicators (e.g. smoking habits and alcohol intake). While ageing is a natural process, there are risk factors which can be controlled in order to prevent the occurrence of VaD such as diet, physical and mental activities, stroke, cardiovascular, hypertension, diabetes, obesity, metabolic, smoking and depression (Vijayan & Reddy, 2016).

2.1.2 Diagnosis of VaD

Generally, the diagnosis starts with the assessment of symptoms and medical history related to VaD, followed by neurophysiological test such as mini-mental state examination (MMSE) and montreal cognitive assessment (MoCA) which act as cognitive screening tools to determine current levels of cognitive status of patients (Dong *et al.*, 2010). However, these tests have not been proven to reliably differentiate VaD from other dementia syndromes, particularly AD. Hence, brain imaging methods specifically magnetic resonance imaging (MRI) and computerized tomography (CT) are being utilized as important supporting tool to diagnose VaD more accurately (Beynon *et al.*, 2012). Imaging changes such as hippocampal sclerosis, white matter changes, infarcts of variable size and hemosiderin (iron-storage complex) deposits indicative of hemorrhages were frequently noticed in VaD patients. Currently, there is no reliable cerebrospinal fluid (CSF) biomarkers for VaD as in AD such as phosphorylated tau and amyloid beta protein level (Korczyn, Vakhapova & Grinberg, 2012). The medical history of patient, brain imaging and the presence of phosphorylated tau and amyloid beta proteins in CSF may help the physician to identify and differentiate patients with VaD from AD. Continuous efforts are still being undertaken to develop non-expensive, reliable and rapid diagnosis methods for early detection of VaD.

2.1.3 Treatment for VaD

At present, there is no pharmaceutical drugs approved by Food and Drug Administration (FDA) for the treatment of VaD (Chang *et al*., 2016; Kumaran, Wahab & Hassan, 2021). This is due to less clinical trials being conducted for VaD compared to AD. Currently, standard treatment mainly focuses on symptomatic management and prevention of additional brain damage to slow down VaD progression.

Since cholinergic deficits are found in both AD and VaD patients, ChEIs approved by FDA for the treatment of AD have been investigated using VaD patients in clinical trials. Based on previous clinical trials, administration of ChEIs have been reported to benefit VaD patients by improving their cognitive function (Malouf & Birks, 2004; Craig & Birks, 2006; Birks, McGuinness & Craig, 2013; Chen *et al.*, 2016). This drug inhibits enzymes that breakdown ACh into acetate and choline, thus conserve the level of ACh and prolong the duration of its action in the CNS (Colovic *et al.*, 2013). Besides that, memantine which is another FDA approved drug for AD treatment have been investigated in VaD patients. Orgogozo *et al.* (2002) and Wilcock *et al.* (2002) reported the administration of memantine improves cognitive function in VaD patients. Memantine plays a role in blocking the binding of glutamate to NMDAR hence counteract glutamate excitotoxicity. The molecular structure of commercially available cholinesterase inhibitors and NMDAR antagonist is presented in Figure 2.1 and the mode of action is summarized in Table 2.1. Furthermore, recognition and control of cardiovascular and cerebrovascular risks using anti-hypertensives, aspirin, statins, anti-diabetes, and lifestyle modification such as regular physical exercise and healthy diet may slow down brain damage progression (Sachdev & Brodaty, 1999). Further preclinical and clinical studies are

required to discover potential new drugs with less side-effect and to validate these drugs for VaD treatments.

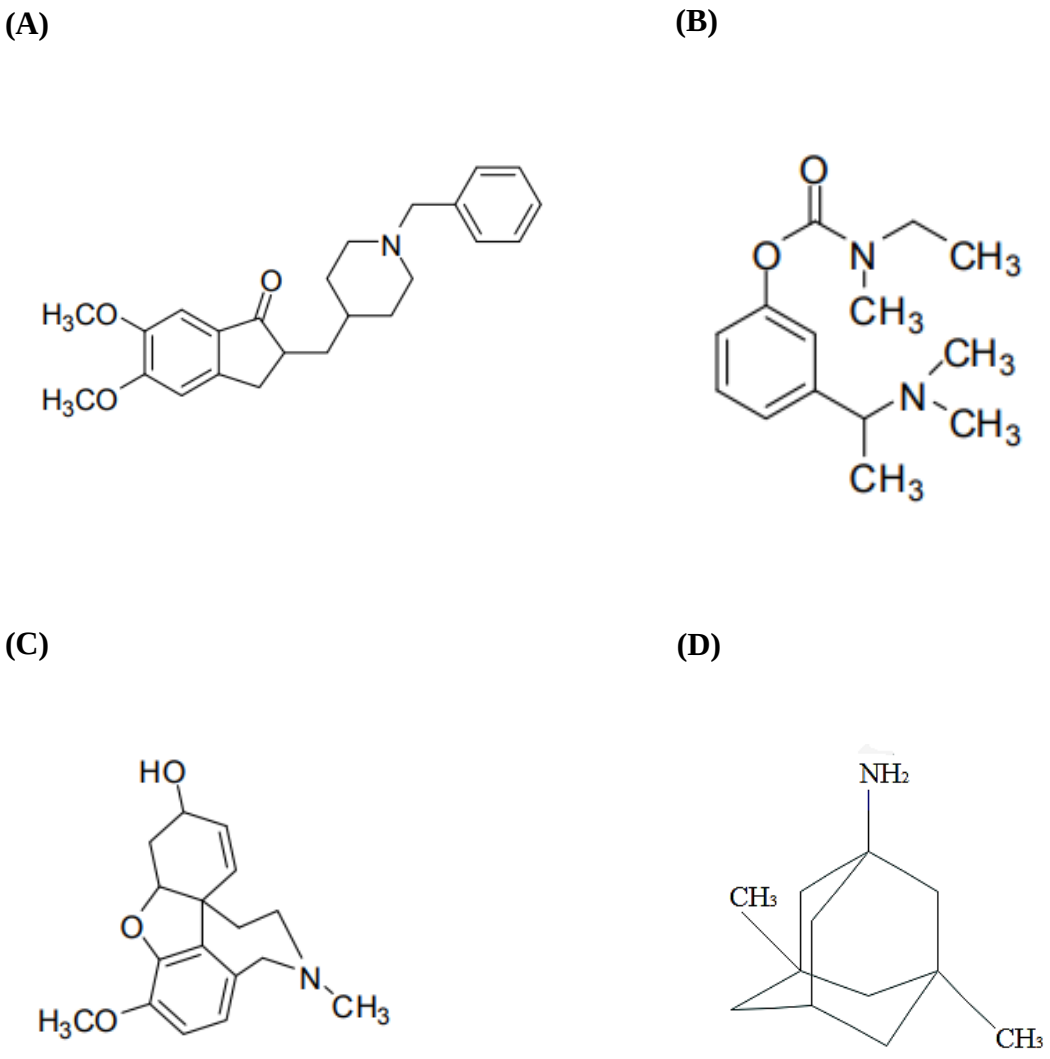

Figure 2.1: Structures of cholinesterase inhibitors; (A) donepezil, (B) rivastigmine, (C) galantamine and NMDAR antagonist; (D) memantine.

Table 2.1: Types of medications recommended in VaD treatment.

Drugs	Mode of action	References
Donepezil (Aricept)	Chemically synthesized piperidine derivative acts as a selective, non-competitive and rapidly reversible inhibitor of acetylcholinesterase (AChE).	Knowles, 2006
Galantamine (Reminyl)	A naturally occuring alkaloid acts as a selective, competitive and reversible inhibitor of AChE.	Wattmo *et al.*, 2013
Rivastigmine (Exelon)	Carbamyl derivative acts as a non-selective, non-competitive and irreversible inhibitor of AChE and butrylcholinesterase (BuChE).	Muller, 2007
Memantine (Ebixa)	Non-competitive blocker of NMDAR binds with moderate affinity to prevent or control excitotoxicity.	Parsons *et al.*, 2013

2.2 Chronic cerebral hypoperfusion (CCH)

Sufficient blood supply to the brain consistent with changes in energy needs are crucial for optimum brain function (Kisler *et al.*, 2017). The brain receives blood supply from bilateral internal carotid arteries (arise from common carotid arteries) and bilateral vertebral arteries (arise from the subclavian arteries). The minor blood vessels branching off these arteries nourish different sections of the brain (Purves *et al.*, 2001). CCH is a term used to describe the condition of prolong insufficient blood supply to the brain to meet metabolic demand which results in brain damage (Puig *et al.*, 2018). CCH plays a major role in provoking neurodegeneration process and leads to dementia, disability and mortality worldwide. Clinical studies have shown that CCH is the common pathological cause of VaD and other neurodegenerative diseases (Zlokovic, 2005; Gorelick *et al.*, 2011; Duncombe *et al.*, 2017; Feng *et al.*, 2020).

The key factors that leads to CCH includes (i) injury to blood vessel developed from artery stenosis or occlusion caused by atherosclerosis, Moyamoya disease, Takayasu's arteritis, arteriovenous malformation and cerebral arteriovenous fistula, (ii) alterations in cerebral hemodynamic such as chronic blood loss, reduced cardiac output due to heart malfunction and persistent hypotension and (iii) increase of blood viscosity due to changes in blood components caused by hyperlipidemia, polycythemia, and hyper-homocysteinemia (Zhao & Gong, 2015).

The detailed pathological mechanisms of CCH leading to cell death and cognitive impairment are not completely understood. However, previous studies have proposed that CCH might promote cell death via (i) glutamate excitotoxicity, (ii) generation of oxidative

stress and (iii) inflammation. These pathological mechanisms may "cooperate" and contribute to the damage of neurons, white matter lesions (WML) and central cholinergic dysfunction during CCH condition and ultimately lead to cognitive impairment and dementia (Kitagawa *et al.* 2005; Farkas, Luiten & Bari, 2007; Adibhatla & Hatcher 2008; Di Lazzaro, *et al.*, 2008; Moretti & Caruso, 2020). The proposed pathological mechanisms in CCH that lead to cell death (Figure 2.2) are described below:

I. **Glutamate excitotoxicity**: Malfunctioning of Na^+/K^+-ATPase due to adenosine triphosphate (ATP) depletion causes prolong depolarization of the neuronal membrane. Consequently, this condition leads to the release of excess glutamate to the extracellular compartments to activate glutamate receptors (Belov Kirdajova *et al.*, 2020). The prolonged activation of glutamate receptors results in excitotoxicity. Intracellular calcium overload via the glutamate receptors initiates activation of phosphatases, lipases, proteases and endonucleases that contribute to cell membrane disruption and DNA fragmentation which finally leads to cell death and WML (Orrenius, Zhivotovsky & Nicotera, 2003; Doyle, Simon & Stenzel-Poore, 2018).

II. **Oxidative stress:** Mitochondrial dysfunction during CCH results in generation of reactive oxygen species (ROS) which in turn causes neuronal cell death. The generated ROS reacts with macromolecules of cells through oxidation and leads to necrosis or apoptosis of the cells (Chong, Li & Maiese, 2005; Redza-Dutordoir & Averill-Bates, 2016; Juan *et al*., 2021). Several experimental studies have shown that oxidative stress play a critical role in CCH-induced

brain damage (Zhang *et al.*, 2015; Zhao *et al.*, 2015; Xu *et al.*, 2016; Guo *et al.*, 2019).

III. **Inflammation**: CCH-induced cognitive impairment is associated with inflammation in rodents (Saggu *et al.*, 2016) and humans (Tarkowski *et al.*, 2003; Kawamoto *et al.*, 2006). CCH leads to infiltration of numerous inflammatory cells, migration of peripheral leukocytes into the brain and activation of microglia. Activation of microglia in cerebral hypoperfused rodents is associated with release of inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1ß and IL-6 which leads to the progression of blood brain barrier (BBB) disruption (Shibata *et al.*, 2004; Harukuni & Bhardwaj, 2006; Lee *et al.*, 2016). Furthermore, the entry of secondary serum substances such as serum proteins, complement component and fibrinogens after BBB damage into the cerebral parenchyma is likely to initiate secondary inflammatory response from resident microglia and further augment WMLs (Ryu & McLarnon, 2009; Venkat, Chopp & Chen, 2017). Swartz *et al.* (2003) reported that WML caused by CCH can disrupt the cholinergic projections from basal forebrain to neocortex, hippocampus and amygdala leading to cognitive dysfunction in VaD patients. During inflammation, TNFα was reported to increase the astrocytic expression of glutaminase, which converts glutamine back to glutamate and acts as a moderator in excitotoxicity promoting events (Milewski *et al.*, 2019).

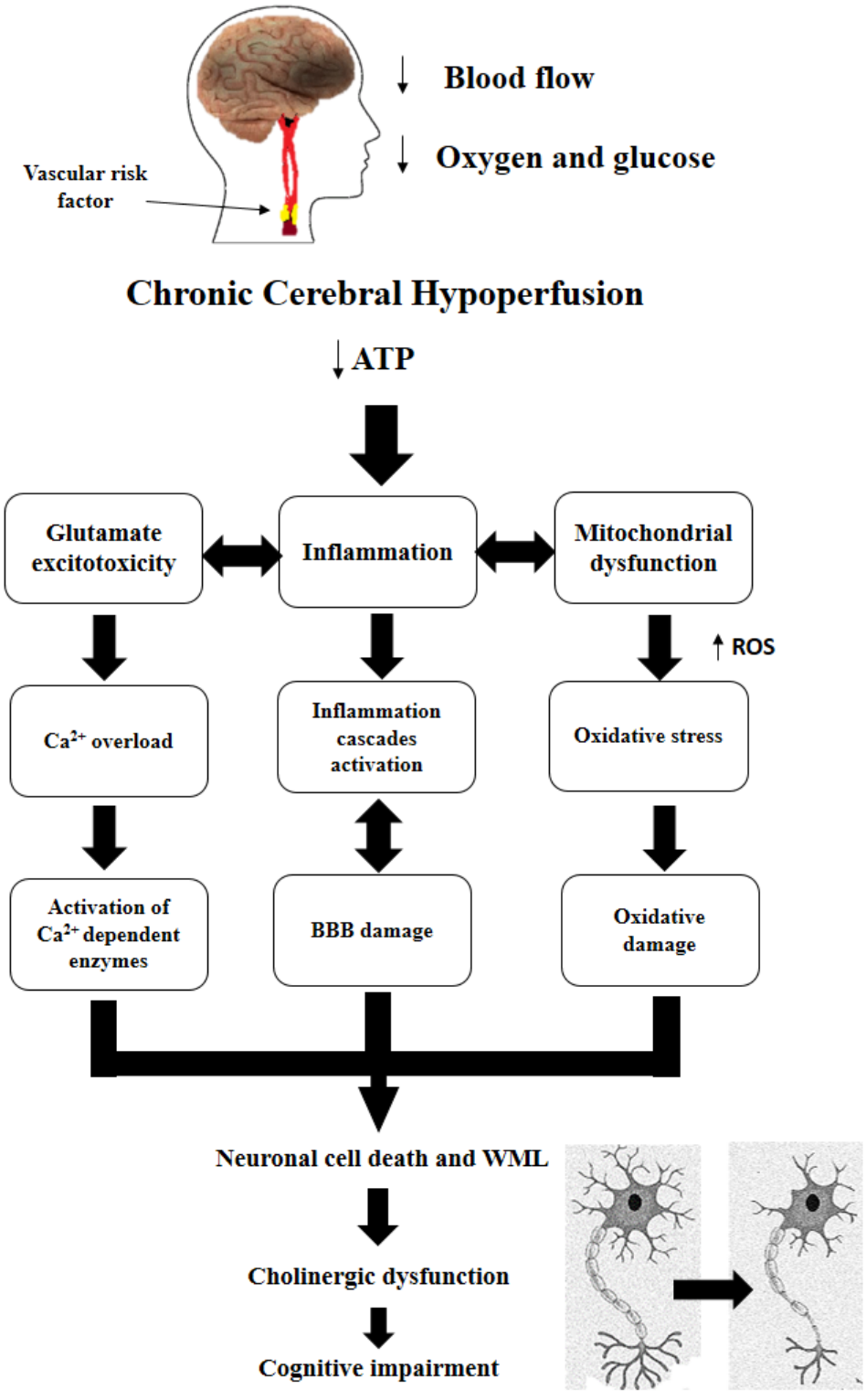

Figure 2.2: Summary of the possible pathological mechanisms involved in CCH-induced cognitive impairment.

2.2.1 Chronic cerebral hypoperfusion animal models

Many animal models have been established to investigate the role of CCH in neurodegenerative processes that leads to cognitive dysfunction in dementia patients. These animal models are developed through occlusion of large arteries that supply blood to the brain in order to mimic CCH condition (Jiwa, Garrard & Hainsworth, 2010). Previous studies have utilized rodents such as rats, mice and gerbils for inducing CCH, however rats are the most suitable species to investigate CCH due to the good reproducibility, its good recovery rate from surgery and complete circle of Willis that allows constant but reduced blood flow after the onset of CCH (Pappas *et al*, 1996; Farkas, Luiten & Bari, 2007). There are two types of global CCH models induced in rats, POBCCA or also known as 2-vessel occlusion (2VO) and 4-vessel occlusion (4VO) (Ahad *et al.*, 2020). Other CCH model includes bilateral common carotid artery stenosis (BCAS) in gerbils (Kudo *et al.*, 1990) or mice (Hase *et al.*, 2017) and unilateral common carotid artery occlusion (UCCAO) in mice (Lee *et al.*, 2020). These protocols are developed to prevent sudden drop in CBF as gerbils and mice lack of fully developed posterior communicating arteries of the circles of Willis (Kelly, McCulloch & Horsburgh., 2001; Sarti *et al.*, 2002). The rat CCH models will be further discussed for the scope of this thesis.

2.2.1(a) 4-vessel occlusion (4VO) model

The 4VO rat model closely resembles the pattern of brain damage seen in humans following a heart arrest. This model is established through electrocoagulation of the vertebral arteries in the alar foramina of the first cervical vertebra, followed by transient occlusion of the common carotid arteries (Lu *et al.*, 2016). Neurodegeneration in the Cornu ammonis 1 (CA1) subfield of the hippocampus and learning impairment in Morris water maze (MWM) test and passive avoidance task were observed in 4VO rats (Jiwa, Garrard & Hainsworth, 2010). The 4VO model is less commonly used due to the technical difficulties. The establishment of this model require 2 days, hence, more time is required as compared to POBCCA model. The most difficult task is the successful electro-cauterization of the vertebral arteries that are hidden underneath the alar foramina. Unsuccessful electro-cauterization may result in incomplete ischemia (Deng & Xu, 2009). The digital subtraction angiography (DSA) is used to visualize the blood flow and confirm the blockage in the artery. In addition, difficulties in gaining access to the vertebral(s) can result in excess muscle trauma and myoglobin urea post-operatively. Besides that, excessive heating of the Cl vertebrate causes damage to the medulla and increases the mortality rate as the animals experience respiratory difficulties due to the medulla damage (Small & Buchan, 2000). Hence a skilled surgeon with good experience is required to establish this model successfully.

2.2.1(b) Two vessel occlusion (2VO) or permanent occlusion of bilateral common carotid artery (POBCCA) model

POBCCA is a well-characterized and widely used animal model to investigate the role of CCH in neurodegenerative processes (Institoris *et al.*, 2007; Kitamura *et al.*, 2012; Jing *et al.*, 2015). POBCCA model is established by the occlusion of both common carotid arteries to reduce blood flow to the brain (Yan & Ai, 2018). There are no technical complications as in 4-VO model. This method is easy to perform in single surgical procedure and the isolation of common carotid artery is much easier due to its location (beside the trachea) and the arteries are easily distinguished from other vessels.

Following the occlusion of the common carotid arteries, three phases of CBF changes have been reported which begins with (i) acute phase: CBF drops sharply and remains low for 2 to 3 days; (ii) chronic phase: last for 8 to 12 weeks, CBF values gradually recover at week 1, but were still significantly lower than the control values. A persistence lower CBF are maintained during chronic phase which closely resemble the condition of reduced CBF in human aging and dementia; (iii) restitution phase: occurs after 3 months whereby CBF begins to normalize or no significant difference compared to control due to compensatory and adaptive mechanisms (Choy *et al.*, 2006). The greatest decrease of blood flow about 60 % in hippocampus and 35–45 % in the cortex and white matter regions as compared to control level were measured in POBCCA rat models (Sopala & Danysz, 2001; Otori *et al.*, 2003). The level of ATP was found to diminish after 5 min of CCH onset and sustained at low levels for 2 weeks but the ATP level returned to the normal level after 8 weeks (Plaschke, 2005).

There are extensive evidences from previous studies at various time points after the induction of POBCCA which reveals patho-mechanisms involved in this model mimics VaD such as generation of ROS and nitric oxide (NO) in the brain due to the depletion of ATP (Nita *et al.*, 2001; Farkas, Luiten & Bari, 2007), activation of microglial and astrocytes (Pappas *et al.*, 1996; Farkas *et al.*, 2004), WML and neuronal damage in CA1 pyramidal neurons of the hippocampus and cerebral cortex (Farkas, Luiten & Bari, 2007) and impairment of cholinergic system such as increase in AChE activity, decrease in ChAT activity and low ACh level (Azam *et al.*, 2018; Bhuvanendran *et al.*, 2019; Al Dera *et al.*, 2019). Consequently, all these mechanisms lead to memory and cognitive dysfunction in POBCCA rats.

POBCCA rats were found to demonstrate learning and memory impairments at week 1 from the onset of CCH (Damodaran *et al.*, 2014). Degeneration of the white matter, cerebral cortex (Otori *et al.* 2003) and hippocampus neurons (Farkas, Luiten & Bari, 2007) were reported between 1 to 2 weeks after CCH. Such degeneration corresponds with learning and memory impairment, which progressively deteriorate with time. POBCCA rats have displayed learning and memory impairment in various behavioral test such as MWM, novel object recognition (NOR), eight-arm radial maze, Y-maze and passive avoidance task (Kumaran *et al.*, 2008; Zhang *et al.*, 2011; Song *et al.*, 2020; Qu *et al.*, 2020).

2.3 Learning and memory

Learning and memory are two interrelated elements involved in cognitive process. Learning is the process of acquiring new skills or knowledge (information), while memory is the storage of the information and experiences that have been learned previously (Squire, 2009). Memory enables human to connect experiences, learn and make sense of lives. Impairment of learning and memory affects the cognitive function and consequently deteriorate the quality life of patient with dementia. The process of memory includes acquisition (encoding of learned information), consolidation (stabilization and storage of information) and retrieval (recall of stored information) (Im *et al.*, 2009).

The learned information is stored in three memory systems based on capacity and duration: (i) sensory memory (SM) that retain large capacity of information but rapidly decaying within few milliseconds (ms), (ii) short-term memory (STM) retain small amount of information for seconds (s) and (iii) long-term memory (LTM) stores unlimited information to be maintained for long periods, even for lifetime. Information from the external environment is first acquired by SM, stored temporarily as STM and finally converted to LTM for long-term storage and retrieval (Tripathy & Ogmen, 2018; Zlotnik & Vansintjan, 2019). The storage of STM relies on existing networks and post-translational modifications, while the storage of LTM is associated with gene expression, *de novo* protein synthesis and formation of new synaptic connections via dendrite growth (Bisaz, Travaglia & Alberini, 2014). The formation of LTM depend on memory consolidation. Memories require reconsolidation several times to achieve permanence in LTM (Tetzlaff *et al.*, 2013).

Memory is classified into two major categories (Figure 2.3), (i) declarative (explicit memory) and (ii) non-declarative (implicit memory) as described below:

I. Declarative memory refers to conscious memory of previously stored experiences, facts and concepts (Ullman, 2004). Declarative memory depends primarily on the integrity of frontal and medial temporal lobe structures, including the hippocampus (Milner, Squire & Kandel, 1998; Brem, Ran & Pascual-Leone, 2013). Explicit memory can be further divided into (a) episodic memory, which stores specific personal experiences and events (time and place) that occur in everyone's life (Dickerson & Eichenbaum, 2010) and (b) semantic memory, which stores general knowledge and information about the world (Battaglia & Pennartz, 2011). Activities such as planning for the future, recalling about the past or reasoning depend on the activation of information stored in semantic memory (Mahon & Caramazza, 2008). Declarative memory involves gradual learning with multiple stimulus and response presentations.

II. Non-declarative memory refers to memory of previously acquired skills, habits and behaviors which can be automatically accessed in unconscious state. Example includes procedural memory that enables one to perform various tasks ranging from simple to complex (e.g. knowing how to drink water from a glass or riding a bike), associative/simple classical conditioning (e.g. emotional/ skeletal musculature response) and non-associative learning (e.g. habituation or sensitization). Non-declarative memory relies mostly on cerebellum, striatum and cortical association areas (Brem, Ran & Pascual-Leone, 2013; Poon & Schmid, 2012).

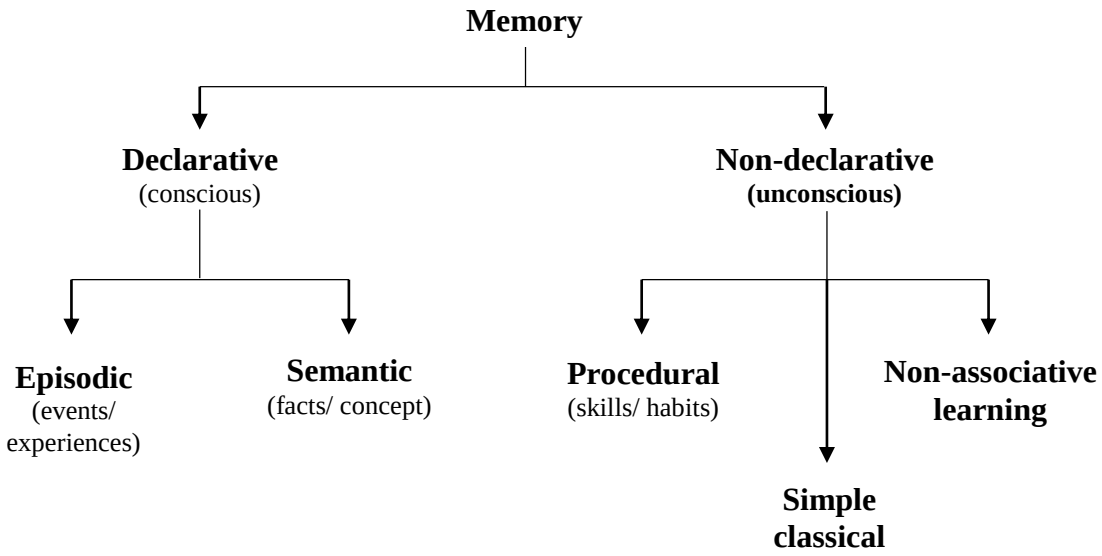

Figure 2.3: Classification of memory.

2.3.1 Synaptic plasticity

Synaptic plasticity refers to the strengthening or weakening of contacts between pre- and post-synaptic neurons due to increasing or decreasing activity respectively and often accompanied by structural alteration of the synapse (Lu, Christian & Lu., 2008). Brain encodes and store information (memory) at the synapse by adjusting the strengths of associations between neurons and there are plenty of evidence that synaptic plasticity plays an important role in learning and memory (Frankland & Bontempi, 2005; Richter & Klann, 2009; Kandel, Dudai & Mayford, 2014). The long-lasting increase of synaptic strength is required for consolidation of both implicit and explicit forms of LTM (Malenka & Nicoll 1999; Marrone 2005; Hawkins *et al.* 2006). The synaptic plasticity is directly regulated by both pre- and post-synaptic neurons through changes of the synaptic machinery such as (i) the quantity of neurotransmitters released from the presynaptic neuron into the synapse, (ii) number of neurotransmitter receptors present on the postsynaptic membrane and (iii) its receptor sensitivity to the released neurotransmitters (Luscher & Malenka, 2012; Mayford, Siegelbaum & Kandel, 2012; Kandel, Dudai & Mayford, 2014).

2.3.1(a) Long-term potentiation (LTP)

LTP is the most popular and generally used physiological model of synaptic plasticity to investigate cellular mechanisms involved in learning and offers an attractive hypothesis on how memories are formed (Abbas, Villers & Ris, 2015; Herring & Nicoll, 2016). Although LTP has been observed in various brain regions such as cortex, amygdala, striatum and cerebellum (Fox, 2002; Lev-Ram *et al.*, 2002; Sigurdsson *et al.*, 2007; Lovinger, 2010), the NMDAR-dependent LTP in CA1 hippocampus is the most widely and well-studied type of LTP. LTP in this brain region is easily inducible and reproducible (Bliss & Collingridge, 1993; Abe & Saito, 2000).

LTP is typically studied *in vitro* and *in vivo* by using electrical stimulation to mimic pre-synaptic action potentials and measuring corresponding post-synaptic responses. Since the discovery of LTP, many studies use high-frequency stimulation (HFS) protocols, 100 Hz, 1 seconds (Bliss & Collingridge 1993). Although effective in generating LTP, 100 Hz is not a rate at which neurons typically fire, hence the search of more physiological naturally occurring firing patterns led to the discovery of theta burst stimulation (TBS). TBS consists of short bursts (4–5 stimuli at 100 Hz) repeated at 5 Hz (Buzsaki, 2002). TBS mimic naturally occurring theta rhythm which is critical for mnemonic processing (Kumar, 2011).

2.3.2 Hippocampal LTP

2.3.2(a) Role of hippocampus in learning and memory

The hippocampus has been recognized to play a vital role in formation of spatial and non-spatial forms of explicit memory (Squire, Stark & Clark, 2004; Pastalkova *et al.*, 2008; Kraus *et al.*, 2013; Macdonald *et al.*, 2013). New information is stored as STM within the hippocampus temporarily, before being transferred to the cerebral cortex for long-term storage (Ivanco & Racine, 2000; Mehta, 2018). Direct and convincing evidence from both human and animal studies supports the role of hippocampus in learning and memory. Moreover, memory problems are frequently linked to hippocampal dysfunction (Snyder *et al.*, 2015).

2.3.2(b) Hippocampus anatomy and circuit

The hippocampus is a lamellar structure that receives highly processed information from a wide range of neocortical regions. Hippocampus consists of CA1–CA3 subfields made up of pyramidal cells and dentate gyrus (DG) with granule cells (Amaral & Witter, 1989; Witter *et al.*, 1989). In addition to excitatory neurons, the hippocampus also contains inhibitory interneurons (Klausberger & Somogyi, 2008).

The tri-synaptic pathways of hippocampus (Figure 2.4) have long been assumed to be the critical circuit for encoding and consolidating information into LTM (Van Strien, Cappaert & Witter, 2009). Information flows in one direction via the tri-synaptic pathways starting from (i) the activated entorhinal cortex (EC) pyramidal neurons sending inputs to DG via perforant path fibers, (ii) the input then passes along the mossy fiber pathway