EFFECTS OF *Centella asiatica* EXTRACT ON LEARNING AND MEMORY OF ADOLESCENT RATS

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UNIVERSITI SAINS MALAYSIA

APEX

UNIVERSITI SAINS MALAYSIA

2017

EFFECTS OF *Centella asiatica* EXTRACT ON LEARNING AND MEMORY OF ADOLESCENT RATS

By

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Thesis submitted in partial fulfillment of the requirement for the degree of

Master of Neuroscience

JUNE 2017

KESAN EKSTRAK *Centella asiatica* DALAM PEMBELAJARAN DAN MEMORI

KEATAS TIKUS REMAJA

Oleh

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Tesis diserahkan untuk memenuhi sebahagian keperluan bagi Ijazah Sarjana

Neurosains

JUN 2017

ACKNOWLEDGEMENTS

I praise and thanks to Almighty Allah for giving me the strength, patience and opportunity to complete the thesis. My greatest thanks to the Universiti Sains Malaysia (USM), Integrated Neuroscience Program (INP) of the School of Medical Sciences for giving me the opportunity to study neuroscience. I acknowledge the financial support through the Ministry of Agriculture Malaysia research grant NKEA Research Grant Scheme NH1014D049 under the research titled: Modulation of the Standardised Extract of *Centella asiatica* (Pegaga) [BECA] on GABA and Glutamate Receptors: Identification of therapeutic intervention in neurodegenerative models which was submitted to USM as Grant no. : 304/CNEURO/6150137/K123 as well as the USM Animal Ethic Committee for their approval (Ethic Approval no. : USM / Animal Ethics Approval/2015/(98)(699)). I would like to express grateful appreciation to my supervisor Prof Dato' Dr. Jafri Malin Abdullah and co supervisor Dr. Sangu Muthuraju for their great leadership, explanation, and valuable comments, guidance, advice, encouragement, patience and endless support during entire research works and thank Prof Dr. Mohd Ilham Adenan from Atta-Ur-Rahman Institute for Natural Product Discovery (AuRins), Universiti Teknologi MARA (UiTM) Puncak Alam for the data on content of the *Centalla asiatica* extract, which is a collaborative work between Universiti Teknologi MARA (UiTM), Prof Dr. Tengku Sifzizul Tengku Muhammad from Universiti Malaysia Terengganu (UMT) and Universiti Sains Malaysia (USM). My deepest gratitude and appreciation is extended to my family members: Father - Mr. Mohd Yusuf Yeo Bin Abdullah, Mother - Mdm Noor Zaiwati Binti Hassan, aunt - Norwani Binti Hassan, sister: Nur Aishah Amira, Nur Atiqah, for their love, support and encouragement in various aspects. Last but not least, special appreciation to staffs of neuroscience department, CRL and histology lab, Animal Research and Service Centre (ARASC), USM, and friends: Chuang Huei Gau, Nurul Atikah and also seniors for their help during my experimental works with their valuable friendship and encouragement.

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LIST OF ABBREVIATIONS AND ACRONYMS

AChE	Acetylcholinesterase
ACh	Acetylcholine
ATP	Adenosine triphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPARs	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AuRins	Atta-ur-Rahman Institute of Natural Product Discovery
ARASC	Animal research and service centre
α	Alpha
β	Beta
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BECA	Bioactive extract of *Centella asiatica*
RECA	Raw extract of *Centella asiatica*
CA	Cornu ammonis
CRL	Centre research of laboratory
Cl	Chloride
ºC	Celcius
CNS	Central nervous system
CaMKll	Calcium/calmodulin dependent protein kinase II
cm	Centimetre
DG	Dentate gyrus
D1R	Dopamine 1 receptor

D2R	Dopamine 2 receptor
Da	Dalton
dH2O	Distilled water
ϒ	Gamma
GABA	Gamma-aminobutyric acid
GABARs	Gamma-aminobutyric acid receptor
Glu	Glutamate
g/mol	Gram/Molar
g	Gram
HRP/DAB	Horseradish peroxidase / 3,3′-Diaminobenzidine
HPLC	High performance liquid chromatography
IHC	Immunohistochemistry
Kainate	2-2-carboxy-3-3-carboxymethyl-4-isopropenyl pyrrolidine
kg	Kilogram
l	Litre
LTP	Long-term potentiation
LTD	Long-term depression
LD 50	Lethal dose 50
MDA	Malondialdehyde
M	Molar
µl	Microlitre
mg	Milligram
mL	Millilitre
mm	Millimetre
mg/dl	Milligram per deciliter

mL/kg	Milligram per kilogram
mPFC	Medial prefrontal cortex
mGluRs	Metabotropic glutamate receptors
NMDA	N-methyl-D-aspartate
NMDARs	N-methyl-D-aspartate receptor
NaH2PO4	Sodium phosphate monobasic
NA2HPO4	Sodium phosphate dibasic
NADH	Nicotinamide adenine dinucleotide
Nm	Nanometre
OFT	Open field test
pH	Potential of hydrogen
PND	Postnatal day
PNS	Peripheral nervous system
PBS	Phosphate-buffered saline
PB	Phosphate buffered
PFA	Paraformaldehyde
PCO	Protein carbonyl
PI	Propidium iodide
%	Percent
Sec	Second
5HT	Serotonin
USM	Universiti Sains Malaysia
UMT	Universiti Malaysia Terengganu
UPM	Universiti Putra Malaysia

IBD, UTM	Institute of Bioproduct Development, Universiti Teknologi Malaysia
UiTM	Universiti Teknologi MARA
US / USA	United State / United State America
WHO	World Health Organization

KESAN EKSTRAK *Centella asiatica* DALAM PEMBELAJARAN DAN MEMORI KEATAS TIKUS REMAJA

ABSTRAK

Centella asiatica, merupakan herba daripada keluarga mackinlayoideae, berasal dari tanah lembap di sekitar kawasan tropika dan subtropika asia yang mempunyai banyak manfaat terhadap kesihatan. *Centella asiatica* adalah tumbuhan herba yang mengandungi fitokimia yang dipercayai mempunyai kesan ke atas fungsi kognitif. Pembelajaran merupakan proses mendapat dan menyimpan maklumat yang diperoleh daripada pengalaman. Memori adalah simpanan kekal yang terhasil daripada maklumat yang dipelajari di mana hippocampus memainkan peranan penting dalam kehidupan mamalia yang bertindak sebagai pengantaraan dalam pembelajaran dan pembentukan memori yang baru. Peringkat remaja merupakan tempoh kritikal bagi tumbesaran proses kematangan otak yang merangkumi perubahan fizikal, psikologi, pembangunan sosial, sistem kognitif dan neurotransmitter. Secara khusus, istilah 'remaja' dalam mamalia mewakili keseluruhan tempoh selepas kelahiran, yang bermula dari tempoh bercerai susu iaitu pada hari ke 21 sehingga kealam dewasa iaitu pada hari ke 60 kelahiran. Sebarang perubahan yang berlaku dalam tempoh tersebut yang disebabkan oleh ejen nootropics boleh membawa kesan yang baik kepada otak terutama dalam fungsi kognitif. Kajian ini dilakukan bagi mengkaji kesan ekstrak *Centella asiatica* dalam peningkatan pembelajaran dan ingatan pada tikus remaja dengan menggunakan penilaian tingkah laku: penilaian ‘open field’ dan ‘water T-maze’. Kajian ini juga mengkaji perubahan morphologi sel neuron menggunakan pewarnaan cresyl violet dan apoptosis. Kami menganalisis ekspresi glutamat reseptor AMPA (amino-3-hidrosi-5-metil-4-α-isoazole-propionik asid) subjenis – GluA1 dan reseptor GABA (Gamma-Aminobutyric acid) subjenis - GABA A α1 menggunakan Immunohistokimia dalam hippocampus haiwan yang sama. Hasil kajian menunjukkan tiada sebarang perubahan yang signifikan dalam aktiviti lokomotor ($p>0.05$). Data ‘water T-maze’ menunjukkan dos 30 mg / kg dengan signifikannya ($p<0.05$) menunjukkan peningkatan dalam fasa pembelajaran, ingatan dan konsolidasi memori, tetapi tidak dalam pembalikan pembelajaran ($p> 0.05$). Data histologi memaparkan tiada perubahan pada morfologi neuron. Analisis Immunohistological mendedahkan bahawa terdapat peningkatan ekspresi reseptor AMPA GluA1 tetapi tidak pada reseptor GABA A α 1 dalam hippocampus CA1 dan CA2. Kesimpulannya, ekstrak *Centella asiatica* telah meningkatkan kebergantungan hippocampus terhadap pembelajaran spatial dan memori pada kebergantungan dos terhadap tikus melalui reseptor AMPA GluA1 dalam kawasan hippocampus CA1 dan CA2.

EFFECTS OF *Centella asiatica* EXTRACT ON LEARNING AND MEMORY OF ADOLESCENT RATS

ABSTRACT

Centella asiatica, herbs from *mackinlayoideae* family, is a native to the wetlands in the tropical and subtropical region of Asia that has many beneficial effect to health. *Centella asiatica* is herbal plant containing phytochemicals that strongly believes has an effect on cognitive function. Learning is an acquisition and storage of information as a consequence of experience. Memory is a relatively permanent storage form of the learned information where the hippocampus is plays the most important roles in mammals in mediation of learning and the formation of new memories. Adolescence stage is the critical development period for maturation of brain processes that encompassed by changes in physical, psychological, social development, cognitive and neurotransmitter system. Specifically, the terminology of 'adolescence' in mammals represents the entire postnatal period, which ranges from weaning postnatal day 21 to adulthood postnatal day 60. Any alteration in this period caused by nootropics agent may bring beneficial effect to the brain especially in cognitive functions. This study investigated the effects of the extract of *Centella asiatica* in enhancement of learning and memory in adolescent rats by using the behavior assessments: open field test and water T-maze test. This study also examined neuronal cell morphological changes using cresyl violet and apoptosis staining. We analysed the expression of glutamate AMPA receptor (amino-3-hydroxy-5-methyl-4-α-isoazole-propionic acid) subtype - GluA1 and GABA receptor (Gamma-Aminobutyric Acid) subtype – GABA A α1 using immunohistochemistry in the hippocampus of the same animals. The result showed no significant changes in locomotor activity ($p>0.05$). Water T maze data showed that 30 mg/kg dose significantly ($p<0.05$) improved in learning, memory and memory consolidation phase but not in reversal learning ($p>0.05$). Histological data displayed that there were no neuronal morphological changes. Immunohistological analysis revealed that there was increased the expression of AMPA GluA1 receptor but not in GABA A α 1 receptor in CA1 and CA2 of the hippocampus. It can be concluded that extract of *Centella asiatica* improved hippocampal dependent spatial learning and memory at dose dependent manner in rats through AMPA GluA1 receptor in CA1 and CA2 regions of the hippocampus.

CHAPTER 1

INTRODUCTION

Plants are promising therapeutic agents because they contain many phytochemicals that have anti-inflammatory, antioxidative as well as anticholinesterase activities. Phytochemicals is a kind of nutrient obtained from food for sustaining life and this chemical scientifically has protective and health improvement properties (Felli, I. C., & Pierattelli, R., 2015; Rasool, M., et al., 2014). *Centella asiatica* is one of the botanical plants that contain many phytochemicals is a tropical plant form family of Apiaceae widely distributed to Southeast Asian countries such as India, china, Sri lanka, Malaysia and Indonesia and also South Africa and Madagascar (Ilkay Erdogan orhan., 2012). There are 8 types of *Centella asiatica* also known as 'pegaga' in their local name that can be found in Malaysia such as 'Pegaga Kampung' 'Pegaga Cina', 'Pegaga Embun' (Khatib, A et al., 2011), 'Pegaga Daun Lebar', 'Pegaga Salad', 'Pegaga Renek' (Hashim, P., 2011), 'Pegaga Gajah' (Wan-Ibrahim, W. I et al., 2010) and 'Pegaga Brunei' (Nayan, D. S., & Ishak, C. F., 2013). *Centella asiatica* ha*s* also commonly known as Gotu Kola (Gray et al., 2017) or Pegaga (Aizada et al., 2017). The major chemical constituent found in *Centella asiatica* are triterpenoids derivative such as asiatic acid, madacassic acid and asiaticoside and flavonoids (Sari et al., 2014, Vasavi et al., 2016; Saoji., et al., 2016). This plant has been widely used in Ayurvedic, African and Chinese traditional medicine (Giribabu et al., 2014, Vasavi et al., 2016). The famous beneficial effect of *Centella asiatica* is in the neuroprotective effect and cognitive function particularly learning and memory (Giribabu et al., 2014; Doknark et al., 2014; Sirichoat., 2015; Gray et al., 2016; Gray et al., 2017).

A characteristic feature of animals and particularly of humans is the ability to alter their behavior on the basis of experience or learning. Learning is the process of acquiring new knowledge about the world and environment meanwhile memory is the process of storage or retention of the acquired knowledge. Memory consist of individual ability to record the sensory stimuli, events, information, as well as retain the information over a short or long period of time, and can be recall when needed (Chakravarthi & Avadhani., 2013). Learning and memory processes involve several regions of brain including cortex, amygdala, cerebellum and hippocampus. The process from memory formation to memory recalling is the series processes that include encoding, memory storage, consolidation and recall. The process of memory formation mechanism is mediated by Long-term potentiation (LTP), induction and expression of synaptic plasticity in which the hippocampus is activated while that all are processed (Yolanda., 2015). The most important role of the hippocampus is its mediation of learning and the formation of new memories and widely known to be the site for encoding and storage of spatial memory in both rodents and humans. The hippocampus is a major component of the brain located inside the medial temporal lobe, beneath the cortical surface. It plays an important role in spatial memory, navigation and long term memory (Chakravarthi & Avadhani., 2013; Shen., 2017).

The hippocampus consist of subregions mainly are areas of CA1, CA2, CA3 and DG with composed of tightly packed pyramidal neuron (Cherubini & Miles., 2015).

There are two types of brain cells involves in learning and memory that is neurons and glial cells. Neurons communicate with others cells by the release of chemical neurotransmitters, which act transiently on postsynaptic receptors (Nuss P., 2015; Fields, R. D et al., 2014; Krebs et al., 2012). There are several neurotransmitter located in the brain. The major excitatory neurotransmitter is glutamate that involved in almost all CNS functions especially in cortical and hippocampal regions that consist of 70% of all excitatory synapses in the central nervous system that utilize glutamate as a neurotransmitter (Danysz & Parsons., 2012), while the major inhibitory neurotransmitter in the CNS are γ-aminobutyric acid (GABA) and it has been estimated that at least one third of all CNS neurons utilize GABA as primary neurotransmitter (Nuss P., 2015). LTP are considered to be a major cellular mechanism underlying learning and memory. Involvement of LTP in the hippocampus required activation of two major ionototrpic glutamate receptors that is α-amino-3-hydroxyl-5-methyl-4-isoxazole propionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors at glutamatergic synapse (Pandey et al., 2015). Glutamate is one of the most important excitatory neurotransmitters in the central nervous system (CNS), generally it mediates fast excitatory transmission across nervous system and plays an important role in various integrative brain functions, as well as in development of the brain (Mukherjee & Manahan-Vaughan., 2013). The inhibitory neurotransmitter γ-aminobutyric acid (GABA) also plays a role as regulator of learning, memory, and synaptic plasticity (Wang et al., 2012).

At birth, the brain is very immature and the brain development is the continuous process begins in intrauterine life that proceeds well after birth and even adolescence. Adolescence is a critical period involves in a transition period between childhood and adulthood, which is important for neurodevelopment that characterized by various changes in anatomy and physiology of the brains (Li et al., 2013) as well as development of behavioral and biological systems (Hsu et al., 2015) and also maturation of neurotransmitter system (Arain et al., 2013). In rodents, the term 'adolescence' represents the entire postnatal period, which approximately ranges from a week after weaning period that is postnatal day 28 (PND 28) to as late as during postnatal day 60 (PND 60) (Li et al., 2013; Hammerslag & Gulley., 2014; Mengler et al., 2014).

During the transition period in adolescent, rodent's hippocampus and prefrontal cortex undergo significant development as well as learning and memory also continue to develop throughout the adolescence period (Uysal et al., 2015). Thus, during critical period of growth and development, the cells can be easily influenced by the nutrition intake (Beheshti., 2015) and any nutritional nourishment in this stage can bring beneficial effect to the brain.

CHAPTER 2

LITERATURE REVIEW

2.1 *Centella asiatica* background

Centella asiatica is one of the botanical plants that belong to Apiaceae family (Saoji et al., 2016; Gray et al., 2017) and a subfamily of Mackinlayoideae. This plant growing mostly in the moist places of plains and foothills of the tropical and subtropical Asian countries such as in Sri Lanka, India, China, Nepal, Pakistan, Indonesia and Malaysia as well as in South Africa, Madagascar, South pacific and Eastern Europe (Aftab et al., 2017; Tripathi et al., 2015; Qureshi et al., 2015; Giribabu et al., 2014; Orhan., 2012). It is widely used as medicinal herbs in African, Ayurvedic and Chinese traditional medicine (Qureshi et al., 2015; Giribabu et al., 2014). *Centella asiatica* or pegaga is commonly known as "Gotu kola, asiatic pennywort, Indian pennywort, Indian water navelwort, tiger herbs and pegagan" (Ghosh et al., 2017; Aizada et al., 2017; Yolanda et al., 2015; Orhan., 2012).

2.2 *Centella asiatica* characteristic

Figure 2.1: ***Centella asiatica*** **plant (Tripathi., 2015)**

Centella asiatica leaves is a prostrate, faintly aromatic, stoloniferous, perennial creeper herb that rooting at the nodes with simple petiolate, palmately lobes leaves attains height up to 15 cm (Ghosh et al., 2016; Tripathi et al., 2015). Specifically, the leaves, 1-3 from each node of stems, borne on pericardial petioles, 2- 6 cm long and 1.5 to 5 cm wide, green in colour, orbicular-reniform with rounded apices which have smooth texture with palmetely veins, sheathing leaf base, crenate margins, glabrous on both sides. The stem is long-stalked, slender, creeping stolon, glabrous, striated and rooting at the nodes (Aftab et al., 2017; Tripathi et al., 2015). They are green to reddish green in colour, and interconnecting one plant to another. The rootstock of this plant consists of rhizomes, growing vertically down with creamish in color and covered with root hairs (Aftab et al., 2017).

2.3 Phytochemical containing in *Centella asiatica*

Centella asiatica is reported to have several types of chemical compounds. Some important chemicals constituents found in *Centella asiatica* are triterpenes and flavonoids. Other chemicals are essential oils, carbohydrates, amino acids, and other compounds, such as vellarin. The major chemicals class found in *Centella asiatica* is triterpene saponosides. The triterpenes include asiatic acid asiaticoside, centelloside, madecassoside, thankuniside, brahmoside, brahminoside, centellose, brahmic, centellic, and madecasic acids, madasiatic acid, betulinic acid, thankunic acid, and isothankunic acid (Nasir et al., 2015; Tripathi et al., 2015; Sari et al., 2014; Orhan., 2012). The Phenols group also important component in the *Centella asiatica* that has potent antioxidant activity. They consist of flavonoids, phenylpropanoids and tannin constituents. *Centella asiatica* also containing vitamins groups such as ascorbic acid, nicotinic acid, and β-carotene as well as mineral group such as calcium, phosphorus, iron, potassium, magnesium, manganese, zinc, sodium, and copper constituent (Tripathi et al., 2015).

2.4 Beneficial effect of *Centella asiatica*

The pharmacological activity of *Centella asiatica* is due to several phytochemicals constituent. *Centella asiatica* was found has an effect of antidepressant activity (Luo et al., 2015; Selvi et al., 2012; Kalshetty et al., 2012), neuroprotective activity (Chanana & Kumar., 2017; Gray et al., 2017, Gray et al., 2016; Nataraj et al., 2016; Giribabu et al., 2014; Rao et al., 2012), anti inflammatory activity, analgesic activity (Qureshi et al., 2015), wound healing activity (Azis et al., 2017; Ruzymah et al., 2012), antimicrobial activity (Vasavi et al., 2016; Netala et al., 2015; Rattanakom & Yasurin., 2015), antidiabetic activity (Abas., 2016), antioxidant activity (Thoo et al., 2013; Abas., 2014), and acethylcholinesterase inhibitor. The most widely known of *Centella asiatica* effect is on central nervous system as well as in learning and memory enhancement activity (Sirichoat et al., 2015; Sari et al., 2014; Yolanda., 2015).

2.5 The central nervous system

Brain and spinal cord is the human central nervous system (CNS). The brain concerned with functions as diverse as thought, language, learning and memory, imagination, creativity, attention, consciousness, emotional experience and sleep. In addition, the brain regulates or modulates visceral, endocrine and somatic functions. The spinal cord serves as important function where it receives much of the sensory information and performs the initial processing of the input. Meanwhile, receptor outside CNS acts as transducers that change physical and chemical stimuli in environment into nerve impulse that are sent to the spinal cord and then the brain, which can read and give meaning to this input. Spinal cord carries all of the motor information that's supplies voluntary muscle and thus, participates directly in control of body movement. It plays a direct role in regulating visceral function, and it serves as a conduit for the longitudinal flow of information to and from the brain (Krebs et al., 2012).

Brain consists of the forebrain from the prosencephalon, the midbrain from the mesencephalon and the hindbrain from the rhombencephalon. The forebrain consists of the cerebral hemisphere and deep structures. The midbrain and hindbrain are collectively referred to as the brainstem and the cerebellum (Krebs et al., 2012). The central nervous system regions including the hippocampus and its subdivisions such as CA1, CA3, and dentate gyrus (DG) (Montagne, A et al., 2015).

2.6 The hipocampus

The hippocampus is a major component of the brain of human and other mammals. It belongs to the limbic system, located bilaterally in the medial temporal lobe, underneath the cortical surface. The hippocampus or more broadly, the hippocampal formation is the curved sheet of cortex folded into the medial surface of the temporal lobe, occupying the floor of the inferior or temporal horn of the lateral ventricle. It is relatively large structure, about 5 cm in length. The hippocampal formation consists of three major parts that is cubiculum, ammon horn and dendate gyrus (Felli & Pierattelli., 2015). Hippocampal contains subregion called as cornu ammonis 1–3 (CA1, CA2, CA3), dentate gyrus (DG), and subiculum (Libby et al., 2012). The most important role of the hippocampus in mammals is its mediation of learning and the formation of new memories. The hippocampus are important for the formation of spatial memory, long term memory and special navigation (Chakravarthi, K., 2013) and it is particularly affected by both normal and pathologic aging impairment as learning and memory performance declines with age (Moorthi et al., 2015; Giribabu et al., 2014).

Hippocampal subregions are different in terms of their cellular nature and organization, as well as their connectivity with the rest of the brain (Fouquet et al., 2012). The hippocampus CA1 contributes to the integration of all sensory and memory inputs. It enables slow and gradual learning and the elaboration of an internal representation of the relations between more than two different items experienced simultaneously (Lavenex, P., & Lavenex, P. B., 2013). CA1 pyramidal neurons is a major output centre from the hippocampus that play critical roles in spatial memory tasks (Li et al., 2012) as well as in memory consolidation of different events, episodic memory, processing spatial information (Barbosa et al., 2012) and encoding (Vivar et al., 2012).

CA2 region are considered as a transition zone since its pyramidal neurons resemble CA3 neurons both in size and dendritic branching patterns and receive inputs from CA3 neurons, similarly to CA1 pyramidal cells. They also receive input from dentate gyrus granule cells, but they lack the thorny excrescences characteristic of the CA3 neurons. In addition to local inputs, CA2 interneurons receive excitation from CA1, CA3, supramamillary body, and the amygdala and later it forms a loop with layer II entorhinal neurons. CA2 region possess important roles in special cognitive and behavioral functions including social recognition memory and temporal coding (Oliva et al., 2016; Mou., 2016).

The hippocampus of CA3 contributes to pattern completion and the rapid and flexible acquisition of spatial memories and episodic memories (Lavenex, P., & Lavenex, P. B., 2013; Cherubini & Miles., 2015).

There are several evidence suggests that the dentate gyrus acts as a preprocessor of incoming information, preparing it for subsequent processing in CA3 (Jonas & Lisman., 2014). The dentate gyrus contributes to pattern separation, treating both spatial and non-spatial information. The postnatal neurogenesis in the dentate gyrus contributes to the encoding of temporal associations, linking the events that happened at the same time and distinguishing the events that happened at different times (Lavenex, P., & Lavenex, P. B., 2013; Jonas & Lisman., 2014).

2.7 Connection circuit among hippocampus subregion in information pathway

Parahippocampal region of the cortex is the first to receive the information from visual, auditory and somatic associative cortexes and then passes through the enthorinal cortex and to the hippocampus proper. Furthermore, the information then passes through three distinct region mainly in the areas of CA1, CA2 and CA3 that composed of tighly packet pyramidal neuron within the hippocampus (Felli & Pierattelli., 2015; Oliva et al., 2016) and these subregions are adjoined by neighbouring areas, the dentate gyrus (DG) (Bonnici et al., 2012; Staples., et al., 2015). Each of this hippocampus subregion plays key roles in different aspects of learning and memory.

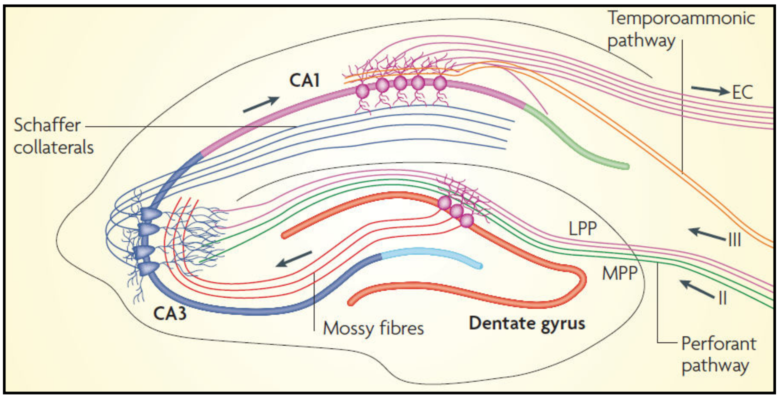

Figure 2.2: Neural circuitry in the rodent hippocampus (Deng et al., 2010)

The hippocampus is mainly organized as a unidirectional circuit starting at the DG, which receives input from the entorhinal cortex via the perforant pathway. The DG projects via the mossy fibers to cells in the CA3, which in turn send projections to the CA1 sector through the Schaffer collaterals. Finally, this circuit is completed as CA1 sends projections to the subiculum, which is the major output region of the hippocampus (Pereira et al., 2013) (Figure 2.2).

2.8 Neurotransmitter

Neurotransmitters are chemicals that carry information from one neuron to another released by presynaptic neurons and are the means of communication at a chemical synapse which is also involved in learning and memory. Neurotransmitter binds to neurotransmitter receptors, which can be coupled with an ion channel such as ionotropic receptors or with an intracellular signaling process such as metabotopic receptors. Neurotransmitters are specific for the receptor they bind to and elicit a specific response in the post synaptic neurons, resulting in either an excitatory or inhibitory signal (Nuss., 2015; Krebs et al 2012).

There are several neurotransmitter located in the brain. Amino acids such as glutamate is the major excitatory neurotransmitter involved in almost all CNS functions especially in cortical and hippocampus regions that consist of 70% of all excitatory synapses in the central nervous system that utilize glutamate as a neurotransmitter (Danysz & Parsons., 2012), while GABA is the major inhibitory neurotransmitter in the central nervous system and it has been estimated least one third of all CNS neurons utilize GABA as their primary neurotransmitter (Nuss., 2015).

The AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor is expressed throughout the mammalian brain and essential for excitatory synaptic transmission. AMPA is classified as ionotropic glutamate receptor acts as excitatory neurotransmitter receptor and mediates the majority of fast excitatory transmission in CNS (Mao et al., 2015). AMPA receptor composed of four subunits heterotetrameric that is GluA1, GluA2, GluA3, and GluA4 (Pandey et al., 2015). Among AMPA receptor subunits, GluA1 is particularly important for structural and functional plasticity, inducing structurally stabilizing effects and increasing synaptic strength. In addition, GluA1 is the main molecular determinant of long-term potentiation (LTP) and synaptic plasticity and therefore of learning and memory (Inta et al., 2014).

The GABA (γ-aminobutyric acid) is a major inhibitory neurotransmitter in CNS. However, during early developmental stage, GABA acts as excitatory in which the processes of neurogenesis, including neuronal proliferation, migration, differentiation, and preliminary circuit-building are implicated by GABA. During early developmental stage, GABA acts as excitatory and implicated in many. However, in the mature CNS, GABA acts as inhibitory. There are two main types of GABA receptors in CNS, the fast acting ionotropic GABA A receptor and slow metabotropic GABA B receptor (Wu & Sun., 2015; Jazvinscak & Vlainic., 2015). Activation of GABA receptors by GABA induces membrane hyperpolarization, and reduces the frequency of the generation of action potentials (Jazvinscak & Vlainic., 2015). The predominant type of GABA receptor in the brains GABA A receptors that composed of two alpha, two beta and one gamma subunit as their homologous subunits (Milenkovic et al., 2013).

Other than glutamate and GABA, acetylcholine is an important neurotransmitter used in the peripheral nervous system (PNS), CNS and neuromuscular junction. There are 2 types of receptors for acetylcholine, that is nicotinic acetylcholine receptors are ionotropic receptors coupled with the nonselective cation channel and the muscarinic acetylcholine receptors that linked to G protein mediated pathways (Krebs et al., 2012). Acetylcholine is an excitatory neurotransmitter at some synapses, such as neuromuscular junction, where the binding of acetylcholine to ionotropic receptors opens cation channels. It is also an inhibitory neurotransmitter at other synapses, where it binds to metabotropic receptor coupled to G proteins that open potassium channels (Tortora, G. J., & Derrickson, B. H., 2011)

On the other hand, biogenic amines are also involves. They are the group of neurotransmitter with an amine group in their structure which is comprises the catecholamines of dopamine, norepinephrine and epinephrine as well as histamine and serotonin. Dopamine involved in many forebrain circuits associated with emotion, motivation, and reward. Its act on G protein coupled receptors and its action can be either excitatory via D1 receptors (D1R) or inhibitory via D2 (D2R) receptors. Norepinephrine is a key neurotransmitter involved in wakefulness and attention. Its act on metabotropic α-adrenergic and β-adrenergic receptor which is both is excitatory. Epinephrine act on the same receptor but its concentration in the central nervous system is much lower than norepinephrine. Histamine binds to an excitatory metabotropic receptor in CNS and involved in wakefulness while serotonin have both excitatory and inhibitory effects (Krebs et al., 2012).

Besides that, adenosine triphosphate (ATP) is also known as energy carrier within cells. However, it is also released by presynaptic neurons as a neurotransmitter. Because it is often released together with other neurotransmitter, it is referred to as a co-transmitter. ATP can be broken down in the synaptic cleft of adenosine, a purine which binds and activates the same receptor as ATP. This purinergic receptor can be either ionotropic or metabotropic. Furthermore, ATP and purines are neuromodulators because they are co-released with other neurotransmitters, and the degree of ionotropic and metabotropic activation will modulate the response to the other neurotransmitter secreted, either enhancing that action or inhibiting it (Krebs et al., 2012) (Figure 2.3).

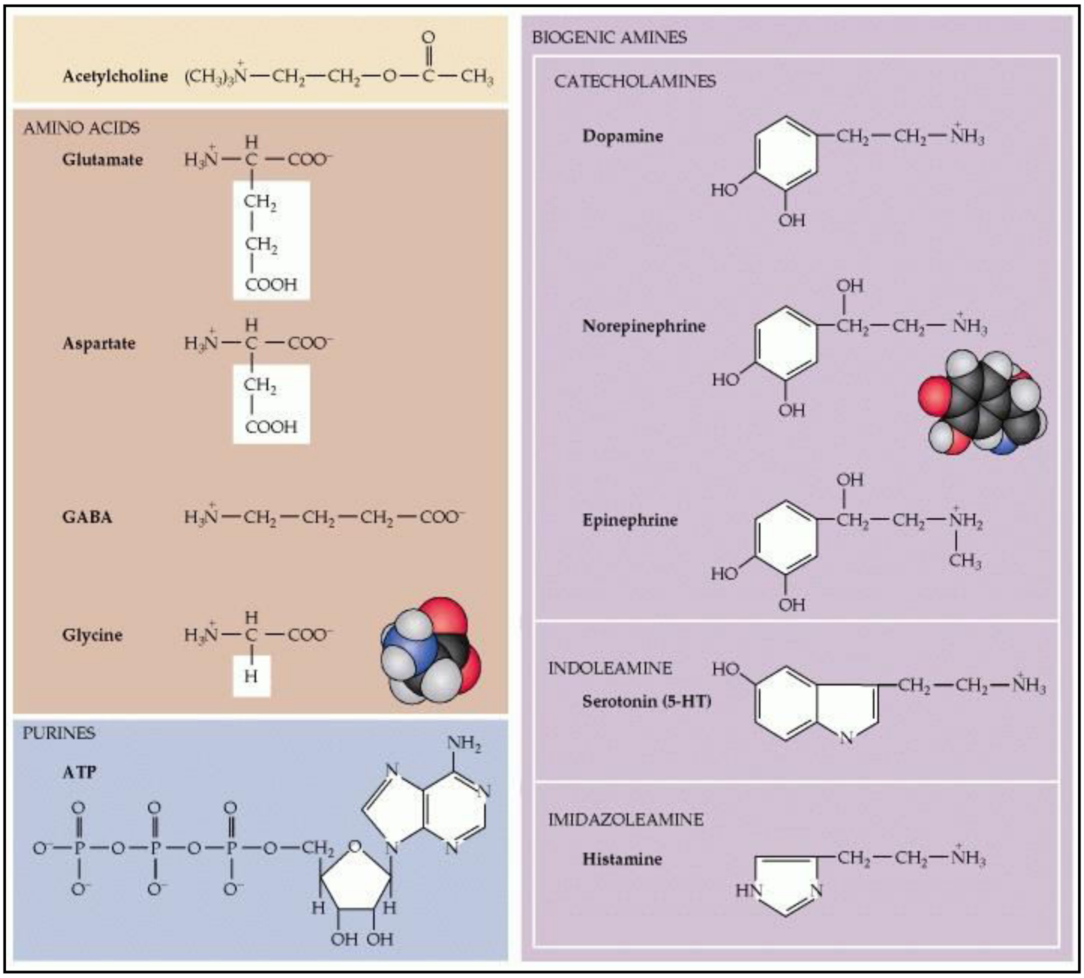

Figure 2.3: Neurotransmitter categories (Purves. D et al, 2001)

2.9 Learning and memory

Learning is a process of acquiring new knowledge while memory is the process of storage or retention information that acquired from the learnt knowledge (Chakravarthi & Avadhani., 2013). The ability of memory including recording of sensory stimulus, events information, and information over a short or long period of time and it can be retrieved at any time when needed (Chakravarthi & Avadhani., 2013). The process from acquisition of information to memory retrieval involves the sequence episode such as encoding, memory storage, consolidation and recall. The process of memory formation mechanism is mediated by long term potentiation

(LTP), induction and expression of synaptic plasticity in which the hippocampus is activated while that all are processed (Yolanda., 2015).

2.10 Types of memory

There are 3 types of memory that is sensory memory, short term memory and long term memory. Sensory memory is an ability to retain impressions of sensory information after the original stimuli have ended that occurs in very short period and later to be transferred to the short term memory. Working memory is a part of short term memory refers to the capacity to maintain this limited amount of information through active rehearsal, usually across a relatively short time interval. Working memory involves transition in between of short term and long term memory and with repetition or rehearsal of working memory may consolidate into long term memory (Jeneson & Squire., 2012; Brady, T. F et al., 2008).

Long term memory consist of 2 types which is explicit memory that involves facts or events are accessible to consciousness and can be expressed explicitly such as remembered event or facts and implicit memory that is not directly accessible to consciousness (Krebs et al., 2012; Deng., et al., 2010). There are 2 form of explicit memory, both of which involve retrieving earlier stored information such as episodic memory and semantic memory. The episodic memory involves the memory for events and includes the ability to learn, store, and retrieve information about experiences that occur in everyday life. These memories include information about the time and place of an event and details about event itself. Meanwhile, semantic memory involves knowledge of fact that has been learned (Krebs et al., 2012).

On the other hand, the implicit memory involves memories that manifest as subconscious behavioral or physiological responses to event or stimuli. Implicit memory includes several forms of learning that occur during performance of a task (Krebs et al., 2012) (Figure 2.4).

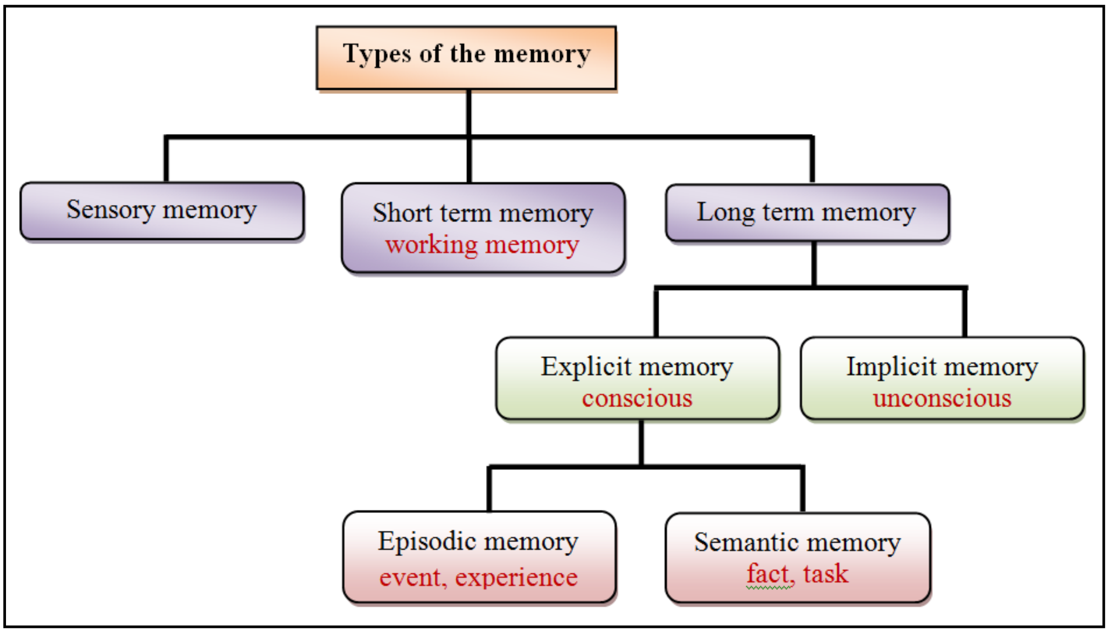

Figure 2.4: Summarization chart - Type of the memory

2.11 Stages of memory-related synaptic changes production

The memory related synaptic changes start with induction of transmitter are being released and binds to AMPA-type glutamate receptors, which then depolarize the postsynaptic region and unblock NMDA-type receptors. In order to increase the size of subsequent excitatory currents, the process continued by expression where the NMDA (N-methyl-D-aspartate) receptors allow calcium to invade and thereby modify AMPA receptors. Consolidation is the final stage where NMDA receptors trigger changes that stabilize the modifications to the AMPA receptors (Kapoor et al., 2013).

2.12 Adolescent

The development of brain in mammals begins during intrauterine life and continues through the adolescent period. The transition period between childhood and adult life known as adolescent that is critical stage for neurodevelopment. During adolescent period, the maturation of neurotransmitter system, brain anatomy and physiology such as hippocampus and prefrontal cortex (PFC) occurs. Besides that, the learning and memory function continue to develop throughout adolescence period. In rodents, the term 'adolescence' represents the entire postnatal period, which ranges from weaning postnatal day 21 (PND 21) and continue to adulthood postnatal day 60 (PND 60) (Uysal et al., 2014; Li et al., 2013). In other hand, the World Health Organization (WHO) defined adolescence in human starts between the ages of 10 and 19 years, with the onset of puberty marking the transition from childhood to adolescence (McCormick et al., 2017).

2.13 Comparison developmental milestone between human and rodents age

The changes in oligodendrocyte maturation state where mitotically active pre-oligodendrocyte, immune system developed and the blood-brain barrier are established during 23 to 32 weeks of gestation for human and postnatal day 1 to 3 for rodents. Moreover, there are several changes occur where the peak of brain growth spurted, peak in gliogenesis occurred, axonal and dendritic density are increasing, changes in the state of maturation of oligodendrocyte and the consolidation of the immune system happened during 36 to 40 weeks of gestation in human and postnatal day 7 to 10 in rodents. The adult brain weighted reaches 90 to 95% during 2 to 3 years old in human and postnatal 20 until 21 in rodents. In addition, the peak in synaptic density and myelination rate also reach more than 50% in adult levels and there are also changes occur in neurotransmitter and receptor at this stage.

Besides that, the fractionation or specialization of prefrontal cortex neural networks (structural maturation) occurs at the age of human 4 to 11 years old and rodent at the postnatal day 25 until 35 where the volume of grey matter and cortical thickness achieves the maximum volume. Meanwhile, during human 12 to 18 years old and rodents 35 to 49 postnatal day, the synapse density are reduced until it reaching a plateau at adult levels and refinement of cognitive-dependent circuitry occurs where ongoing myelination cause increasing white matter volume and fractional anisotrophy. Finally, at the age of human 20 years old and postnatal day 60 of rodents, neurotransmitters and synaptic density achieved adult levels and declining in grey matter plus with ongoing myelination occurs. Summary on (Table 2.1) (Semple et al., 2013).

Human	Rodent	Developmental milestones
23–32 wk gestation (pre-term infant)	pnd 1–3	Oligodendrocyte maturation state changes—predominance of mitotically active pre-OLs[a].
		Immune system development.
		Establishment of the blood-brain barrier.
36–40 wk gestation (term infant)	pnd 7–10	Peak brain growth spurt.
		Peak in gliogenesis.
		Increasing axonal and dendritic density.
		Oligodendrocyte maturation state changes–switch to a pre-dominance of immature OLs.
		Consolidation of the immune system.
2–3 year old	pnd 20–21	Brain reaches 90–95% of adult weight.
		Peak in synaptic density at 50%> adult levels.
		Peak in myelination rate.
		Neurotransmitter and receptor changes.
4–11 year old	pnd 25–35	Fractionation/specialization of prefrontal cortex neural networks (structural maturation).
		Maximum volume of grey matter and cortical thickness.
12–18 year old	pnd 35–49	Reduced synapse density, reaching a plateau at adult levels.
		Refinement of cognitive-dependent circuitry. Ongoing myelination; increasing white matter volume and fractional anisotrophy.
20 years +	pnd 60+	Adult levels of neurotransmitters.
		Adult levels of synaptic density.
		Ongoing myelination and declining grey matter.

Table 2.1: Summary of developmental processes across comparable ages in humans and rodents (Semple et al., 2013).

2.14 Hypothesis

We hypothesised that the extract of *Centella asiatica* may promote improvement in learning and memory, as well as increase the level of GABA A α 1 and AMPA GluA1 activity.

2.15 Rational of study

Centella asiatica is a popular herbal containing beneficial nutrition that has been used as antidepressant, neuroprotective, anti inflammatory, analgesic, wound healing, antimicrobial, antidiabetic, antioxidant, and acethylcholinesterase inhibitor. Moreover, the cognitive function including learning and memory enhancing properties of *Centella asiatica* have been well documented. The medicinal benefits of *Centella asiatica* is due to phytochemicals constituent mainly are triterpenes and flavonoids. The major active component triterpenes is asiatic acid (Tripathi et al., 2015). Adolescence characterized by critical developmental stage is transitional period that occurs between childhood and adulthood characterized by several changes in brain anatomy and physiology (Hammerslag & Gulley., 2014). Thus, treatment with *Centella asiatica* during active brain growth can produce long lasting beneficial effect to the brain. However, there are very less studies and data describing the potential cognitive enhancer effect by crude extract of *Centella asiatica* in learning and memory on adolescent rats. Moreover, there are no previous studies that using both low dose and high dose of crude extract of *Centella asiatica* in learning and memory in adolescent age rats. The purpose of this study is to investigate the effects of the extract of *Centella asiatica* towards learning and memory function that has been carried out on adolescent age rats since active brain growth occurs in the adolescent period.

2.16 Objectives

2.16.1 General objectives

To study the effects of the extract of *Centella asiatica* on hippocampus dependent spatial learning and memory of adolescent Wistar rats

2.16.2 Specific objectives

1. To assess the effects of the extract of *Centella asiatica* on locomotor activity using open field test as well as on learning and memory of adolescent Wistar rats in water T-maze test.
2. To examine the morphological changes in hippocampus of the rats after 2 weeks administration of the extract of *Centella asiatica* using cresyl violet, and apoptosis staining.
3. To analyze the level of glutamate receptor (AMPA subtype - GluA1) and GABA receptor (GABA subtype – GABA A α_1) in hippocampus after 2 weeks administration of the extract of *Centella asiatica* using Horseradish peroxidase/ 3,3′-Diaminobenzidine (HRP/DAB).

CHAPTER 3

MATERIALS AND METHOD

3.1 Ethical approval

The animal ethics in this study was carried out following the approval from the Animal Ethic Committee USM on 2015, USM / Animal Ethics Approval/2015/(98)(699). This was for the Universiti Sains Malaysia (USM) sub-grant titled Modulation of the Standardised Extract of *Centella asiatica* (Pegaga) [BECA] on GABA and Glutamate Receptors: Identification of therapeutic intervention in neurodegenerative models for Dr. Jingli Zhang and Prof Dato' Dr. Jafri Malin Abdullah which is part of a collaborative grant from the Ministry of Agriculture of Malaysia working together with Universiti Teknologi MARA (UiTM) and Universiti Malaysia Terengganu (UMT).