Effects of Honey Produced by Stingless Bee (*Heterotrigona itama*) on Spatial Memory and Learning in Female Albino Mice

BY

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Kesan Madu yang dihasilkan Oleh Lebah Tanpa Sengat (*Heterotrigona itama*) Terhadap Memori Spatial dan Pembelajaran dalam Tikus Betina Albino

OLEH

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LIST OF ABBREVIATIONS

AChE	: Acetylcholinesterase
ACTB	: β actin
Akt	: Protein kinase B
AMPAR	: α-amino-3-hydroxy-5-methyl receptor
ARASC	: Animal Research and Service Centre
BDNF	: Brain-derived Neurotropic Factor
CBP	: CREB-binding protein
CPE	: Carboxy peptidase E
Cpu	: Caudate putamen
CREB	: cAMP response element-binding protein
ERK	: Extracellular signal-regulated kinases
GABA	: γ- amino butyric acid
HD	: Hungtinton disease
Itpr1	: Inositol 1,4,5 triphosphate receptor 1
JNK	: c-Jun amino (N)-terminal kinases
Kb	: Potassium Channel
LGE	: Lateral ganglionic eminence
LTD	: Long Term Depression
LTM	: Long term memory
LTP	: Long Term Potentiation
Map2k3	: Mitogen-activated protein kinase kinase 3
MAPK	: Mitogen-activated protein kinase
mBDNF	: Mature brain derived neurotropic factor

mGluR	: Metabotropic glutamate receptor
MSN	: Medium sized spiny neurons
MWM	: Morris water maze
Nab	: Sodium Channel
NLK	: Nemo-like kinase
NMDAR	: N-methyl-D-aspartate receptor
NOR	: Normal object recognition
NT	: Neurotrophin
OFT	: Open field test
$p75^{NTR}$	: Pan neurotrophin
PI3K	: Phosphatidylinositol-4,5-biphosphate 3-kinase
PIP2	: Phosphatidylinositol 4,5-biphsphate
PLC γ	: Phospholipase C γ
Shc	: Src homology 2-containing protein
STM	: Short term memory
Str	: Striatum
TGN	: Trans golgi network
Trk	: Tropomysin-related kinase
TrkB	: Tyrosine kinase receptor B
UPC2	: Uncoupling proteins 2
VPA	: Valproate
VZ/SVZ	: Ventricular/ Subventricular

LIST OF SYMBOLS

α	: Alpha value; Significance level
β	: Beta
ºC	: Celsius; Unit of temperature
cm	: Centimetre
cm^2	: Centimetre square
δ	: Delta
γ	: Gamma
g	: Gram
kg	: Kilogram
µM	: Micro meter
mm	: Millimetre
mg	: Milligram
mL	: Millilitre
min	: Minutes
n	: Number
%	: Percentage
pH	: P*otential of hydrogen*
p	: *p*-value
s	: Seconds

ABSTRAK

Kesan Madu Lebah *Heterotrigona itama* pada Memori Spatial dan Pembelajaran ke Atas Tikus Albino Betina

Dalam abad ke 21, penurunan daya ingatan dan pembelajaran adalah salah satu isu kesihatan yang penting. Kesan penggunaan madu ke atas pembelajaran dan daya ingatan telah dikaji sejak sedekad dahulu. Oleh kerana sumber madu di negara-negara tropika sangat terhad, madu lebah kelulut dicadangkan sebagai alternatif. Berdasarkan kajian saintifik, kesan positif penggunaan madu lebah ke atas pembelajaran dan daya ingatan masih berkurangan. Oleh yang sedemikian, kajian ini dijalankan bertujuan untuk menilai kesan madu lebah kelulut *(Heterotrigina itama)* ke atas daya ingatan tikus. Tikus betina albino yang berumur dua hingga tiga bulan dibahagikan kepada lima kumpulan dengan setiap kumpulan dirawat dengan kepekatan madu yang berbeza (750 mg/kg dan 2000 mg/kg) selama 7 dan 35 hari. Ujian tingkah laku dengan menggunakan penerokaan medan terbuka telah digunakan untuk menyiasat spatial memori. Selepas ujian tingkah laku, tikus dikorbankan, dan bahagian striatum pada otak diambil untuk proses pewarnaan menggunakan hematoxylin dan eosin (H&E). Selain itu, gen yang berkaitan dengan memori melibatkan *Bdnf, Map2k3* dan *Itpr1*diuji menggunakan *reverse transcriptase chain reaction polimerase (RT-PCR)*. Hasil kajian menunjukkan terdapat perubahan ujian tingkah laku bergantung kepada dos yang diberi ($p \leq 0.05$). Berdasarkan keputusan, madu suplemen 2000/mg/kg yang berikan untuk jangka tujuh hari menunjukkan peningkatan dalam memori dan pembelajaran pada tikus betina berbanding madu yang diberikan 750 mg/kg mengikut jumlah kemasukan ke pangkalan rumah. Namun, apabila jumlah masa di luar pangkalan

rumah diambil kira, tikus yang dirawat selama 35 hari menunjukkan pengurangan masa di luar pangkalan rumah berbanding dengan tikus yang dirawat selama tujuh hari. Suplemen madu lebah kelulut tidak menyebab perubahan neurodegenerasi pada striatum dan terdapat kenaikan pada *Bdnf*, *Map2k3* dan *Itpr1* gen dalam otak yang diperolehi daripada laluan neurotropik (BDNF). Secara kolektif, data dalam kajian ini memberikan bukti awal yang mencadangkan bahawa rawatan madu kelulut *(Heterotrigona itama)* dapat meningkatkan daya ingatan dan pembelajaran dalam tikus albino betina

Kata Kunci: madu *Heterotrigona itama,* striatum, pembelajaran, memori, tikus albino betina, BDNF

ABSTRACT

Effects of Honey Produced by Stingless Bee (*Heterotrigona itama*) on Spatial Memory and Learning in Female Albino Mice

In the 21st century, memory and learning decline is one of the most important health care issues. The use of the effect of honey on memory and learning were investigated since the last decade. To date, sources of honey are limited in tropical countries therefore, stingless bee honey is proposed as an alternative. Stingless bees are found in most subtropical and tropical regions of the world. They are highly eusocial bees and are identified with over 500 species worldwide Scientific research on specific positive effects of stingless bee honey on learning and memory are still lacking. Hence, this study intends to evaluate the effects of stingless honey bee (*Heterotrigona itama*) honey on memory in female albino mice. Female albino mice aged two to three months were divided into four groups with each group treated with different concentration of honey (750 mg/kg and 2000 mg/kg) for a duration of 7 and 35 days respectively and a control group. Open field exploration test was used to access spatial memory. Following behavioral tests, the mice was sacrificed, and the striatum was harvested for hematoxylin and eosin (H&E) staining. Lastly, genes linked with memory were tested through reverse transcriptase polymerase chain reaction (RT-PCR). The current findings indicated that there are changes in the dose-dependent manner ($p \leq 0.05$). From behavioral analysis, 2000 mg/kg of stingless honey bee supplementation for seven days showed improvement in the memory and learning of female mice compared to 750 mg/kg of stingless bee honey during total entries to the home base. However, when compared to the total time outside the home based, mice

treated for a duration of 35 days spent less time outside the home base as compared to a duration of seven days indicating that mice treated for a longer duration adapted quickly to the environment thus showed a reduce in anxiety as compared to mice treated with stingless bee supplementation for a shorter duration of seven days. Stingless honey bee supplementation did not cause any neurodegenerative changes in the striatum and there was an upregulation of *Bdnf, Map2k3* and *Itpr1* genes from the brain derived neurotropic (BDNF) pathway. Collectively, the data in this study provides preliminary evidence suggesting that stingless bee honey (*Heterotrigona itama)* treatment enhances spatial memory and learning performances in female albino mice.

Keywords: *Heterotrigona itama.*, striatum, learning, memory, female albino mice, BDNF

CHAPTER 1

INTRODUCTION

Stingless bees (Meliponini *sp.*) are found in most subtropical and tropical regions of the world. They are highly eusocial bees and are identified with over 500 species worldwide (Biluca, et al., 2016; Chuttong et al., 2016). In Malaysia, the bees are known as 'Lebah Kelulut'. The multiflora honey produced by the stingless bees are stored in resin pots within the nests. The two main species of stingless bees being reared in Malaysia are the *Geniotrigna thoracica* and *Heterotrigona itama* (Abu Bakar et al., 2017). Also, stingless bees uses resins of different plant species for chemical defences, food and nest construction (Salim et al., 2012). Stingless bees are important for the environmental integrity as their colonies are affected by local disturbance and tree health (Salim et al., 2012).

There is an increasing number of supporting scientific studies in both animal and human models suggesting the benefits of honey in memory and learning (Al-Rahbi et al., 2014; Azman, et al.,2015; Othman et al., 2015; Othman et al., 2011; Rao et al.,2016). Previous findings mention that presence of oxidative properties can slow down or stop the process of oxidative stress in the cells leading to the development of degenerative and neurological diseases such as Parkinson's and Alzheimer diseases (Yaacob et al., 2018). The composition of honey differs in contributions of the beekeepers, environmental aspects, climates and plant types (Akanmu et al., 2011; Nweze et al., 2016). Honey is an important part of traditional medicine and is contains

about 200 substances including 25 different sugars functioning independently in the human metabolism. In addition, honey also contains proteins, acids, minerals and a number of minor components such as colloids, minerals, flavour, vitamins, aroma substances, enzymes, amino acids, pollen, sugar alcohols, and pigments (Akanmu et al., 2011; Nweze et al., 2016). Stingless bee honey is a type of honey produce by most stingless bees. It is used extensively for anti-inflammatory, antimicrobial, colorectal cancer and has antioxidant properties (Yaacob et al., 2018). In Malaysia, the *Kelulut* bee honey has high medicinal values (Rahman et al., 2015; Yaacob et al., 2018) despite that, majority of previous literature have been conducted using honey from the *Apis* species (Nweze et al., 2016).

Living organisms requires memory as an essential feature. Hence, memory marks multiple similar events or a certain experienced to enable an organism to produce a more appropriate or a faster reaction. This is termed 'learning' as organisms need to evaluate the memory against stored background information (Witzany, 2018). Therefore, learning and memory are cognitive functions that encompasses of many subcomponents that are mediated through neuronal plasticity (Brem et al., 2013; Giese & Mizuno, 2013). Brain's functional organization and physical changes are altered by thinking and learning according to the theories of neuroplasticity (Galván, 2010). Animal studies suggest that cortical morphology is influenced by an increased size of the soma and nucleus of neurons, capillary and glia dimensions and when animals are exposed to enriched environments. The increase of cortical grey matter results alterations in the strength of existing connections and the formation of new connections by dendritic spine growth (Galván, 2010).

The striatum has an involvement in basal ganglia and as such plays a role in the complex forms of memory and learning (Leonibus et al., 2005; Lovinger, 2010). A manipulation in this region affects the processing of spatial information from a variety of learning tasks (Leonibus et al., 2005). The neurotrophin brain-derived neurotrophic factor (BDNF) controls plasticity and synaptic transmission, and promotes neuronal differentiation and survival (Caldeira et al., 2007; Cunha, 2010). BDNF is studied most extensively in the adult central nervous system (CNS), as it has shown to provide an important role in long-term potentiation (LTP). LTP is a form of synaptic plasticity which is considered a cellular model for long-term memory (LTM) formation (Cunha, 2010). Therefore, the present study aims to investigate the effects of honey produced by stingless bees (*Heterotrigona itama*) on spatial memory and learning in female albino mice focusing on the behavioural and gene expression aspect of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Comparison between Honey Bees and Stingless Bees

Honey is a sweet and vicious substance produced by honeybees through the secretion of plants and nectar of blossoms or through the excretions of plant sucking insects in which honeybees (*Apis* sp.) with specific substance of their own collect, combine or transform honey leaving it in the honey comb to mature and ripen (Abeshu & Geleta, 2016; Abu Bakar et al., 2017) while the stingless bee honey known as *Kelulut* produces multiflora honey that stores in clusters of small resin pots near the bee nests (Figure 2.1) (Abu Bakar et al., 2017). Honey has simple sugars and is absorbed directly into bloodstream without digestion making it often eaten as an energy food (Abeshu & Geleta, 2016). Table 2.1 compares the differences between honey bees and stingless bees.

Table 2.1 Differences between Honey Bees and Stingless Bees

Characteristics	**Honey Bees**	**Stingless Bees**
Types of bee honey in Malaysia	Tualang honey	Kelulut honey
Produced by	*Apis dorsata*	*Meliponini sp.*
Honey storage	Honey combs (Kek et al., 2014)	Honey pots (Kek et al., 2014)

Figure 2.1 Resin pots from the stingless bee honey. The multiflora honey is stored in clusters in small resin pots

2.2 Stingless Bees

A considerable amount of literature has been published on stingless bee honey identifying them as Meliponine. The first written references mentioning Meliponine bees are from the German and Spanish explorers in South and Central America and in Europe in the 16th century following reports from Australia in the 17th century (Biluca et al., 2016; Chuttong et al., 2016; Hrncir et al., 2016). About 250 stingless bee honey are identified throughout the Indo-Burma-Malayan and Australian region and Neotropical regions of the world (Figure 2.2) (Rahman et al., 2015).

In Malaysia, there are about 35 species of stingless bee identified. The four most popular cultured species are the *Geniotrigona thoracica*, *Lepidotrigona terminate*, *Trigona leaviceps* and *Heterotrigona itama* (Baharuddin et al., 2014). Duarte et al., (2014) mentions that molecular markers plays a role in the estimation of genetic diversity and the level of population structure. As such, microsatellites successfully characterize genetic variability at the inter- and intraspecific level in different Meliponine species.

Figure 2.2 Stingless Bee. The bee is identified as Meliponine.

2.3 Stingless Beekeeping and Honey Harvesting

Stingless bee keeping is known as meliponiculture. In Malaysia, it is recorded that meliponiculture has been established in an ancient undertaking albeit during the Malacca Sultanate era (Basrawi et al., 2017; Chuttong et al., 2016). However, honey hunting often leads to destructions of nests and trees. Since pots with pollen are found in honey storage area; during the harvest, stingless bee honey is squeezed out of the storage pots resulting in a large amount of pollen getting lost during the harvest (Ramli et al., 2017; Sommeijer, 1999). Also, the emptied nest material is thrown away and the nest cavity in log-hive unreachable as the log has narrow openings at the end. When stingless bee honey is collected from natural colonies, part of the brood may be destroyed, and the colonies can suffer badly because of the harvest (Ramli et al., 2017; Sommeijer, 1999). Death in pupae development is caused when there is a rise in hive temperature causing a detrimental towards the growth of colony. Thermostatic effects promote thermoregulation in natural hives, where shades along with wood insulation

are able to keep the consistency of the hive temperature (Ramli et al., 2017). Therefore a project from Mustafa et al., (2017) establishes stingless beekeeping as an alternative approach to promote sustainable environment via espousal of pollination by the bees and to stimulate rural transformation in generating income.

Mustafa-Hive (MH) is utilized; a main innovation from USM with collaboration from Honeygold Laboratory as an industrial partner and from DRONESS – a non-government organization (NGO) (Mustafa et al., 2017). In traditional log hive rearing techniques, a topping compartment is attached to the log to enable honey production. Mustafa-Hive is an acronym of meliponiculture that is used once a colony is obtained from a log. When colonies enter Mustafa-Hive, colonies can be split into two. This process occurs repeatedly once colonies are settled making this a sustainable system for stingless beekeeping. The hive is also equipped with honey cassettes that induce monolayer honey pods making honey to be easily harvested using suction pumps. Also, the Mustafa-Hive reduces the intrusion and disturbance into the colony upon harvesting (Ramli et al., 2017). Mustafa-Hive confirms the sustainability of the new beekeeping industry through quality improvement, sustainable farming methods and standardization of production (Mustafa et al., 2017).

The study by Mustafa et al., (2017) results in the induced formation of monolayer honey pots and colony survival. The Mustafa-Hive module enables hygienic and absolute stingless bee honey extractions from honey cassette thus indirectly promotes the development of the stingless bee industry and encourages sustainable meliponiculture. Hence, the current study uses honey obtained and provided from the Mustafa-Hive rearing system.

2.4 Physicochemical Properties of Stingless Honey Bees

Studies on the physicochemical properties on the Malaysian stingless bee honey were reported by Abu Bakar et al., (2017) and Kek et al., (2014). The physicochemical properties from previous studies identifies colour intensity; a reliable parameter that specifies the presence of pigments that represents antioxidants such as flavonoids and carotenoids. The Kelulut honey had ABS_{450} values at ranges 1029.00-2103.17 mAU. ABS_{450} is an indication for the presence of flavonoids and carotenoids in stingless bee honey. The range from the study was greater that the various types of Malaysian honey produced by the *Apis* sp reported by Khalil et al., (2011). A higher value of ABS_{450} in Kelulut honey provides a higher content of colour pigments as compares to other Malaysian honey (Keng et al., 2017). The colour of honey is black and sometimes red or green hues, but they usually vary naturally from light yellow to amber to dark amber (Abu Bakar et al., 2017; Santos do Nascimento et al., 2015).

The dark amber of honey describes a higher mineral content than lighter ones (Sgariglia et al., 2010). From the US Department of Agriculture-approved standards, the colour values from the study classifies *H. itama* honey with a dark amber colour while *G. thoracica* honey as amber. According to Kek et al., (2014), *Kelulut* had the highest colour intensity suggesting high antioxidants. Overall, there is a higher value of phenolic content in Malaysian honey in both *Kelulut* and *Apis* sp. This is predictable as composition and properties of honey is affected greatly by various factors such as biodiversity and collection season in Malaysia, conditions of honey, mode of storage, nectar source and harvest technology (Kek et al., 2014).

Also, reports from Keng et al., (2017) mentioned that the tested stingless bee honey samples ranges from pH 3.29 to pH 3.71. The high acidity of the stingless bee honey analysed in the study was due to the presence of hydrogen peroxide (H_2O2) and high levels of organic acids is responsible for the stability against microbial spoilage and flavour (Santos do Nascimento et al., 2015).

Moisture influences specific weight, viscosity, crystallization, maturation, conversation and taste of honey (Santos do Nascimento et al., 2015). The Kelulut honey from a previous study reported to have a moisture content of 21.4-31.59%. Similar study reported that Kelulut honey had a higher moisture content than honey of *Apis* sp. in Malaysia (14.86-19.06%) (Keng et al., 2017).

Electrical conductivity is a parameter that depends on the floral source of honey and is closely related to the concentration of proteins, minerals and organic acids (Santos do Nascimento et al., 2015). Ash content is associated with electrical conductivity of honey revealing presence of mineral content (Biluca et al., 2016; Santos do Nascimento et al., 2015). Keng et al., in 2017 reported that Kelulut honey was in range of 0.22-0.41% of the ash content which were within values of previous studies (0.03-1.23). In addition, hydroxymethylfurfural (HMF) is used as an indicator for the quality of honey. When honey is subjected to inadequate storage conditions, addition of invert sugars or high temperature, the HMF content increases resulting in aging of honey (Santos do Nascimento et al., 2015).

2.5 Effects of Honey on Memory and Learning

Several studies show honey gives beneficial effects on memory and learning in clinical trials and animal studies (Azman et al., 2015; Mijanur et al., 2014; Othman et al., 2015). Rao et al. in 2016 reported that Tualang honey showed a significant activity against cerebral hypoperfusion, which is one of the numerous factors contributing to Alzheimer's disease (AD). Following a study on 102 healthy postmenopausal women, participants showed improvement in their immediate memory after receiving Tualang honey. The study concludes that the result was similar with memory enhancement found in women receiving oestrogen plus progestin therapy (Othman et al., 2015). Al-Himyari, (2009) found that components in honey may prevent other cognitive diseases and dementia after a long-term study on the efficacy of honey in treating dementia in humans.

One of the most common types of studies on animals provides a correct dosage of treatment which is proportional to the surface area of the test animals rather than the body weight. In other words, smaller animals generally require a larger dosage on a milligram per kilogram basis (mg/kg) (Wojcikowski & Gobe, 2014). For instance, in the study of Chepulis et al., (2009) a long-term feeding of honey, sugar-free and sucrose diet may provide some effects on spatial memory and anxiety on rats. A too small a dosage is used, may result in no observed effects and for the scientific community interested in drug discovery hence, it is often difficult for scientist to choose the optimal dose in animal experiments testing the benefits of honey for example (Wojcikowski & Gobe, 2014). Emerging research has documented that rats fed with different honey concentration (10, 20, and 40%) resulted in a reduce in anxiety

and has excitatory effects on the central nervous system (CNS), particularly at the highest non-sedative dose (Oyekunle et al., 2010).

In animal studies, Tualang honey reduces depressive symptoms and improves memory induced by loud noise. Rats treated with Tualang honey produced better memory scores compared to the control rats (Azman et al., 2015). Honey-fed rats showed better spatial memory and significantly less anxiety throughout all stages compared with the control group of rats. In addition, as assessed by object recognition tasks, the spatial memory of honey-fed rats was significantly higher during later months compared to sugar-free and sucrose diet (Chepulis et al., 2009; Mijanur Rahman et al., 2014). Also, glial cells responds to honey therapy in addition to neural effects as honey shows a neuroprotective effect in ischemic model in rats (Mijanur Rahman et al., 2014).

2.6 Fundamentals of Memory

Memory has different stages or phases according to Bekinschtein et al., in 2014 (Figure 2.3). Long term memory (LTM) is divided into declarative memory and procedural memory. Declarative memory is further categorized into sematic memory (facts) and episodic memory (experiences/events) (Izquierdo et al., 2006; Omizzolo et al., 2014). Episodic memory captures information following an order of events and is linked to spatial encoding. In addition, Buzsaki & Moser, (2013) states that egocentric network contributes to episodic memory. However, sematic memory is suggested to be an extension of the spatial encoding which is memory of places as well as facts. It is tested as allocentric memory and learning in rodents (Vorhees & Williams, 2014).

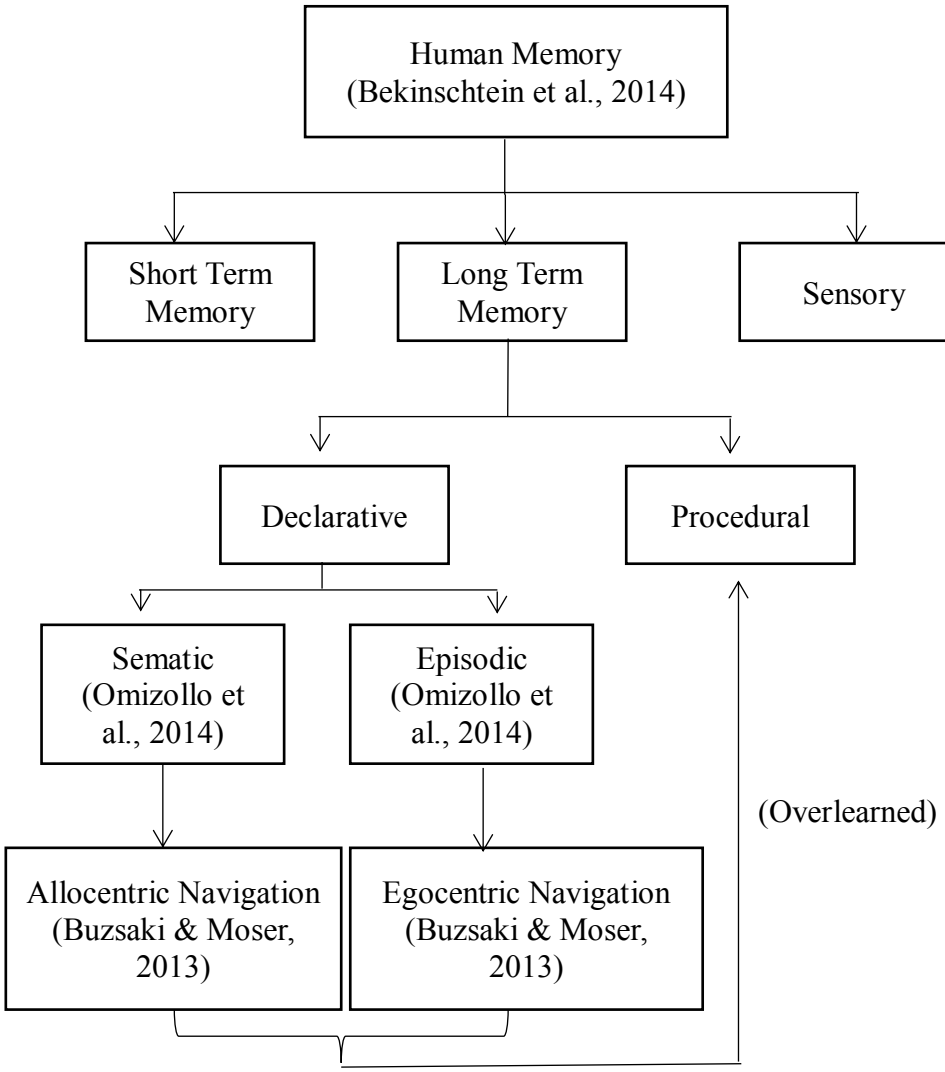

Figure 2.3 Summarization of Human Memory

There are at least two distinct types of navigations; egocentric and allocentric navigations. Egocentric navigation requires the ability to operate using internal cues (Vorhees & Williams, 2014). Egocentric navigations are further divided into path integration and route-based navigation. Path integration relies on rate of turns, signposts and vector addition and movements whereas route-based navigation follows a path of specific set of rules with the order of turns recalled. As an example, humans retrace their steps when blindfolded (Eilam et al., 2003; Vorhees & Williams, 2014).

Allocentric spatial memory is refer to mnemonic representations that focuses viewpoint-invariant relations among items, as well as fixed relations between local environment and items (Fidalgo & Martin, 2016). Previous author has mentioned the roles of the hippocampus in allocentric spatial memory (Fidalgo & Martin, 2016).

2.7 Brain Region Mediating Navigation

The striatal complex is the largest of the basal ganglia nuclei (Figure 2.4) and is located at the base of the forebrain and is important for learning and decision making. It has been generally thought from a functional point of view, that the striatum is involved in the motivational process and the control of motor function (Leonibus et al., 2005; Shiflett & Balleine, 2011). The striatum receives projections from the entire cortical and passes it to the nuclei of basal ganglia. The medium-sized spiny neurons (MSNs) mediates most striatal functions, which comprises of 5% of interneurons with the rest being striatum. MSNs migrates to the striatum during embryogenesis. Also, MSNs are born in the ventricular/subventricular zones (VZ/SVZ) of the lateral ganglionic eminence (LGE), that uses γ-amino butyric acid (GABA) as a transmitter (Baydyuk & Xu, 2014). It has been demonstrated that impairments at the striatum induce deficits in many learning tasks that require the processing of spatial information (Leonibus et al., 2005).

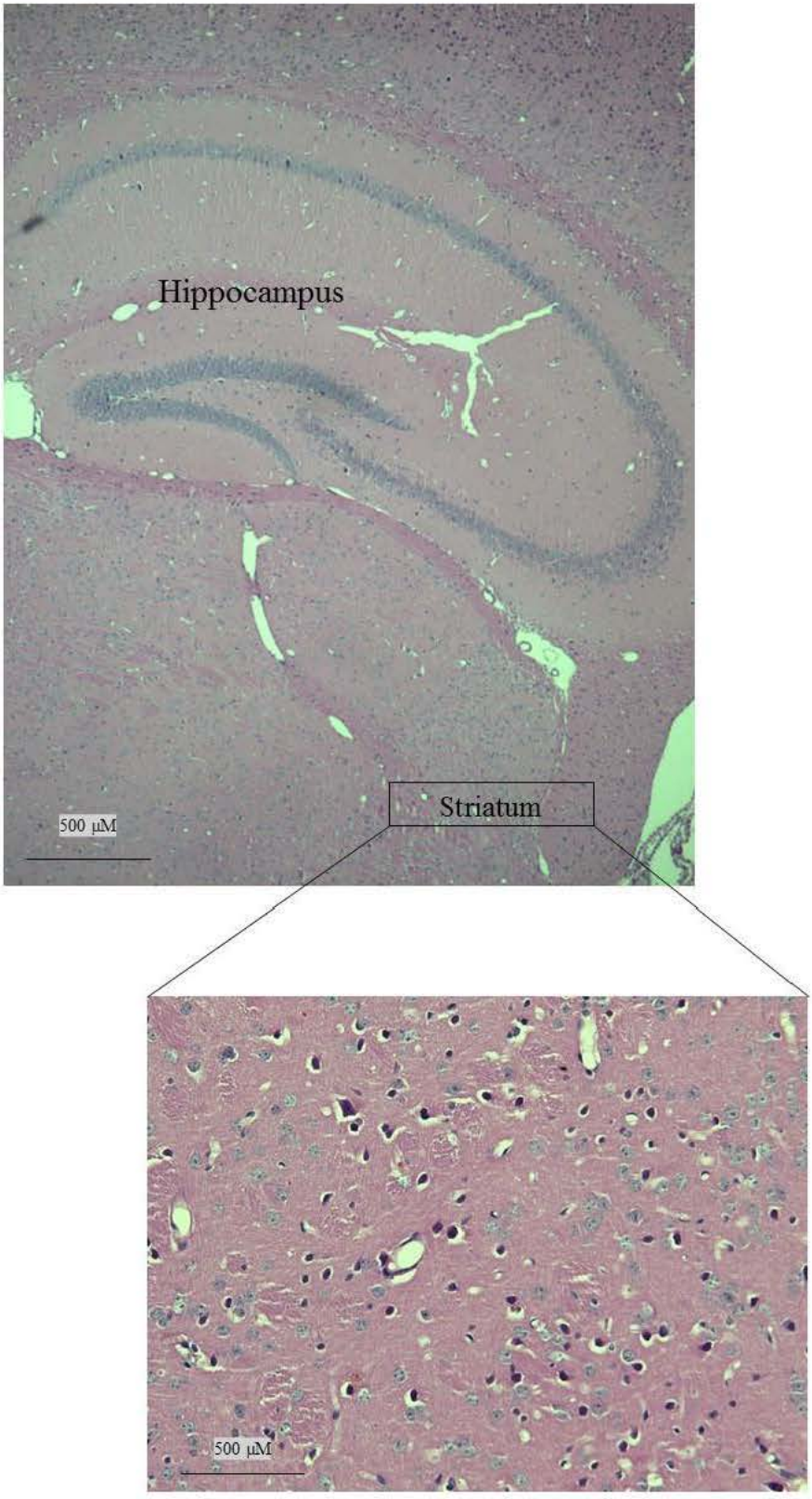

Figure 2.4 Brain region portraying hippocampus and striatum of mouse. The coronal section of the brain is stained with hematoxylin and eosin (H&E) staining under 100 X magnification. Coronal sections of the striatum were obtained through the guidance of Franklin & Paxinos, (2008) from Bregma -0.58 mm to -1.06 mm.

Leonibus et al., (2005) shows that pharmacological influences of the striatum affect the performances in the radial maze, morris water maze (MWM) and in tasks of spatial displacements. There is substantial evidence that the striatum has roles in mediating spatial memory and learning. Izquierdo et al., (2006) suggests that animal behavioural studies have both a formation of habit and a declarative component; all of which closely mimic human situations of daily life.

The procedural version is depended on the striatum. Rodents must learn their way in a non-spatial version of the MWM to the platform through exploration of the tank in which no visual cues are presented. A procedural learning is also found in the spatial version of this task: *i.e.*, to swim to escape; an acquired habit. The declarative information (the one oriented by spatial cues) on where to escape depends on the underlying knowledge of swimming to escape. People with striatal impairment have difficulty in retracing the circle relative as compared to people without striatal impairment. Interestingly, habits occur when the memorized operations are overlearned. When this occurs, procedural or implicit memory occurs when the location of the memory is shifted within the brain (Izquierdo et al., 2006).

2.8 Molecular Signalling in Memory

2.8.1 Neurotrophin

Recent studies have shown that striatal neurons are dependent on neurotropin (NT) for their survival, growth and proper functions (Baydyuk & Xu, 2014). Leal et al., (2015) mentions that activation of $p75^{NTR}$ and tropomyosin-related kinase (Trk) receptors mediates physiological responses to NT. The NT family comprises of BDNF, nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5). Leal

et al., (2015) also states that NT3 binds to TrkC, BDNF and NT4/5 binds to TrkB and lastly NGF binds to TrkA. NT signalling through TrkB receptors regulates proliferation, cell survival, dendrite and axon growth and the fate of neural precursors (Bathina & Das, 2015; Murray & Holmes, 2011).

2.8.2 Brain Derived Neurotropic Factor

Brain derived neurotropic factor (BDNF) is abundant in brain regions and is an important mediator of neuronal plasticity. Also, BDNF bridges experience with enduring change in neuronal function because it shows a remarkable activity-dependent regulation of secretion and expression (Bliss & Cooke, 2011; Calabrese et al., 2014; Walker et al., 2011). Deletion of either the *Bdnf* or *TrkB* gene leads to dendritic degeneration, neuronal loss and cell decline in the excitatory neurons of the dorsal forebrain. In addition, BDNF plays a huge role in LTP- a cellular substrate for memory and learning by modulating plasticity and synaptic function (Baydyuk & Xu, 2014).

Lovinger in 2010 realizes that long term synaptic plasticity differs in different striatal sub regions, with LTD predominating in caudal striatum and dorsolateral striatum and LTP more prevalent in rostral and dorsomedial striatum. By regulating the phosphorylation of NMDAR, BDNF enhances glutamatergic synaptic transmission post-synaptically thereby enhancing NMDAR activity. BDNF causes changes in the synaptic strength in an activity-dependent synaptic plasticity where it is thought to underlie learning and memory formation (Caldeira et al., 2007).

BDNF participates in early phases of LTD and LTP through co-release with presynaptic glutamate (Bliss & Cooke, 2011). Accordingly, the activity-dependent synaptic plasticity includes early- and late phases of LTP induced by high-frequency stimulation (Caldeira et al., 2007). In other words, spatial memory explicitly encodes, stores, identifies and recalls particular arrangement or spatial information of specific routes or objects; including related spatial mental images in the location (Kessels et al., 2001). In animals, spatial learning is a representative for declarative memory as it comprises a flexible spatial cognitive (Ferbinteanu, 2016).

The roles of BDNF in the CNS depends on $p75^{NTR}$; a receptor that interrelates with the precursor pro-BDNF and the TrkB receptor that is responsible for the functions of BDNF (Bekinschtein et al., 2014). The activation of the p75 receptor enables in the change of the subunit composition of the N-methyl-D-aspartate (NMDA)-type receptor (NMDAR) that promotes the induction of LTD while TrkB, whose activation results in the induction of LTP (Bliss & Cooke, 2011). BDNF is synthesized in the endoplasmic reticulum (ER). Pro BDNF (precursor protein) travels through the trans-Golgi network (TGN) and Golgi body and is sorted by vesicles in the presence of lipid raft that is linked with a sorting receptor carboxy peptidase E (CPE). Eventually pro BDNF is transported into activity-dependent secretion by post-synaptic dendrites. An active mature BDNF (mBDNF) is formed when the terminal domain of pro-BDNF is cleaved by a different protein convertase enzyme (Bathina & Das, 2015; Leal et al., 2015).

Mitochondria not only plays an important role in plasticity and synaptic plasticity, but it also generates adenosine triphosphate (ATP) (Geisler et al., 2017).

Mitochondrial uncoupling proteins (UPC) are inner mitochondrial membrane proteins that reactive mitochondrial Ca^{2+} levels, regulate energy metabolism and reactive oxygen species production (Barnstable et al., 2017). Hence, UCP2 acts as a neuroprotector and a neuromodulator in the CNS (Barnstable et al., 2017). Among the signalling pathways that affects mitochondrial function are BDNF and glutamate; both in which plays important roles in learning, memory and synaptic plasticity (Geisler et al., 2017).

Reinhart et al., (2015) addresses that Pro-BDNF protein is encoded with one 3' protein coding exon from the BDNF gene with eight 5' non-coding exons (exons I-VIII) is regulated by alternative promoters. Eleven different BDNF transcripts in rodents and humans are results from the alternative splicing of each 5' non-coding exon to the common 3 coding exons. In addition, the transcripts throughout the neuronal subtypes and the brain are expressed differently however they eventually translate the same mBDNF protein. BDNF binds with TrkB leading it to the autophosphorylation and dimerization of tyrosine residues in the intracellular domain of the receptor. BDNF regulates cation channels such as Kb channels and Nab channels. Not only that, BDNF affects protein synthesis by translational and transcriptional mechanism and modulated ligand gated gates.

BDNF signalling pathways activates either one or both CREB-binding protein (CBP) and CREB transcription factors; regulating expressions of gene encoding proteins involving in stress resistance, cell survival and neural plasticity (Figure 2.5) (Bathina & Das, 2015). There are three major signalling pathways that is activated by BDNF which are extra-cellular signal-regulated kinase ½ (ERK ½), PLC-γ, and

phosphatidylinositol 3-kinase (PI3K) (Baydyuk & Xu, 2014; Bekinschtein et al., 2014; Cunha, 2010; Yamada & Nabeshima, 2003).

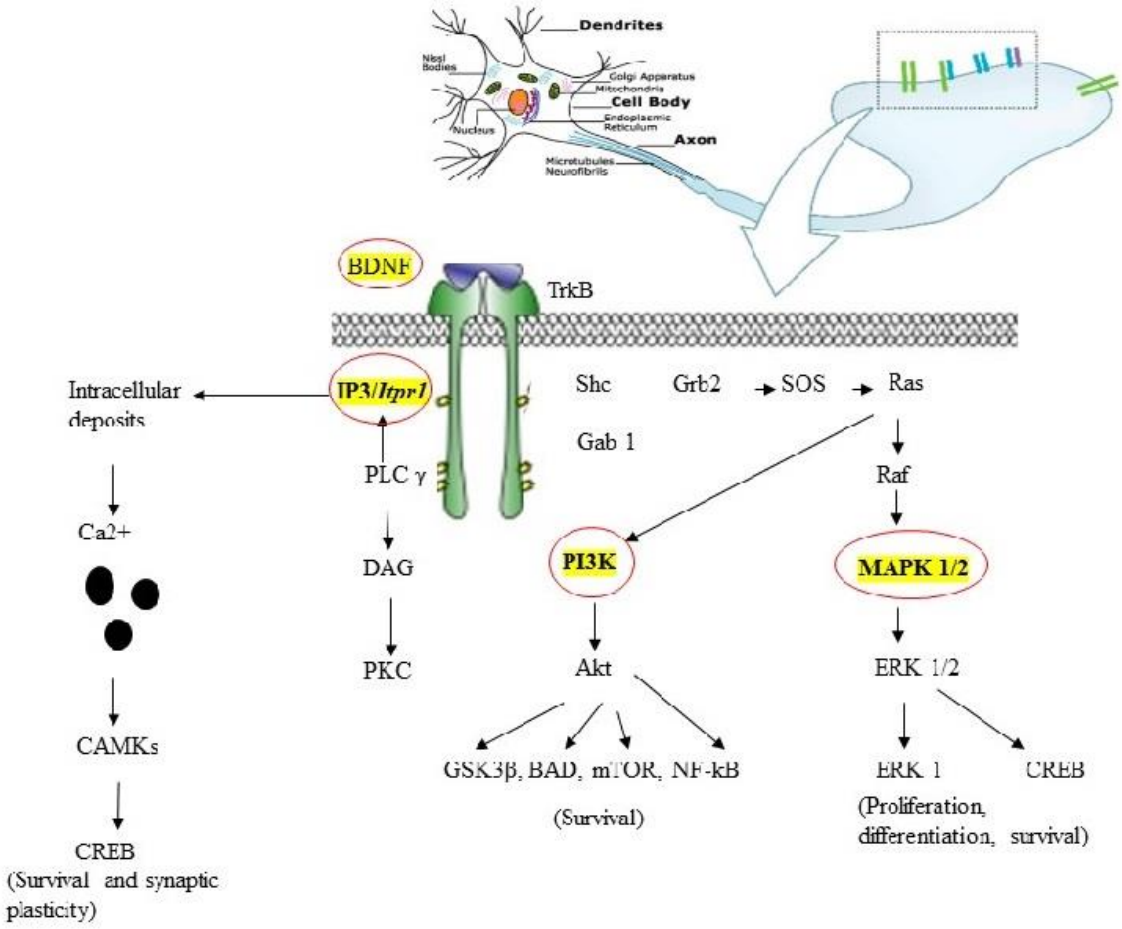

Figure 2.5 Schematic of BDNF signalling pathways. (Image summarized and modified from Bathina & Das, 2015; Tejeda & Díaz-Guerra, 2017. Date retrieved 4, February 2018).

2.8.2.1 PLC/DAG/IP3 Pathway

Inositol 1,4,5 triphosphate receptor (IP_3R) has a molecular weight of 313000 Da. Mikoshiba in 2015 reports that IP_3R has IP_3 binding site at the N-terminal portion and transmembrane region existed at the C-terminal portion through intensive biophysical and biochemical analysis. When BDNF docks with Trk receptor, adapter

protein PLC-γ is phosphorylated causing a breakdown of membrane lipids to IP3 promoting an increase in diacylglycerol (DAG) and intracellular Ca^{2+} concentration (Fedorenko et al., 2014). DAG regulates protein kinase C; a requirement in neurite outgrowth for the MAPK/ERK signal (Bathina & Das, 2015; Baydyuk & Xu, 2014). A reduced IP3 formation following a reduction in intracellular Ca^{2+} release might be related to the blocking of PLC-γ signalling thus inhibiting LTP (Murray & Holmes, 2011).

IP_3R molecules are expressed in a cell type-specific manner and is bind to each isoform in an isoform specific manner. As the size of IP_3R is long, especially between the transmembrane channel pore region and the IP_3 binding site, therefore three different platforms of IP_3R may offer the interactions between various molecules. Not only that, abnormality of signalling trajectory and IP_3R subtype results in various disease state (Mikoshiba, 2015). An example of IP_3R subtype is the IP_3R1. IP_3R1 is involved in neural plasticity and neurite extension. According to Higo et al., (2010), blockage of IP_3R1 caused by ER stress results in brain damage. Evidently, each IP_3R type plays a significant role in determining its trajectory of cell function and signalling and a role as a signalling hub by association with various molecules.

2.8.2.2 Ras/MAPK/ERK Pathway

Mitogen-activated protein kinases/Extracellular signal-regulated kinases (MAPK/ERK) has an important role in neurogenesis; a common mediator underlying both improvement of cognition and memory and learning formation (Bathina & Das, 2015; Garthe et al., 2016; Peng et al., 2010). Fourteen MAPKs has been characterized

into seven groups in mammals. These type of protein coverts extracellular stimuli into a wide range of cellular responses. Atypical MAPKs consist of nonconforming particularities and comprises of nemo-like kinase (NLK), extracellular signal-regulated kinases 3/4 (ERK 3/4) and ERK7. However, conventional MAPKs consists of c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38 isoforms (α, β, γ and δ), ERK5 and ERK1/2. MAPK, MAPKK and MAPKKK are a set of three evolutionarily conserved, sequentially acting kinases that is composed from each group of conventional MAPKs (Cargnello & Roux, 2011; Giese & Mizuno, 2013).

When TrkB binds to BDNF, it results in auto-phosphorylation and dimerization of tyrosine residues forming a docking site for phospholipase C (PLC) and src-homology 2-domain containing adaptor protein (Shc). Shc bounds to adapter protein Grb 2 and when it is docked to the receptor by guanine nucleotide-releasing factor SOS, Ras activates the Ras/MAPK-ERK pathway, PLC pathway and PI3K pathway (Bathina & Das, 2015; Murray & Holmes, 2011; Peng et al., 2010).

2.8.2.3 IRS/PI3K/AKT Pathway

Other pathways downstream to BDNF includes activation of protein kinase B (Akt), PI3K and insulin receptor substrate -1 (IRS-1/2). Fear condition induces PI3K/Akt signalling by activating TrkB and increasing the BDNF protein expression. This is important for fear long term memory (LTM) formation. The amygdala plays a role in the PI3K/Akt signalling in the LTM formation, while this pathway is unnecessary in the hippocampal LTM formation (Giese & Mizuno, 2013). Following the study of Giese & Mizuno, (2013). ERK activation is induced when retrieval of

contextual fear LTM causes the activation of PI3K/Akt signalling in the hippocampus. Besides roles in memory, Ras plays a major role in apoptosis. Also, PI3K pathway is crucial in the activation of pro-survival genes that is responsible for cell survival. An inhibition of any part of the Ras-PI3K-Akt pathway knowingly reduces survival of sympathetic neurons in the presence of NGF (Bathina & Das, 2015; Manning & Toker, 2017).

2.9 Animal model in Assessing Memory and Learning

Several animal models of memory and learning has been developed recently. For example animals learn to suppress exploratory tendency to avoid aversive stimuli in the step-down inhibitory avoidance test (Kolata et al., 2008). The paradigm is frequently used and is well established to access learning and hippocampus-dependent memory (Izquierdo et al., 2006) in rats and mice. The test is usually an association made between an aversive stimulus (a footshock) and a movement (stepping down) in a particular context that takes place in seconds leading to a robust memory (Izquierdo et al., 2006).

Not only that, the Y-maze with external cues is also a test for measuring hippocampus-dependant/spatial memory. This paradigm is based on the innate tendency of the rodent to remember a rewarding environment or a location of safe and to explore a new environment (Paul et al., 2009). The experimental animals are usually maintained on a water-restricted or food-restriction diet and are trained to choose specific arm of the maze to receive food or water reinforcement. Also, the experimental animal were shown to create a cognitive map in the test maze, which is

to locate the target within the maze and is a strategy of real global representation of external environment (Paul et al., 2009)

In addition, the open field (OF) is the most popular test in animal psychology for the use in behavioural phenotyping in rodents, mainly laboratory mice and rats (Figure 2.6). The test animal is commonly regarded as a primary index and is introduced into an illuminated and plain arena; observed for time length ranging from a few minutes to repeated exposures for several hours (Eilam, 2003; Kalueff et al., 2007). Other studies have considered that drug-treated rats are organized to a key location- the home base and that spatio-temporal structure of OF behaviour is normal. The assumption of these studies is that the trips, stops and the home-base are essential for locomotor behaviour in the OF that highlight the changes it undergoes in psychological or genetic preparations and the structure of behaviour (Eilam, 2003).

Dvorkin et al., (2008), states that the home-base acts as an attractor in two ways: first, within a roundtrip, the home base applies a gradually increasing attraction on the rat to return to it, and second, the stops the rat takes upon visiting it. It is revealed in previous work on rats that home-base is the most ideal place in the location upon examinations of the spatial distribution of stops. Therefore, memory and recognition of home-base location is implicated when the both forms of attraction are acted on; a locational memory. The author highlighted that locational memory in the study implies familiarity and spatial recognition, consequently also reflects spatial memory.

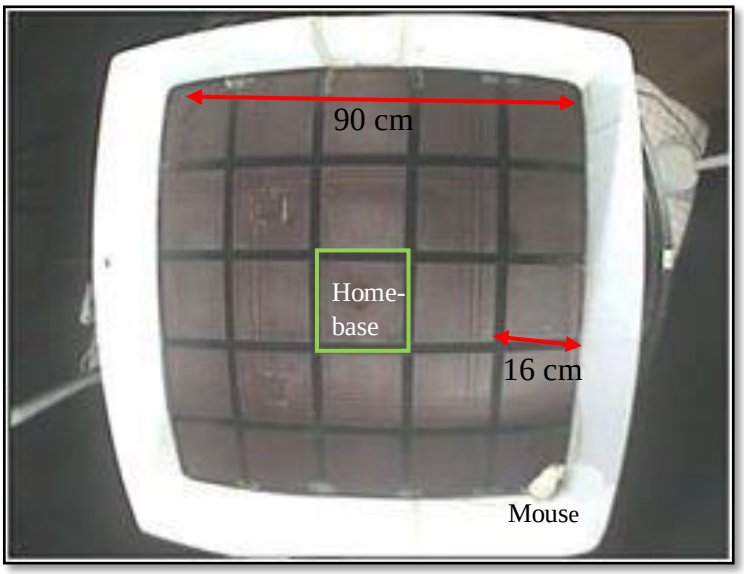

Figure 2.6 Set up of Open Field Test

2.10 Rational of the Study

Memory and learning decline is one of the most important health care issues. While considering honey bee population like Tualang (*Apis* sp.) is declining due to pesticides, lodging and timber activities, an alternative source from stingless bee honey is sought after. Tualang honey has been thoroughly studied on the effects of memory and learning however, the mechanism of stingless bee honey supplementation for the improvement of memory and learning through the brain derived neurotropic factor pathways (BDNF) have not been identified. Some studies have suggested a role of stingless bee honey in the enhancement of memory and learning, although this have not been observed consistently. Even though there is strong evidence of stingless bee honey supplementation in memory and learning exposure, but a link between memory and learning through the BDNF pathways and stingless bee honey supplementation has not been shown.