CHARACTERISATION OF TUALANG HONEY SILVER NANOPARTICLES (THSN) AND NEUROPROTECTIVE EFFECT OF TUALANG HONEY AND THSN ON KAINIC ACID-INDUCED NEURODEGENERATION IN MALE RATS' HIPPOCAMPUS

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LIST OF ABBREVIATIONS

4-HNE	4-hydroxynonenal
AD	Alzheimer's diseases
Ag^{+}	Silver ion
Ag^{0}	Metallic silver
$AgNO_3$	Silver nitrate
AIF	Apoptotic-inducing factor
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
AMPA	a-amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
ARASC	Animal Research and Service Centre
ATP	Adenosine triphosphate
BAD	BCL2 associated agonist of cell death
BAK	BCL2 antagonist/killer 1
BAX	BCL2 associated X
BDNF	Brain-derived neurotrophic factor
BH3	Bcl-2 homology 3
BID	BH3 interacting domain death agonist
BIM	Bcl-2-like protein 11
BSLT	Brine shrimp lethality test
Ca^{2+}	Calcium ions
CA1	Cornu ammonis 1

CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
Caspase-3	Cysteinyl aspartate specific proteinases-3
CAT	Catalase
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
CNS	Central nervous system
COX	Cyclooxygenase
CRL	Central research laboratory
CXCL10	C-X-C motif chemokine ligand 10
dH_2O	Distilled water
DLB	Dementia with Lewy Bodies
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent one-step process assay
FADD	FAS-associated death domain
FAS	Fas cell surface death receptor
FDA	Food and Drug Administration
$FeCl_3$	Ferric Chloride
FESEM	Field emission scanning electron microscope analysis
FGS	First generalised seizure
FJC	Fluoro Jade C
$FeSO_4$	Ferrous sulphate
FRAP	Ferric reducing antioxidant power

FT-IR	Fourier transform infrared spectroscopy
GABA	Gamma-aminobutyric acid
$GABA_AR$	Gamma-aminobutyric acid type A receptors
GFAP	Glial fibrillary acidic protein
GGT	Gamma-glutamyl transpeptidase
GMF	Glia maturation factor
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-33	Interleukin-33
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HMF	Hydroxymethylfurfural
HO•	Hydroxyl radicals
HRP	Horseradish peroxide
iNOS	Inducible nitric oxide synthase
i.p.	intraperitoneal
i.v.	intravenous
IP_3	Inositol 1,4,5-trisphosphate
KA	Kainic acid
KARs	Kainate receptors

KH_2PO_4	Potassium dihydrogen phosphate
LFT	Liver function test
LSPR	Localised surface plasmon resonance
LTP	Long-term potentiation
MAO	Monoamine oxidase
MDA	Malondialdehyde
MetR	Metabotropic glutamate receptors
MOMP	Mitochondrial outer membrane permeability
mTOR	Mammalian target of rapamycin
MPTP	Mitochondrial permeability transition pores
MWM	Morris water maze
Na^+	Sodium ions
Na_2HPO_4	Disodium hydrogen phosphate
$NaBH_4$	Sodium borohydride
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic
NAIP	Neuronal apoptosis inhibitor protein
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptors
NORT	Novel object recognition test
NO	Nitric oxide
NOS	Nitric oxide synthase
NOx	Total nitrate/nitrite

NOX	NADPH oxidase
Nrf2	Nuclear factor erythroid 2–related factor 2
NSAIDs	Non-steroidal anti-inflammatory drugs
$^1O_2$	Singlet oxygen
$O_2^{-\bullet}$	Superoxide anion radicals
$ONOO^-$	Peroxynitrite anions
OFT	Open field test
PARP	Poly adenosine diphosphate-ribose polymerase
PBS	Phosphate buffer saline
PCO	Protein carbonyl
PD	Parkinson's disease
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PNS	Peripheral nervous system
PTP	Permeability transition pore
RFT	Renal function test
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
s.c.	subcutaneous
SGOT	Serum glutamic-oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive substances

TEM	Transmission electron microscope analysis
TGF-β	Transforming growth factor beta
TH	Tualang honey
THSN	Tualang honey silver nanoparticles
TIMP-1	Tissue inhibitor matrix metalloproteinase 1
TLE	Temporal lobe epilepsy
TNF-α	Tumour necrosis factor-alpha
TNFR2	Tumour necrosis factor receptor type II
TPM	Topiramate
TPTZ	2,4,6-Tripyridyl-S-triazine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
USM	Universiti Sains Malaysia
UVB	Ultraviolet B
UV-Vis	Ultraviolet-visible spectrometry
XO	Xanthine oxidase
XRD	X-ray diffraction
WHO	World Health Organisation
w/v	Weight per volume

PENCIRIAN NANOPARTIKEL PERAK MADU TUALANG DAN KESAN NEUROPROTEKTIF MADU TUALANG DAN NANOPARTIKEL PERAK MADU TUALANG TERHADAP NEURODEGENERATIF YANG DIARUHKAN OLEH ASID KAINIK PADA HIPOKAMPUS TIKUS JANTAN

ABSTRAK

Neurodegeneratif merupakan suatu ciri kebanyakan penyakit kronik melibatkan sistem saraf pusat yang mengakibatkan kemerosotan terhadap struktur dan fungsi neuron. Eksperimen aruhan eksitotoksisiti dalam keadaan neurodegeneratif oleh asid kainik (KA) telah dikaitkan dengan pelbagai mekanisme termasuk tekanan oksidatif, tindak balas keradangan yang berlebihan, dan apoptosis. Madu Tualang (TH), kaya dengan antioksidan semulajadi, dan semakin dikaji sebagai alternatif untuk mencegah beberapa penyakit neurodegeneratif. Walaupun banyak kajian telah menekankan faedah TH, penggunaan nanopartikel perak yang disintesis daripada TH masih terhad. Oleh itu, penyelidikan ini bertujuan untuk menilai kesan neuroprotektif TH dan nanopartikel perak TH (THSN) terhadap neurodegeneratif yang diaruhkan oleh KA dalam hipokampus tikus. THSN telah disintesis dan dicirikan oleh spektrometri penyerapan (UV-Vis), meter belauan serbuk sinar (XRD), spektroskopi inframerah transformasi fourier (FTIR), mikroscopi imbasan pancaran medan (FESEM) dan mikroskopi penghantaran elektron (TEM). Sebanyak 288 ekor tikus Sprague Dawley jantan telah dibahagikan secara rawak kepada tiga fasa eksperimen (termasuk kajian tingkah laku, pengukuran biokimia dan kajian histologi). Dalam setiap fasa, 96 ekor tikus telah dibahagikan kepada lapan kumpulan utama (n = 12/kumpulan utama): kawalan, THSN 10 mg, THSN 50 mg, KA sahaja, KA + TH, KA + THSN 10 mg, KA + THSN 50 mg, dan

KA + Topiramate (TPM). Setiap kumpulan utama telah dibahagikan lagi kepada subkumpulan 24 jam dan 5 hari, yang terdiri daripada 16 subkumpulan (n = 6/subkumpulan). Tikus telah diberikan air suling, TH (1.0 g/kg), THSN (10 mg/kg atau 50 mg/kg), atau TPM (40 mg/kg) secara oral sebanyak lima kali setiap 12 jam. Suntikan di bawah kulit menggunakan larutan salina atau KA (15 mg/kg) diberikan selepas 30 minit daripada rawatan oral yang terakhir. Sebelum haiwan dikorbankan, penilaian tingkah laku telah dijalankan menggunakan ujian lapangan terbuka dan ujian pengecaman objek novel. Analisis biokimia, toksikologi, dan histologi dilakukan ke atas hipokampus pada 24 jam dan 5 hari selepas aruhan KA. Pemberian KA kepada tikus mengakibatkan sawan, perubahan aktiviti lokomotor, dan kemerosotan ingatan. Selain itu, KA menyebabkan peningkatan tekanan oksidatif (seperti yang dibuktikan oleh peningkatan ketara dalam aras MDA, PCO, dan NOx dan penurunan ketara pada CAT, SOD, GSH, dan TAS), aras TNF-α (penanda keradangan saraf), aktiviti caspase-3 (penanda apoptosis), dan seterusnya neurodegeneratif di dalam hipokampus. Keputusan untuk ujian fungsi ginjal dan hati menunjukkan bahawa THSN tidak menyebabkan kesan toksik yang ketara terhadap haiwan. Jelas bahawa, pra-rawatan dengan TH dan THSN telah meningkatkan kependaman sawan, memulihkan ingatan yang telah merosot, dan mengurangkan tekanan oksidatif, keradangan saraf, apoptosis, dan kerosakan pada neuron di dalam hipokampus model tikus neurodegeneratif yang diaruhkan oleh KA. Kesimpulannya, TH dan THSN menunjukkan kesan neuroprotektif terhadap neurodegeneratif yang diaruhkan oleh KA melalui sifat antioksidan, anti-radang dan anti-apoptosis. Kajian klinikal lanjut perlu dijalankan untuk menggunakan TH dan THSN sebagai agen neuroprotektif yang berpotensi.

CHARACTERISATION OF TUALANG HONEY SILVER NANOPARTICLES (THSN) AND NEUROPROTECTIVE EFFECT OF TUALANG HONEY AND THSN ON KAINIC ACID-INDUCED NEURODEGENERATION IN MALE RATS' HIPPOCAMPUS

ABSTRACT

Neurodegeneration is a feature of many chronic disorders of the central nervous system that result in the deterioration of neuronal structure and function. Experimental induction of excitotoxicity-mediated neurodegeneration by kainic acid (KA) has been associated with various mechanisms, including oxidative stress, excessive inflammatory response, and apoptosis. Tualang honey (TH), which contains a powerful natural antioxidant, is increasingly studied as an alternative prevention for several neurodegenerative diseases. Despite the numerous studies highlighting the benefits of TH, the application of silver nanoparticles synthesised from TH remains limited. Thus, this research aimed to evaluate the neuroprotective effects of TH and TH silver nanoparticles (THSN) against KA-induced neurodegeneration in the rat hippocampus. THSN was synthesised and characterised by UV-Visible (UV-Vis) spectroscopy, X-ray Diffraction (XRD), Fourier Transform Infrared (FTIR) spectroscopy, Field Emission Scanning Electron Microscope (FESEM), and Transmission Electron Microscope (TEM). A total of 288 male Sprague Dawley rats were randomised into three experimental phases (including behavioural assessment, biochemical measurement, and histological studies). In each phase, 96 rats were randomly divided into eight major groups (n = 12/major group): control, THSN 10 mg, THSN 50 mg, KA only, KA + TH, KA + THSN 10 mg, KA + THSN 50 mg, and KA + Topiramate (TPM). Each major group was subdivided into

24 h and five days subgroups, comprising 16 subgroups (n = 6/subgroups). The rats were given distilled water, TH (1.0 g/kg), THSN (10 mg/kg or 50 mg/kg), or TPM (40 mg/kg) orally, five times at 12 h intervals. Subcutaneous injections of saline solution or KA (15 mg/kg) were given 30 min after the last oral treatments. Before the animals were euthanised, behavioural assessments were conducted using the open field test and a novel object recognition test. Biochemical, toxicological, and histological analyses were performed on the hippocampus at 24 h and 5 days following KA induction. The KA administration on rats resulted in seizures, alteration in locomotor activity, and memory impairment. Additionally, KA increased oxidative stress (as evidenced by significant increases in MDA, PCO, and NOx levels and significant decreases in CAT, SOD, GSH and TAS levels), TNF-α level (neuroinflammatory marker), caspase-3 activity (apoptosis marker), and subsequent neurodegeneration in the hippocampus. The result for renal and liver function test showed that THSN caused no significant toxic effect on animals. Notably, TH and THSN pre-treatment increased the seizure latency, improved memory deficits, and reduced oxidative stress, neuroinflammation, apoptosis, and neuronal damage of rats' hippocampus in the KA-induced neurodegeneration model. In conclusion, TH and THSN exerted their neuroprotective effects against KA-induced neurodegeneration via antioxidant, anti-inflammatory, and anti-apoptotic properties. Further clinical studies need to be conducted to establish TH and THSN as a potential neuroprotective agent.

CHAPTER 1

INTRODUCTION

1.1 Research background

Neurodegeneration is characterised by the progressive loss and deterioration of neural structures and functions within the brain or spinal cord over time, which ultimately results in neuronal cell death and central nervous system (CNS) dysfunction (Ou *et al.*, 2021). It is a feature of rapidly rising chronic diseases such as epilepsy. As reported by the World Health Organisation (WHO), epilepsy affects an estimated 50 million people globally, which makes it one of the most prevalent neurological illnesses (World Health Organisation, 2019). Meanwhile, according to the recent study by Fong et al. (2021), the prevalence of lifetime epilepsy among the Asian countries, particularly in Malaysia is 7.8 per 1000 persons. Excitotoxicity is considered a key pathological condition in which neuronal cells are injured and die as a result of neurotransmitters overstimulation, such as glutamate. Excitotoxic neuronal death has been shown to be associated with acute and chronic neurodegenerative disorders, such as Alzheimer's disease (AD) (Ong *et al.*, 2013), hypoxia/ischemia (Kim *et al.*, 2017a), Parkinson's disease (PD) (Iovino *et al.*, 2020), amyotrophic lateral sclerosis (ALS) (Dong *et al.*, 2021), and temporal lobe epilepsy (TLE) (Yang *et al.*, 2021).

Recently, several studies have investigated the role of excitotoxicity in neurodegenerative rat models induced by neurotoxic agents such as kainic acid (KA) (Lu *et al.*, 2020; Sairazi *et al.*, 2018). KA is a potent neurotoxicant isolated from

Digenea simplex (a species of red algae) (Maeno *et al.*, 2019). It is widely used for seizure induction and to explore the mechanisms of excitotoxicity and neurodegeneration in biological research. For example, the administration of KA to animal models (such as rodents) results in seizures, behavioural changes, oxidative stress, glial activation, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, inflammatory mediator production, and apoptosis (Liu *et al.*, 2015; Riljak *et al.*, 2015; Sairazi *et al.*, 2018; Sairazi *et al.*, 2017b; Torres-Peraza *et al.*, 2013; Wang *et al.*, 2018; Xu *et al.*, 2014; Xue *et al.*, 2017). On top of that, the KA-induced animal model is useful in identifying selective neurodegeneration in the brain contributed by excitotoxicity in the thalamus, hippocampus, and piriform cortex, and amygdala (Sairazi *et al.*, 2017b; Sakurai *et al.*, 2015; Zhu *et al.*, 2018).

Basically, the role of the hippocampus is important in the brain's ability to learn and remember, hence it is associated with numerous neurodegenerative illnesses such as PD and AD (Calabresi *et al.*, 2013; Ramezani *et al.*, 2016). Kim *et al.* (2017b) reported that hippocampal oxidative damage has been linked to KA-induced neurotoxicity *in vivo*. In another study, it was found that domoic acid, which is similar to KA in its structure, caused neuronal death in the rat hippocampal region as early as day one of administration, and the damage got worse by the fifth day (Ananth *et al.*, 2003). The distribution and selective sensitivity of the KA receptors in the brain are related to KA-induced selective vulnerability in hippocampal neurons (Wang *et al.*, 2005).

Several underlying biochemical events can lead to neurodegeneration, including oxidative stress, dysfunction of mitochondria, neuroinflammation, apoptosis, and misfolded protein aggregation (Kempuraj *et al.*, 2016; Nakamura *et al.*, 2012; Radi *et al.*, 2014). Previous studies showed that oxidative stress in animals

of KA-induced excitotoxicity was resulted by an increase in production of nitric oxide (NO) and thiobarbituric acid reactive substances (TBARS), as well as a reduction in total antioxidant status (TAS) (Sairazi *et al.*, 2017a; Swamy *et al.*, 2014a). Additionally, it has been documented that neuroinflammatory mechanisms also contributed to the series of events. This eventually leads to neuronal degeneration (Chen *et al.*, 2016) and the production of inflammatory cytokines, especially tumour necrosis factor-alpha (TNF-α), which is involved in neuronal pathology (Olmos and Lladó, 2014). Furthermore, other mechanisms like apoptosis, have been shown to cause neuronal death as demonstrated by elevated cysteinyl aspartate specific proteinases-3 (caspase-3) levels in animal and human studies (Feng *et al.*, 2018; Płóciennik *et al.*, 2015). Therefore, these events are the primary targets in combating neurodegenerative illnesses.

Recent studies have demonstrated that KA-induced excitotoxicity may serve as a model for explaining the pathways behind oxidative stress, neuroinflammation, and apoptosis in neurodegenerative disorders (Sairazi *et al.*, 2018; Swamy *et al.*, 2014a; Ullah *et al.*, 2014). Due to these events, antioxidants play a vital role as the protective agents that can minimise the damaging effect caused by excitotoxic agents such as KA. Utilising natural antioxidants as a remedy for neurodegenerative diseases has become more popular in recent years. Many natural products contain antioxidants that can protect against neuronal degeneration and seizures (Sairazi *et al.*, 2015). Moreover, various phytochemical compounds have free radical scavenging properties and activate key antioxidant enzymes in the brain, therefore protecting the brain damage caused by oxidative stress (Mijanur Rahman *et al.*, 2014).

Honey, a natural antioxidant, has been used for generations to treat multiple illnesses and is considered a memory-boosting food supplement (Mishra, 2011). Honeys have variable composition due to differential floral sources, geographical origin, total phenolic content, and water percentage (Frankel *et al.*, 1998; Mohamed *et al.*, 2010). In recent decades, honey has been investigated for its antidiabetic, antibacterial, antifungal, anti-inflammatory, wound healing, reproductive, gastroprotective, hepatoprotective, antitumour, and antioxidant effects (Benlyas *et al.*, 2016; Devasvaran and Yong, 2016; El-Haskoury *et al.*, 2019; Haron and Mohamed, 2016; Header *et al.*, 2016; Hemmati *et al.*, 2015; Kageyama *et al.*, 2018; Kolayli *et al.*, 2020; Zhao *et al.*, 2017). Furthermore, several important constituents in honey, including polyphenols and flavonoids, have displayed neuroprotective roles by ameliorating oxidative stress, attenuating neuroinflammation, enhancing cognitive abilities, and preventing neuronal damage caused by neurotoxins (Filomeni *et al.*, 2012; Han *et al.*, 2012; Xia *et al.*, 2015). Moreover, previous findings have shown that bee propolis can restore NO and TNF-α levels, activity of glutamine synthetase and caspase-3, as well as oxidative stress in KA-mediated excitotoxicity (Swamy *et al.*, 2014a; Swamy *et al.*, 2014b). Honey exerts its neuroprotective effects at various stages of neurodegeneration, especially in early events (Mijanur Rahman *et al.*, 2014). Nevertheless, its protective neuropharmacological mechanism has not been fully elucidated.

Tualang honey (TH) is commonly utilised for food supplements and in folk remedies in Malaysia. It is a wild multifloral honey produced by Asian rock bees (*Apis dorsata*). TH was chosen for this study since literature has highlighted its superior efficacy over other types of honey due to its richness in polyphenols and flavonoids (Kishore *et al.*, 2011). Furthermore, prior research have shown that

supplementation of TH results in memory improvement (Azman *et al.*, 2015; Othman *et al.*, 2011) and alleviated locomotor activity in animals of KA-induced oxidative stress (Sairazi *et al.*, 2017b). Interestingly, the role of antioxidant properties of TH in neuroprotective activity against excitotoxicity in KA-induced rats' brain model has been reported in recent studies (Sairazi *et al.*, 2018; Sairazi *et al.*, 2017b). Additionally, TH has also been explored in nanotechnology fields and was found to be a good reducing and stabilising agent and, importantly, function as a precursor in nanoparticles by-products (Haiza *et al.*, 2013; Hasim *et al.*, 2020b; Phuna *et al.*, 2020).

Nanotechnology has captured the attention of scientists all over the world in recent decades as a means of discovering and exploring new properties and capabilities of nanoparticles which have an overall dimension of less than 100 nm. As a result, they developed ways to control and exploit nanoparticles in various fields such as medicine and biopharmaceuticals, for example, as carriers for drug delivery (Ge *et al.*, 2014; Iravani, 2011). Nowadays, nanoparticles are one of the treatment options for several neurological disorders like AD, PD, and vascular dementia (Spuch *et al.*, 2012). In addition, nanoparticles containing antioxidants are becoming a promising method for mitigating oxidative stress, with potential implications as a treatment and prevention of neurodegenerative illnesses (Sandhir *et al.*, 2015). Silver nanoparticles, for example, have been shown to have excellent free radical scavenging, antimicrobial, and anti-inflammatory activities (Jain *et al.*, 2019; Kailasa *et al.*, 2019; Kokila *et al.*, 2015).

Therefore, this present study was carried out to investigate the mechanisms of action of TH and silver nanoparticles synthesised using TH (THSN) following KA-induced neurodegeneration in the rat hippocampus, focusing on their antioxidant,

anti-inflammatory, and anti-apoptotic properties. The neuroprotective effects of TH and THSN were evaluated in this study by assessing the levels of oxidative status markers [i.e., malondialdehyde (MDA), protein carbonyl (PCO), total nitrate/nitrite (NOx)], catalase (CAT), superoxide dismutase (SOD), reduced to oxidised glutathione ratio (GSH/GSSG), and total antioxidant status (TAS)], inflammatory marker (TNF- α), and apoptotic marker (caspase-3) activity. Lastly, the extent of neurodegeneration in the hippocampus was evaluated through histological (Flouro Jade C (FJC) and cresyl violet) staining.

1.2 Justification of the study

Although the exact pathogenesis of neurodegenerative disorders is limited, there are indications that the mechanisms of excitotoxicity participate in neurodegeneration. Thus, understanding the pathways involved in excitotoxicity is importance for the future clinical treatment in neurodegenerative diseases. Excitotoxicity can be induced *in vivo* using KA, which allows mechanisms of neurodegenerative processes and diseases to be investigated, particularly epilepsy. KA receptors hyperexcitation is responsible for the majority of seizure induction and neuronal death, thus serving as a promising target for therapeutic intervention directed against excitotoxicity, which might delay the progress of neurodegeneration.

Several conventional drug therapies such as anticonvulsant drugs have been approved by the Food and Drug Administration (FDA) to alleviate disease progression by reducing the impact of neurodegeneration. However, as people become more aware of the potency and adverse effects of synthetic drugs, there is an increasing interest in natural product remedies with minimal side effects upon long-term use. Nowadays, it is estimated that almost 80% of people in developing

countries still rely on traditional medicine for their main health care (Tran *et al.*, 2020). Traditional medicine from natural products, such as honey, has rich sources of phytochemical ingredients, and the pharmacological effects of medicinal plants have been considered as a promising future treatment for the management of health care, particularly for neurodegenerative illnesses.

TH, a natural product with powerful antioxidant properties, has been reported to possess neuroprotective activity against neurological disorders by ameliorating oxidative stress, inflammatory reactions, apoptosis, and the extent of neurodegeneration following KA-induced excitotoxic injury in the rats' piriform cortex, cerebellum, and brainstem (Sairazi *et al.*, 2018; Sairazi *et al.*, 2017a; Sairazi *et al.*, 2017b). However, the effect of TH on the hippocampus has yet to be determined. It has been proposed that the effectiveness of traditional medicine in clinical trials has been less impressive, partly due to the low bioavailability of the compounds. The incorporation of nanoparticles into a delivery system for natural products would be a major advance in the efforts to improve their therapeutic effects.

Experimental research has recently demonstrated that nanoparticles can significantly enhance the bioavailability of natural products both *in vitro* and *in vivo* (Li *et al.*, 2022), thereby making them more powerful and effective than raw materials. Nanotechnology has proven its capability to manipulate particles in order to target specific areas of the cell and body and regulate drug delivery (Raj *et al.*, 2021; Wu and Tang, 2018). In addition, nanoparticles containing antioxidants are becoming a promising method for mitigating oxidative stress, with potential implications as a treatment and prevention of neurodegenerative illnesses (Sandhir *et al.*, 2015). This has raised the possibility that nanoparticles that interfere with glutamatergic neurotransmission may be clinically beneficial in treating

neurodegenerative diseases. Efforts to develop novel treatments using THSN for KA-induced neurodegeneration models which target specific pathomechanisms are currently being pursued. To date, it is not known whether there are protective effects of TH and THSN on the level of oxidative stress markers, neuroinflammation, apoptosis, and morphology in the hippocampus of KA-induced excitotoxicity mediated neurodegeneration model. Therefore, the present study was carried out to investigate the mechanisms of action of TH and THSN following KA-induced neurodegeneration in the rat hippocampus.

1.3 Research objectives

1.3.1 General objective

To assess the characterisation of THSN and the association between the assessments of behavioural changes, estimation of toxicity parameters, biochemical markers (oxidative stress, excessive inflammatory response, apoptosis,), and histopathological study pertain to neurodegeneration and neuroprotective effect of TH and THSN in male rats' hippocampus.

1.3.2 Specific objectives

1. To synthesise and characterise the silver nanoparticles by using TH.
2. To evaluate the seizure, locomotor activity, and memory of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
3. To determine the toxicity parameters in the blood serum of male rats pre-treated with THSN versus related controls after 24 h and 5 days of saline solution administration.

4. To determine the biochemical markers of oxidant/antioxidant levels, inflammatory and apoptosis in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
5. To assess the neuronal loss in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.

1.4 Hypothesis of the study

1.4.1 Null hypothesis

1. There is no significant change in the seizure, locomotor activity, and memory of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
2. There is no significant change in toxicity parameters (LFT and RFT) in the blood serum of male rats pre-treated with THSN versus related controls after 24 h and 5 days of saline solution administration.
3. There is no significant change in the biochemical markers of oxidant/antioxidant levels, inflammatory and apoptosis in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
4. There is no significant change in the neuronal loss in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.

1.4.2 Alternative hypothesis

1. There is a significant change in the seizure, locomotor activity, and memory of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
2. There is a significant change in the toxicity parameters (LFT and RFT) in the blood serum of male rats pre-treated with THSN versus related controls after 24 h and 5 days of saline solution administration.
3. There is a significant change in the biochemical markers of oxidant/antioxidant levels, inflammatory and apoptosis in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.
4. There is a significant change in the neuronal loss in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration.

1.5 Research questions

1. How to synthesise and what are the characteristic properties of silver nanoparticles synthesised using TH?
2. Is there any change in the seizure, locomotor activity, and memory of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration?
3. Is there any change in the toxicity parameters (LFT and RFT) in the blood serum of male rats pre-treated with THSN versus related controls after 24 h and 5 days of saline solution administration?

4. Is there any change in biochemical markers of oxidant/antioxidant levels, inflammatory and apoptosis in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration?
5. Is there any change in the neuronal loss in the hippocampus of KA-induced male rats pre-treated with TH and THSN versus related controls after 24 h and 5 days of KA administration?

CHAPTER 2

LITERATURE REVIEW

2.1 Nervous tissue

Nervous tissue, also called as neural tissue, is the main tissue component of the nervous system. Nervous system, composed by the central nervous system (CNS) and peripheral nervous system (PNS), is implicated in the communication with both the external and internal environment of the organism by responding to chemical and physical stimuli (Mtui *et al.*, 2020). Nervous tissue comprised of two types cells namely the neuroglia and neuron, which are involved in providing nutrients and transmitting nerve impulses respectively (Caruso Bavisotto *et al.*, 2019). Neuroglia and neurons are more complex in their shapes and sizes than cells in other tissues.

2.1.1 Neuroglia

Neuroglia, glial cells or simply glia, are the most abundant cells in nervous tissue (over 90%) (Uemura, 2015). It represents the most numerous cell family in the CNS with 5 to 10 glial cells per neuron or 350 billion cells per brain (Jennes, 2017). Neuroglia is generally small in size and only their nuclei are clearly seen in routine histological preparations (Uemura, 2015). Neuroglia of the CNS can be divided into macroglia and microglia. The macroglia includes oligodendrocytes, astrocytes, and ependymoglial cells that originate from the ectoderm, while the microglial cells derive from the yolk sac and they are found in the CNS during early embryonic development (Janowska *et al.*, 2019).

Neuroglia retains their ability to divide, provide, metabolic, and structural support for neurons. This glial capacity to divide is essential for their structural and functional support of neurons by simply holding neurons together (Uemura, 2015). They also maintain brain plasticity and protect the brain for functional recovery from injuries (Liu *et al.*, 2020). Dysfunction of neuroglia may promote neurodegeneration, an eventually, the retraction of neuronal synapses, which leads to cognitive deficits (Henstridge *et al.*, 2019). They are less electrically excitable than neurons and do not form chemical synapses (Jennes, 2017).

2.1.2 Neuron

Neuron is highly specialised, excitable cell that contains a cell body or soma which includes the perikaryon (the region around the nucleus) that is the central site of synthesis and degradation of proteins, lipids, and carbohydrates (Jennes, 2017). Neuron is an essential cellular element in the CNS and specialised in information processing by receiving, processing, and transfering impulses from one neuron to the next via synapses (the specialised contact areas between two neurons) (Uemura, 2015). Neurons can be categorised according to their size, shape, neurochemical characteristics, location, and connectivity, which determine their particular functional role in the brain (Hof *et al.*, 2014).

There are five general categories of neurons: (1) inhibitory local circuit neurons (e.g., GABAergic interneurons in the cerebral and cerebellar cortex); (2) inhibitory projection neurons (e.g., medium spiny neurons of the basal ganglia or Purkinje cells of the cerebellar cortex); (3) excitatory local circuit neurons (e.g., spiny stellate cells of the cerebral cortex); (4) excitatory projection neurons (e.g.,

pyramidal neurons in the cerebral cortex); and (5) neuromodulatory neurons (e.g., dopaminergic neurons of the substantia nigra) (Hof *et al.*, 2014).

Neurons stop dividing within a few months after birth (Uemura, 2015). Therefore, if nerve damage involves cell bodies in the adult animal, resulting neuronal death will permanently change the structure and functions of the affected areas (Uemura, 2015). The neuronal death in nervous tissue, especially hippocampus, has been identified as a potential contributor to neurological diseases (Chavoshinezhad *et al.*, 2019; Zhang *et al.*, 2020).

2.2 Hippocampus

2.2.1 Location and structure

The hippocampus is a brain structure located within the medial temporal lobe and forms part of the limbic system. The hippocampus is characterised by a curved sheet of cortex that is folded into the medial surface of the temporal lobe. Hippocampal transverse sections in the rodent brain appear as two interlocking "C" shapes with three different subfields, including the dentate gyrus, the hippocampus proper, and the subiculum (Figure 2.1) (Gendelman and Ikezu, 2008; Knierim, 2015).

The pattern of afferent termination is a characteristic of hippocampus circuitry. Major hippocampal afferents originate from the entorhinal cortex, and ipsilateral and contralateral hippocampal subfields synapse on the dendrites of the principal cells in a laminated pattern. Afferents also have a laminar organisation in the hippocampal proper. Another important feature of the hippocampus is intrinsic circuitry. Information that flows through the hippocampus proceeds from the dentate

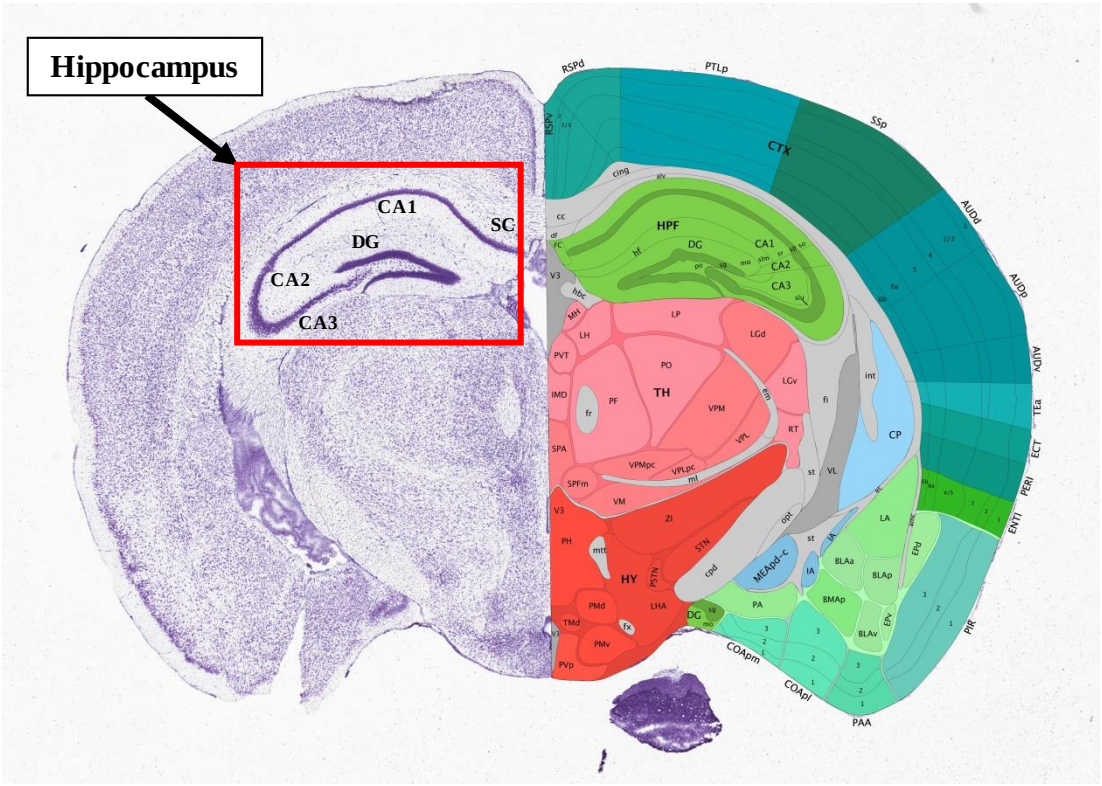

Figure 2.1 A coronal section of the brain in a mouse. The section shows the location of the hippocampus and its subfields (Image is downloaded from Allen Institute of Brain Science, 2021). Hippocampus proper: CA1, CA2, CA3; dentate gyrus: DG; subiculum: SC.

gyrus (DG) to Cornu ammonis 3 (CA3), Cornu ammonis 1 (CA1), and subiculum, forming the principal intrinsic trisynaptic circuit. Cornu ammonis 2 (CA2) represents a small portion of the hippocampus and is located between the CA1 and CA3 regions (Patestas and Gartner, 2016).

2.2.1 (a) The hippocampus proper

Hippocampus proper is a structure in the brain's temporal lobe that plays a major role in learning and memory processes. Hippocampus proper has been implicated in several major brain disorders, including epilepsy and Alzheimer's disease (AD) (Chatzikonstantinou, 2014; Mufson *et al.*, 2015). The hippocampus proper is a U-shaped fold of the cortex that consists of (cornu Ammonis, CA) CA1, CA2, and CA3 fields. It has three distinct layers: an outer molecular layer; a middle pyramidal layer; and an inner polymorphic layer (stratum oriens) (Schultz and Engelhardt, 2014) as shown in Figure 2.2.

The CA1 field is composed of densely packed, medium-sized cells. Meanwhile, the CA2 and CA3 fields are adjacent to the CA1 field, which has larger, less densely packed cells. The CA2 pyramidal cells can be distinguished from CA3 pyramidal cells by the absence of thorny spines on the proximal apical dendrites in Golgi preparations but not by Nissl-staining (Gendelman and Ikezu, 2008). Sensory information enters the hippocampus proper mostly via the perforant pathway, which consists of neuronal axons in layers II and III of the entorhinal cortex. First, the perforant path axons terminate on the dendrites of the dentate gyrus granular cells. Then, the information passes from the dentate gyrus to the CA3, CA1, and subiculum, producing the hippocampal intrinsic trisynaptic circuit (Gendelman and Ikezu, 2008).

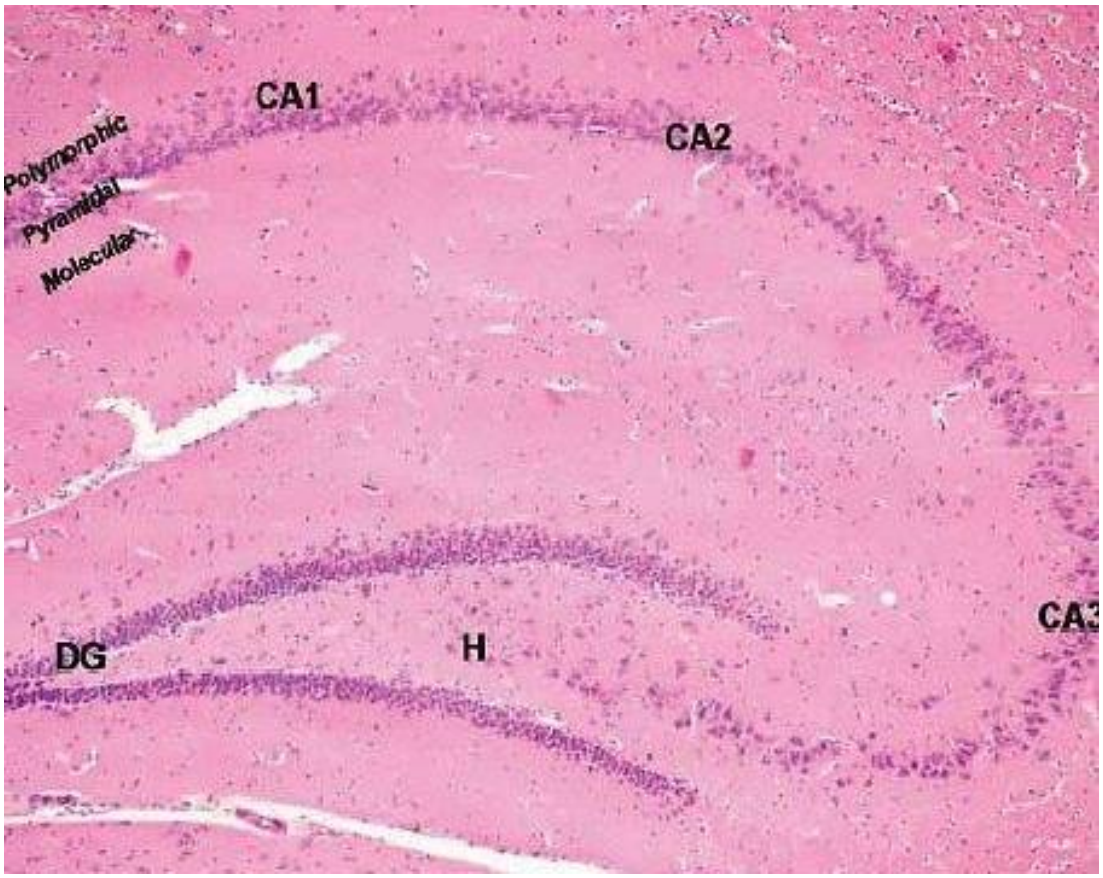

Figure 2.2 The image showing three layers (polymorphic, pyramidal, and molecular) of hippocampus. Magnification: 100 times. Dentate gyrus: DG; Hilus: H (Abd El-Aleem et al., 2008).

2.2.1 (b) Dentate gyrus

The dentate gyrus is one of the few brain regions where neurogenesis occurs. The dentate gyrus is divided into three layers: an outer molecular layer; a middle granule cell layer; and an inner polymorphic layer (Figure 2.3). The polymorphic layer is also known as the hilus of the dentate gyrus or the CA4. The granule cell layer comprises densely packed granule cells, which are the principal neurons in the dentate gyrus. These granule cells have small and spherical cell bodies (Patestas and Gartner, 2016).

The dentate gyrus projects only to CA3 through the mossy fibres within the hippocampal formation. The mossy fibres are the axons of dentate gyrus granule cells that extend from the dentate gyrus to the CA3 pyramidal cells, forming their major output. In addition, mossy fibres synapse on CA3 neurons, which display distinctive terminal boutons with various transmitter release sites and post-synaptic densities. Furthermore, several granule cells are able to synapse onto a single CA3 pyramidal cell. This pathway has been extensively explored as a model for the functional involvement of kainate receptors (KARs) in synaptic plasticity (Nair *et al.*, 2021).

2.2.1 (c) Subiculum

Like the CA fields of the hippocampus proper and the dentate gyrus, the subiculum also has similar features. In principle, the subiculum can be divided into three layers: a molecular layer; an enlarged pyramidal cell layer; and a polymorphic layer (Figure 2.4). The molecular layer is the most superficial layer and is continuous with the stratum radiatum and the stratum lacunosum-moleculare of the adjacent hippocampal

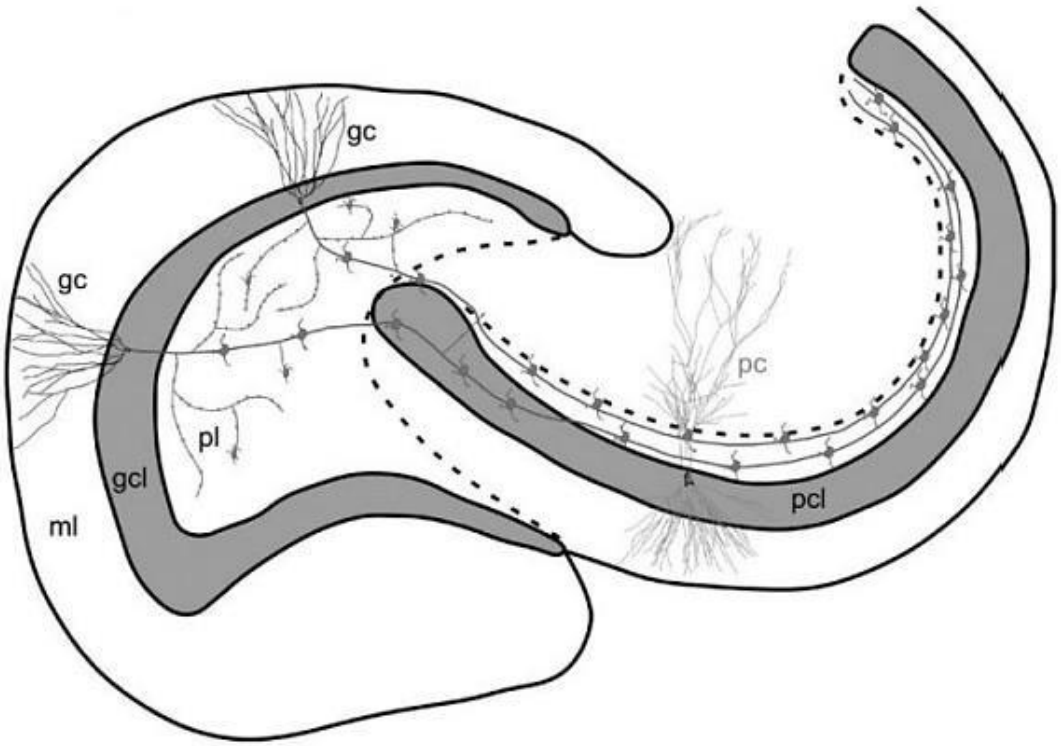

Figure 2.3 The illustration showing three layers (molecular layer, granule cell layer, and polymorphic layer) of dentate gyrus. Molecular layer: ml; Granule cell layer: gcl; Polymorphic layer: pl; Granule cell: gc; Pyramidal cell: pc (Amaral et al., 2007).

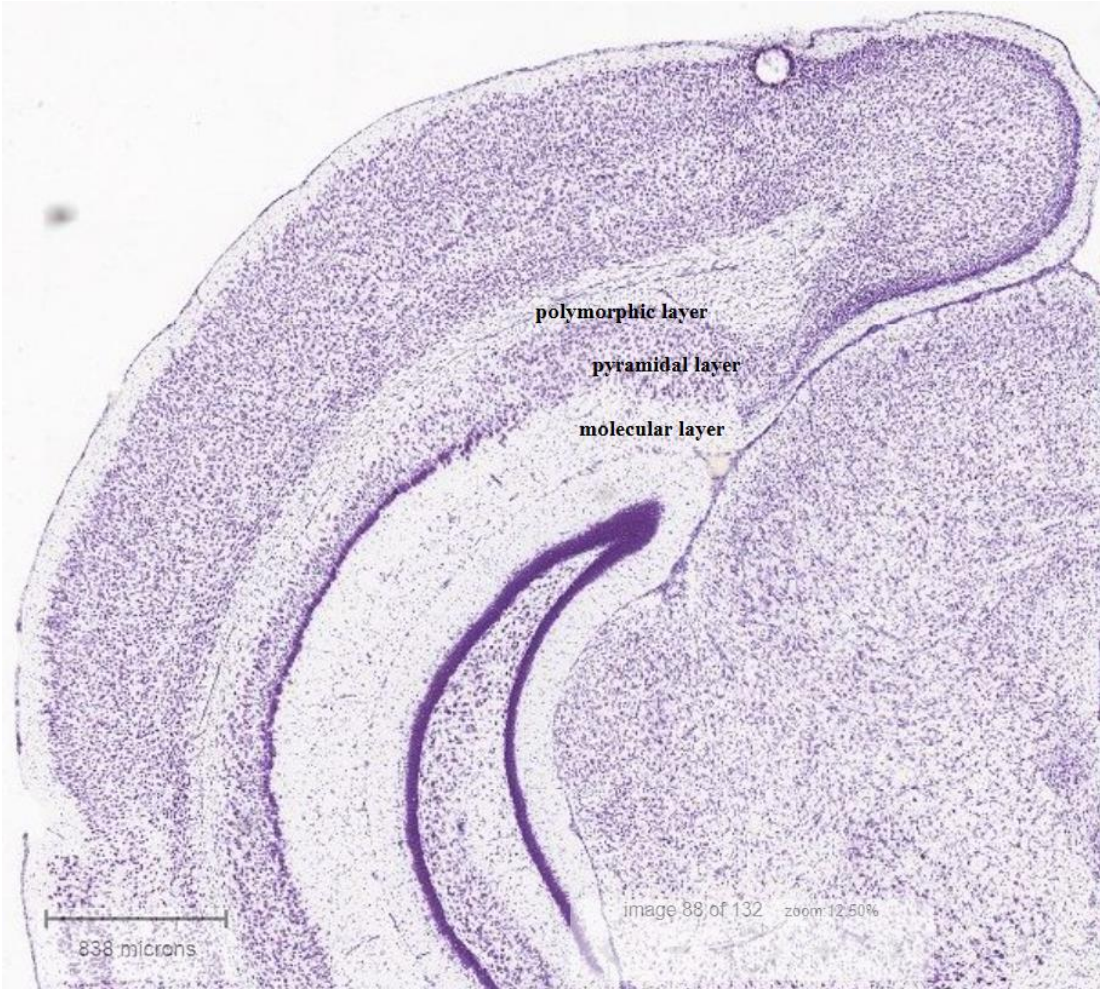

Figure 2.4 The image showing three layers (molecular layer, pyramidal layer, and polymorphic layer) of subiculum. Scale bar: 838 microns. (Image is downloaded from Allen Institute of Brain Science, 2023).

CA1 area. The pyramidal cell layer, which is flanked by the molecular layer and the polymorphic layers, is thicker than the hippocampal pyramidal cell layer (Matsumoto et al., 2019). The subiculum and CA1 of the hippocampus proper overlap at their borders, forming a complex transitional zone. The subiculum is important source of hippocampal projections to nuclei in the medial diencephalon, which are crucial for human memory and rodent spatial learning, highlights the subiculum's significance for learning and memory (Aggleton and Christiansen, 2015).

2.2.2 Role of hippocampus in memory and learning

Hippocampus is well known to play an important role in the consolidation of short-term memory to long-term memory. Hippocampal damage frequently impairs the formation of new memories (anterograde amnesia) and events that occurred shortly before the damage (retrograde amnesia), but access to older memories is preserved. This is because long-term memories are not stored in the hippocampus but in other parts of the brain. It has been shown that the hippocampus is involved in storing and processing spatial memory. This is supported by an animal study demonstrating the rate of firing of hippocampal neurons was correlated with the location of the animal in a test environment (Deshmukh and Knierim, 2013).

The discovery prompted the hypothesis that the hippocampus may function as a cognitive map, as supported by many lesion and unit recording studies (Banta Lavenex *et al.*, 2014; Knierim, 2015). For instance, rats with hippocampal lesions exhibit learning impairment in the radial arm, T-maze, and Morris water maze, in which rats have difficulty in finding the hidden platform (Ros-Simó *et al.*, 2013; Teixeira *et al.*, 2014; Topuz *et al.*, 2020). Furthermore, neuroimaging studies on humans have demonstrated that the hippocampus is active during spatial navigation.

This indicates that the hippocampus contributes to the encoding and retrieval of spatial information in humans (Baumann and Mattingley, 2021; Zeidman and Maguire, 2016). The molecular basis of memory formation and storage by the hippocampus is thought to involve long-term potentiation (LTP) (Aziz et al., 2019). LTP is the repetitive activation of excitatory synapses in the hippocampus, which increases synaptic strength lasting for hours to a lifetime (Kennedy, 2016). As a result of LTP, new dendritic spines and synaptic connections are formed, which contribute to the formation of long-term memory (Basu and Lamprecht, 2018).

The structural and functional changes in the hippocampus are also particularly affected by dementia and other neurodegenerative disorders as characterised by cognitive and emotional dysregulation (Gulyaeva, 2019). Studies have suggested an involvement of CA2 subfield in cognition and cognitive decline in dementia with Lewy bodies (DLB) (Liu *et al.*, 2019; Pang *et al.*, 2019). Despite the lack of atrophy in comparison with AD, DLB CA2 shows increased microglial activation in association with cognitive decline (Imamura *et al.*, 2005; Mak *et al.*, 2017). These findings suggest an important role for the CA2 in some forms of memory, as well as its unique lack of vulnerability to damage, particularly in the early stages of disease. These properties make the CA2 an important area to consider in relation to neurodegenerative diseases.

2.3 Neurodegeneration

2.3.1 Definition of neurodegeneration

According to Przedborski *et al.* (2003), the prefix “neuro-” refers to nerve cells (also known as neurons), whereas “degeneration” refers to the process of losing structure or function. Therefore, neurodegeneration is the gradual deterioration of nerve

structure or function in the brain or spinal cord. Neurodegeneration occurs in any pathological condition affecting neurons, particularly in neurodegenerative diseases. Furthermore, this condition is present in the normal ageing process (Giacalone *et al.*, 2015).

2.3.2 Mechanisms of neurodegeneration

The mechanisms of neurodegeneration are present in normal physiological and pathological conditions. However, the action is highly exerted in diseases (Guerra-Araiza *et al.*, 2013), leading to severe damage and functional loss. Several mechanisms have been identified that contribute to the neurodegenerative process, such as oxidative stress generated by high reactive oxygen species (ROS), excessive neuroinflammatory response, excitotoxic effects by excitatory amino acids, and dysregulation of cellular energy metabolism (Niedzielska *et al.*, 2016). These neurodegenerative processes can damage proteins, lipids, and nucleic acids as well as possibly activate the mitochondrial permeability transition pore, which further stimulates free radical production, results in energy failure, and release of pro-apoptotic factors (Cowan *et al.*, 2019; Radi *et al.*, 2014; Redza-Dutordoir and Averill-Bates, 2016). The neurodegenerative process may also be triggered by genetic factors related to an intrinsic susceptibility, aging, and environmental factors (Guerra-Araiza *et al.*, 2013).

2.3.2 (a) Mechanism of oxidative stress in neurodegeneration

Oxidative stress is a condition caused by an imbalance between ROS production (i.e., oxidant) and the body's defence systems (i.e., antioxidant). It is a state of antioxidant system dysfunction that results in excessive ROS generation that may lead to cellular damage (Gandhi and Abramov, 2012). Oxidative stress can be elicited either by

excessive ROS production from exogenous processes (infections, ionising radiation, alcohol consumption, and smoking) or endogenous processes (mitochondrial respiratory chain) (Niedzielska *et al.*, 2016). Oxidative stress is directly associated with neuronal damage, neuroinflammation, mitochondrial dysfunction, decreased sensitivity to neurotransmitters, and excitotoxicity, all of which can result in neurodegeneration (Giacalone *et al.*, 2015).

Several reactive molecules and free radicals produced from molecular oxygen are referred to as ROS. Most ROS are generated as byproducts during mitochondrial electron transport during aerobic respiration (de Araújo *et al.*, 2016; Floyd and Hensley, 2002). Examples of ROS include superoxide anions radicals ($O_2^{-\bullet}$), hydroxyl radicals ($HO^{\bullet}$), singlet oxygen ($^1O_2$), peroxynitrite anions ($ONOO^-$), and NO (Niedzielska *et al.*, 2016; Popa-Wagner *et al.*, 2013). In contrast, some ROS are not free radicals, such as hydrogen peroxide (H_2O_2), due to the absence of unpaired electrons (Kurutas, 2015). These ROS molecules may cause structural and functional changes in cell membranes, proteins, lipoproteins, enzymes, hormones, and genetic materials. For instance, oxidative degradation of lipids (lipid peroxidation) leads to the decomposition of polyunsaturated fatty acids that give rise to compounds such as 4-hydroxynonenal (4-HNE) and MDA. These compounds react with and modify the structure and function of deoxyribonucleic acid (DNA) or protein molecules (Albarracin *et al.*, 2012; Fritz and Petersen, 2013).

ROS are typically produced in small amounts in all tissues and immediately eliminated by antioxidant systems. At physiological concentrations, this ROS performs important regulatory and mediator functions. However, a chain of radical reactions is induced when ROS are present in excess, thus raising the potential for