BEHAVIOURAL AND HIPPOCAMPAL NEUROPLASTICITY EFFECTS OF A BIOACTIVE FRACTION FROM *CLITORIA TERNATEA* LINN. ROOT EXTRACT IN A RAT MODEL OF CHRONIC CEREBRAL HYPOPERFUSION

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UNIVERSITI SAINS MALAYSIA

2022

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by

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Thesis is submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

November 2022

ACKNOWLEDGEMENT

First and foremost, I am grateful to Allah S.W.T for giving me the strength, health, patience and knowledge to complete this work successfully.

I would like to express my deepest appreciation to my main supervisor, Associate Professor Dr. Zurina Hassan for allowing me to conduct this project. I am very grateful to her for the excellent supervision, guidance and never-ending support towards the completion of my project and thesis. Sincere thanks for the time that she spent on reading and indispensable suggestions offered to improve the thesis. Special thanks to my co-supervisors; Associate Professor Dr. Vikneswaran Murugaiyah, Dr. Ahmad Tarmizi Che Has and Dr. Lim Gin Keat for their assistance, idea and invaluable guidance especially in designing my research project.

I am also grateful to Professor Dr. Vicknasingam Balasingam Kasinather (Director Centre for Drug Research) for the facilities provided during this research project. My special appreciation to Nelson Chear Jeng-Yeou for his excellent guidance especially in chemical analysis. I am indebted for the time he spent on chromatography and spectroscopic analyses. Many thanks to the staffs Centre for Drug Research who contributed to the success of this study, especially Puan Siti Najmi Syuhadaa, Cik Noorul Hamizah, Encik Hilman Sulaiman, Encik Zamri and Puan Sharena.

I extend my heartfelt gratitude to my beloved mother, Bunga Lasia Mat Daud and siblings, Ahmad, Asmat, Asmah, Kasman, Hirman, Hirma, Imran, Fatimah, Era and Salasiah for their unlimited love, motivation as well as financial support during this PhD journey. My sincere thanks to my beloved friends Muhammad Eshal Dzulkapli, Muhammad Hazim, Mohamad Azmeer Effendy, Nur ‘Izzati Mansor, Tiang

Ning, You Cheik Yee, Kesevan Rajahkumaran, Tan Ai Fein, Rima Atria, Aiman Nadhirah, Muhammad Zulfadhli, Fatin Hilyani, Azlan Abd. Rahim, Tuan Mohammad Faisal, Ridhuan, Nur Sa'adah, Nur Syafiqah, Mohamad Hafifi, Nadia Saref, Dinesh Ramachandran, and Azlin Saref for their support throughout this wonderful journey.

I would like to express my special thanks to Dr. Siti Rafidah Yusuf, Advisor of Journal Club for the opportunity given to facilitate this society. Not forgetting to all members of the Journal Club for the endless support and for making this journey meaningful. Finally, I would like to acknowledge the USM RUI Grant (1001/CDADAH/8012302) and the Institute for Postgraduate Studies for the financial funding.

Thank you, may Allah bless all of us.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
/	per
<	Less than
=	Equal
>	More than
^{o}C	Degree of Celsius
cm	Centimeters
g	Gram
h	Hour
Hz	Hertz
I	Length
i.p.	Intraperitoneal
kDa	Kilodalton
kg	Kilogram
KHz	Kilohertz
m	Meter
M	Molar
M	Muscarinic
m/z	mass-to-charge ratio
mA	Milliampere
mg	Milligram
mg/mL	Milligram per millilitre

min	Minutes
mL	Milliliters
mm	Millimetres
mM	Millimolar
ms	Millisecond
n	Number of samples
nm	Nanometre
O^2	Oxygen
p	Probability value
p.o	Per os
R^2	Coefficient of determination
sec	Second
U/mL	Unit per millilitre
µg/mL	Microgram per millilitre
µL	Microlitres
µm	Micrometre
x	Multiply
2VO	Two-vessels occlusion
3xTg-AD	Triple transgenic Alzheimer's disease
4VO	Four-vessels occlusion
5HT	5-hydroxytryptamine
α	Alpha
β	Beta
γ	Gamma

δ	Delta
ε	Epsilon
θ	Theta
π	Pi
$Å^2$	Angstroms squared
A	Absorbance
ABRC	Analytical Biochemistry Research Centre
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Anterior posterior
APP	Amyloid-beta precursor protein
AR	Analytical reagent
ARASC	Animal Research and Service Centre
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BuChE	Butyrylcholinesterase
C	Sample concentration
CA	Cornu ammonis
CA1	Cornu ammonis 1

Ca^{2+}	Calcium
CA3	Cornu ammonis 3
CAMKIIα	Calcium/calmodulin kinases II alpha
cAMP	Cyclic adenosine monophosphate
CBF	Cerebral blood flow
CCA	Common carotid arteries
CCH	Chronic cerebral hypoperfusion
CD_3OD	Deuterated methanol
CI	Cognitive impairment
Cl^-	Chloride
CLA	Clitorienolactone A
CNS	Central nervous system
CREB	cAMP response element-binding protein
CT	*Clitoria ternatea*
CTRF	*Clitoria ternatea* root fraction
CVD	Cerebrovascular diseases
DG	Dentate gyrus
DH	Dorsal hippocampus
DNA	Deoxyribonucleic acid
DTNB	5,5’-dithiobis (2-nitrobenzoic acid)
E	East
E/I	Excitatory/inhibitory
EC	Entorhinal cortex
ECA	External carotid artery

ELISA	Enzyme-linked immunosorbent assay
E-LTP	Early long-term potentiation
ERK	Extracellular regulated kinase
EtOAC	Etyl-acetate
FA	Formic acid
fEPSP	field Excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid A
$GABA_B$	γ-aminobutyric acid B
GAD	Glutamic acid decarboxylase
GPCRs	G protein-coupled receptors
gprot/L	Gram protein per litre
H_2O_2	Hydrogen peroxide
HCL	Hydrochloride
HIF-1	Hypoxia-inducible factor-1
HPLC-DAD	High-performance liquid chromatography coupled diode array detector
HRP	Horseradish peroxidase
I/O	Input/output
IC_{50}	Half-maximal inhibitory concentration
ICA	Internal carotid artery
ICH	International Conference on Harmonisation
IgG	Immunoglobulin G
iGluR	Ionotropic glutamate receptor
I-LTP	Initial long-term potentiation

LC-QTOF-MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
LD_{50}	Lethal dose 50 %
LDS	Lithium dodecyl sulphate
L-LTP	Late long-term potentiation
LOD	Limit of detection
LOQ	Limit of quantification
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptors
MAGUKs	membrane-associated guanylate kinases
MCAo	Middle cerebral artery occlusion
MCA	Middle cerebral artery
MeOH	Methanol
Mg^{2+}	Magnesium
mGluR	Metabotropic glutamate receptor
ML	Mediolateral
mRNA	Messenger ribonucleic acid
MWM	Morris water maze
N	North
NA	Noradrenaline
nAChR	Nicotinic acetylcholine receptors
NMDA	N-methyl D-aspartate acid
OFT	Open-field test
PAT	Passive avoidance task

PBOCCA	Permanent bilateral occlusion of common carotid arteries
PFA	Paraformaldehyde
pH	Potential of hydrogen
PKA	Protein kinase
PKC	Protein kinase C
PPF	Paired pulse facilitation
ppm	Parts per million
PSD-95	Postsynaptic density protein 95
PVDF	Polyvinylidene fluoride
rcf	Relative centrifugal force
RSD	Relative standard deviation
S	South
SD / σ	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error mean
SI	Selectivity index
SVD	Small vessel disease
SYNP	Synaptophysin
TBS	Theta-burst stimulation
TBST	Tris-buffered saline containing tween 20
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography
TMS	Tetramethylsilane

UCCAO	Unilateral common carotid arteries occlusion
US	Unpleasant stimulus
USA	United State of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V	Volt
v/v/v	volume per volume per volume
VaD	Vascular dementia
VCI	Vascular cognitive impairment
veh.	Vehicle
VH	Ventral hippocampus
W	West
w/v	Weigh per volume
WM	White matter

LIST OF APPENDICES

KESAN TINGKAHLAKU DAN NEUROPLASTISITI HIPOKAMPUS FRAKSI BIOAKTIF EKSTRAK AKAR *CLITORIA TERNATEA* LINN. DALAM MODEL TIKUS SEREBRUM HIPOPERFUSI KRONIK

ABSTRAK

Terapi farmakologi sangat disyorkan untuk mengurangkan keterukan sindrom tingkah laku dan psikiatri demensia, serta untuk membalikkan penurunan kognitif secara berkesan dalam pesakit demensia bergejala. Bukti saintifik mengenai pendekatan berorientasikan kognisi untuk merawat kecacatan kognitif menggunakan produk berasaskan semulajadi ditemui dengan baik. Oleh itu, tesis ini menyiasat kesan fraksi bioaktif daripada ekstrak akar *Clitoria ternatea* (CT) dalam tingkah laku dan neuroplastisitas hipokampal dalam model tikus hipoperfusi serebrum kronik (CCH). Pendekatan fraksinasi berpandukan bioasai telah dijalankan untuk menghasilkan pecahan bioaktif akar CT. Sebagai permulaan, serbuk akar CT diekstrak menggunakan pengekstrakan berjujukan kekutuban pelarut yang berbeza-beza. Kemudian, ekstrak telah disaring untuk bioaktiviti mereka menggunakan kajian *in vitro* dan *in vivo*. Ekstrak kloroform akar CT selanjutnya tertakluk kepada fraksi dan sebatian pengasingan menggunakan kromatografi lajur untuk menghasilkan pecahan bioaktif dan sebatian aktif. Untuk penilaian kawalan kualiti, kandungan clitorienolactone A (CLA) dalam fraksi bioaktif akar CT (255 mg/g) dan ekstrak aktif (150 mg/g) ditentukan menggunakan kromatografi cecair berprestasi tinggi ditambah dengan analisis pengesanan tatasusunan diod. Dalam kajian ini, kesan fraksi bioaktif akar CT pada prestasi tingkah laku dan keplastikan sinaptik hipokampal telah dinilai dalam model haiwan hipoperfusi serebrum kronik yang disebabkan oleh oklusi dua hala kekal arteri karotid biasa (PBOCCA). Pemberian satu oral fraksi bioaktif akar CT (10, 20, dan 40 mg/kg) dengan ketara meningkatkan pengekalan ingatan dalam tugas

pengelakan pasif dan membalikkan pembelajaran spatial dan defisit ingatan semasa ujian berselirat air Morris selepas induksi PBOCCA. Di samping itu, fraksi bioaktif akar CT (10, 20, dan 40 mg/kg) dan CLA (10 mg/kg) secara berkesan membalikkan penindasan LTP dalam tikus PBOCCA semasa rakaman elektrofisiologi. Pemendapan plak amiloid-beta dalam hipokampus model tikus PBOCCA telah diterbalikkan selepas rawatan dengan fraksi bioaktif (40 mg/kg) dan CLA (10 mg/kg). Tahap neurotransmiter yang memodulasi proses pembelajaran dan ingatan dinilai menggunakan asai ELISA. Keputusan menunjukkan bahawa fraksi bioaktif akar CT dan CLA secara dramatik memulihkan disregulasi glutamat, asetilkolin, GABA, serotonin, dan noradrenalin dalam hipokampus tikus PBOCCA. Ekspresi protein isyarat sinaptik molekul dalam hipokampus juga diukur menggunakan analisis blot barat. Keputusan mendedahkan bahawa pemberian fraksi bioaktif (40 mg/kg) dan CLA (10 mg/kg) secara dramatik memulihkan regulasi rendah AMPAR, NMDAR, CREB, BDNF, PSD-95, dan synaptofisin berikutan induksi PBOCCA, kecuali untuk ekspresi ERK dan CAMKII. Secara kesimpulannya, rawatan komponen bioaktif ekstrak akar CT meningkatkan prestasi tingkah laku dan neuroplastisiti hipokampus dalam tikus PBOCCA melalui pelbagai mekanisme molekul. Seterusnya, fraksi ini berpotensi untuk dibangunkan sebagai ubat nootropik pada masa hadapan untuk memerangi demensia vaskular.

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ABSTRACT

Pharmacological therapy is highly recommended for reducing the severity of the behavioural and psychiatric syndromes of dementia, as well as for effectively reversing cognitive decline in symptomatic dementia patients. Scientific evidence on cognition-oriented approaches for treating cognitive impairment using natural-based products is well discovered. Therefore, the thesis investigates the effects of bioactive fraction from *Clitoria ternatea* (CT) root extract in the behavioural and hippocampal neuroplasticity in chronic cerebral hypoperfusion (CCH) rats model. The bioassay-guided fractionation approach was carried out to produce the CT root bioactive fraction. To begin with, CT root powder was extracted using sequential extraction of solvent varying polarity. Then, the extracts were screened for their bioactivity using *in vitro* and *in vivo* studies. The CT root chloroform extract was further subjected to fractionation and isolation compound(s) using column chromatography to produce the bioactive fraction and active compound(s). The amount of clitorienolactone A (CLA) in the CT root bioactive fraction (255 mg/g) and active extract (150 mg/g) was quantified using high-performance liquid chromatography and diode array detection analysis for quality control purposes. In the present study, the effects of CT root bioactive fraction on behavioural performance and hippocampal synaptic plasticity were evaluated in an animal model of chronic cerebral hypoperfusion induced by permanent bilateral occlusion of common carotid arteries (PBOCCA). A single oral administration of CT root bioactive fraction (10, 20, and 40 mg/kg) significantly

improves memory retention in the passive avoidance task and reverses the spatial learning and memory deficits during the Morris water maze test after PBOCCA induction. In addition, CT root bioactive fraction (10, 20, and 40 mg/kg) and CLA (10 mg/kg) effectively reversed the suppression of LTP in PBOCCA rats during electrophysiological recordings. The deposition of amyloid-beta plaque in the hippocampus of the PBOCCA rats model was reversed after treatment with the bioactive fraction (40 mg/kg) and CLA (10 mg/kg). The neurotransmitter level that modulates the learning and memory process was evaluated using ELISA assay. The results demonstrated that CT root bioactive fraction and CLA dramatically restored glutamate, acetylcholine, GABA, serotonin, and noradrenaline dysregulation in the hippocampus of PBOCCA rats. The expression of molecular synaptic signalling proteins in the hippocampus was also measured using western blot analysis. The results reveal that administering bioactive fraction (40 mg/kg) and CLA (10 mg/kg) dramatically restored the downregulation of AMPAR, NMDAR, CREB, BDNF, PSD-95, and synaptophysin following PBOCCA induction, except for ERK and CAMKII expressions. Conclusively, treatment of the bioactive component of CT root extract improved behavioural performance and hippocampus neuroplasticity in PBOCCA rats via multiple molecular mechanisms. As a result, this fraction has the potential to be developed as a nootropic drug in the future to combat vascular dementia.

CHAPTER 1

INTRODUCTION

1.1 Background

Chronic cerebral hypoperfusion results from persistently decrease cerebral blood flow (CBF) to the brain, and it is thought to be a significant cause of dementia-related cerebrovascular diseases. Prolonged episodes of low CBF caused brain dysfunction and neuronal death (Tameem & Krovvidi, 2013). Cerebral blood arteries are permanently endowed with highly effective regulatory mechanisms that ensure adequate oxygen and nutrients. However, during chronic cerebral hypoperfusion (CCH), these processes become malfunctioning and are unable to compensate for the reduction in CBF, resulting in brain damage (Kunz & Iadecola, 2009).

Severe cerebral hypoperfusion may lead to acute infarction within 3 hours through necrosis of neuronal cells (Deb et al., 2010). On the other hand, chronic cerebral hypoperfusion has been linked to neurodegeneration for months to years due to neuronal apoptosis without an infarction (Broughton et al., 2009). Cerebral hypoperfusion has emerged as a primary cause of cognitive deficits and neuronal death processes leading to dementia. Alzheimer's disease is the most common dementia, while vascular dementia (VaD) contributes about 20% of all dementia cases (Rizzi et al., 2014; Wolters & Ikram, 2019). Look into the basis of vascular hypothesis, VaD is caused by a decrease in cerebral blood flow, which causes hypoxia and permeability of the Blood-Brain Barrier (BBB), resulting in a prolonged neurotoxic effect that promotes amyloid-beta deposition and neurodegeneration in certain areas of the brain, including the hippocampus, cerebral cortex, basal ganglia, and cerebellum (Bell et al., 2010). In the pre-clinical study, permanent bilateral occlusion of common carotid

arteries (PBOCCA) or two-vessels occlusion (2VO) induced CCH significantly decreased CBF globally around 66 % in the cortex and 48 % in the hippocampus (Neto et al., 2005). However, after four weeks of post-2VO, reduction of CBF was found to be 35-45 % in the cortex and white matter region and 60 % in the hippocampus (Sopala & Danysz, 2001; Otori et al., 2003). In a separate study, cognitive impairment after four weeks of post-2VO was observed in animal behavioural (Damodaran et al., 2014). These phenomena paved the way for the development of VaD as its condition similarly happens, paving the way to develop VaD as its condition similarly occurs in ageing and dementia states.

Hippocampus is crucial for learning and memory, yet its precise role remains elusive. Long-term potentiation (LTP) is a significant indicator of the efficacy of excitatory synaptic transmission following the delivery of high-frequency electric stimulation. Additionally, LTP provides critical insight into the cellular mechanisms underlying learning and contributes to understanding of how memories are processed (Kumar, 2011). LTP is primarily induced by stimulating glutamatergic synapses between perforant path fibres originating in the entorhinal cortex and granule cells of the dentate gyrus projected to CA3-CA1 pyramidal neurons via the Schaffer collateral pathway (Abraham et al., 2019; Baltaci et al., 2019). Besides, the mechanism of LTP is also contributed by the modulation of several neuromodulators or neurotransmitters including noradrenergic, serotoninergic and cholinergic systems (Kumar, 2011).

The therapeutic interventions of VaD are more challenging as its characterised as a multifactorial disease. There are three cholinesterase inhibitors: donepezil, galantamine and rivastigmine that recommended for clinical use widely (Kaduszkiewicz et al., 2005). However, the adverse event of the cholinesterase inhibitors needs to be considered as it may causes nausea, diarrhea, vomiting as well

as muscle cramps (Regenold et al., 2018). Moreover, unsuccessful of other neuroprotective drugs such as calcium channel blocker: nimodipine and flunarizine, glutamate antagonist drugs: zonampanel, selfotel, and traxoprodil, serotonin agonist (repinotan), and piracetam in pre-clinical and clinical trials has become more complicated (Xu & Pan, 2013).

Therefore, it drove an idea to study an alternative treatment to neuroprotective drugs using the natural-based plant product: *Clitoria ternatea* (CT) in a rat model of chronic cerebral hypoperfusion. CT aqueous root extract has been shown to improve memory and learning by elevating acetylcholine content, acetylcholinesterase activity, and dendritic arborisation of amygdala neurons (Rai et al., 2001; Rai et al., 2002; Rai et al., 2005). A previous study found that the methanolic extract of CT root significantly increased the memory performance in the chronic cerebral hypoperfusion rat model. Moreover, the extracts also increased glutamate-induced calcium and ameliorated synaptic function, which may contribute to its improving effect on cognitive behaviour (Damodaran et al., 2018). It has been determined that methanolic extracts of CT root and leaf are safe to be consumed in rats (Kamilla et al., 2012; Karta et al., 2013). Altogether, this thesis presents a comprehensive investigation of CT root extracts' ability to improve memory and learning functions in the CCH rat model. The experimental design in this thesis is summarised in Figure 1.1.

1.2 Problem statement

Chronic cerebral hypoperfusion is a brain injury caused by decreased blood flow, oxygen, and nutrient supply. As for the consequences, some brain regions vulnerable to ischemic damage, such as the hippocampus, are affected, resulting in cognitive impairment. Currently, systemic reviews and meta-analyses of the

effectiveness of cholinesterase inhibitors such as donepezil, rivastigmine, and galantamine show no conclusive proof of improving cognitive test scores or slowing the progression of cognitive decline. As a result, the medicinal plant may offer health benefits as an alternative therapy for VaD. CT Linn is one of the medicinal plants that deserves more attention because it is one of the ingredients in 'Medha Rasayana,' rejuvenating ingredients used in traditional Ayurvedic medicine to treat neurological syndromes (Mukherjee et al., 2008). To date, both *in vivo* and *in vitro* studies of CT root extracts have been studied comprehensively and was proven to be beneficial for learning and memory functions (Rai et al., 2001; Rai et al., 2002; Rai et al., 2005; Rai et al., 2010; Damodaran et al., 2018; Damodaran et al., 2020). Hence, in this study, bioactive fraction from CT root extracts was investigated its cognitive-enhancing properties to reverse cognitive decline and restore the memory that provides significant value as a therapeutic strategy targeting VaD.

1.3 Hypothesis

Bioactive fraction from CT root extracts have potential to reverse learning and memory deficits in the rat model of CCH. In addition, the neurotransmitters and molecular synaptic signalling proteins are significantly expressed in the brain of CCH rats. Collectively, it is anticipated that finding from the study would contribute to understanding of behavioural performance and neuroplasticity effects of bioactive fraction in the PBOCCA rat model induced CCH.

1.4 Objectives

1.4.1 General objectives

The thesis aims to study the behavioural and hippocampal neuroplasticity effects of a bioactive fraction from *Clitoria ternatea* Linn. root extract in a rat model of chronic cerebral hypoperfusion.

1.4.2 Specific objectives

1. To produce the CT root extracts and bioactive fraction from CT root using bioassay-guided fractionation approach.

2. To quantify the amount of CLA in CT root extracts and bioactive fraction using validated HPLC-DAD method for quality control assessment.

3. To evaluate the effects of CT root extracts and bioactive fraction on behavioural performances and *in vivo* hippocampal neuroplasticity in the CCH rat model.

4. To quantify the level of amyloid-beta plaque in the hippocampus of the CCH rat model following a single administration of bioactive fraction from CT root extracts using ELISA analysis.

5. To quantify the level of neurotransmitters that modulates learning and memory following a single administration of bioactive fraction from CT root extracts using ELISA analysis

6. To measure the expression of molecular synaptic signalling proteins that mediate synaptic plasticity in the hippocampus of the CCH rat model following a single administration of the bioactive fraction from CT root extracts using western blot analysis.

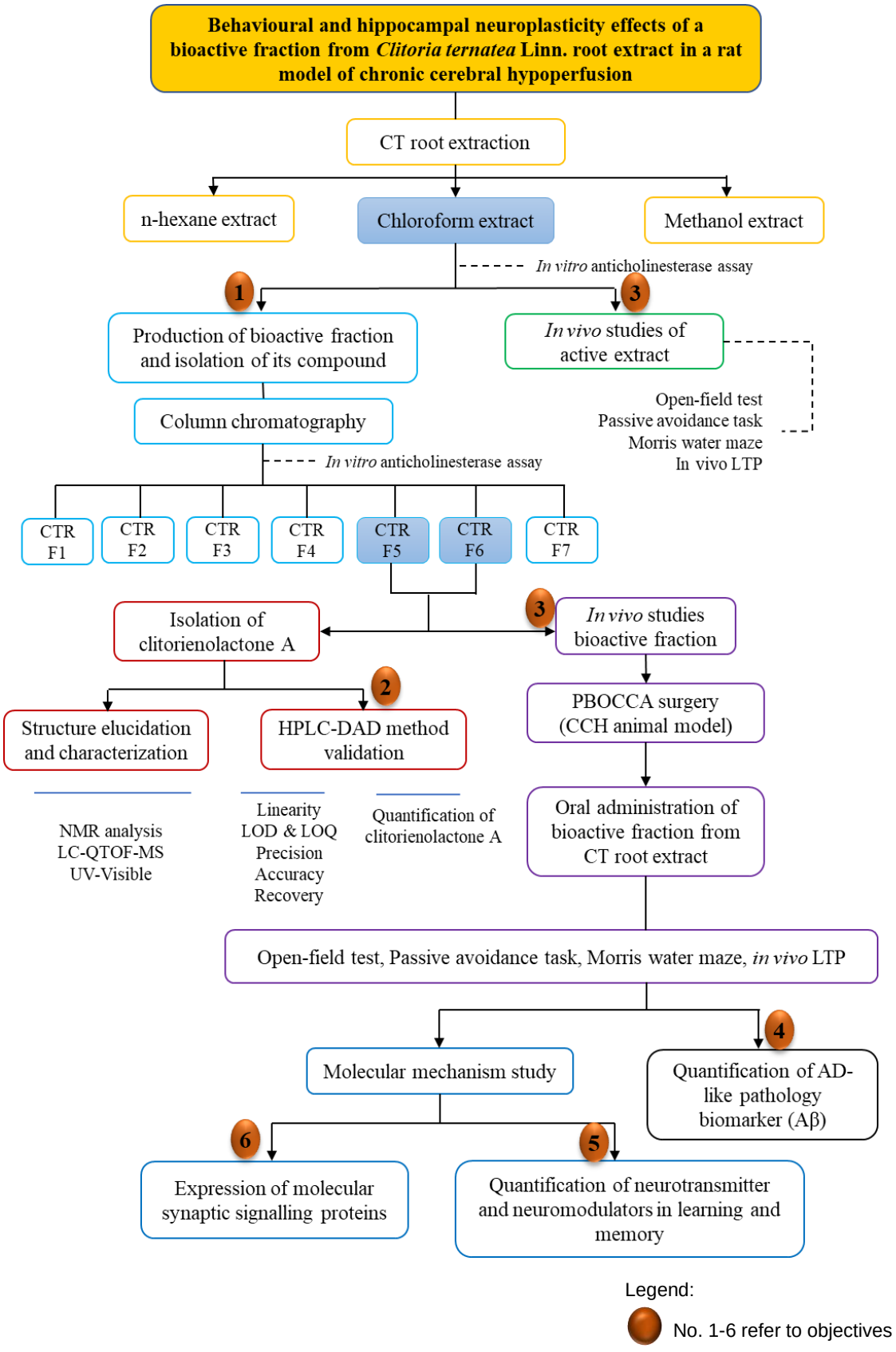

Figure 1.1: Thesis workflow

CHAPTER 2

LITERATURE REVIEW

2.1 Vascular dementia (VaD)

Vascular dementia (VaD), which is frequently associated with Alzheimer's disease (AD), is the second most common form of dementia in elderly persons and is thought to be caused by vascular factors. VaD is caused by various vascular aetiologies that damage the brain's blood vessels and impair their capability to produce the brain with sufficient oxygen and nutrients (Román et al., 2004). One of the primary hallmarks of VaD, which is characterised by cognitive decline, is multiple minor infarcts linked to hypertension. Identifying clinical subgroups of VaD was a crucial step in developing current vascular aetiology-based pathological classification systems. Several years ago, the concepts and characterizations of dementia and cognitive impairment (CI) caused by cerebrovascular diseases were investigated comprehensively (Khan et al., 2016).

VaD is a condition that can be caused by ischemia or haemorrhagic events. The cognitive syndrome is characterised by impaired social or physical performance (Román et al., 2004). The term "vascular cognitive impairment" (VCI) refers to any disease that has a vascular origin or reduces brain blood flow. It also includes a gradual, progressive clinical course as well as the contribution of vascular diseases (O'Brien et al, 2003).

The investigation of neuropathological changes has enabled the clinical diagnostic criteria for cerebrovascular diseases associated with cognitive impairment to be refined (Hachinski et al., 2006). Diagnosis of VaD or VCI pathologically involves a crucial examination of clinical or phenotypic characteristics and event

timing. Neuropathological alterations are difficult to define due to their diverse location and association with other neurodegenerative diseases. The vascular blockage presence of bleeding, the distribution of arterial regions, and the arteries' size contribute to memory impairment and lead to VaD. Numerous brain areas have been implicated with VaD, including the hippocampus (Khan et al., 2016).

2.1.1 Neurochemical changes of VaD

VaD is linked to a variety of cellular signalling and regulatory pathways, such as apoptosis, autophagy, oxidative stress, and inflammation. Patients with dementia exhibit a preferentially suppressed neuro-inflammatory response, consistent with vascular pathogenesis instead of Alzheimer's disease. According to reports, VaD is undergoing transmitter-specific modifications. Choline acetyltransferase activity was decreased in patients with multi-infarct dementia or VaD (Sharp et al., 2009). Separately, the activities of choline acetyltransferase were reduced significantly in the frontal and temporal cortices of people with cerebral autosomal arteriopathy with subcortical infarcts, ranging from 60 % to 70% degradation. (Keverne et al., 2007). Additionally, patients with VaD who also concurrent AD pathology have a more marked decline in cholinergic function (Román & Kalaria, 2006). On the other hand, infarct dementia was associated with a significant increase in cholinergic activity in the frontal brain (Sharp et al., 2009). Monoamine deficiencies, including 5-hydroxytryptamine (5HT), have been observed in patients with VaD's in basal ganglia and neocortex (Gottfries et al., 1994). However, to compensate for the 5HT deficiency, 5-HT(1A) and 5-HT(2A) receptors appear to be elevated in the temporal cortex of patients with multi-infarct VaD (Elliott et al., 2009), revealing distinctions in the brain chemical pathology of VaD syndromes and implying the possibility of manipulating VaD with new therapeutics.

2.1.2 Treatment of VaD

Treatment for VaD includes nootropics, vasodilators, antithrombotic agents and various natural products had been studied. However, these drugs have had mostly negative results in the clinical phase (Romàn, 2000). Studies have shown that propentofylline, a glial modulator, can help patients with dementia with cognitive impairment. After 48 weeks, there was a significant improvement compared to the placebo group (Kittner et al., 1997). Another promising and well-tolerated treatment is memantine, a moderate affinity non-competitive N-methyl-D-aspartate receptor antagonist. On the other hand, memantine has been shown to improve the MMSE (mini-mental state examination), functional levels, and care reliance of patients with VaD compared to placebo group. However, the cognitive therapy effect of memantine was found to be significantly greater in patients suffering from small vessel disease. (Wilcock et al., 2002).

There is conclusive evidence that cholinergic pathways play a part in the development of VaD. The post-mortem activity of choline acetyl-transferase was significantly lower in VaD patients in various brain areas, including the caudate putamen, hippocampus, and temporal lobe. This translates to a 40 % loss of the cholinergic system rather than a 70 % loss in AD patients (Court & Perry, 2003). In a separate study, the decrement of cholinergic biomarkers such as acetylcholine was found in the hippocampus, neocortex, and cerebrospinal fluid of stroke-prone spontaneously hypertensive rats, which well correlate with learning and memory decline (Kimura et al., 2000). Similar to Alzheimer's disease therapy, it is reasonable to speculate that enhanced cholinergic transmission may be a rational approach to treating VaD. As a result, three known acetylcholinesterase inhibitors were approved to be used for the treatment of Alzheimer's disease: donepezil (Black et al., 2003;

Wilkinson et al., 2003), rivastigmine (Moretti et al., 2003), and galantamine (Erkinjuntti et al., 2002) (Table 2.1).

Table 2.1: Summary of vascular dementia treatment in clinical phase

Drugs	Mode of action and benefit	References
Memantine	NMDAR antagonist receptors; improve cognition and behaviour	Wilcock et al., 2002; Areosa et al., 2005
Galantamine	Cholinesterase inhibitor; increase transmission of acetylcholine; improve cognition and behaviour	Erkinjuntti et al., 2002; Bullock et al., 2004
Donepezil	Cholinesterase inhibitor; increase transmission of acetylcholine; improve cognition and behaviour; most significant drug for the treatment of dementia	Black et al., 2003; Wilkinson et al., 2003
Rivastigmine	Cholinesterase inhibitor; increase transmission of acetylcholine; improve cognition and behaviour	Moretti et al., 2003

2.2 Chronic cerebral hypoperfusion

Chronic cerebral hypoperfusion or CCH is a critical mechanism underlying the initiation of vascular disorders associated with high blood pressure, hyperglycemic, and coronary heart disease. These factors affect the cerebral vascular system, decreasing blood flow to the brain (Meyer et al., 2000; Valerio Romanini et al., 2013). CCH's significance in dementia has risen to the fore in neurology research (Ruitenberg et al., 2005; Roh & Lee, 2014). Patients with mild to severe intracranial artery stenosis experience a more rapid decline in cognitive and functional abilities than those without the condition (Zhu et al., 2014). Previous research suggested that CCH may contribute to neurodegeneration by causing neuronal physiological failure,

the formation of reactive oxygen species, and the release of pro-inflammatory cytokines by activated microglia, all of which damage neurons and contribute to white matter lesions. Several studies have been conducted (Kitagawa et al., 2005; Farkas et al., 2007; Bang et al., 2013; Ahad et al., 2020). As a result of the preceding findings, it can be hypothesised that CCH may cause the cognitive impairment associated with cerebrovascular disease.

2.2.1 Possible mechanisms of CCH to neurodegenerative diseases

VaD is a significant contributor to dementia disorder. It is characterised as having difficulties in planning, judgement and memory, and reduced blood flow to the brain and subsequently caused neuronal death. VaD is challenging to diagnose owing to the large number and variety of lesions and their sites in the brain. Chance factors for vascular illnesses such as stroke, hypertension, high cholesterol, and smoking increase the risk of VaD. As a result, reducing these risk factors can help reduce the likelihood of having VaD. There are several possible mechanisms of induction of CCH towards neurodegenerative diseases, such as VaD and AD: (1) oxidative stress, (2) synaptic dysfunction, (3) neurodegeneration, (4) accumulation of amyloid-beta aggravation, (5) phosphorylation of tau protein, and (6) white matter lesion and activation of glial cell (Figure 2.1).

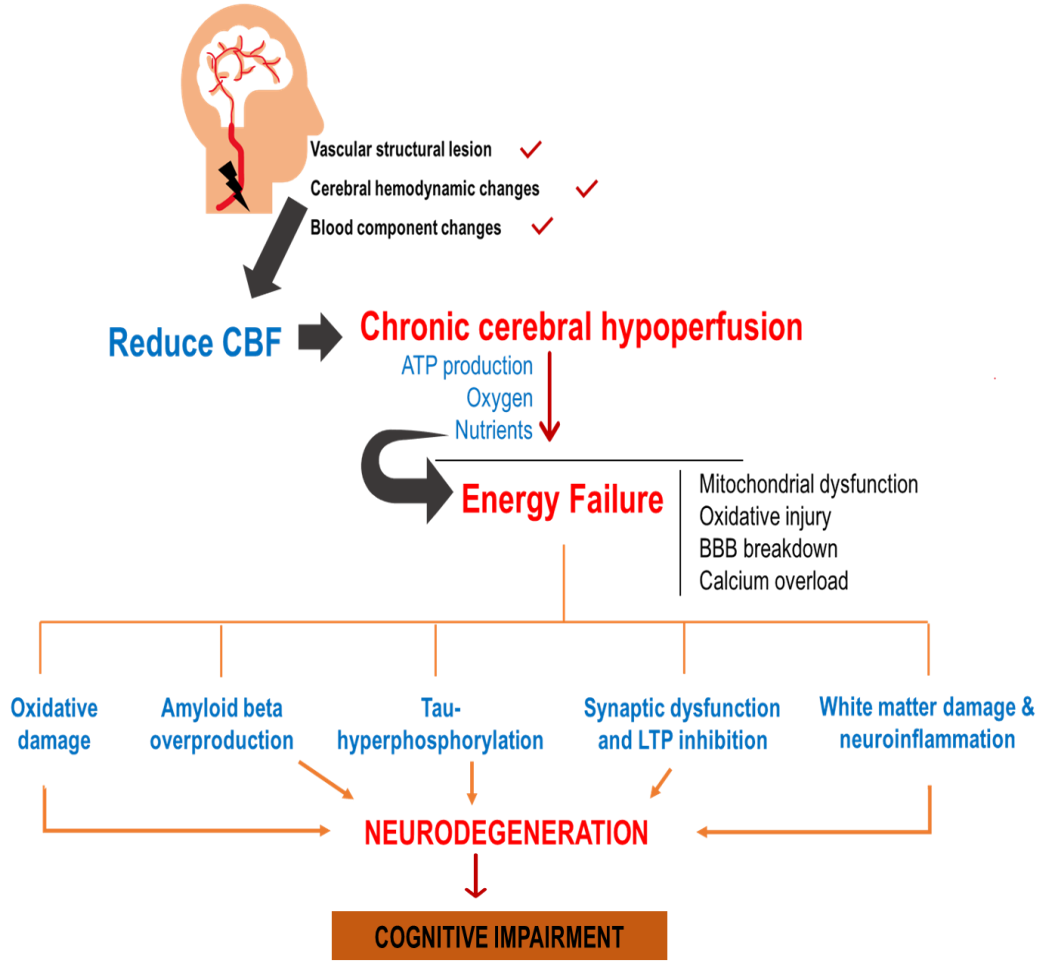

Figure 2.1: Summary of the possible mechanism by which CCH promotes/causes neurodegeneration. CCH can initiate several pathways that result in oxidative stress, amyloid-beta overproduction, hyperphosphorylation of tau, synaptic dysfunction, white matter lesion, and neuroinflammation. Adapted from Zhao & Gong, 2015, *Cellular and Molecular Neurobiology*, 35(1), 101–110.

2.2.1(a) Oxidative stress

Oxidative stress occurs when the systemic expression of reactive oxygen species exceeds the capacity of the biological process to detoxify the reactive intermediates or repair the damage rapidly. Degradation of neuronal cells can cause harm by generating peroxides and free radicals, which can wreak havoc on virtually all cell components, including proteins, lipids, and DNA. Numerous human disorders, including Alzheimer's disease, are caused by oxidative stress (Valko et al., 2007; Dean et al., 2011). CCH may impair mitochondrial function and suppress protein synthesis,

upsetting the balance of antioxidants and reactive oxygen species and resulting in oxidative damage (Orsucci et al., 2013). According to Liu et al. (2012), they discovered an increase in malondialdehyde, a lipid peroxidation end product, caused spatial learning and memory deficits following three-vessel occlusion. In another study, permanent bilateral occlusion of the common carotid arteries (PBOCCA) resulted in cholinergic dysregulation. It leads to oxidative damage, which is associated with learning and memory impairment (Xi et al., 2014). Taken together, it is hypothesised that oxidative injury may play a role in neurodegeneration.

2.2.1(b) Synaptic dysfunction

Synapses are such primary components of transmitting information between neurons. They are made up of the presynaptic, synaptic cleft, and postsynaptic membranes. The brain's normal functioning, including cognitive performance, is dependent on synaptic integrity. Numerous synaptic proteins can be used to investigate synaptic integrity, including synapsin, synaptophysin, and post-synaptic density protein (PSD) 95. In the brains of VaD patients, changes in the levels of these synaptic proteins have been observed (Sinclair et al., 2015). A previous study reported a decrease in PSD-95 and synaptophysin levels in the brain of a rat five weeks after PBOCCA surgery, most notably in the hippocampus (Wang et al., 2010). Thus, these synaptic changes may underpin memory impairment on a biological level.

Long-term potentiation (LTP) in the CCH rats' model can be used to study the CCH-induced impairment of neuronal plasticity. *In vivo* hippocampal synaptic plasticity done by Damodaran et al. (2018) showed significant LTP impairment following PBOCCA-induced CCH. Similar finding by Azam et al. (2018), rats that underwent PBOCCA surgery impaired the hippocampal synaptic plasticity.

Altogether, it can be concluded that induction of CCH causes alteration in synaptic transmission due to loss of neuronal integrity, subsequently leading to cognitive impairment.

2.2.1(c) Neurodegeneration

Synaptic and neuronal damage in Alzheimer's disease is closely related to the severity of dementia symptoms and has also been observed following chronic cerebral hypoperfusion (Wang et al., 2010; Zhao et al., 2014). For example, after 27 weeks of induction, chronic cerebral hypoperfusion in animal models revealed apoptotic morphology in hippocampal pyramidal neurons. Indeed, the degree of working memory impairment is strongly related to the number of apoptotic neurons in the hippocampus, suggesting that apoptotic loss of pyramidal neurons may contribute to the memory impairment correlated with CCH (Yoshioka et al., 2011; Hattori et al., 2014; Lee et al., 2020). Furthermore, hippocampal atrophy with pyknotic and apoptotic cells was observed in the brain after eight months of CCH induction by PBOCCA. (Nishio et al. 2010).

The neuronal loss in the CA1 regions, along with degradation of acetylcholine levels in the cortex and striatum, were observed following four months of PBOCCA surgery. As a result, the neuronal loss caused by CCH may result from a prolonged period of neuronal excitation (Ni et al. 1995). In addition, fluoro-Jade staining reveals a significant number of degenerative neurons in the hippocampus and cerebral cortex of CCH rats after unilateral typical carotid arteries occlusion (UCCAO), particularly in the granule cells of the crest of the dentate gyrus (Zhao et al. 2014). Additionally, this study indicates that the dentate gyrus is the region that is the most susceptible to CCH-induced neurodegeneration. Interestingly, they found that CCH-induced

neurodegeneration may be triggered or exacerbated by abnormal tau hyperphosphorylation.

2.2.1(d) Accumulation of amyloid-beta aggravation

Amyloid-beta **(**Aβ), a 36–43 amino acid peptide, is the primary component of the amyloid plaques observed in the AD brain. Aβ is produced via proteolytic cleavage of amyloid precursor protein (APP) by γ -secretases. Aβ pathological deposition occurs in AD patients' brain parenchyma, vascular structure, and transgenic animal models with APP mutations (Kokjohn & Roher, 2009; Elder et al., 2010). CCH has been reported to accelerate the deposition of Aβ. CCH generated by PBOCCA can promote Aβ fibrillisation and deposition in the intracellular compartment, accelerating the degenerative alterations associated with AD in transgenic mice (APPSwe/Ind) one month after PBOCCA (Kitaguchi et al., 2009). Numerous studies have demonstrated that CCH and other hypoxia-induced circumstances enhance APP processing by β- and γ-secretases (Zhiyou et al., 2009; Koike et al., 2010; Pluta et al., 2013). A proposed mechanism by which CCH enhances APP processing and results in Aβ accumulation is that it promotes HIF-1 (hypoxic-inducible factor 1) expression, which subsequently binds to the promoter of β-secretase, increasing its expression (Zhang et al., 2007).

2.2.1(e) Hyperphosphorylation of tau

The tau protein, which is associated with microtubules, becomes excessively hyperphosphorylated in the brains of people with neurodegenerative illnesses, commonly referred to as tauopathies. It has been established that aberrant tau hyperphosphorylation is required for neurodegeneration in AD and, most likely, other tauopathies (Iqbal et al., 2013). Numerous variables can trigger tau hyperphosphorylation. One of these causes could be CCH-induced impairment of

brain glucose metabolism, as this impairs tau O-GlcNAcylation (post-translational modification of intracellular proteins), resulting in tau hyperphosphorylation (Liu et al., 2009). Furthermore, hyperphosphorylation of Tau and increased Aβ production appear to be highly susceptible to cerebral hypoperfusion. According to Koike et al. (2010), a single modest cerebral hypoperfusion had dramatic and long-lasting effects on tau hyperphosphorylation and Aβ overproduction in transgenic AD disease models in animal. Thus, it is plausible to infer that repeated transient cerebral ischemia may play a role in AD development.

2.2.1(f) Glial activation and white matter lesion

Both human and animal models of CCH show white matter degeneration and glial cell activation (Fernando et al., 2006; Scherr et al., 2012). The greater the area of white matter lesions, the greater the extent of ischemia damage (Kitaguchi et al., 2009). In rat models, the corpus callosum appears to be more severely damaged by CCH than the striatum. CCH has been shown to activate more glia in the corpus callosum compared to the control group (Yoshizaki et al., 2008). Damage to the brain's white matter is directly linked to the activation of the brain's glial cells (Nakaji et al., 2006). CCH-induced white matter damage has been linked to two possible mechanisms: constant cerebral ischemia damages the white matter by increasing the oxidative stress and reactive oxygen species levels (Shibata et al., 2004). It also damages the blood-brain barrier, allowing inflammation to enter the brain parenchyma and setting off an immune response that results in the release of numerous serine proteases. Second, CCH (Farkas et al. 2005).

2.2.2 Animal model of CCH

Animal models are an effective tool to discover mechanisms and therapeutic strategies to combat human illnesses. In recent decades, several animal models of stroke have been employed intensively to discover ischemia processes and therapeutic development (Liu et al., 2009). There are two types of ischemic: global and focal ischemic stroke. Compared to global ischemia, focal ischemia is more appropriate for representing human stroke. On the other hand, global ischemic is relevant in global brain damage caused by cardiac arrest and resuscitation. These models are created through mechanical occlusion of blood vessels as shown in Figure 2.2 or various embolisation techniques (Li & Zhang, 2021)

Pulsinelli and Brierley (1979) developed the 4VO model in unanesthetized rats. They discovered bilateral hemisphere ischemic brain damage with highly predictable brain damage. In a separate study, the 4VO model for forebrain ischemia using the anatomical foundation for the vertebral artery was found to be highly repeatable (Toda et al., 2002). The model has numerous advantages, including ease of preparation, a high predicted ischemia neuronal injury rate, and a low frequency of seizures. The most serious drawbacks are the lengthy time required to complete a two-stage surgical procedure and the permanent occlusion of vertebral arteries. Furthermore, animals require enhanced postoperative care due to the high death rate and frequent complications (Panahian, 2001).

In 1972, the 2VO model was initially introduced by Eklof and Siesjo in slightly anaesthetised rats and had been modified on several occasions since (Eklöf & Siesjö, 1972). Permanent blockage of bilateral carotid arteries might serve as a model for neurodegenerative disorders associated with persistent cerebral hypoperfusion

(Bayat & Haghani, 2017). According to Raval et al. (2009), induction of 2VO induced global cerebral ischemia results in histopathological damage to the susceptible areas in the brain that are vulnerable to hypoxic, including the hippocampus, striatum, and neocortical regions. On the other hand, chronic cerebral hypoperfusion by permanent ligation of carotid arteries may impair cognitive behaviour and synaptic plasticity inhibition. For example, Damodaran et al. (2014) showed significant spatial learning and memory impairment after four weeks of 2VO surgery. The result is consistent with the synaptic plasticity inhibition following induction of 2VO induced CCH (Azam et al., 2018; Tiang et al., 2020). The advantages of utilising the 2VO model include the following: (1) one-stage surgery; (2) highly reproducible ischemic damage; (3) high animal survival rate for studies of reperfusion injury; and (4) suitability for studies of physiological, molecular, biochemical, and behavioural changes during the post-ischemic phase.

The middle cerebral artery (MCA) and its branches are estimated to be responsible for approximately 70 per cent of infarcts in humans with ischemic stroke. Occlusion of the MCA (MCAo) is most commonly used in rodent models of ischemic stroke, as demonstrated by 40% of more than 2,600 studies on neuroprotection following ischemic stroke (Fluri et al., 2015; Wang et al., 2020). Because it does not necessitate a craniectomy, the MCAo model is technically less invasive. The common carotid artery (CCA) is temporarily blocked, a suture is inserted directly into the internal carotid artery (ICA), and the suture is advanced until it cuts off blood flow to the middle cerebral artery (MCA) (Fluri et al., 2015). MCAo induces focal ischemic after 30 minutes, which causes a striatal infarct, as this part of the brain is more susceptible to ischemic damage. After an hour of MCAo, the striatum and cortex are damaged. 40% of hemispheric infarcts have been found after 24 hours of MCAo.

Using this model has the advantages of mimicking human ischemic stroke, reproducibility, low invasiveness, and clearly defined ischemic lesion location (Fluri et al., 2015).

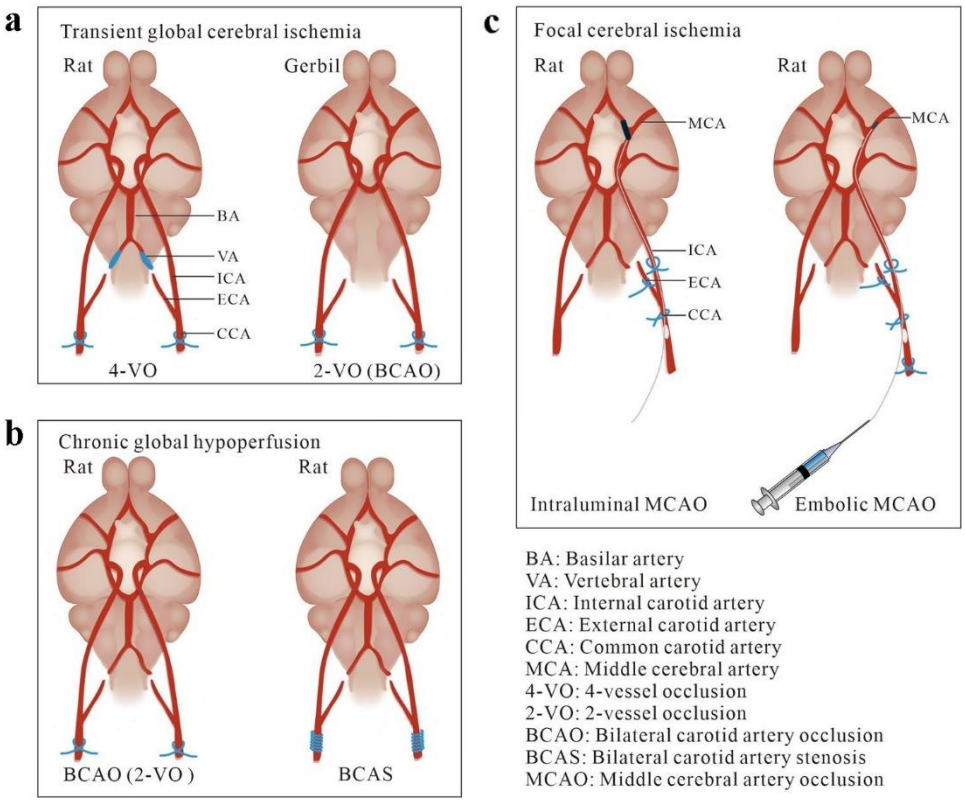

Figure 2.2: Schematic diagram of chronic cerebral hypoperfusion rat model. a) The transient global cerebral ischemia by 4VO or 2VO, b) chronic cerebral hypoperfusion by 2VO, and c) focal cerebral ischemia by MCAO. Adapted from Tuo et al., 2021, *Journal of Molecular Neurosciences*, 71, 1.

2.3 Learning and memory

Cognitive function is a broad word that encompasses a variety of functions, including memory, association, language, attention, idea generation, and problem-solving. There are two types of memories: short-term (working memory) and long-term memory as shown in Figure 2.3. Short-term memory is limited in capacity and only lasts a few seconds to a minute. In contrast, long-term memory can store a more significant amount of information for an indefinite time. Long-term memory can be

classified into two types which are explicit (declarative) and implicit (non-declarative). Declarative memory responds to the inquiry "what," and it encompasses knowledge of facts such as locations, objects, and people, as well as their meanings (Brem et al., 2013).

Moreover, declarative memory is further subdivided into episodic and semantic memories. Declarative memory defines as subjectively experienced events that are context-specific, such as time and location. In comparison, semantic memory refers to knowledge of these facts taken independently of the context they were acquired (Sharma et al., 2010). The hippocampus area (CA fields, dentate gyrus, and subicular complex) is part of a system that physically connects structures in the medial temporal lobe critical for mammalian memory (Squire et al., 2004). In humans, primates, and rodents, injury to this area decreases performance on various learning and memory tests (Eichenbaum & Cohen, 2001).

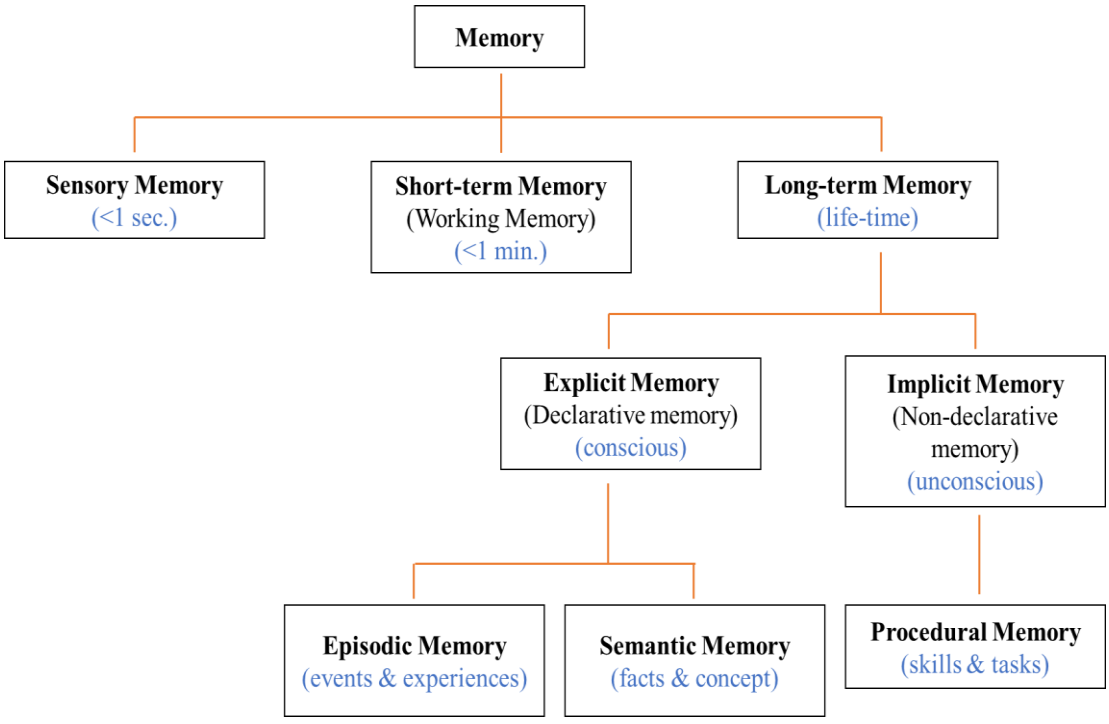

Figure 2.3: Classification of memory. Adapted from Brem et al., 2013, *Handbook of Clinical Neurology*, 116, 693–737.

2.3.1 Behavioural test for learning and memory

The most noticeable symptom of neurodegenerative diseases is the increasing loss of cognitive integrity caused by the hippocampus's loss of neurons and synapses. As a result, the capacity of an actual neurodegenerative animal model to reliably replicate the behavioural alterations observed in human patients is a "must-have" attribute. Behavioural activities in animals are often separated into associative and operant learning tasks for studying cognition. Cues in the environment are used in associative learning tasks to condition a specific response in the animal. The type of memory being examined is used to classify cognitive activities into further categories: (1) spatial memory (Morris water maze, radial arm maze), (2) contextual memory (passive avoidance task, fear conditioning), (3) working memory (Y-maze, T-maze), (4) novelty (object recognition), and (5) Motor and exploratory activities (open-field test) (Bryan et al., 2009). The present study has focused on the motor and exploratory activity, passive avoidance task and Morris water maze, which will be described in detail in the following sub-chapter.

2.3.1(a) Open-field test

An open-field test (OFT) is commonly used to assess motor function in an open field by measuring spontaneous activity. Several variables can be measured, including distance travelled, time spent moving, rearing, and change in activity over time. Some results, such as faeces, centre time, and activity during the first 5 mins, are likely to indicate emotionality, such as anxiety (Hrnkova et al., 2007; Kraeuter et al., 2019). The arena of OFT can take any geometric shape, although the most frequent are circular and rectangular. The box can be made of any washable material, including

metal, plastic, or plywood. A standard size (for rats) is 50 centimetres high, 40 x 60 centimetres rectangular, or 40-60 centimetres circular (Seibenhener & Wooten, 2015).

2.3.1(b) Passive avoidance task

Passive avoidance is a similar type of learning task to contextual fear conditioning in that it is based on associative emotional learning (LeDoux, 2000). On the other hand, the step-through task is a single-trial emotional memory experiment that incorporates fear conditioning and an instrumental response, such as an animal's active choice, to avoid entering a dark compartment associated with an aversive event. Passive avoidance acquisition is quantified by a significant increase or decrease in step-through latency. Thus, passive avoidance is straightforward, as both the safe and noxious compartments are well defined, as is the appropriate adaptive response is to avoid entering the dark chamber (Ögren & Stiedl, 2010).

2.3.1(c) Morris water maze

The Morris water maze was created to evaluate spatial or location learning and will be referred to as the Morris water maze throughout this section (MWM). Morris first described the fundamental processes in 1984 and later added information and procedures for evaluating related forms of learning and memory (Morris, 1984). MWM performance has been used to investigate learning and memory and the relationship between MWM performance, neurotransmitter systems, and medication effects. In addition, MWM performance is related to long-term potentiation (LTP) and NMDA receptor activity; it is a critical technique for studying hippocampal circuitry (Sullivan, 2010).

2.3.2 Neurotransmitters and neuromodulators involved learning and memory

As highly specialised human brain processes, learning and memory entail a complex combination of neurotransmitters and cellular events. The most frequently accepted learning and memory model entails attention, acquisition, storage, and retrieval. Each of these actions requires the interaction of neurotransmitters such as dopamine, acetylcholine, norepinephrine, glutamate, gamma-aminobutyric acid (GABA), and serotonin though specific neurotransmitters have been shown to predominate. In addition, long-term memory development is a result of neuroplastic cellular activities. Understanding the mechanisms of learning and memory facilitates drug discovery and aids in comprehending the activities of various currently available medications (Myhrer, 2003).

This thesis has focused on the transmission of glutamate, acetylcholine, GABA, serotonin and epinephrine systems modulating learning and memory, which have been highlighted in the Figure 2.4. The details of the highlighted neurotransmitters as discussed in the following section.

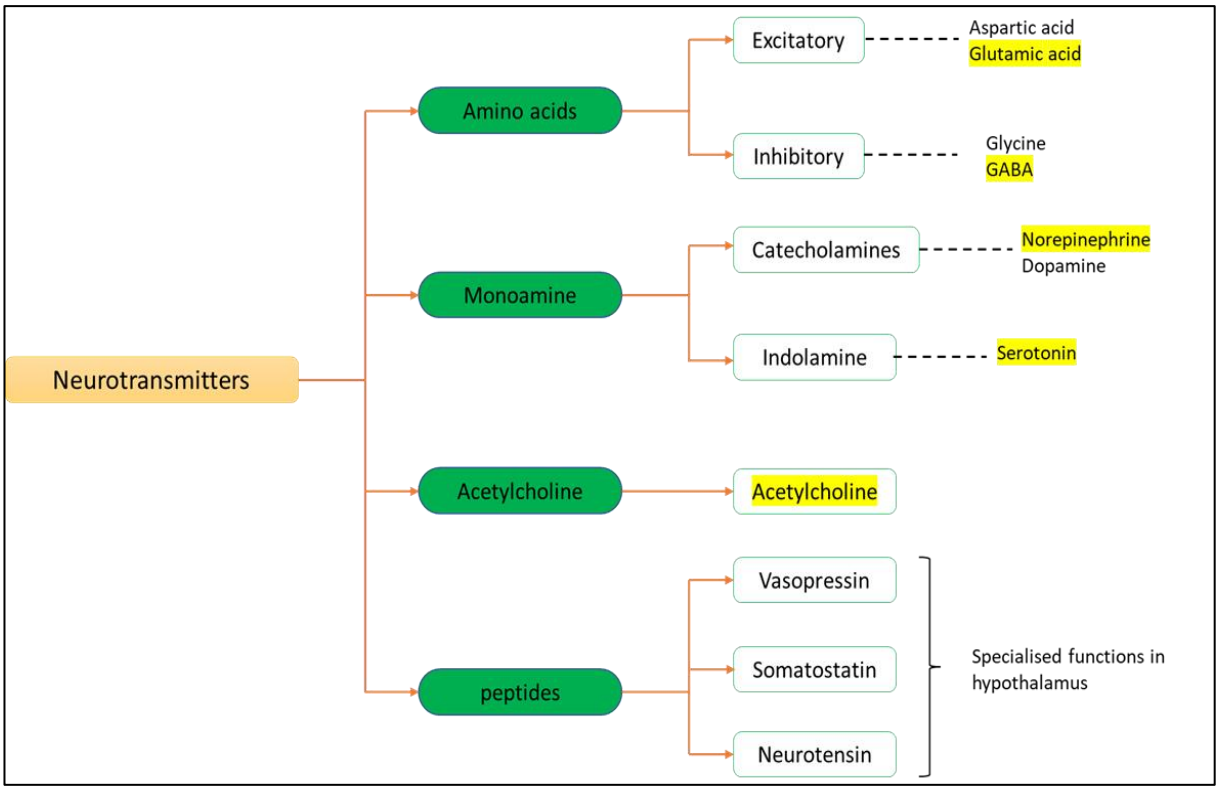

Figure 2.4: Classification of the neurotransmitters. Adapted from Myhrer, 2003, *Brain Research Review*, 41(2-3), 268–287.

2.3.2(a) Glutamatergic system

Glutamate, an excitatory amino acid, is the most abundant amino acid transmitter in the central nervous system (CNS), important in learning and memory. Glutamate binds to both ionotropic and metabotropic glutamate receptors (iGluRs and mGluRs). iGluRs mediate rapid transmission. They are classified into three subtypes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainic acid receptors (Traynelis et al., 2010). mGluRs mediate slower transmission and are more modulatory by their very nature. mGluRs are thought to have seven transmembrane areas in common with other G protein-coupled receptors (Crupi et al., 2019). The role of glutamate in memory is mainly involved with LTP, a memory storage mechanism. Bliss & Lomo (1973) were first described LTP as a synaptic and cellular mechanisms model that may underpin memory