STUDIES OF *CLITORIA TERNATEA* LINN. ROOT EXTRACT ON BEHAVIOURAL EFFECTS AND *IN VIVO* HIPPOCAMPAL SYNAPTIC PLASTICITY IN A RAT MODEL OF CHRONIC CEREBRAL HYPOPERFUSION

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celsius
β	Beta
>	Greater than
±	Plus minus
=	Equal to
n	Number of animals
R^2	Correlation coefficient
x	Multiplication
<	Lesser than
A	Ampere
a.m.	Ante meridiem
et al.	And other
eV	Electron volt
cm	Centimetre
g	Gram
g/L	Gram per litre
h	Hour (s)
Hz	Hertz
i.e	That is
L	Litre
m/z	Mass to charge ratio
mA	Miliampere
m	Metre

mm	Millimetre
mL	Millilitre
ms	Milliseconds
min	Minute (s)
mg/mL	Milligram per millilitre
mg/kg	Milligram per kilogram
mL/kg	Millilitre per kilogram
mL/min	Millilitre per minute
mmol/L	Millimole per litre
µl	Microlitre
µmol/L	Micromole per litre
µg/mL	Microgram per millilitre
U/L	Units per litre
s	Second(s)
ACh	Acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
ALP	Alkaline phosphatase
ALT	alanine aminotransferase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Anteroposterior
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBB	Blood-Brain Barrier
BCAS	Bilateral common carotid artery stenosis

BDNF	Brain-derived neurotropic factor
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
CA1	Cornu Ammonis 1
CaMKII	Ca^{2+} /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CBF	Cerebral blood flow
CCH	Chronic cerebral hypoperfusion
ChAT	Choline acetyltransferase enzyme
CID	Compound identification
CNS	Central nervous system
CREB	cAMP response element binding protein
CT	*Clitoria ternatea* Linn.
DAB	3, 3'-diaminobenzidine
DPX	Di-N-Butyle Phthalate in Xylene
DSM	Diagnostic and Statistical Manual
DTNB	(5,5'-Dithiobis(2-nitrobenzoic acid)
EI	Electron ionization
E-LTP	Early LTP
ERK	Extracellular regulated kinase
FDA	Food and Drug Administration
fEPSP	Field excitatory post synaptic potential
GC-MS	Gas chromatography-Mass Spectrometry
GFAP	Glial fibrillary acidic protein
HDBB	Horizontal limb of the diagonal band of Broca
H & E	Hematoxylin and eosin

HFS	High-frequency stimulation
HPTLC	High performance thin layer chromatography
ICH	International Conference on Harmonisation
IL-1β	Interleukin-1β
I-LTP	Initial LTP
i.p.	Intraperitoneally
LD_{50}	Lethal Dose 50
L-LTP	Late LTP
LOD	Limit of detection
LOQ	Limit of quantification
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
ML	Mediolateral
MMP	Matrix metalloproteases
NBM	Nucleus basalis magnocellularis
NIST	National Institute of Standard and Technology
NMDA	N-methyl-D-aspartate
OD	Optical density
OECD	Organization for Economic Co-operation and Development
Oxo	Oxotremorine
PBOCCA	Permanent, bilateral occlusion of the common carotid arteries
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
pH	Potential of Hydrogen
PIP_2	Phosphatidylinositol 4, 5-biphosphate

PKA	Protein kinase A
PKC	Protein kinase C
Physos	Physostigmine
p.o.	Per os
CV	Coefficient of variation
RE	Relative error
ROS	Reactive oxygen species
Scopo	Scopolamine
SD	Sprague Dawley
SEM	Standard error mean
SIM	Selected ion monitoring
STM	Short-term memory
TBS	Theta-burst stimulation
TNF-α	Tumor necrosis factor alpha
UCCAO	unilateral common carotid artery occlusion
USM	Universiti Sains Malaysia
V	Ventral
VAChT	Vesicular acetylcholine transporter
VaD	Vascular dementia
Veh	Vehicle
v/v	Volume over volume
w/w	Weight over weight

KAJIAN MENGENAI EKSTRAK AKAR *CLITORIA TERNATEA* LINN. KE ATAS KESAN-KESAN TINGKAH LAKU DAN *IN VIVO* KEPLASTIKAN SINAPTIK HIPOKAMPUS DI DALAM MODEL TIKUS SEREBRUM HIPOPERFUSI KRONIK

ABSTRAK

Clitoria ternatea Linn. (CT) adalah tumbuhan asli yang berasal dari negara tropika seperti Malaysia. Bahagian akar tumbuhan telah dikaji secara meluas untuk aktiviti farmakologinya. Tetapi sumbangannya terhadap peningkatan pembelajaran dan memori serta *in vivo* keplastikan sinaptik di dalam model tikus serebrum hipoperfusi kronik (CCH) masih belum diteroka. Untuk tujuan kawalan kualiti, kandungan taraxerol (0.15% ± 0.01) dalam ekstrak metanol akar CT telah ditentukan menggunakan kaedah analisis kromatografi-jisim spektrometri gas (GC-MS) yang disahkan. Dalam penyelidikan ini, kaedah pembedahan oklusi dwisisi umum arteri karotid secara kekal (PBOCCA) telah digunakan untuk menghasilkan model CCH dalam tikus. Rawatan akut dan kronik (28 hari) ekstrak akar CT pada dos 200 dan 300 mg/kg, p.o. menyebabkan peningkatan dapatan semula memori dalam prosedur penghindaran pasif. Kesan-kesan ekstrak akar CT dinilai lagi dalam prosedur berselirat air Morris. Hasil kajian menunjukkan bahawa rawatan ekstrak akar CT (200 dan 300 mg/kg, p.o.) memulihkan gangguan pembelajaran spasial dan memori rujukan yang disebabkan oleh CCH. Rawatan kronik ekstrak akar CT (200 dan 300 mg/kg, p.o.) juga didapati mengurangkan kerosakan neuron yang disebabkan oleh CCH di bahagian CA1 hipokampus. Peningkatan aktiviti asetilkolinesterase (AChE) dalam korteks frontal dan hipokampus tikus PBOCCA telah dikurangkan secara ketara dengan rawatan ekstrak akar CT pada dos tinggi (300 mg/kg, p.o.). Dalam

penyelidikan keplastikan sinaptik, ekstrak akar CT (200 dan 300 mg/kg, p.o.) memulihkan penindasan *in vivo* hipokampus potensiasi jangka panjang (LTP) pada kolateral Schaffer CA3-CA1 sinaps yang diinduksi oleh CCH. Keputusan ini menunjukkan bahawa ekstrak akar CT boleh memberi kesan terhadap fungsi pembelajaran dan memori tikus PBOCCA melalui fasilitasi LTP. Ekstrak akar CT tidak menunjukkan peningkatan pada memori spasial dan LTP hipokampus dalam tikus PBOCCA yang telah dirawat dengan skopolamina (antagonis reseptor muskarinik; 1.0 mg/kg, i.p.). Dalam kajian ketoksikan, dos berulang ekstrak akar CT (100, 200 dan 300 mg/kg, p.o.) didapati selamat pada tikus PBOCCA selepas 28 hari rawatan. Kesimpulannya, ekstrak metanol akar CT memulihkan defisit pembelajaran dan memori serta gangguan *in vivo* keplastikan sinaptik di dalam model tikus CCH, dengan itu menyokong potensi terapeutik ekstrak akar CT dalam rawatan pembuluh demensia (VaD).

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ABSTRACT

Clitoria ternatea Linn. (CT) is a native plant in tropical countries like Malaysia. The root part of the plant has been studied extensively for its pharmacological activities. However, its contribution towards learning and memory enhancement together with *in vivo* synaptic plasticity in a chronic cerebral hypoperfusion (CCH) rat model has yet to be explored. For quality control purposes, taraxerol content (0.15% ± 0.01) in the methanolic extract of CT root was determined using validated gas chromatography-mass spectrometry (GC-MS) analytical method. In the present study, the permanent bilateral occlusion of common carotid arteries (PBOCCA) surgery method was employed to develop CCH model in rats. The acute and chronic (28 days) treatment of CT root extract at doses of 200 and 300 mg/kg, p.o. resulted in a significant enhancement in memory retention of the passive avoidance task. Effects of CT root extract were further assessed in the Morris water maze task. The results demonstrate that CT root extract (200 and 300 mg/kg, p.o.) treatment restored spatial learning and reference memory impairments induced by CCH. Chronic treatment of CT root extract (200 and 300 mg/kg, p.o.) was also found to diminish CCH-induced neuronal damage in the CA1 region of the hippocampus. An increased acetylcholinesterase (AChE) activity in the frontal cortex and hippocampus of the PBOCCA rats was significantly inhibited by the CT root extract at a high dose (300 mg/kg, p.o.). In synaptic plasticity study, CT root extract (200

and 300 mg/kg, p.o.) restored the CCH-induced *in vivo* hippocampal long-term potentiation (LTP) suppression at the Schaffer collateral CA3-CA1 synapse. These results indicate that CT root extract may affect learning and memory functions in PBOCCA rats via LTP facilitation. CT root extract (300 mg/kg, p.o.) did not show any improvement on spatial memory and hippocampal LTP in scopolamine (muscarinic receptor antagonist; 1.0 mg/kg, i.p.) pre-treated PBOCCA rats. In toxicity study, repeated doses of CT root extract (100, 200 and 300 mg/kg, p.o.) were found to be safe in PBOCCA rats after 28 days treatment. In conclusion, the methanolic extract of CT root extract restored the learning and memory deficits and *in vivo* synaptic plasticity impairment in a CCH rat model, thus supporting the therapeutic potential of CT root extract in the treatment of vascular dementia (VaD).

CHAPTER 1

INTRODUCTION

1.1 Background

Dementia, a brain disorder that affects the elderly, is characterized by the deteroriation of cognitive performance. The affected person will be unable to live independently. The most common form of dementia is Alzheimer's disease (AD). Next is vascular dementia (VaD), making up approximately 20% of all dementia cases. VaD occurs when vascular pathological alterations damage blood vessels and reduce blood flow to the brain. As a result, there is a gradual decrease in cognitive functions and behaviour. VaD is becoming more prevalent causing major health, social and economic issues. Drugs that are presently being used to counter this disease, memantine, galantamine and donepezil have shown modest therapeutic effects. But it should be noted that these drugs also create adverse effects (Baskys and Hou, 2007; Kalaria et al., 2008; Prince et al., 2013).

Since time immemorial, medicinal plants have been used to treat various diseases. Among them is the *Clitoria ternatea* Linn. (CT), a plant known to treat neurological disorders and enhance intellect. Locally, its name is 'bunga telang' (Butterfly pea) whilst in India, it is referred to as 'Shankapushpi' (Mukherjee et al., 2008). To date, there are a number of scientific studies on animal models employing CT root extract to improve learning and memory function in chemically or electroshock induced memory impairment conditions (Taranalli and Cheeramkuczhi,

2000; Vyawahare et al., 2006). However, these studies are insufficient to provide evidence-based use of the plant as a potential therapeutic agent for the treatment of VaD. In order to demonstrate its efficacy in treating VaD, a chronic cerebral hypoperfusion (CCH) animal model is required.

CCH is considered an important factor that leads to the decline in cognitive ability, paving the way for VaD to develop. The reduction of cerebral blood flow creates a condition of CCH that promotes neurodegeneration by activating a cascade of neuropathological events. This leads to neuronal cell damage and contributes to white matter lesions, cholinergic dysfunction and, subsequent cognitive decline, a trend observed in VaD (Swartz et al., 2003; Farkas et al., 2007; Liu and Zhang, 2012). Numerous studies have confirmed that permanent, bilateral occlusion of the common carotid arteries (PBOCCA) in rats is a well-established CCH model which could trigger pathophysiological changes in the brain resulting in neuronal damage, cholinergic dysfunction and cognition deficits that resemble VaD (Ni et al., 1995; Farkas et al., 2007; Liu and Zhang, 2012; Cechetti et al., 2012). Therefore in this thesis, detailed work was undertaken to explore the beneficial effects of CT root extract on learning and memory functions using a CCH rat model induced by PBOCCA surgery method.

1.2 Problem statement

VaD is the second most common cause of dementia that characterized by a progressive cognitive function decline. CCH is considered a notable risk factor that contributes to neurodegeneration and memory dysfunction in neurological diseases such as VaD. The discovery and development of effective treatment for this disease is challenging one. Unfortunately, no drug has been approved by FDA for VaD treatment. As VaD being a heterogeneous disease, herbal medicines which characterized by multicomponent and multitarget approach could provide viable therapies for VaD. *Clitoria ternatea* Linn. (CT) is an herbal plant that has been reported to treat neurological disorders and used as a memory booster in folk medicine. CT root extract has been shown to alleviate learning and memory functions in several animal models of memory impairment. In view of this, using a CCH animal model, the memory restoring potential of CT root extract was investigated as to whether this herbal plant extract could be a potential therapeutic strategy for treatment of VaD.

1.3 Scope of study

CT root extract has been reported to improve learning and memory functions in chemically (*i.e.* scopolamine and streptozotocin) or electroshock induced memory impairment conditions. Therefore; it seems that CT root extract may have positive effects on memory impairments in a rat model of CCH. In this present study, beneficial effects of CT root extract on learning and memory functions will be assessed using passive avoidance task and Morris water maze in CCH rat model

induced by PBOCCA surgery method. The behavioural performance is indirectly associated with motor function. In order to ascertain this possibility, the open-field test will be performed to examine the effect of CT root extract on spontaneous locomotor activity and exploratory behaviour in rats.

Synaptic plasticity is the cellular basis of learning and memory processes in the brain. The reductions in cognitive ability are often accompanied by synaptic plasticity impairment in neurodegenerative-related disorders such as VaD. It can be proposed that restoring synaptic plasticity dysfunction may result in greater cognitive improvements. Therefore, this study aims to determine the effect of CT root extract on hippocampal LTP inhibition in PBOCCA rats using *in vivo* electrophysiological recording.

Cognitive decline induced by CCH is associated with the degeneration of basal forebrain cholinergic neurons. Currently, enhancements of cholinergic transmission via cholinesterase inhibitors have become one of the potential therapeutic strategies for VaD treatment. In view of this, the present study aims to determine the effect of chronic CT root treatment on *ex vivo* cholinesterase activity in the brain samples. This study also further intended to investigate the involvement of the cholinergic system in mediating the effects of CT root extract against hippocampal-dependent spatial memory deficit in Morris water maze and LTP suppression in PBOCCA rats.

The rationale behind this study is based on the memory enhancing properties of CT root extract and growing evidences of its effectiveness in several animal models. Therefore, it can be hypothesized that CT root extract may help with CCH-

induced learning and memory impairment in PBOCCA rats. Overall, the findings discover from the present study would provide the basis for developing CT root extract as a potential therapeutic agent for the treatment of VaD.

1.4 Objectives

1. To quantify the amount of taraxerol in methanolic extract of CT root by using validated GC-MS method for quality control assessment.

2. To study the real time course of motor, and learning and memory functions following the CCH induced by PBOCCA method in several behavioural tests, *i.e.* open-field test, passive avoidance task and Morris water maze.

3. To evaluate the effects of acute and chronic (28 days) oral administration of CT root extract on motor activity, and learning and memory functions in PBOCCA rats using open-field test, passive avoidance task and Morris water maze.

4. To examine changes in neuronal damage and glial fibrillary acidic protein (GFAP)-positive astrocytes expression following chronic oral treatment of CT root extract in PBOCCA rats.

5. To determine the effects of chronic administration of CT root extract on *ex vivo* brain cholinesterase activity in PBOCCA rats.

6. To investigate the toxicity of chronic oral administration of CT root extract in PBOCCA rats.

7. To study the effects of CT root extract on synaptic plasticity (*in vivo* LTP) in hippocampal mediate learning in PBOCCA rats.

8. To elucidate the involvement of cholinergic system in mediating the effect of root extract on learning and memory processes in the Morris water maze task and *in vivo* hippocampal LTP.

CHAPTER TWO

LITERATURE REVIEW

2.1 Dementia

Globally, dementia is a chronic geriatric disease. A recent epidemiological study mentioned that in 2010, the number of aged people (>60 years) suffering from dementia was 35.6 million. This value is expected to increase to 65.7 million in 2030 and on to 115.4 million in 2050 respectively. Interestingly, most of these patients are from the low and middle income countries (Prince et al., 2013). In Malaysia, the number of people with dementia has been estimated to increase from 0.12 million people in 2015 to 0.59 million people by the year 2050 (Alzheimer's disease International Report, 2014).

In 2013, the American Psychiatric Association's Diagnostic and Statistical Manual (DSM-5) redefined "dementia, delirium, amnestic and other geriatric cognitive disorders" as "neurocognitive disorder". The DSM-5 diagnosis of major neurocognitive disorder corresponds to dementia, requires substantial impairment in one or more cognitive domains which interfere with independence in everyday activities (Diagnostic and Statistical Manual, 2013). Clinically, dementia is caused by brain dysfunction and characterized by a progressive cognitive function decline without impairment in consciousness (McKhann et al., 1984; Prince et al., 2013). Impairment of memory, thinking, comprehension, calculation, learning, language,

and judgement have been further linked to changes in emotional control, social behaviour and motivation (World Health Organization, 1992).

Dementia is caused by various underlying diseases and brain disorders. Therefore, each type of dementia is characterized by a specific group of signs, symptoms and underlying neuropathological features. AD, the most common form of dementia, manifests itself with the accumulation of amyloid plaques and neurofibrillary tangles in the brain (Ballard et al., 2011). VaD, the second most prevalent type results from various types of vascular-related diseases. The incidence rate of VaD has been reported to be 6 to 12 cases per 1000 people over 70 years of age annually (Hébert and Brayne, 1995; van der Flier and Scheltens, 2005). Apart from AD and VaD, diseases such as Lewy bodies (Byrne, 1997), Huntington's disease (Ho et al., 2003), Parkinson's disease (Zesiewicz at al., 2006), Multiple sclerosis (Chiaravalloti and DeLuca, 2008) and Human Immunodeficiency Virus (HIV) (Sacktor and Robertson, 2014) can lead to dementia. For the scope of the present thesis, VaD will be described here.

2.1.1 Vascular dementia

Vascular Dementia (VaD) is a progressive disease that affects human cognitive performance. In VaD, various vascular etiologies damage blood vessels and reduce blood flow to the brain, as a result causing deprivation of oxygen and essential nutrients and causes neuronal death. This then leads to cognitive impairment as seen in dementia patients. Symptoms seen in VaD patients are depression, forgetfulness, slow thinking, anxiety, and disorientation. In addition, VaD patients also lose their

abilities in problem-solving, working memory, planning, reasoning, thinking, judgement, and completing tasks (Venkat et al., 2015; Khan et al., 2016).

With VaD being a heterogeneous disease, there is a variation in its clinical presentation and cognitive profile depending on the origin and type of vascular occlusion, arterial territories network, haemorrhage, and size of vessel (Figure 2.1). As an example, subcortical dementia comes around through small vessel disease characterized by periventricular white matter ischemia and lacunar strokes. Cortical dementia on the other hand is caused by large vessel disease which can cause multiple cortical infarcts (Román, 2002; Khan et al., 2016). Strategic infarct dementia, some other forms of VaD, are caused by focal ischemia lesions at those parts of the brain which are associated with normal cognitive functions, and mixed dementia which is made up of both vascular and Alzheimer's lesion (Wallin et al., 2003; Erkinjuntti, 2008).

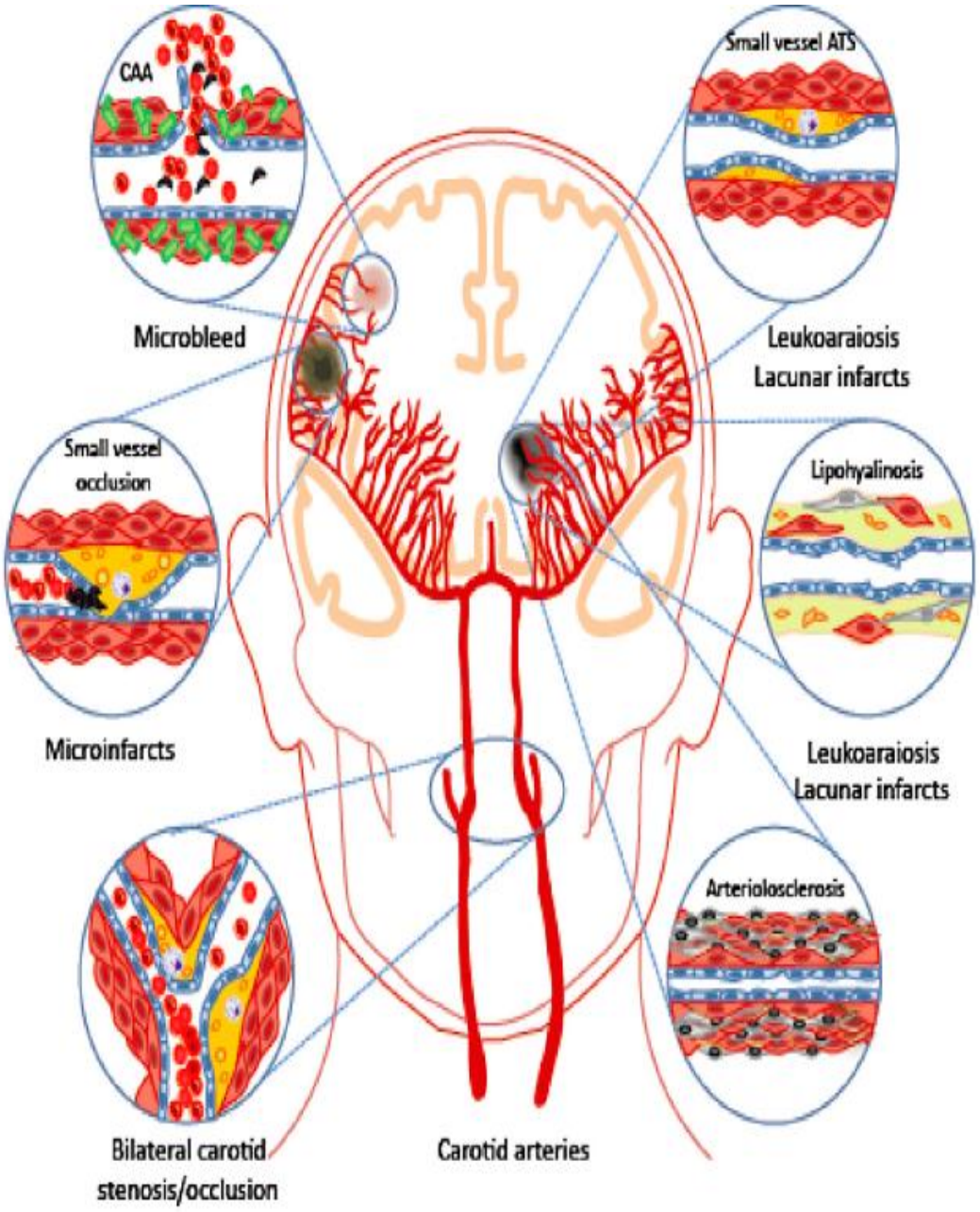

Figure 2.1: Vascular lesions leading to VaD.
Abbreviations: CAA, cerebral amyloid angiopathy; ATS: atherosclerosis. Reprinted with permission from The pathobiology of vascular dementia by Iadecola, C, 2013 Neuron, 80, p. 844-866. Copyright 2017 by Elsevier.

To date, there is no drug that has been approved by the Food and Drug Administration (FDA) for the treatment of VaD. Confounding this is the fact that there is a shortage of studies on arresting this disease. Presently, the mode of treatment is only by preventing the progression of cognitive deterioration in VaD patients. Memantine is an N-methyl-D-aspartate (NMDA) receptor antagonist, approved by FDA for AD treatment. Two studies reported that this drug improve cognitive functions in VaD patients (Orgogozo et al., 2002; Wilcock et al., 2002). However, there are side effects related to other NMDA receptor antagonists such as MK-801, phencyclidine, and ketamine. These cause hallucinations, drowsiness and cognitive loss. As a result, further clinical studies are underway to identify the efficacy of memantine in the treatment of VaD (Lipton, 2004; Baskys and Hou, 2007).

Clinical trials done using acetylcholinesterase inhibitors including donepezil, galantamine, rivastigmine and huperzine A have shown that there is an improvement in cognitive function and daily activities in patients suffering from mild to moderate VaD (Moretti et al., 2002; Erkinjuntti et al., 2002; Xu et al., 2012b). Three of these clinical trials have indicated that donepezil is well tolerated in humans and improves cognitive function (Wilkinson et al., 2003; Black et al., 2003; Román et al., 2010). But, this drug creates gastrointestinal side- effects, as well as insomnia, malaise and dizziness together with agitation and aggression (Dunn et al., 2000). Clinical trials on nimodipine, a calcium channel antagonist, have failed to show a significant effect on cognitive improvement and functional status in patients with VaD (Pantoni et al., 2000).

Herbal medicines such as *Ginkgo biloba, Bacopa monnieri, Ginseng, Curcuma longa* and *Centella asiatica* have been reported for their neuroprotective and cognition-enhancing effects in animals and humans (Chang et al., 2016). With Ginkgo *biloba* extract, there is an improvement in cognitive function of patients with VaD but its clinical outcomes are inconsistent (van Dongen et al., 2000; Ahlemeyer and Krieglstein, 2003; Jiang et al., 2013). Clinical efficacy of other herbal medicines for VaD treatment cannot be validated because of limited clinical research. Considering the clinical side effects of these drugs and insufficient evidence on clinical efficacy of herbal medicines, the FDA has not approved them for VaD treatment (Baskys and Hou, 2007; Wang et al., 2009).

2.1.2 Chronic cerebral hypoperfusion (CCH)

The human brain comprises only 2% of total body mass but it consumes about 20% of the oxygen and 25% of the glucose consumed by the human body in order for it to conduct normal cerebral function (Belanger et al., 2011). The brain is highly dependent on proper cerebral blood flow in order for it to receive a constant supply of oxygen and energy substrates. Even a slight disruption in cerebral blood flow will have detrimental effects and if this is prolonged, the end result would be brain death. (Hossmann, 1994; Kunz and Iadecola, 2009).

When there is a sustained shortage of cerebral blood flow to the brain, CCH takes place. Conditions associated with this include carotid stenosis or occlusion, arteriovenous malformations, and cerebral small vessels disease (Nencini et al., 1993; Tatemichi et al 1995; Sarti et al., 2002b). Factors that cause CCH in humans

are advanced age as well as disorders that affect the cerebral vascular system including cardiovascular disorders, diabetes, generalized atherosclerosis, hypertension and smoking (Meyer et al., 2000; de la Torre, 2012). According to previous studies, CCH can cause lesions in selective and highly vulnerable regions of the brain especially the periventricular white matter, basal ganglia and hippocampus leading to a progressive decline in cognitive functions, as observed in VaD patients (Liu and Zhang, 2012; Venkat et al., 2015). Moreover, in VaD patients, white matter lesions induced by CCH can interrupt the cholinergic projections from the basal forebrain to other parts of the brain causing impairment in attention and executive function (Swartz et al., 2003).

CCH has been associated with neurodegeneration through the production of reactive oxygen species (ROS), neuronal energy failure, pro-inflammatory cytokines secreted by activated microglial cells and glutamate-induced excitotoxicity. These events interact with each other thereby contributing to neuronal damage. The latter leads to white matter lesion and cholinergic dysfunction leading to memory impairment and development of VaD (Baskys and Blaabjerg, 2005; Farkas et al., 2007; Wang et al., 2007 Liu and Zhang, 2012). CCH can cause mitochondrial dysfunction and protein inhibition leading to imbalance of antioxidants and ROS ratio resulting in oxidative injury to vascular endothelial cells, glia and neuronal cells. This in turn could impair vascular functioning and neurovascular coupling, conditions would then reduce the efficacy of the cerebral blood flow leading to white matter damage (Liu and Zhang, 2012; Zhao and Gong, 2015).

CCH may also initiate a cascade of inflammatory activities like peripheral leukocytes recruitment, microglia and astrocyte activation. These react by secreting

pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β) and matrix metalloproteases (MMP), causing neuronal death and blood–brain barrier (BBB) disruption thereby inducing secondary brain damage through the generation of chronic inflammation ultimately leading to cognitive loss (Liu et al., 2005; Wang et al., 2007; Wang et al., 2010a).

Oxygen and glucose deprivation during the early stages of cerebral hypoperfusion result in the failure of energy production and the depletion of adenosine triphosphate (ATP) leading to membrane depolarization and increased neuronal glutamate release. This in turn over activates the NMDA receptors allowing for the excessive influx of Ca^{2+} into the nerve cells initiating cell death signalling cascades through the activation of several degradative enzymes such as proteases and endonucleases. Finally, neuronal cell death takes place (Baskys and Blaabjerg, 2005; Weber, 2012). Taken together, these studies indicate that CCH may underlie cognitive decline in the development of VaD in the elderly (Figure 2.2).

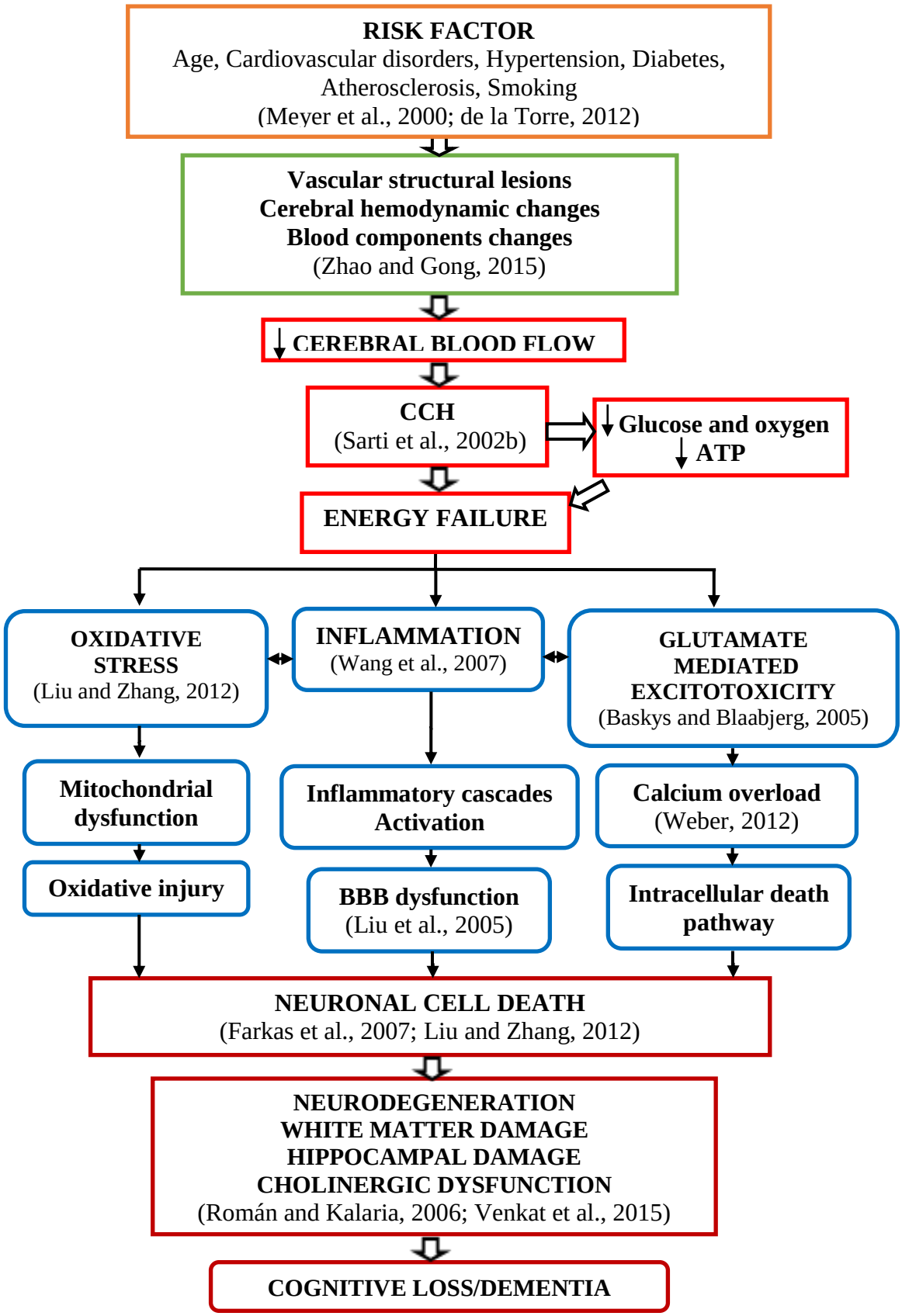

Figure 2.2: Summary of the possible pathways involved in CCH-induced cognitive impairments/dementia. Abbreviations: CCH, chronic cerebral hypoperfusion; ATP, adenosine triphosphate; BBB, blood–brain barrier.

2.1.3 Animal models of CCH

Several animal models on CCH have been developed to create ischemic injuries with various degrees of severity in the brain allowing for detailed examinations of the metabolic, cellular and molecular mechanisms as well as behavioral consequences of disrupted cerebral circulation. Techniques such as occlusion or stenosis of specific and also large arteries that supply blood to the brain would create conditions of cerebral hypoperfusion and trigger pathophysiological changes, and as a consequence, lead to neuronal damage and cognitive impairment (Farkas et al., 2007; Bacigaluppi et al., 2010; Jiwa et al., 2010).

The CCH model has been developed using several different species including dogs, cats and non-human primates. Nevertheless, rodents (rats, gerbils, and mice) are the most preferred because firstly, their cerebrovascular anatomy and physiology closely resembles those of higher species (Ginsberg and Busto, 1989). There are a number of proposed CCH models including bilateral common carotid artery stenosis (BCAS) in gerbils or mice, unilateral common carotid artery occlusion in mice (UCCAO), and permanent, bilateral occlusion of common carotid artery (PBOCCA) or 2-vessel occlusion in rats (Shibata et al., 2004; Farkas et al., 2007; Yoshizaki et al., 2008).

2.1.3(a) Bilateral common carotid artery stenosis (BCAS)

Gerbils and mice lack fully developed posterior communicating arteries of the Circle of Willis. Occlusion of carotid arteries leads to a severe drop in cerebral blood flow. Therefore, carotid stenosis is required to achieve CCH in these animal species (Kelly et al., 2001; Sarti et al., 2002b). BCAS model has been developed using wire microcoils of varying internal diameters (0.22 mm, 0.20 mm, 0.18 mm and 0.16 mm) in C57B1/6 mice. Pathologically, following BCAS, these mice developed white matter lesions, BBB disruption, microglial and astrocyte proliferation, and working memory deficit. But there were no grey matter ones (Shibata et al., 2004; Nakaji et al., 2006; Shibata et al., 2007). However, reference memory deficit, metabolic abnormalities and neuronal damage in hippocampal were only observed at 8 months after BCAS (Nishio et al., 2010). Additionally, this model is exclusive to the C57B1/6 strain due to the variability of cerebral blood flow after BCAS in other strains (Shibata et al., 2004).

2.1.3(b) Unilateral common carotid artery occlusion (UCCAO)

Another technique to produce CCH in mice, UCCAO, employs occlusion of the right common carotid artery. This results in white matter damage in the corpus callosum, activation of the microglia, elevation in pro-inflammatory cytokines and non-spatial working memory impairment (Yoshizaki et al., 2008). In the experiments that had been conducted, the rodents exhibited motor function deficit, anxiety and mild cognitive impairment. There was also dysregulation of synaptic proteins and mild

neurodegeneration (Zhao et al., 2014). There is a limitation to this model in that histological infarct is not readily detectable and not well characterized.

2.1.3(c) Permanent, bilateral occlusion of common carotid artery (PBOCCA)

PBOCCA is a well-characterized CCH animal model which has been widely used in experimental studies (Wang et al., 2000; Xu et al., 2012a). Besides, PBOCCA using rats is an appropriate animal model to represent conditions of CCH. The rat models here had a complete Circle of Willis developing persistent but reduced blow flow after the onset of PBOCCA (Farkas et al., 2007). This method is easy to perform in single surgical procedure, and results in the reduction of cerebral blood flow to approximately 35-45% in the cortical and white matter areas and to 60% in the hippocampus of the control level (McBean and Kelly, 1998; Farkas et al., 2007).

PBOCCA leads to early cerebral blood flow reduction and gradually starts to recover after 1 week (Tsuchiya et al., 1992; Otori et al., 2003). Previous findings have shown that PBOCCA caused a slight reduction in cerebral blood flow after 8 weeks to 3 months (Ohta et al., 1997; Otori et al., 2003). Finally, it returns to normal level after 6 months because vertebrobasilar arterial system provides compensatory blood flow via the Circle of Willis to the brain (Choy et al., 2006; Farkas et al., 2007). An extensive number of studies have demonstrated white matter lesions (Ni et al., 1995; Farkas et al., 2004; Choi et al., 2016), microglia and astrocytes activation (Pappas et al., 1996; Vicente et al., 2009; Cechetti et al., 2012), cholinergic dysfunction (Ni et al., 1995; Tanaka et al., 1996), and abnormal synaptic plasticity

(Wang et al., 2010b) in the hippocampal Cornu Ammonis 1 (CA1) region and cortex at various time points after the onset of PBOCCA.

With PBOCCA model, progressive neuronal damage occurs within selectively vulnerable areas such as the CA1 pyramidal neurons of the hippocampus and neocortex (Ohtaki et al., 2006). Pathological assessment demonstrated neuronal damage in the CA1 region appears from 7 days after surgery and persists for up to 3 months (Ni et al., 1994; Cechetti et al., 2012). Other studies also noted hippocampal neuronal loss after 6 months of PBOCCA surgery in rats (Pappas et al., 1996; Bennett et al., 1998). Accompanying these pathological changes is the progressive cognitive impairment such as seen in VaD patients and indicated by the number of behavioural tests, including the Morris water maze, 8-arm radial maze, object recognition task and T maze (Ni et al., 1994; Sarti et al., 2002a; Vicente et al., 2009; Cechetti et al., 2012). In summary, these findings support the fact that PBOCCA using rats is a suitable animal model of CCH with robust application in the development of potential therapeutic targets to treat cognitive impairment in VaD patients.

2.2 Learning and memory

Learning and memory are one of the most important studied subjects in the field of neuroscience. Behaviour involves a continuous flow of information through several independent brain systems. The systems process the information. The output, either directly or indirectly, controls behaviour. Under certain conditions, information being processed may change the neural systems and alter the processing of similar information in future events at the same time changing the corresponding output of the system. These conditions cause an alteration in behaviour attributing to a process called ‘learning’ and subsequently leading to the inference of the existence of ‘memory’ (White and McDonald, 2002).

Memory involves processes such as acquisition, consolidation and retrieval (Abel and Lattal, 2001). Acquisition refers to memory formation, consolidation refers to when the memory is stabilized and retrieval is recall of the stored information. In summary, learning reflects a complex set of neural processes. It involves acquisition of information and behavioural modification associated with this. Memory on the other hand is defined as the recall of such behavioural modification (Deiana et al., 2011).

2.2.1 Types of memory

Memory is typically classified based on content (declarative or non-declarative), duration (short-term or long-term) or its nature which could be archival (short- and

long-term memories) or transient (working memory) (Izquierdo et al., 1999; Deiana et al., 2011) (Figure 2.3).

Declarative (explicit) memory is the ability to retain facts and events which can be brought to mind and described verbally. This kind of memory is dependent on structures and connection in the medial temporal lobe and midline diencephalon. In amnesia, this memory is impaired. Declarative memory can be further sub-divided into semantic memory and episodic memory. Semantic memory allows one to recollect facts and knowledge not related to a certain experiences. Episodic memory on the other hand is the capacity to recollect experiences in terms of their elements (what), location (where) and temporal occurrence (when). One example of episodic memory is the spatial type. This class of memory gathers information on the surroundings and their spatial orientations. As for non-declarative memory, it is defined as unconscious memory involving habit formation and skill learning. This type of memory occurs as a modification within specialized performance systems and is revealed through reactivation of the systems within which the learning process initially occurred (Squire, 1992; Squire, 2004; Deiana et al., 2011).

In 1966, McGaugh propounded the 'three memory trace system' concept involving immediate memory, short-term memory (STM) and memory that consolidates slowly and is relatively permanent (long-term memory, LTM). The first refers to the memory system that lasts for a very short period like a second or few minutes. This kind of memory basically works as an online system because it relies on persistent electrical activity for its presence. Meanwhile, STM refers to memory that is established in a few seconds or minutes and persists for several hours whilst

LTM represents memory that lasts at least 24 h and requires gene activation and protein synthesis (Izquierdo et al., 1999).

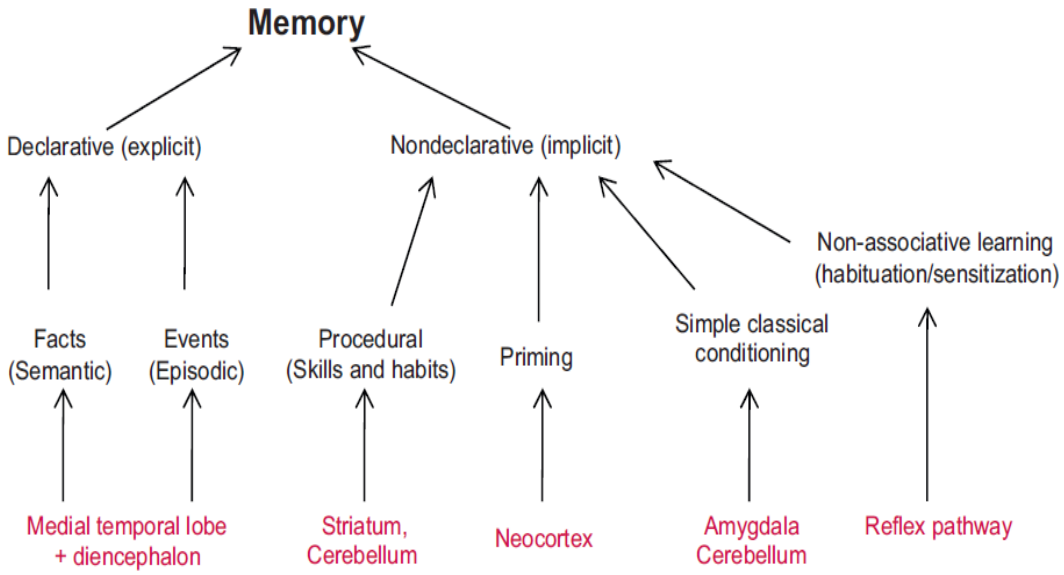

Figure 2.3: A taxonomy of memory systems. Reprinted with permission from The cholinergic system and spatial learning by Deiana, S., Platt, B., Riedel, G, 2011, *Behavioural Brain Research*, 221,389-411. Copyright (2011) by Elsevier.

2.2.2 Behavioral tests for learning and memory

To study learning and memory, behavioral paradigms need to be developed in order to characterize the fundamental behaviors associated with memory and its underlying mechanisms. Basically, behavioural tasks are divided into associative or operant learning tasks. Associative learning tasks use cues in the environment to condition a specific response in the animal. The operant learning task requires the animal to make a particular response to a specific stimulus to achieve a certain result (Bryan et al., 2009).

Cognitive associated tasks are further classified according to the type of memory including spatial memory (Morris water maze, radial arm maze), contextual memory (passive avoidance learning, fear conditioning), working memory (Y-maze, T-maze), novelty (object recognition) or activity (open-field test) (Bryan et al., 2009). There being a wide variety of behavioural tests available to assess cognition function in rodents, the present thesis has focused on the open-field test, passive avoidance task and Morris water maze. These will be described in more detail in the following sections.

2.2.2(a) Open-field test

One test that was developed in 1934 to study emotional behavior in rats is the open-field-test, the invention of Calvin S. Hall. Presently, it is used to study exploratory behavior and motor function of animals (Patti el al., 2005; Rao et al., 2005) as well as anxiety-related matters pertaining to the animals (Prut and Belzung, 2003). In this test, general motor activity are assessed by measuring locomotor activity (sum of

stereotypes movement and locomotion of animal across and through the field) and total distance travelled (cm) during a test session (Sweatt, 2010). The test works by placing the animal in the centre of a circular or square open field. Video technology or a laser gridded arena is then used to monitor its movement. This is a very simple, effective and reliable test with a great amount of robustness together with minimum animal handling (Bryan et al., 2009; Samson et al., 2015).

2.2.2(b) Passive avoidance task

The passive avoidance task, a single-trial behavioural paradigm, uses contextual fear conditioning in rodents to measure associative learning (Ögren et al., 2008). In this task, the animals are kept in a 2 compartment apparatus -1 compartment lit whilst the other dark and associated with an aversive event (foot shock). Experience received during training ensures that the animal avoids entering the dark compartment and remains in the well-lit one. The step-through latency (latency to enter into dark compartment) indicates the animal's cognitive ability to associate the dark compartment with the aversive event (Bryan et al., 2009).

The central components of the limbic system such as the amygdala and hippocampus are considered to be involved in contextual learning of passive avoidance task (Lorenzini et al., 1996; McGaugh, 2004; Baarendse et al., 2008). The advantages of this method are that it is a rapidly learned task, and memory can be assessed easily and reliably after a substantial time period following training. The passive avoidance task being a single-trial task ensures that the drug effect can be timed, and allows for studies on time-dependent processes in learning and

memory, *i.e.* consolidation, acquisition and retrieval of memory (Gold, 1986; Ögren et al., 2008).

2.2.2(c) Morris water maze

The Morris water maze also known as 'water maze' was first established by Richard Morris in 1984 to test spatial learning and memory in rats. The test depends on distal cues that are subsequently processed, consolidated and retrieved in order for the rats to successfully navigate from starting points along the wall of the water maze to the hidden platform located below the water's surface.

In the Morris water maze test, spatial learning is assessed during training trials. Reference memory is measured by the rat's preference for the former platform area during the absence of the platform. A visible platform task is used to measure non-spatial strategies and visual acuity in rodents (Morris, 1984; Vorhees and Williams, 2006; Terry, 2009). A number of studies have suggested that Morris water maze is a spatial learning task that is hippocampus-dependent (Morris et al., 1982; Tsien et al., 1996; Martin et al., 2005). Supporting this view is reversible inactivation of the hippocampus using lidocaine impaired Morris water maze performance in rats (Broadbent et al., 2006).

Importantly, Morris water maze offers several advantages. It does not require a pre-training period and restriction of food unlike the radial arm maze and T-maze (Deacon and Rawlins, 2006; Vorhees and Williams, 2014). Furthermore, it provides reliable results with a wide range of tank configurations, experimental procedures and species i.*e.* mice, rats and humans (Astur et al., 2002; Vorhees and Williams,