ELUCIDATING THE MECHANISMS OF LIPOPOLYSACCHARIDE-INDUCED COGNITIVE IMPAIRMENT IN RATS TREATED WITH TUALANG HONEY AND ITS METHANOLIC EXTRACTION

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2020

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by

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Thesis submitted in fulfilment of the requirements

for the degree of

Doctor of Philosophy

March 2020

ACKNOWLEDGEMENTS

Alhamdulillah. Praise to ALLAH the Almighty.

First and foremost I would like to express my sincerest gratitude to my supervisor, Dr. Idris bin Long and my co-supervisors, Assoc. Prof. Dr. Rahimah binti Zakaria and Assoc. Prof. Dr. Zahiruddin bin Othman, for giving me an opportunity to be their postgraduate student, for the countless advice and encouraging guidance, for their patience and knowledge they have shared in completing this thesis, throughout my study period at the Universiti Sains Malaysia, Kubang Kerian, Kelantan.

I would like to express gratitude to Ministry of Higher Education (MoHE), Malaysia for my sponsorship through the MyBrain15 scholarship scheme (MyPhD), and Fundamental Research Grant Scheme (FRGS) (203/PPSP/6171174) for funding of this study. Also thank you to Biomedicine laboratory staffs, and not to forget Dr Saidi bin Jaafar, director of Animal Research and Service Centre (ARASC) and their staffs for providing me with the help and facilities in animal care and part of laboratory works. Thanks also go to Dr Wan Muhammad Amir and Dr Anis Kausar for their help in workshop and the guidance in selection of statistical analysis for my study. Also, I have been blessed with a cheerful group of fellow postgraduate students.

Last but not least, very special thanks to my father (Wan Yaacob Wan Abdul Hamid), my mother (Rozamah Aman), my lovely wife and children (Nurul Izzati Hamzan, Wan Nur Hanania, Wan Muhammad Ikhwan and Wan Muhammad Hazeem) and my siblings and relatives; of whom without failed, have provide me something much greater in all these years, the love.

Jazakallahukhairankathira.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percentage
<	less than
>	more than
°C	celcius
µL	microlitre
µmol	micromolar
4-PL	four-parameter logistic
ABTS	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)
Abs	absorbance
ACh	acetylcholine
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
ApoE4	apolipoprotein E type 4 allele
APP	amyloid precursor protein
ARASC	Animal Research and Service Centre
ATP	adenosine triphosphate
Aβ	beta amyloid
BACE-1	beta-secretase 1
BBB	blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CA	*cornus ammonis*
Ca^{2+}	calcium ion
CAT	catalase
CEQ	catechin equivalents
ChAT	choline acetyltransferase
cm	centimetre
CNS	central nervous system
COX-2	cyclooxygenase-2
CSF	cerebrospinal fluid
Cu^{3+}	copper ion
DG	dentate gyrus
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl

DPX	dibutylphthalate polystyrene xylene
EATH	ethyl acetate extract of Tualang honey
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EOAD	early-onset Alzheimer's disease
FAD	familial Alzheimer's disease
FAMA	Federal Agricultural Marketing Authorities
FDA	Food and Drug Administration
$Fe^{2+/3+}$	iron ion
FRAP	ferric reducing antioxidant power
g	gram
g	gravitational forces
GAE	gallic acid equivalents
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
h	hour
HMF	5-hydroxymethyl furfural
HPA	hypothalamic–pituitary–adrenal
HRP	horseradish peroxidase
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IC_{50}	50% of inhibition concentration
IDV	integrated density values
IFN-γ	interferon gamma
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
ITH	gamma-irradiated Tualang honey
IκBα	inhibitor of kappa B
iNOS	inducible nitric oxide synthase
JAK	janus kinase
kDA	kilodalton
kg	kilogram
kGy	kilogray
L	litre

LOAD	late-onset Alzheimer's disease
LPS	lipopolysaccharide
LTP	long-term potentiation
M	molar
MAPK	mitogen activated protein kinase
mg	microgram
Mg^{2+}	magnesium ion
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
MTH	methanolic extract of Tualang honey
MWM	Morris water maze
Na^{+}	sodium ion
NADPH	nicotinamide adenine dinucleotide phosphate
NFT	neurofibrillary tangle
NF-κB	nuclear factor-kappa B
ng	nanogram
nm	nanometre
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOR	novel object recognition
NOS	nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion
OD	optical density
$OH^{\bullet}$	hydroxyl radical
$ONOO^{-}$	peroxynitrite
PBS	phosphate buffer saline
pg	picogram
PG	prostaglandin
PLA_2	phospholipase A_2
psen1	presenilin 1
psen2	presenilin 2

PUFA	polyunsaturated fatty acid
R^2	coefficient of determination
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
sAPPα	soluble alpha-amyloid precursor protein
SD	Sprague Dawley
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
sec	second
SEM	standard error mean
SOD	superoxide dismutase
SPE	solid phase extraction
SPSS	Statistical Package for the Social Sciences
STAT3	activator of transcription 3
STZ	streptozotocin
TBH	tetrahydrobiopterin
TBS	tris buffer saline
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TFC	total flavonoid content
TGF-β	transforming growth factor-beta
TGN	trans-golgi network
TH	Tualang honey
TLR-4	toll-like receptor 4
TMB	3,3'5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor-alpha
TPC	total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
TXB_2	thromboxane B_2
USM	Universiti Sains Malaysia
UV/VIS	ultraviolet and visible
V	voltage

PENJELASAN MENGENAI MEKANISME GANGGUAN KOGNITIF AKIBAT LIPOPOLISAKARIDA DALAM TIKUS YANG DIRAWAT DENGAN MADU TUALANG DAN EKSTRAK METANOLNYA

ABSTRAK

Alzheimer (AD) merupakan sejenis penyakit yang boleh disifatkan sebagai penurunan fungsi kognitif secara progresif. Pencarian penambah baik fungsi kognitif daripada derivatif produk semulajadi semakin mendapat perhatian. Madu Tualang (TH) yang dihasilkan oleh lebah liar (*Apis dorsata*) merupakan salah satu sumber antioksida semulajadi dan dikatakan mempunyai banyak kesan positif terhadap kesihatan manusia, termasuklah fungsi kognitif. Empat jenis sampel madu seperti TH, TH yang telah didedahkan kepada sinar gamma, ekstrak metanol (MTH) dan etil asetat dari TH telah diuji pada kajian peringkat awal dan didapati bahawa MTH menunjukkan potensi antioksida yang paling tinggi. Justeru itu, kajian ini dilakukan bertujuan untuk menghuraikan mekanisme oleh TH dan MTH terhadap gangguan kognitif pada tikus yang disebabkan oleh lipopolisakarida (LPS), dan juga membuat perbandingan dengan memantine, salah satu ubat standard yang digunakan untuk merawat AD. Sejumlah 100 ekor tikus jantan Sprague Dawley dibahagikan secara rawak kepada 5 kumpulan: (i) kawalan, (ii) LPS yang tidak dirawat, (iii) LPS yang dirawat dengan 200 mg/kg TH, (iv) LPS yang dirawat dengan 150 mg/kg MTH and (v) LPS yang dirawat dengan 10 mg/kg memantine. Semua rawatan diberikan secara suntikan intraperitoneal sekali dalam sehari selama 14 hari. LPS telah diberikan kepada tikus-tikus pada hari keempat daripada hari-hari rawatan untuk mewujudkan tindakbalas keradangan yang menyebabkan gangguan fungsi-fungsi kognitif. Setelah itu, fungsi memori ruangan dan pengecaman dinilai dengan ujian rintangan air

Morris (MWM) dan ujian pengecaman objek baru (NOR). Setelah 24 jam ujian tingkahlaku, semua tikus dikorbankan dan tisu hipokampus dikeluarkan. Kemudian, tahap ekspresi protein akibat keradangan di dalam tisu hipokampus ditentukan dengan kaedah imunohistokimia dan Western blot. Aras enzim-enzim antioksida, kerosakan oksidatif dan pengumpulan amiloid dinilai dalam hipokampus yang dihomogenasikan dengan menggunakan ELISA komersial kit. Akhir sekali, pewarnaan Nissl ke atas hirisan hipokampus dilakukan untuk menganalisa bilangan sel-sel neuron. Pemberian LPS kepada tikus-tikus telah menyebabkan kemerosotan pada memori ruangan dan pengecaman. LPS juga telah menyebabkan peningkatan yang signifikan pada keradangan saraf yang dibuktikan melalui peningkatan pada aras ekspresi COX-2, iNOS and TNF-α dalam tisu hipokampus. Terdapat juga penurunan yang ketara pada aras enzim-enzim antioksida seperti CAT, GPx, GR dan SOD, diiringi peningkatan aras MDA dan pengumpulan amiloid selepas pemberian LPS. Di samping itu, LPS juga telah mengurangkan bilangan sel positif Nissl di semua bahagian pada hipokampus. Rawatan dengan TH dan MTH selama 14 hari berupaya menambahbaik memori ruangan dan pengecaman. Tambahan pula, TH dan MTH berjaya menurunkan keradangan saraf, tekanan oksidatif, pengumpulan amiloid serta meningkatkan bilangan sel positif Nissl dalam hipokampus. Kesan perlindungan saraf oleh TH dan MTH didapati setanding dengan rawatan memantine. Kesimpulannya, kajian ini menunjukkan bahawa TH dan MTH berupaya meningkatkan memori ruangan dan pengecaman melalui sifatnya yang mempunyai anti keradangan, antioksida, anti amiloid serta membantu meningkatkan bilangan sel-sel saraf pada tikus-tikus yang mengalami gangguan kognitif disebabkan oleh LPS. Oleh itu, TH dan MTH berpotensi untuk digunakan sebagai terapi alternatif untuk mencegah daripada kemerosotan memori dalam pesakit AD.

ELUCIDATING THE MECHANISMS OF LIPOPOLYSACCHARIDE-INDUCED COGNITIVE IMPAIRMENT IN RATS TREATED WITH TUALANG HONEY AND ITS METHANOLIC EXTRACTION

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive loss of cognitive functions. The search for cognitive enhancers from natural products derivative has gained much attention. As a natural antioxidant source, Tualang honey (TH) produced by wild bees (*Apis dorsata*) is expected to have a positive influence on human health, including cognitive function. Four types of honey such as raw TH, irradiated TH, TH methanolic (MTH) and ethyl acetate extract were tested in the preliminary study and it was found that MTH exhibited the highest antioxidant potentials. Therefore, this study aimed to elucidate the mechanism of TH and MTH on lipopolysaccharide (LPS)-induced cognitive impairment in rats, and compare with memantine, one of the standard drugs used in the management of AD. A total of 100 male Sprague Dawley rats were randomly divided into 5 groups: (i) control, (ii) untreated LPS, (iii) LPS treated with 200 mg/kg TH, (iv) LPS treated with 150 mg/kg MTH and (v) LPS treated with 10 mg/kg memantine. All treatments were administered intraperitoneally once daily for 14 days. LPS was injected into the rats at Day 4 of the treatments to create neuroinflammatory responses that cause cognitive dysfunctions. Spatial and recognition memory functions were assessed using Morris water maze (MWM) and novel object recognition (NOR) tests. Twenty four hours after completing behavioural tests, all rats were sacrificed and hippocampal tissues were removed. Localization and expression of inflammatory markers in the hippocampal tissue were

determined by immunohistochemistry analysis and Western blot. The levels of antioxidant enzymes, oxidative damage and amyloid deposition in rats' hippocampal homogenates were determined by commercially available ELISA kits. Nissl staining of hippocampal slices was employed to determine neuronal count. The administration of LPS to rats resulted in spatial and recognition memory impairment. LPS also caused a significant increase in neuroinflammation as evidenced by the increase in COX-2, iNOS and TNF-α expression in hippocampal tissues. There was a marked reduction in the levels of antioxidant enzymes such as CAT, GPx, GR and SOD with the concomitant increase in MDA level and amyloid deposition following LPS administration. LPS also caused reduction in the number of Nissl positive neurons in all hippocampal regions. TH and MTH treatments for 14 days significantly improved spatial and recognition memory. Besides, TH and MTH were able to reduce neuroinflammation, oxidative stress, amyloid deposition as well as improve Nissl positive neuronal count in the hippocampus. The neuroprotective effects of TH and MTH were found to be comparable with memantine. In conclusion, this study showed that both TH and MTH could prevent LPS-induced spatial and recognition memory impairment via its anti-inflammatory, antioxidant and anti-amyloidogenic properties. Both TH and MTH have the potential to be used as alternative therapy to protect against memory decline in AD patients.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Neurodegenerative disorder such as Alzheimer's disease (AD) is common among older people and characterized by progressive cognitive deficits and behavioural disturbances (Barnes *et al.*, 2015). The neurological changes associated with AD include beta-amyloid (Aβ) aggregation, hyperphosphorylation of tau protein, brain mitochondrial dysfunction, cholinergic deficits, neuroinflammation and oxidative stress (Klafki *et al.*, 2006). The changes evolve gradually over decades before the clinical symptoms become apparent in affected individuals (Mendiola-Precoma *et al.*, 2016).

Extensive studies proposed that sustained increase in various proinflammatory mediators such as tumour necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide (iNOS) in the central nervous system (CNS) are closely related to neuronal damage and memory impairments (Agostinho *et al.*, 2010; Bernhardi *et al.*, 2015). Moreover, the overproduction of proinflammatory mediators will lead to high production of free radicals, thereby causing oxidative stress. Enhanced oxidative damage also has been reported in the brain of AD patients at early stage of mild cognitive impairment and in individuals with early signs of dementia (Kim *et al.*, 2015a).

Lipopolysaccharide (LPS) injection has been widely used to create a non-transgenic model for AD. LPS is a component of gram-negative bacterial cell wall, induces a neuroinflammatory reaction, impairs memory function and elevates oxidative stress by increasing TNF-α, COX-2, iNOS and other proinflammatory mediators in the hippocampus when administered systemically (Lee *et al.*, 2008; Krstic *et al.*, 2012; Hoogland *et al.*, 2015). This peripheral inflammatory *milieu* can be relayed to the CNS through vagus nerve activation, brain endothelial cell activation and circumventricular organs (Hansen *et al.*, 2000; Konsman *et al.*, 2002). LPS activates microglia leading to the production of proinflammatory cytokines within the brain (Erridge *et al.*, 2002).

Cytokine receptors are distributed throughout the brain with high densities in the hippocampus. Hence, the hippocampus is thought to be particularly prone to immune-related alterations (Yin *et al.*, 2013). The interaction between proinflammatory cytokines and neuronal elements during development may alter the brain in a way that makes it more vulnerable to LPS-induced hippocampus-dependent memory impairments (Deng *et al.*, 2012). LPS also has been shown to damage neurons by causing oxidative stress through depletion in the levels of antioxidant enzymes which foster free radical generation (Zhu *et al.*, 2007). In fact, LPS-induced inflammation in the hippocampus demonstrates severe learning and memory deficits in a variety of behavioural tasks (Frank-Cannon *et al.*, 2009).

Although neuroinflammation is associated with neuropathological and cognitive deficits, clinical trials using non-steroidal anti-inflammatory drugs (NSAIDs) have not been encouraging, and in some cases accelerated progression of the disease (Martin *et al.*, 2008). Clinical uses of cholinesterase inhibitors and memantine have been promising in treating mild to moderate AD. However, while these therapies help to maintain neuronal function, they do not provide a significant impact on the reversal of the disease process (Folch *et al.*, 2018). Therefore, the use of natural products exhibiting antioxidant and anti-inflammatory activities for treating AD-related cognitive impairments has been suggested in recent studies (Ajibola, 2015; Arulselvan *et al.*, 2016; Shal *et al.*, 2018).

Tualang honey (TH) has a relatively better antioxidant and anti-inflammatory activities compared to Manuka and other Malaysian honey (Ahmed *et al.*, 2018). Other pharmacological effects of TH include antidiabetic, anticancer, antihypertensive, hepatoprotective, antibacterial and facilitates wound healing (Tan *et al.*, 2009; Erejuwa *et al.*, 2010a; Erejuwa *et al.*, 2012a; Devasvaran and Yong, 2016; Ahmed and Othman, 2017) Recently, TH has been demonstrated to improve memory performances and hippocampal morphology in aged rats (Azman *et al.*, 2015; Kamarulzaidi *et al.*, 2016). Despite numerous studies that demonstrated the beneficial effects of TH, its neuroprotective roles in LPS-induced neuroinflammation and cognitive impairment remains unexplored. Therefore, the present study aimed to evaluate the possible neuroprotective effects of TH and its methanolic extract on LPS rat model and to compare with memantine, one of the standard treatments for AD.

1.2 Rationale of the study

AD is the most common form of dementia and possibly contributes to 60–70% of dementia cases (Armstrong, 2013). The impairments associated with this disease are devastating for the patients and their families. Due to the ageing profile of the population, AD is creating an enormous strain on the health care system, families and the federal budget. Unfortunately, there are no effective treatment for AD patients and recent clinical trials showed inconsistent results (Imtiaz *et al.*, 2014). Since inflammation and oxidative stress are key processes in the development and progression of AD, a natural product that possesses anti-inflammatory and antioxidant activity is considered to be a promising approach to prevent or delay the disease progression.

In Malaysia, TH has been traditionally consumed to cure various ailments and as a supplement. Therapeutic values of TH have been reported due to the presence of its phenolic compounds. They act as excellent antioxidants, anti-inflammatory, cognitive enhancer, cholinesterase and β-secretase inhibitors and prevent Aβ peptide accumulation and its fibril formation (Bhullar and Rupasinghe, 2013; Figueira *et al.*, 2017; Omar *et al.*, 2017). Most of the researchers use pure or honey cocktail for the treatment, whereas the use of polyphenolic compounds extracted from honey has not been extensively studied. Therefore, in the present study pure TH and its methanolic extract were evaluated for their neuroprotective effects in LPS-induced male rats. Given the properties of TH, the present study hypothesized that TH and its methanolic extract can improve detrimental effects caused by LPS-induced rats comparable to memantine. The potential neuroprotective effects could be delineated through their antioxidant and anti-inflammatory properties.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study was to evaluate the possible neuroprotective effects of TH and its methanolic extract in LPS-induced rat and to compare with memantine. The possible underlying mechanisms involved would also be elucidated.

1.3.2 Specific objectives

1. To evaluate the effect of TH and its methanolic extract on spatial and recognition memory in LPS-induced rat.

2. To determine the effect of TH and its methanolic extract on localization as well as expression of proinflammatory proteins in LPS-induced rat's hippocampus.

3. To measure the effect of TH and its methanolic extract on the level of antioxidant enzymes and oxidative damage in LPS-induced rat's hippocampus.

4. To determine the effect of TH and its methanolic extract on the level of amyloid deposition in LPS-induced rat's hippocampus.

5. To measure the effect of TH and its methanolic extract on the neuronal count in LPS-induced rat's hippocampus.

CHAPTER 2

LITERATURE REVIEW

2.1 Neurodegenerative diseases

Neurodegenerative disease is a general term for a range of neurological disorders which primarily affects neurons in the CNS. It is characterized by the progressive loss of neuronal structure that resulted in the neuronal cell death, which leads to neurodegeneration (Frank-Cannon *et al.*, 2009). Neurons normally cannot easily regenerate or repair themselves, so when they become damaged or die they cannot be replaced by the body. This is why mature neurons of the nervous system are postmitotic (Frade and Ovejero-Benito, 2015).

Acute neurodegeneration is a condition in which neurons are rapidly damaged and commonly die in response to a sudden insult or traumatic events, such as head injury, cerebral or subarachnoid haemorrhage, ischaemic brain damage and stroke (Trendelenburg, 2008). Meanwhile, chronic neurodegeneration is a condition in which neurons in the nervous system sustain a neurodegenerative process that typically starts slowly and worsen over time with multifactorial causes, resulting in progressive neuronal cell death (Lull and Block, 2010). Examples of chronic neurodegenerative diseases are AD, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, temporal lobe epilepsy, frontotemporal dementia and the spinocerebellar ataxias. These diseases are diverse in their pathophysiology with some causing memory and cognitive impairments and others affecting a person's ability to move, speak and breathe (Gao and Hong, 2008; Lull and Block, 2010; Chen *et al.*, 2016).

2.1.1 Alzheimer's disease

AD was first described in 1907 by a German physician named Alois Alzheimer, is currently the main cause of dementia in elderly people, accounting for 60-70% of cases (Armstrong, 2013). The global prevalence of dementia was estimated at 44 million and is predicted to double every 20 years (Mendiola-Precoma *et al.*, 2016). AD can be categorized into early- and late-onset. Early-onset AD (EOAD) typically begins between the ages of 30 and 60 years, and accounts for fewer than 5% of AD cases. The more common form, late-onset or sporadic AD (LOAD), presents after the age of 65 (Imtiaz *et al.*, 2014; Mendiola-Precoma *et al.*, 2016). As the world's population is constantly aging, the number of vulnerable individuals is growing, especially among the very old. It has been estimated that around 81.1 million people worldwide will suffer from AD by 2040 (Mayeux and Stern, 2012; Bondi *et al.*, 2017). The disease has been the sixth leading cause of death across all ages and the fifth leading cause of death in those aged above 65 years (Brookmeyer *et al.*, 2007). According to Alzheimer's disease Foundation Malaysia (2016), it is predicted that currently there are about 50,000 people in Malaysia who are suffering from this disease.

2.1.2 Risk factors and symptoms

Age is the most significant risk factor for AD, with a risk of 10% for persons older than 65 years and nearly 50% for those older than 85 years (Yiannopoulou and Papageorgiou, 2013; Malve, 2016). Apart from age, various studies have assessed the relationship between metabolic syndrome as a whole and the risk of AD or cognitive decline. Most of these investigations demonstrated a positive association between the presence of this syndrome and cognitive dysfunction (Raffaitin *et al.*, 2009; Solfrizzi

et al., 2010). There appears to be little difference in risk between men and women, but overall, more women suffer from AD because they generally live longer than men. Another risk factor for AD is genetic autosomal dominant familial AD (FAD), which is typically an EOAD, has been linked to mutations in genes encoding presenilin1 (*psen1*) and presenilin2 (*psen2*) (Kumar-Singh *et al.*, 2006; Cacquevel *et al.*, 2012; Tanzi, 2012; Cheng *et al.*, 2015). Inheritance of the apolipoprotein E type 4 allele (*ApoE4*) may also increase the risk of developing sporadic LOAD, though *ApoE4* alone is neither necessary nor sufficient to cause AD (Rossor *et al.*, 1996; Schachter and Davis, 2000; Sadigh-Eteghad *et al.*, 2012; Karch *et al.*, 2014). Moreover, recent finding shows that poor lifestyle factors such as smoking or alcohol consumption may also play a role in the development of AD (van Praag, 2018). Meanwhile, some studies have shown a link between Alzheimer's disease and major head injury (Fleminger *et al.*, 2003; Hovens *et al.*, 2014; Ramos-Cejudo *et al.*, 2018).

The common initial symptoms are forgetfulness and difficulties with routine tasks. As the disease progresses, patients develop more severe memory loss, speech impairment, visual and spatial deficits, and loss of coordination and fine motor control (Kalaria *et al.*, 2008; Li *et al.*, 2014; Barnes *et al.*, 2015). Besides the cognitive, sensory, and motor deficits caused by the disease progression, there are a number of behavioural and psychological symptoms related to AD. These symptoms include agitation and aggression, wandering, disturbances in the sleep cycle, depression, anxiety, delusions and hallucinations (Shaji *et al.*, 2009; Cerejeira *et al.*, 2012; Kolanowski *et al.*, 2017). AD reduces the life expectancy of those affected, with average survival time after diagnosis of 5-7 years (Fitzpatrick *et al.*, 2005; Ganguli *et al.*, 2005).

2.1.3 Hallmarks of AD

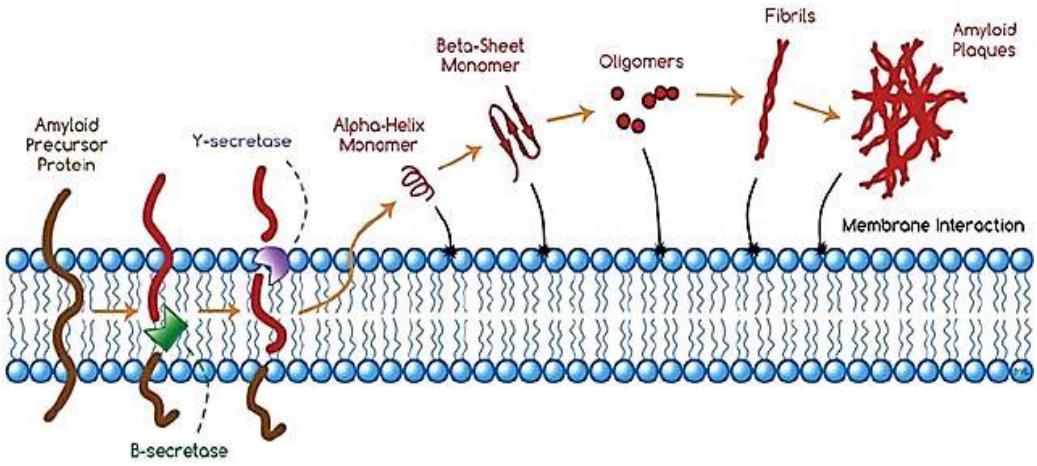
The pathogenesis of AD is complex and is likely to involve several cellular and molecular mechanisms (Skaper, 2012). However, Aβ was identified as a leading candidate to trigger AD. Monomer Aβ is about 4 kDa peptide which is produced by the intramembrane proteolytic cleavage of bigger protein, known as amyloid precursor protein (APP) (Humpel, 2011). APP is synthesized in the endoplasmic reticulum and then transported through the Golgi apparatus to the trans-golgi network (TGN) where the highest concentration of APP is found in neurons at steady state. Under normal state, APP from TGN can be transported in secretory vesicles to the cell surface where it can be proteolytically cleaved directly by enzymes α-secretase and then γ-secretase, producing a soluble molecule (sAPPα) which may have neuroprotective effects (Dawkins and Small, 2014).

Under pathological state, APP is sequentially cleaved by β-secretase 1 (BACE-1) and γ-secretase, producing a set of Aβ peptides of varying lengths from 38 to 43 residues depending on the position of the cleavage with γ-secretase (Murphy and LeVine, 2010) [Figure 2.1(A)]. These Aβ peptide fragments have been found to play a critical role in the amyloid plaque characteristic of AD pathology. However, most predominantly formed fragments are $A\beta_{1-40}$ and $A\beta_{1-42}$ (Holtzman *et al.*, 2011). $A\beta_{1-40}$ is soluble, less neurotoxic, and predominantly found in the healthy brain. Meanwhile, $A\beta_{1-42}$ is highly neurotoxic, has a greater propensity to aggregate and predominantly found in brains with AD pathology, despite the fact that only two amino acids differ from each other (El-Agnaf *et al.*, 2000). Evidences supporting the participation of Aβ in AD pathogenesis is based on genetic study of FAD patients reporting that mutations in the genes *psen1* and *psen2* (genes encoding enzymes that

cleave APP to produce Aβ peptides) can cause an aggressive early-onset form of AD, leading to an increase in the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$ peptides produced as well as enhanced aggregation tendency (Bentahir *et al.*, 2006; Kumar-Singh *et al.*, 2006; Cacquevel *et al.*, 2012).

In addition to the presence of amyloid plaques, the other marker of AD is the intracellular deposition of neurofibrillary tangles (NFT) which consists of aggregated hyperphosphorylated forms of the microtubule-associated tau protein (Šimić *et al.*, 2016). Tau protein is documented to have a dominant role in the stabilization of neuronal microtubules, thus providing the tracks for intracellular transport. In healthy brain, balance between microtubule-associated phosphatase and kinases have been shown to maintain both phosphorylation and dephosphorylation states of tau (Gong and Iqbal, 2008). In AD, upregulation of kinases and downregulation of phosphatase trigger hyperphosphorylation of tau protein that generates insoluble pair helical filaments and tangled clumps, and culminates in the formation of NFT (Dolan and Johnson, 2010; Iqbal *et al.*, 2010) [Figure 2.1(B)]. Changes in Tau protein might affect stabilization of microtubules, thus are likely to impair axonal transport, leading to synaptic dysfunction and neurodegeneration. The exact molecular mechanism by which NFT deposition causes plausible synaptic dysfunction and neuronal death is still not established (Ittner and Götz, 2010). Investigations with mouse models suggest that the role of NFT in AD may be lower than Aβ since Aβ plaque pathology was not developed in a tau transgenic mouse model, whereas Aβ formation in APP transgenic mice was found to induce hyperphosphorylation of tau, leading to the formation of NFT (Götz *et al.*, 2004).

(A)



(B)

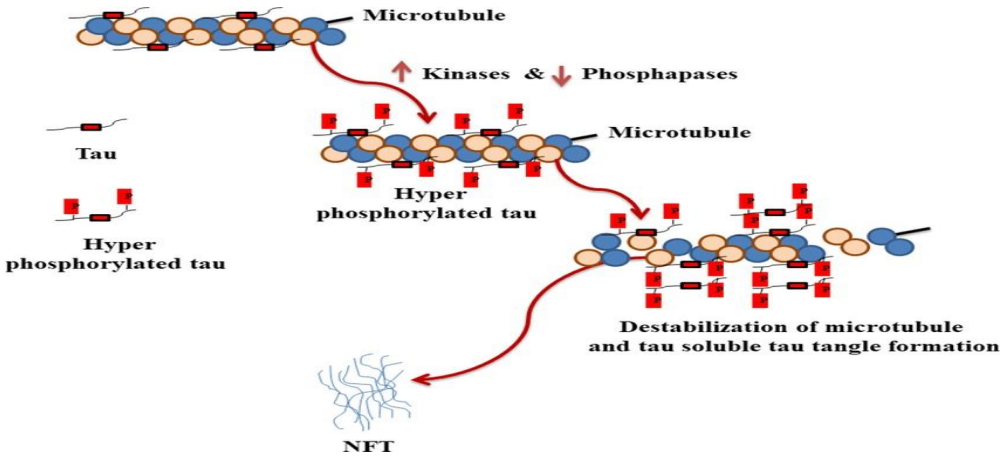


Figure 2.1: Aβ plaques and NFT formations are hallmark deposits of AD. (A) The major protein component of the plaques is Aβ that results from proteolytic cleavage of APP. BACE-1 generates the amino terminus of Aβ, and γ-secretase defines its length. Aβ forms toxic oligomeric aggregates that then deposit as plaques. (B) In AD, tau is hyperphosphorylated and dissociates from microtubules, causing them to depolymerize. Tau is then deposited in aggregates such as NFT. Adapted from Drolle *et al.* (2014) and Kumar *et al.* (2018).

2.1.4 Treatments

AD is complex, and it is unlikely that any one drug or other intervention will successfully treat it. Current approaches focus on helping people maintain mental function, manage behavioural symptoms, and slow down the disease progression (Grill and Cummings, 2010). At present, five drugs are approved by the Food and Drug Administration (FDA) for use in AD cases. Cholinesterase inhibitors such as galantamine, rivastigmine, donepezil and tacrine are prescribed for mild to moderate AD. These drugs may help reduce some symptoms and help control some behavioural symptoms (Mendiola-Precoma *et al.*, 2016). It is not fully understood how cholinesterase inhibitors work to treat AD, but research indicates that they prevent the breakdown of acetylcholine (ACh), a brain chemical believed to be important for memory and thinking. However, as AD progresses, the brain produces less and less ACh and therefore cholinesterase inhibitors may eventually lose their effect (Tumiatti *et al.*, 2008; Wang *et al.*, 2015).

The other drug that is clinically used for treating AD is memantine, which had been used in this study as a positive control. Memantine is a moderate-affinity, uncompetitive, voltage-dependent N-methyl-D-aspartate (NMDA) receptor antagonist with fast on/off kinetics (Robinson and Keating, 2006). Memantine appears to work by regulating the activity of glutamate, a major excitatory amino acid neurotransmitter involved in information processing, storage and retrieval. Glutamate plays an important role in learning and memory by triggering NMDA receptors to let a controlled amount of calcium ion (Ca^{2+}) into a nerve cell. The calcium helps create the chemical environment required for information storage (Hynd *et al.*, 2004). Excess glutamate, on the other hand, overstimulates NMDA

receptors so that they cause the opening of voltage-gated ion channels and allow too much Ca^{2+} influx into the nerve cells. This process is called glutamate excitotoxicity (Mehta *et al.*, 2013a). The resulting Ca^{2+} overload is particularly neurotoxic, leading to activation of several enzymes including phospholipases, endonucleases, proteases, protein kinases and inducible nitric oxide synthase (iNOS). The above-mentioned enzymes will enhance the degradation of proteins, membranes and nucleic acids (Wang and Reddy, 2017). Moreover, excessive Ca^{2+} could also result in reactive oxygen species (ROS) generation, mitochondrial dysfunction, oxidative stress and inflammatory responses (Guo *et al.*, 2013). These processes ultimately lead to neuronal cell death, which can be necrotic or apoptotic.

Memantine may protect cells against excess glutamate by partially blocking NMDA receptors, including the pathological influx of Ca^{2+} and oxidative stress in postsynaptic neurons, while preserving physiological transmission, which is essential for normal cellular function (Figure 2.2). In turn, this reduces the levels of neuronal damage and alleviates the symptoms of the disease (van Marum, 2009). The efficacy of memantine 20 mg/day has been examined in seven randomised, double-blind, placebo-controlled, multicentre trials of 12–28 weeks' duration in patients with mild to severe AD (Folch *et al.*, 2018). Since memantine is a low-affinity antagonist, it blocks the NMDA receptors but it is rapidly displaced from it, avoiding prolonged receptor blockade and the related negative side effects on learning and memory that have been reported in high-affinity NMDA receptor antagonists (Danysz and Parsons, 2003). Memantine also has suitable safety and tolerability limits indicating a good therapeutic margin. Another advantage of memantine is that it only interacts with the channel when it is pathologically activated under an excessive glutamate concentration in the synaptic cleft (Robinson and Keating, 2006).

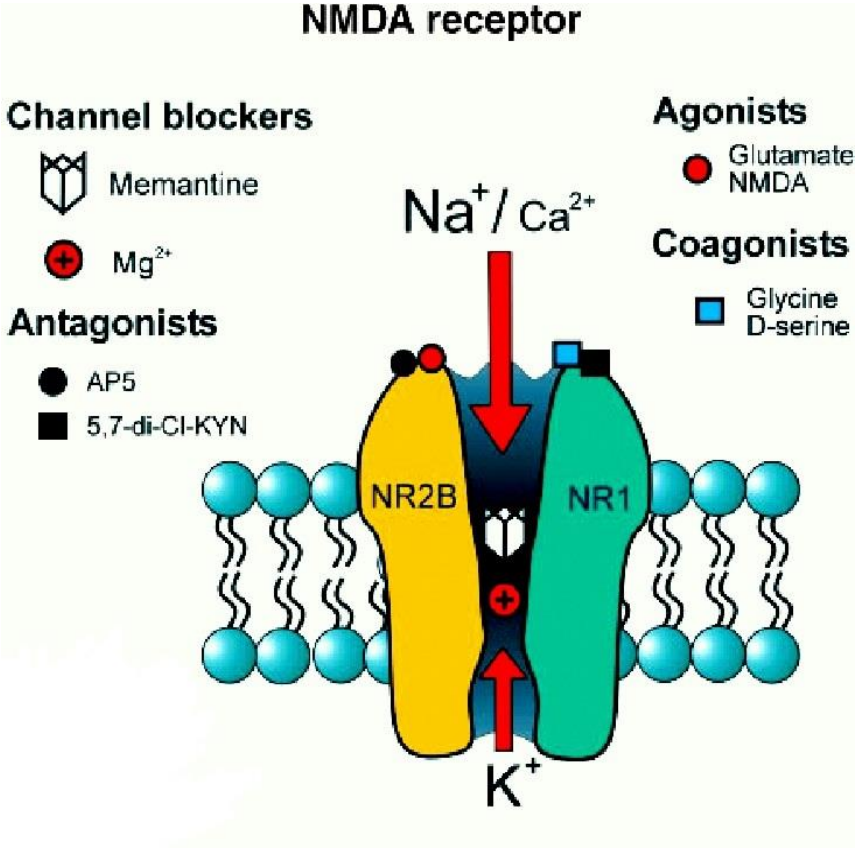


Figure 2.2: Schematic diagram of the NMDA receptor and the channel blocking site for memantine. The drug may protect cells against excess glutamate by partially blocking NMDA receptors, including the pathological influx of Ca^{2+} and oxidative stress in postsynaptic neurons. Adapted from Danysz and Parsons (2003).

2.2 Possible molecular mechanisms of neurodegeneration in AD

The characteristic features of AD are the appearance of extracellular Aβ plaques and NFT in the intracellular environment, neuronal death and the loss of synapses, all of which resulted in cognitive decline in a progressive way (Armstrong, 2013). There are epidemiological, clinical, and experimental data that support several hypotheses of AD pathogenesis. The cholinergic hypothesis suggests that there is a reduction in the activity of choline acetyltransferase (ChAT) and ACh levels in areas such as the hippocampus and cerebral cortex (Mufson *et al.*, 2008; Contestabile, 2011; Ferreira-Vieira *et al.*, 2016). Brain mitochondrial dysfunction has been proposed as the first pathogenic event leading to neurodegeneration (Guo *et al.*, 2013). Other hypotheses are associated with blood-brain barrier (BBB) disruption, decrease in cerebral blood flow as well as hypometabolism (Mendiola-Precoma *et al.*, 2016). In addition, it is widely accepted that neuroinflammation and oxidative stress can have both detrimental and beneficial influences on the neural tissue (Agostinho *et al.*, 2010; Barrientos *et al.*, 2015; Kim *et al.*, 2015a). Thus, the involvement of neuroinflammation and oxidative stress in the onset and progress of neurodegenerative process in AD is becoming increasingly recognized.

2.2.1 Neuroinflammation and AD

Inflammatory response in the CNS usually referred to as neuroinflammation, has been a matter of particular interest in the last two decades. In general, an acute neuroinflammatory response is beneficial to the CNS, since it contributes to the repair of damaged tissue and to diminish further injury (Rogers, 2008). In most cases, acute neuroinflammation will cease once the threat has been eliminated and homeostasis has been restored (Glass *et al.*, 2010). On the contrary, chronic

neuroinflammation is an often self-perpetuating response that persists long after an initial toxic insult and can trigger abnormal brain functions (Lyman *et al.*, 2014; Shabab *et al.*, 2017). The sustained release of inflammatory factors might perpetuate the neuroinflammatory process activating additional glial cells, which causes further release of proinflammatory and neurotoxic factors, contributing to neuronal dysfunction and consequently to pathology.

2.2.2 Glial cells activation and inflammatory cytokines production

The first indication of neuroinflammation is glial cells activation, mainly microglia. They are the resident of brain macrophages which play a vital role in an organism's defence and tissue repair (Bernhardi *et al.*, 2015). Microglia becomes activated upon the exposure to pathogens, tissue damage, neurotoxins, abnormal stimulation, infection or injury. At this point, they can even attack healthy neurons either physically, by secreting apoptosis factors or via phagocytosis (Graeber and Streit, 2010). Thus, microglia are the critical cells in brain inflammation and inflammatory neurodegenerative diseases. After being activated, microglial cells will release numerous oxidants and activate various genes and proteins, including proinflammatory cytokines such as interleukin-1 beta (IL-1β), IL-6, TNF-α, COX-2, complement factors, ROS and iNOS, which can cause neuronal dysfunction and death (Carson *et al.*, 2006; Glass *et al.*, 2010; Spencer *et al.*, 2012; Bernhardi *et al.*, 2015) (Figure 2.3). In terms of chronic neuroinflammation, these cells can remain activated for extended periods, releasing cytokines and neurotoxic molecules that give rise to long-term neurodegeneration (Gao and Hong, 2008).

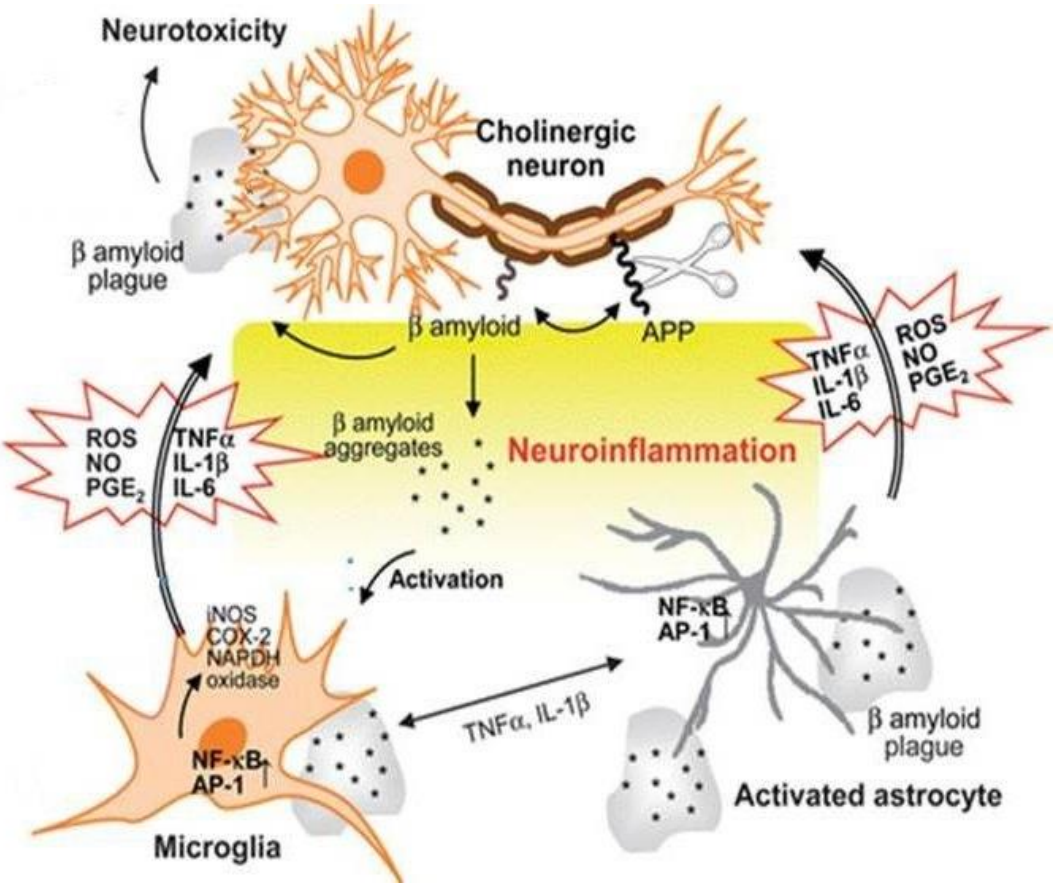


Figure 2.3: Schematic representation of the role of glial cells in the pathophysiology of AD. Numerous stimuli can activate microglia and astrocyte, which cause the increased production of proinflammatory cytokines, intracellular Aβ and Tau aggregation, leading to synaptic loss and neuronal death. Adapted from Shal *et al.* (2018).

In the brain of AD patients, increased levels of several cytokines and chemokines have been observed, including IL-1β, IL-6, TNF-α, IL-8, COX-2, transforming growth factor-beta (TGF-β), macrophage inflammatory proteins-1α, as compared with age-matched control subjects (Katsuse *et al.*, 2003; Fujimi *et al.*, 2007; Morimoto *et al.*, 2011). Moreover, the presence of high levels of inflammatory factors in the plasma such as IL-12, IL-16, IL-18 and TGF-β was identified as a possible predictor of conversion from mild cognitive impairment (MCI) to AD (Motta *et al.*, 2007). A study had reported that increased cytokines production, mainly interferon-gamma (IFN-γ) and TNF-α can enhance Aβ deposition through the upregulation of BACE1 or the reduction of Aβ clearance and degradation by glial cells (Yamamoto *et al.*, 2007). In other studies, proinflammatory cytokines and ROS might change the substrate selectivity and activity of kinases/phosphatases leading to tau hyperphosphorylation at pathological sites (Arnaud *et al.*, 2006; Sy *et al.*, 2011). Thus, this provides a framework to suggest that neuroinflammation might be a link between Aβ deposition and the formation of NFT. Studies performed *in vitro* with cultured microglia isolated from human or rodent brains also indicated that Aβ exposure triggers an inflammatory-like response, which includes an increased production of proinflammatory cytokines, reactive oxygen and nitrogen species and other neurotoxic factors (Jang and Surh, 2005; Sultana *et al.*, 2005; Garção *et al.*, 2006; Szaingurten-Solodkin *et al.*, 2009). These studies demonstrate that Aβ accumulation and proinflammatory cytokines are interconnected and regulate each other which contribute to the recruitment of activated microglia that can further secrete several proinflammatory cytokines and ROS, accelerating the neurodegenerative process in AD.

2.2.3 COX-2, iNOS and TNF-α in neuroinflammation pathway

Cyclooxygenase-1 and COX-2, also known as prostaglandin endoperoxide synthases, are the two enzymes that play a central role in the inflammatory cascade. Both these two isoforms are encoded by different genes, but share 60% homology in their amino acids sequence and have comparable kinetics (Morita, 2002). The pathways of COX-1 and COX-2 are associated with neuroinflammation and neurodegeneration. They catalyse the same reaction of deoxygenation of arachidonic acid, released from membrane phospholipids by phospholipase A_2 (PLA_2) to yield prostaglandin G_2 (PGG_2). PGG_2 is further converted to prostaglandin H_2 (PGH_2) in a peroxidase reaction. PGH_2 is then transformed into prostaglandin E_2 (PGE_2) which is a neuroinflammatory mediator (Aïd and Bosetti, 2011; Lima *et al.*, 2012). However, these two isoforms also show individual differences. COX-1 is normally constitutively expressed in most tissues and thought to be involved in homeostasis. Meanwhile, COX-2 is barely detectable under normal physiological conditions, but can be induced rapidly and transiently by proinflammatory mediators and mitogenic stimuli (Aïd and Bosetti, 2011; Nørregaard *et al.*, 2015), thereby mediating deleterious effects in the neurodegenerative disorders.

In the CNS, COX-2 is mainly detected in the perinuclear, dendritic and axonal domains of neurons, particularly in hippocampus, cortex, amygdala and dorsal horn of the spinal cord of both human and rodent (Aïd and Bosetti, 2011). COX-2 also has been implicated in critical physiological functions such as neurotransmitter release, synaptic transmission, blood flow regulation and sleep/wake cycle (Yang and Chen, 2008; Chung *et al.*, 2010; Kirkby *et al.*, 2018). Indeed, previous studies have reported that COX-2 expression is elevated in AD

brain patients (Ho *et al.*, 1999; Pasinetti, 2002; Fujimi *et al.*, 2007) and that condition is correlated with clinical dementia (Ho *et al.*, 2001). Moreover, *in vitro* study suggests that COX-2 upregulation in the AD brain may be mediated by the exposure of neurons to Aβ which may contribute to Aβ neurotoxicity (Wang *et al.*, 2014).

There are three isoforms of nitric oxide synthase (NOS) that account for nitric oxide (NO) production, namely endothelial NOS (eNOS), neuronal NOS (nNOS) and iNOS (Brown, 2007). All NOS isoforms catalyse the conversion of arginine and equimolar molecules of oxygen in the presence of various cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (TBH), into NO and equimolar amounts of citrulline. Unlike other isoforms, iNOS is hardly expressed and usually induced under pathological disorders (Yuste *et al.*, 2015). Various stimuli such as TNF-α, IL-1β and IFN-γ can induce the expression of iNOS. In prior studies, iNOS-expressing microglia was found in the senile plaques of AD patients (Haas *et al.*, 2002; Katsuse *et al.*, 2003; McCann *et al.*, 2005). Once expressed, iNOS produces high levels of NO continuously.

Excessive NO production, as evoked by inflammatory signals, has been associated with neuronal dysfunction and cell death mechanisms. Moreover, excessive NO synthesis under neuroinflammation give rise to the formation of reactive nitrogen species (RNS), resulting in nitrosative stress in the brain (Tripathi *et al.*, 2007; Brown, 2010; Asiimwe *et al.*, 2016). In addition, the combination of NO and free radicals like the superoxide anion will result in the formation of highly reactive peroxynitrite. Peroxynitrite can then stimulate nitration of many proteins, induce lipid peroxidation and cause DNA damage. At low concentration, NO and

superoxide are essential for the normal physiological regulation of the nervous system and play an important role in synaptic plasticity, learning and memory (Paul and Ekambaram, 2011; Hardingham *et al.*, 2013; Džoljić *et al.*, 2015). However, at high concentrations, they are key cytotoxic molecules.

In the healthy CNS, TNF-α has regulatory functions on key physiological processes such as learning and memory, synaptic plasticity, food and water intake, sleep and astrocyte-induced synaptic strengthening (Krueger, 2008; Beste *et al.*, 2010; Olmos and Lladó, 2014). However, TNF-α also is a potent proinflammatory cytokine that plays a central role in initiating and sustaining the inflammatory response. In response to injury or disease, glial cells, particularly microglia release large amount of TNF-α, together with other proinflammatory cytokines such as IL-1β, IL-6, IFN-γ, prostaglandins, ROS and complements proteins. Excessive production of TNF-α will trigger the activation of microglia which then initiate the synthesis of more TNF-α and other pro-inflammatory cytokines in the brain. This insult may then result in reactive microgliosis to begin a self-propelling and vicious cycle of neuronal damage. It is proposed that reactive microgliosis may underlie the progressive and long-lasting neuroinflammation and neurodegeneration of numerous neurodegenerative diseases (Block *et al.*, 2007; Schmid *et al.*, 2009; Olmos and Lladó, 2014). In fact, TNF-α can be found at sites of brain injury, and thus has been described as an indicator of damage. TNF-α has also been detected in plaques of post-mortem AD brains, and increased levels of TNF-α correlate with progressive hallmark symptoms of AD (Tansey *et al.*, 2007).

Additionally, TNF-α can induce glutamate-mediated excitotoxicity either by inhibiting glutamate transport on astrocytes or by increasing the localization of ionotropic glutamate receptor to synapse (Pickering *et al.*, 2005). This will lead to massive extracellular glutamate concentrations, allowing excessive Ca^{2+} influx that result in neuronal death. In another study, TNF-α activates nuclear factor-kappa B (NF-κB) signalling resulting in an increase in Aβ synthesis through BACE-1 transcription (Chen *et al.*, 2012a). Elevated TNF-α is also directly associated with cognitive impairment (Belarbi *et al.*, 2012). In animal models, high levels of TNF-α are seen in the AD brain as compared to controls, and behavioural deficits in these models are mitigated by blocking TNF-α (Gabbita *et al.*, 2012). This suggests that inflammatory cytokine release and its effects are closely related to AD pathology.

2.2.4 Oxidative stress and AD

Oxidative stress can possibly be viewed both as a cause, and as a consequence, of neuroinflammation. Oxidative stress occurs as a result of imbalance between the productions of free radicals including ROS, RNS and chlorinated compounds and the antioxidant defence (Thanan *et al.*, 2015). Approximately 95-98% of free radicals such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and peroxynitrite ($ONOO^-$) are formed in mitochondria as by-products of cellular respiration (Federico *et al.*, 2012). Despite the fact that oxygen is needed for survival, the brain is sensitive to oxygen metabolic activities that produce ROS. Studies indicate that 2-5% of the total oxygen consumed by mitochondria isolated from the brain produces ROS (Floyd and Hensley, 2002; Murphy, 2009). Other sources of ROS are NADPH oxidase, which is responsible for the formation of

$O_2^{\bullet-}$ from O_2, xanthine oxidases, cyclooxygenases and lipoxygenases, NOS and cytochrome P450s (Riganti *et al.*, 2008; Friedman, 2011; Biswas, 2016).

The brain is particularly susceptible to oxidative damage. It accounts for only 2% of body weight but consumes about 20% of total body oxygen, which results in a relatively high load of ROS during oxidative phosphorylation in relation to its tissue mass. Moreover, the brain contains a large amount of polyunsaturated fatty acids (PUFA) and has a relatively limited antioxidant capacity compared to the other organs (Floyd and Hensley, 2002; Bonda *et al.*, 2010; Cobley *et al.*, 2018). The production of high free radicals may interact with DNA, proteins, lipids, carbohydrates and components of mitochondria, leading to oxidative damage of these biomolecules and cellular dysfunction (Liu *et al.*, 2017). Additionally, oxidative stress can trigger the overexpression of iNOS and the activation of constitutive nNOS, leading to an increased NO production via the enzymatic conversion of arginine to citrulline. NO is a vital signalling and redox factor in the brain that is implicated in controlling synaptic activity and also in neurodegenerative process, such as inflammatory responses (Law *et al.*, 2001; Brown, 2010).

Accumulating evidences have reported that presence of prolonged oxidative stress is a characteristic of AD brains in addition to the established pathology of Aβ plaque and NFT (Yan and Baolu, 2013). It has been demonstrated that the levels of malondialdehyde (MDA), 4-hydroxynonenal and F2-isoprostanes, which resulted from lipid peroxidation, and markers of oxidative damage to DNA and RNA such as 8-hydroxy-2´-deoxyguanosine and 8-hydroxyguanosine, are elevated in AD brains. Production of protein oxidation such as carbonyl derivatives and 3-nitrotyrosin are

also increased in multiple brain regions and cerebrospinal fluid (CSF) of patients with AD or MCI (Nunomura *et al.*, 2006; Madeo and Elsayad, 2013; Yan and Baolu, 2013; Biswas, 2016).

Furthermore, oxidative stress can also cause misprocessing of APP to form amyloidogenic products. Several *in vitro* studies show a relationship between energy-related metabolic stress with high levels of secreted APP mediated by β-secretase, thereby giving rise to the production of amyloidogenic fragments (Borghi *et al.*, 2007; Tamagno *et al.*, 2008; Daulatzai, 2016; Muche *et al.*, 2017). On the other hand, aggregation of redox-active metal ions for example copper, iron or zinc to Aβ peptide further catalyse the production of ROS and may contribute to oxidative damage on both the Aβ peptide itself and on surrounding molecules such as proteins, lipids, carbohydrates and nucleic acids (Cheignon *et al.*, 2017). There is also evidence that Aβ itself can be oxidized via the formation of peptidyl radicals, resulting in enhanced aggregation (Butterfield *et al.*, 2013). The aggregation between amyloidogenic fragments in turn, accelerates existing molecular cascades associated with oxidative stress.

Numerous studies have indicated that the ROS system is correlated with the pathology that results from neuroinflammation (Agostinho *et al.*, 2010; Quintanilla *et al.*, 2012; Hsieh and Yang, 2013). Oxidative burst is believed to be one of the earliest biochemical events that result in the inflammatory activation of microglia. Activated microglia produce abundant of $O_2^{\bullet-}$ and H_2O_2 by activating NADPH oxidase (Rojo *et al.*, 2014). Moreover, ROS has been shown to be capable of modulating gene expression via activating kinases signalling, including mitogen-activated protein