THE NEUROPROTECTIVE EFFECT OF MINOCYCLINE VIA TLR-4/NF-κβ SIGNALLING PATHWAY IN LIPOPOLYSACCHARIDE - INDUCED COGNITIVE IMPAIRMENT IN MALE RATS

ENTESAR YASEEN ABDO QAID

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By

ENTESAR YASEEN ABDO QAID

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%	percentage
<	less than
>	more than
°C	Celsius
μL	microlitre
μmol	micromolar
3NP	3-nitropropionic acid
4-PL	Four-parameter logistic
ABTS	2,2’-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)
Abs	absorbance
ACh	acetylcholine
AD	Alzheimer’s disease
AgCM	astrocyte-conditioned medium
Akt	a serine/threonine protein kinase isoform
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	anti-psychotic
ApoE4	apolipoprotein E type 4 allele
APP	amyloid precursor protein
ARASC	animal research and service centre
ATF-1	activating transcription factor 1
ATP	adenosine triphosphate
Aβ	beta amyloid
BACE-1	beta-site APP-cleaving enzyme
BBB	blood-brain barrier

BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdala
BSA	bovine serum albumin
CA	cornus ammonis
Ca^{2+}	calcium ion
CAT	catalase
CD 14	cluster differentiation antigen 14
CD40L	CD40 ligand
CEQ	catechin equivalents
CGC	cerebellar granule cells
ChAT	choline acetyltransferase
Cm	centimetre
CMS	chronic mild stress
CNS	central nervous system
COX-2	cyclooxygenase-2
CREB	cyclic-AMP response element binding
CREM	cAMP response element modulator
CRP	c-reactive protein
CSF	cerebrospinal fluid
Cu^{3+}	copper ion
CUMS	chronic unpredictable mile stress
CUS	chronic unpredictable stress
DAB	3, 3’-Diaminobenzidine
DG	dentate gyrus
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl

DPX	dibutylphthalate polystyrene xylene
DNP	diabetic neuropathic pain
DNPH	dinitrophenylhydrazine
E.coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPO	erythropoietin
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FDA	food and drug administration
$Fe^{2+/3+}$	iron ion
FRAP	ferric reducing antioxidant power
G	gram
GAE	gallic acid equivalents
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSK3β	glycogen synthase kinase-3
GSSG	oxidized glutathione
H_2O	water
HFD	high fat diet
HMF	5-hydroxymethyl furfural
HAAO	hydroxyanthranilic acid dioxygenase
HBMVEs	human brain microvascular endothelial cells
HD	Huntington's disease

HI	hypoxic ischaemia
HIV	Human immunodeficiency virus
HMGB1	high mobility group box 1
HO-1	heme oxygenase-1
HPA	hypothalamic–pituitary–adrenal
Hr	Hour
HRP	horseradish peroxidase
Iba-1	ionized calcium-binding adapter molecule1
I/R	Ischaemic/reperfusion
IC50	50% of inhibition concentration
i.c.v.	intracerebroventricular
ICR	institute of cancer research
IDE	insulin-degrading enzyme
IDO	indoleamine 2, 3 dioxygenase
IDV	integrated density values
IFN-γ	interferon gamma
IFS	inescapable foot shock
IHC	Immunohistochemistry
IkB	inhibitor of NF-$_{kB}$
IKK	inhibitor kappa B kinase
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IP	interferon inducible protein
IR	insulin receptor
IRAK	interleukin-1 receptor-associated kinase

I/R	ischaemic/ reperfusion
kDA	kilo dalton
Kg	kilogram
L	litre
LBP	lipopolysaccharide binding protein
LDH	lactate dehydrogenase
LH	learned helplessness
LITAF	LPS-induced stimulation of TNF-α factor
LOX-1	lectin-like oxidized low density lipoprotein receptor-1
LPS	lipopolysaccharide
LTD	long-term depression
LTM	long-term memory
LTP	long-term potentiation
M	molar
MAPK	mitogen activated protein kinase
MCI	mild cognitive impairment
MCP	monocyte chemoattractant protein
MD2	myeloid differentiation protein 2
MDA	malondialdehyde
MDD	major depressive disorder
MEK	mitogen-activated protein kinase kinase
Mg	microgram
Mg^{2+}	magnesium ion
MHC	major histocompatibility complex
Min	minute
MIN	minocycline
MIP	monocyte chemoattractant protein

mL	millilitre
Mm	millimetre
mM	millimolar
MM	memantine
mPFC	medial prefrontal cortex
MPH	methylphenidate
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MWM	Morris water maze
Na+	sodium ion
NAc	nucleus accumbens
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ND	neurodegenerative diseases
NE	North-East
NFT	neurofibrillary tangle
NF-$_{kB}$	nuclear factor-kappa B
Ng	nanogram
NGF	nerve growth factor
NLRP3	nod-like receptor protein 3
Nm	nanometre
NMDA	N-methyl-D-aspartate
NMRI	Naval Medical Research Institute
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOR	novel object recognition
NOS	nitric oxide synthase

NOX	NADPH oxidase
NP	Neuropathic pain
NPCs	neural progenitor cells
Nrf-2	nuclear factor erythroid 2–related factor 2
NSAIDs	non-steroidal anti-inflammatory drugs
NT	neurotrophin
NW	North-West
O_2	oxygen
$O2^{\bullet-}$	superoxide anion
oAβ	Aβ oligomers
OBX	olfactory bulbectomized
OD	optical density
$OH^{\bullet}$	hydroxyl radical
ONOO-	peroxynitrite
OPC	oligodendrocyte progenitor cells
p38 MAPK	p38 mitogen-activated protein kinase
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PBMCs	peripheral blood mononuclear cells
PCO	protein carbonyl
PD	Parkinson’s disease
PDN	painful diabetic neuropathy
Pg	picogram
PG	prostaglandin
PGE_2	prostaglandin E_2
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2

PI3K	phospho-inositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLA2	phospholipase A2
PCO	protein carbonyl
POCD	postoperative cognitive dysfunction
PSD-95	Post-synaptic density protein 95
psen1	presenilin 1
psen2	presenilin 2
PTSD	post-traumatic stress disorder
PUFA	polyunsaturated fatty acid
PV	parvalbumin
PVDF	polyvinylidene difluoride
QUIN	quinolinic acid
R^2	coefficient of determination
RANTES	regulated upon activation normal T-cell expressed secreted
RARs	retinoic acid receptors
RM ANOVA	Repeated measure ANOVA
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAP	serum amyloid P
sAPPα	soluble alpha-amyloid precursor protein
SCI	spinal cord injury
SD	Sprague Dawley

SD	sleep deprivation
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel
SE	South-East
Sec	second
SEM	standard error mean
Shh	sonic hedgehog
SIRT1	sirtuin 1
SOD	superoxide dismutase
SPE	solid phase extraction
SPSS	statistical package for the social sciences
STAT3	activator of transcription 3
STM	short-term memory
STZ	streptozocin
SW	South-West
Syp	synaptophysin
TBARS	thiobarbituric acid reactive substance
TBI	traumatic brain injury
TBS	tris buffer saline
TBST	tris buffer saline-Tween 20
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TGF-β	transforming growth factor-beta
TG-SDS	tris/glycine/SDS buffer
TH	tyrosine hydroxylase
TLR-4	toll-like receptor 4
TNF-α	tumour necrosis factor-alpha
T-PERTM	tissue protein extraction reagent with HaltTM protease and

	phosphatase inhibitor cocktail
TRAM	TRIF-related adaptor molecule
TrkB	tropomyosin receptor kinase B
TRIF	TIR-domain-containing adapter-inducing interferon-β
USM	Universiti Sains Malaysia
V	voltage
VTA	ventral tegmental area
WB	Western blot
WDR	wide dynamic range

KESAN NEUROPROTEKTIF OLEH *MINOCYCLINE* MELALUI LALUAN ISYARAT TLR-4/NF- κβ PADA TIKUS JANTAN YANG MENGHIDAPI KEMEROSOTAN KOGNITIF YANG DISEBABKAN OLEH LIPOPOLISAKARIDA.

ABSTRAK

Proses neuroinflamasi melibatkan pengaktifan sel glial dan laluan isyarat t*oll-like receptor-4/faktor nuklear kappa B* (TLR-4/NF-κB) telah dikaitkan dengan kemerosotan kognitif. *Minocycline*, telah menunjukkan potensi dalam mengawal pengaktifan sel glia dan mengurangkan neuroinflamasi. Kajian ini bertujuan untuk menyelidiki mekanisma molekular *minocycline* ke atas penurunan kognitif yang disebabkan oleh lipopolisakarida (LPS) dalam hipokampus tikus Sprague-Dawley (SD) jantan dewasa, dibandingkan dengan *memantine*. Lima puluh ekor tikus SD dibahagikan secara rawak kepada lima kumpulan: i) kawalan yang dirawat dengan normal salin, ii) LPS (5 mg/kg) yang dirawat dengan normal salin, iii) LPS (5 mg/kg) yang dirawat dengan *minocycline* 25 mg/kg, iv) LPS (5 mg/kg) yang dirawat dengan *minocycline* 50 mg/kg, dan v) LPS (5 mg/kg) yang dirawat dengan *memantine* 10 mg/kg. Semua rawatan diberikan sekali sehari selama dua minggu secara intraperitoneal. LPS disuntik secara intraperitoneal sekali pada hari ke-5 ekperimen untuk memulakan tindak balas neuroinflamasi yang mendorong kemerosotan pembelajaran dan ingatan. Selepas itu, Ujian labiran air Morris (MWM)) dan ujian pengecaman objek novel (NORT) digunakan untuk menilai pembelajaran dan ingatan (ruang serta pengecaman). Setelah itu, tikus dikorbankan dan hipokampus diekstrak. Imunohistokimia dan Western blot dijalankan untuk mengukur ekspresi penanda mikroglia dan astrosit, protein-protein keradangan, neurotropik dan transkrip. Tahap molekul oksidatif (malondialdehid (MDA) dan protein karbonil (PCO)), enzim

antioksidan (katalase (CAT) dan superoksida dismutase (SOD)) dan protein tau terfosforilasi diukur menggunakan kit ELISA. Pewarnaan merah Congo dan ungu Cresyl digunakan untuk menentukan pengumpulan amiloid dan bilangan neuron. Rawatan *minocycline* selama dua minggu melindungi daripada gliosis, neuroinflamasi, tekanan oksidatif, pengumpulan amiloid, pembentukan protein tau terfosforilasi, kehilangan neuron dan kemerosotan pembelajaran serta ingatan. Menariknya, kesan neuroprotektif *minocycline* adalah setanding dengan kesan *memantine* dan bergantung kepada dos, semakin tinggi dos *minocycline*, semakin banyak kesan neuroprotektif yang ditunjukkan. Kesimpulannya, kajian ini membuktikan bahawa *minocycline* berpotensi untuk digunakan sebagai terapi pencegahan dan terapeutik alternatif untuk melindungi daripada kemerosotan kognitif yang berkait dengan penyakit neurodegeneratif pada manusia. Mekanisma multifaset *minocycline* meliputi sifat anti-glial, anti-inflamasi, antioksidan, anti-amiloidogenik,dan anti-tau protein, yang secara efektif memperbaiki kemerosotan pembelajaran dan ingatan yang disebabkan oleh LPS. Penemuan ini memerlukan penyelidikan lebih lanjut di peringkat ujian klinikal terhadap *minocycline* sebagai terapi alternatif untuk kemerosotan kognitif.

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ABSTRACT

Neuroinflammatory processes involving glial cell activation and the toll-like receptor 4/nuclear factor kappa B (TLR-4/NF-κB) signalling pathway have been implicated in cognitive impairment. Minocycline, has shown potential for regulating glial cell activation and reducing neuroinflammation. This study aimed to investigate the molecular mechanism of minocycline in lipopolysaccharide (LPS)-induced cognitive decline in the hippocampus of adult male Sprague-Dawley (SD) rats, comparing it to memantine. Fifty SD rats were randomly assigned to five groups: i) control treated with normal saline, ii) LPS (5 mg/kg) treated with normal saline, iii) LPS (5 mg/kg) treated with 25 mg/kg minocycline, iv) LPS (5 mg/kg) treated with 50 mg/kg minocycline, and v) LPS (5 mg/kg) treated with 10 mg/kg memantine. All treatments were administered once daily for two weeks via the intraperitoneal route. On day 5 of the experiment, intraperitoneal LPS injection was performed to induce a neuroinflammatory reaction and provoke learning and memory dysfunction. Morris Water Maze (MWM) and novel object recognition task (NORT) were used to assess learning, spatial memory, and recognition memory. Then, the rats were sacrificed, and hippocampal tissue was collected. Immunohistochemistry and Western blot were conducted to evaluate the expression of microglia and astrocyte markers, inflammatory, neurotrophic, and transcriptional proteins. The levels of oxidative molecules (malondialdehyde (MDA) and protein carbonyl (PCO)), antioxidant enzymes (catalase (CAT) and superoxide dismutase (SOD)), and phosphorylated tau proteins were measured using ELISA kits. Congo red and Cresyl violet staining were

employed to assess amyloid accumulation and neuronal count. Minocycline treatment for two weeks conferred protection against LPS-induced gliosis, neuroinflammation, oxidative stress, amyloid accumulation, phosphorylated tau protein formation, neuronal loss, and learning and memory dysfunction. Notably, the neuroprotective effects of minocycline were comparable to memantine and exhibited dose-dependent with higher minocycline doses yielding greater neuroprotection. In conclusion, this study demonstrates that minocycline possesses potential preventive and therapeutic benefits against cognitive decline associated with neuroinflammatory diseases in humans. Its multifaceted mechanisms encompass anti-glial, anti-inflammatory, antioxidant, anti-amyloidogenic, and anti-tau protein properties, effectively ameliorating LPS-induced learning and memory impairments. These effects of minocycline were dose-dependent-the higher the dose, the better effects and comparable to memantine effects. These findings warrant further investigation of minocycline as an alternative therapy for cognitive decline in clinical settings.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Neurodegenerative diseases (NDs) are chronic gradual progressive diseases commonly observed in the elderly population. Additionally, they are marked by high rates of recurrence, non-recovery and chronicity. NDs are characterised by deterioration of learning and memory function. The most common NDs are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Perry, 2004; Cunningham et al., 2009; Hou et al., 2017).

Neuroinflammation, oxidative stress, neurotrophic factor alteration, and neuronal damage are the underlying molecular mechanisms of NDs. Furthermore, amyloid accumulation and the formation of phosphorylated tau protein have been identified as pathological features of Alzheimer's disease. Before clinical symptoms appear, neurologic damage and dysfunction develop gradually and progressively (Perry, 2004; Cunningham et al., 2009; Hou et al., 2017; Teleanu et al., 2022).

Several studies have shown that sustained activation of microglia and astrocytes markers such as ionised calcium-binding adaptor molecule-1 and glial fibrillary acidic protein (Iba-1 & GFAP), stimulation of the toll-like receptor/nuclear factor kappa B (TLR-4/NF-kB) pathway, elevation of the proinflammatory mediators such as tumour necrosis factor-alpha (TNF-α) and cyclooxygenase (COX)-2, amyloid accumulation, and phosphorylated tau protein formation is closely correlated with downregulation of brain-derived neurotrophic factor (BDNF)/ cAMP response

element-binding protein (CREB) pathway, neuronal damage and cognitive impairment (Perry, 2004; Cunningham et al., 2009; Hou et al., 2017; Teleanu et al., 2022). Furthermore, neuroinflammation induces the production of free radicals, such as thiobarbiturates, protein carbonyl (PCO), and malondialdehyde (MDA) that cause DNA and lipid peroxidation as well as a decrease in antioxidant enzyme levels and activity, such as catalase (CAT) and superoxide dismutase (SOD). This phenomenon is known as oxidative stress and it has been observed in the brains of ND patients and reported as early pathologic changes in AD-associated dementia (Dugger and Dickson, 2017; Hansson, 2021; Yaseen et al., 2021; Rauf et al., 2022).

Lipopolysaccharides (LPS) administration in animals is one of the widely used non-transgenic animal models of neuroinflammatory disease. LPS is derived from the cell wall of the Gram-negative bacteria named *E.coli*. It causes gliosis (glial cell overactivation), neuroinflammation, oxidative stress, amyloid accumulation and the formation of phosphorylated tau protein, which disrupts synaptic transmission, causes neurologic damage and impairs learning and memory function. LPS-induced neuroinflammation is initially mediated by glial cells activation, which then stimulates the release of additional proinflammatory cytokines and mediators (Espinosa-Oliva et al., 2011; Badshah et al., 2016; Hossain et al., 2018; Cásedas et al., 2019).

TLR-4 and cytokines receptors are abundant in hippocampal neurons and microglial cells, respectively. The hippocampus is the most vulnerable organ to oxidative stress and immune-related changes. The interaction between neuronal elements and proinflammatory cytokines and mediators makes the hippocampus extremely vulnerable to LPS-induced neuronal damage, which results in hippocampal-

dependent learning and memory impairment. In several animal models, LPS has been shown to cause hippocampal neuronal damage and impair learning and memory function by activation of neuroinflammatory and oxidative stress pathways (Sheng et al., 2003; Batista et al., 2019; Domínguez-Rivas et al., 2021).

Since neuroinflammation has been implicated in the pathogenesis of NDs, several clinical trials are being conducted to introduce anti-neuroinflammatory agents capable of alleviating/preventing neuroinflammation. One of these trials used non-steroidal anti-inflammatory drugs (NSAIDs) to treat neuroinflammation but did not recommend them as neuroinflammation therapy because they accelerated disease progression in some cases (Ibrahim et al., 2012).

Minocycline (microglial inhibitor) is a second-generation semi-synthetic tetracycline antibiotic that has been used for decades as an antibacterial medication. It acts as an anti-inflammatory, antioxidant, anti-amyloid and anti-tau protein activities. Minocycline also has neuroprotective effects such as anti-apoptotic, anti-depressant and memory-enhancing properties. Recently, it has been established that minocycline enhances neurogenesis and cognitive function in several studies (Tomás-Camardiel et al., 2004; Zhu et al., 2014; El-Shimy et al., 2015; Zhang et al., 2019; Sun et al., 2020).

Several clinical and experimental studies have shown that minocycline has neuroprotective properties. As a result, the use of minocycline as an anti-inflammatory and antioxidant drug has sparked considerable interest in the treatment of neuroinflammatory disease-induced cognitive impairment (Lee et al., 2004; Filipovic and Zecevic, 2008; Zhang et al., 2019).

Despite several reports demonstrating its beneficial neuroprotective effects, its neuroprotective effects against LPS-induced neuroinflammation and learning and memory impairment have yet to be thoroughly investigated. Therefore, this study aims to elucidate the potential neuroprotective effects of minocycline on LPS male rats' model of neuroinflammation in comparison to memantine, an approved symptomatic drug for AD by food and drug administration (FDA).

1.2 Rationale of the study

NDs are characterised by chronic progressive impairment of cognitive function and are most common in the elderly population. They are devastating health problems for patients and their families and they put a strain on families, the health- care system and the federal budget. Unfortunately, there is no cure for these diseases and recent experimental and clinical trials have yielded inconclusive results. Because neuroinflammation, oxidative stress and amyloid accumulation all play important roles in the pathogenesis of NDs, introducing therapy that is anti-inflammatory, antioxidant and anti-amyloid and tau protein appears to be a promising approach to protecting/preventing the progression of NDs.

Minocycline has been extensively studied as a neuroprotective treatment. It has anti-inflammatory, antioxidant, anti-amyloid and tau protein, anti-apoptotic, neurogenesis enhancer, anti-depressant and cognitive enhancer properties. Many clinical and experimental experiments were conducted to demonstrate its neuroprotective properties against brain injury and dysfunction. The current study hypothesised that minocycline when compared to memantine, can alleviate the

detrimental effects of LPS and these therapeutic benefits are attributed to its anti-inflammatory, antioxidant and anti-amyloid capabilities.

1.3 Objectives of the study

1.3.1 General objectives

The general objective of this study was to elucidate the molecular mechanisms underlying minocycline's neuroprotective effects on the TLR-4/NF-kB signalling pathway in LPS-injected rats. Additionally, the neuroprotective effects of minocycline and memantine were compared.

1.3.2 Specific objectives

1. To determine the effects of minocycline on food intake and body weight in LPS-injected rats in comparison to memantine.

2. To evaluate the effects of minocycline on learning and spatial memory as well as recognition memory in LPS-injected rats in comparison to memantine.

3. To determine the effects of minocycline on localisation and expression of inflammatory proteins (TLR-4 & NF-kB), microglia and astrocyte markers (Iba-1 & GFAP) as well as and proinflammatory proteins (TNF-α & COX-2) in the hippocampus of LPS-injected rats in comparison to memantine.

4. To determine the effects of minocycline on localisation and expression of neurotrophins (BDNF) and transcriptional factor (CREB) in the hippocampus of LPS-injected rats in comparison to memantine.

5. To determine the effects of minocycline on oxidative molecular damage involving, protein carbonyl and malodialdehyde (PCO & MDA) and antioxidant enzymes including, catalase and superoxide dismutase (CAT & SOD) levels in the hippocampus of LPS-injected rats in comparison to memantine.

6. To evaluate the effects of minocycline on amyloid accumulation and phosphorylated tau protein level in the hippocampus of LPS-injected rats in comparison to memantine.

7. To determine the effects of minocycline on neuronal count in the hippocampus of LPS-injected rats in comparison to memantine.

1.4 Hypothesis of this study:

This study hypothesized that:

1. Minocycline administration reduces food intake and body weight in LPS-injected rats.

2. Minocycline administration improves learning, spatial memory, and recognition memory in LPS-injected rats.

3. Minocycline administration alters the localization and expression of inflammatory proteins (TLR-4 & NF-kB), microglia and astrocyte markers (Iba-1 & GFAP) as well as and proinflammatory proteins (TNF-α & COX-2) in the hippocampus of LPS-injected rats

4. Minocycline administration influences the localization and expression of neurotrophins (BDNF) and transcriptional factor (CREB) in the hippocampus of LPS-injected rats.

5. Minocycline administration reduces oxidative molecular damage (PCO & MDA) and enhances antioxidant enzyme (CAT & SOD) levels in the hippocampus of LPS-injected rats.

6. Minocycline administration decreases amyloid accumulation and phosphorylated tau protein level in the hippocampus of LPS-injected rats.

7. Minocycline administration preserves neuronal count in the hippocampus of LPS-injected rats. The conceptual framework of this hypothesis has been illustrated in Fig. 1.1.

The null Hypothesis of this study:

1. Minocycline administration has no effect on food intake and body weight in LPS-injected rats.

2. There is no relationship between minocycline administration and improvements in learning, spatial memory, and recognition memory in LPS-injected rats.

3. Minocycline administration has no effect on the localization and expression of inflammatory proteins (TLR-4 & NF-kB), microglia and astrocyte markers (Iba-1 & GFAP) as well as and proinflammatory proteins (TNF-α & COX-2) in the hippocampus of LPS-injected rats

4. Minocycline administration has no effect on the localization and expression of neurotrophins (BDNF) and transcriptional factor (CREB) in the hippocampus of LPS-injected rats.

5. There is no relationship between minocycline administration and the reduction of oxidative molecular damage (PCO & MDA) and enhancement of antioxidant enzyme (CAT & SOD) levels in the hippocampus of LPS-injected rats.

6. Minocycline administration has no effect on amyloid accumulation and phosphorylated tau protein level in the hippocampus of LPS-injected rats.

7. There is no relationship between minocycline administration and the preservation of neuronal count in the hippocampus of LPS-injected rats.

1.5 Conceptual framework

It has been demonstrated that the administration of LPS causes negative outcomes in rats, including increased food intake and body weight. Cognitive deficiencies such as poor learning, recognition memory, and spatial memory are also brought on by it. LPS stimulates glial cells and initiates neuroinflammatory processes, which leads to the activation of inflammatory proteins like TLR-4 and nuclear factor kappa B (NF-kB). Moreover, it stimulates the synthesis of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2) and tumor necrosis factor-alpha (TNF-α). Additionally, oxidative stress—which is defined by elevated levels of oxidative molecular damage and decreased antioxidant enzyme activity—is linked to LPS-induced neuroinflammation. Moreover, the administration of lipopolysaccharides (LPS) results in the build-up of amyloid plaques and phosphorylated tau proteins, which are pathological indicators of neurodegenerative illnesses. Additionally, it causes downregulation of CREB/BDNF signalling pathway and neuronal loss in in the hippocampus.

On the other hand, taking minocycline has advantages. In rats given LPS injections, it lowers body weight and food consumption. Treatment with minocycline enhances cognitive function by addressing deficiencies in learning, recognition memory, and spatial memory. In the hippocampal regions, it affects the production and distribution of glial cell markers such glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) as well as inflammatory proteins like TLR-4 and NF-kB. Moreover, minocycline affects transcriptional factors such as cAMP response element-binding protein (CREB) and neurotrophins, specifically brain-derived neurotrophic factor (BDNF), in terms of both expression and location.

Furthermore, by lowering oxidative molecular damage and raising levels of antioxidant enzymes, minocycline treatment lowers oxidative stress. It maintains the number of neurons and upregulates CREB/BDNF signalling pathway in the hippocampal region of rats given lipopolysaccharide injections while reducing the buildup of amyloid and phosphorylated tau protein.

This theoretical paradigm clarifies the harmful consequences of administering LPS to rats and emphasizes the possible therapeutic benefits of minocycline in reducing LPS-induced neuroinflammation, oxidative stress, cognitive decline, and neurodegenerative processes. The conceptual framework of this hypothesis has been illustrated in Fig. 1.1.

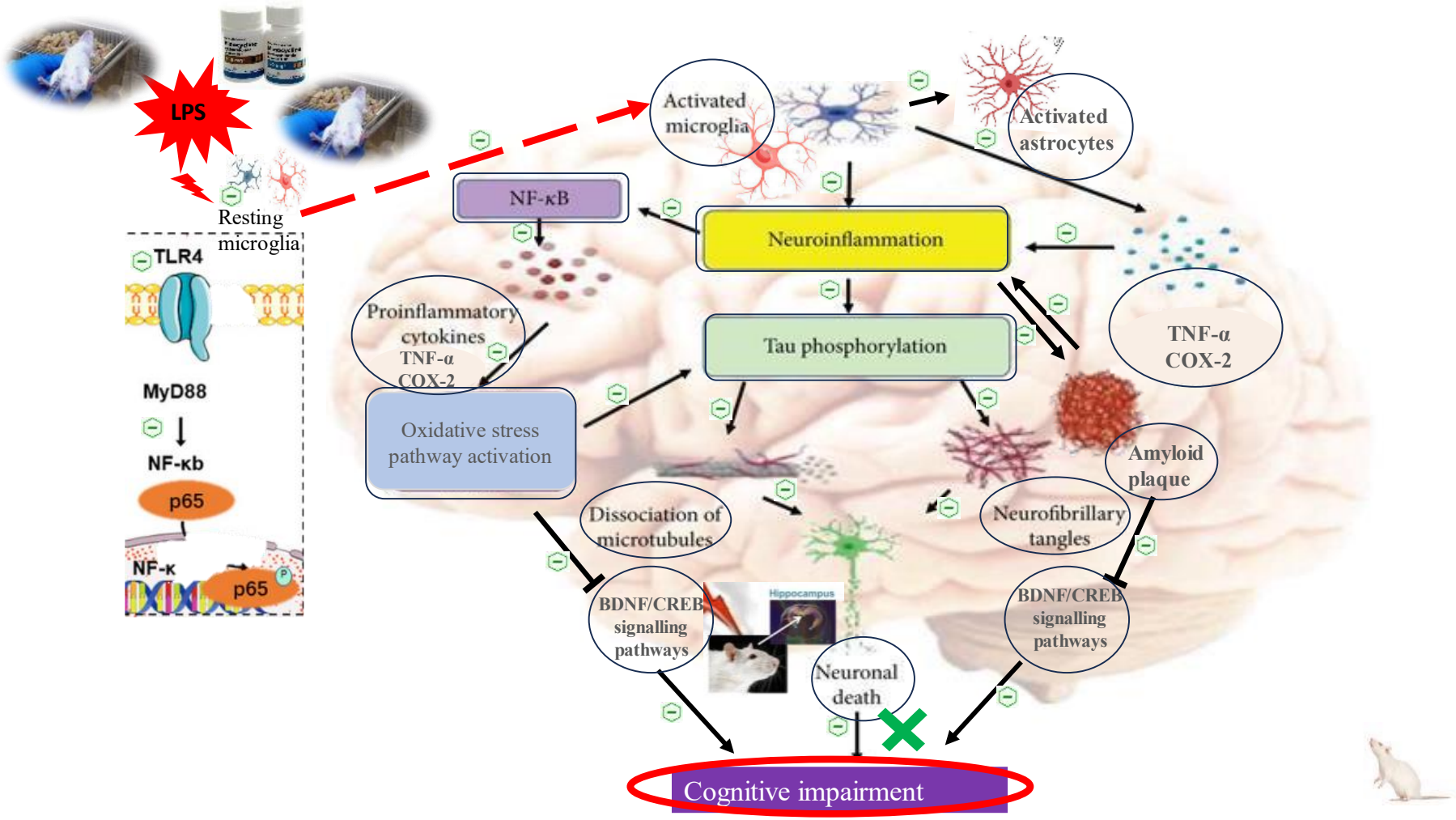

Figure 1.1: The conceptional framework for the proposed mechanism of actions of minocycline against LPS-induced cognitive impairment.

=inhibit
= stimulate
= inhibit

CHAPTER 2

LITERATURE REVIEW

2.1 Nervous tissue

Nervous tissue is a specialized type of tissue found in the nervous system, which is responsible for coordinating and transmitting signals throughout the body. It is composed of two main types of cells: neurons and glial cells. Neurons are the fundamental units of the nervous system. They are highly specialized cells that generate, transmit, and receive electrical and chemical signals, allowing for communication within the nervous system. Neurons consist of a cell body, dendrites, and an axon. The cell body contains the nucleus and other organelles, while dendrites receive signals from other neurons. The axon carries the signals away from the cell body to other neurons, muscles, or glands (Prinz et al., 2021).

Glia, or glial cells, are non-neuronal cells that provide support and protection to neurons. They play crucial roles in maintaining the structural integrity of the nervous system and ensuring its proper functioning. Glial cells include astrocytes, oligodendrocytes, and microglia. Microglia are a type of glial cell that act as the immune cells of the central nervous system (CNS). They play a vital role in monitoring and protecting the CNS by recognizing and responding to pathogens, injury, and inflammation. Microglia are responsible for immune surveillance, removing cellular debris, and modulating inflammatory responses. They have a unique ability to change their morphology and function in response to various stimuli, allowing them to maintain a healthy environment within the CNS (Borst et al., 2021).

2.2 Neurodegeneration

The central nervous system (CNS) is a complicated organ in the human body that is poorly understood. The brain is the primary important organ in animals, controlling all other organ functions through neural connection and signalling transduction. Many neuroinflammatory diseases disrupt this neural circuit and connection. Chronic progressive cognitive decline, which is a common pathological feature of various NDs, has been linked to neuroinflammation, oxidative stress, amyloid deposition, phosphorylated tau protein formation, synaptic loss and neuronal death (Teleanu et al., 2022).

Neurodegeneration is characterised by changes in brain structure and function as well as a decrease in neuronal survival and an increase in neuronal death. There is still no explanation to explain why neuronal cells cannot regenerate. Additionally, there is no medicinal agent that can enhance neural tissue regeneration or treat NDs (Teleanu et al., 2022).

On the other hand, novel multi-target development therapies for NDs that inhibit Ca^{2+} overload, excitotoxicity, endoplasmic reticulum stress and apoptosis are being studied to treat ND-related comorbidity. The pathogeneses of NDs are supported by a wealth of experimental, epidemiological and clinical evidence. Glial cells and neurons degenerate in NDs leading to their clinical manifestations. In normal physiologic conditions, microglial cells facilitate communication and interaction between immune cells and the brain. However, this connection is impaired in chronic neuroinflammation, which leads to neurodegeneration (Kempuraj et al., 2016).

As mentioned earlier, the NDs include MS, AD, PD, HD and ALS. MS is autoimmune, inflammatory, demyelinating and NDs characterised by demyelination, axonal degeneration, infiltration of neuronal cells and neuronal death. Clinical symptoms of MS include depression, cognitive impairment, spasticity, fatigue, bladder and sexual dysfunction and pain, whereas pathological features include degeneration of demyelinated neurons and death of oligodendrocytes (Crayton and Rossman, 2006). The pathologic hallmark of this disease is the migration of T-cells via blood brain barrier (BBB) followed by activation of mast cells and T-cells, gliosis, demyelination, neuronal degeneration and death. Microglial activation triggers the production and release of inflammatory mediators such as cytokines, phagocytosis and free radicals such as reactive oxygen species and reactive nitrogen species (ROS & RNS) (Crayton and Rossman, 2006).

The second most common ND is PD. The hallmark of this disease is the death of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies in neuronal tissue. Activation of glial cells and inflammatory mediators have been linked to the aetiology and pathology of PD. Neuronal death stimulates neuroinflammation in a vicious cycle. Glial cells, which present abundantly in substantia nigra and dopaminergic neurons are vulnerable to its neurotoxicity. The Lewy bodies stimulate immunological factors, inflammatory mediators and glial cells, which in turn initiate and exacerbate neurodegeneration in the dopaminergic neuron in substantia nigra. Motor and non-motor symptoms are clinical indications of PD. Muscle stiffness, bradykinesia and resting tremor are examples of motor symptoms, whereas cognitive decline, mental problems and olfactory and autonomic dysfunction are examples of

non-motor symptoms. Degeneration of dopaminergic neurons in the substantia nigra is a patho-histologic characteristic of PD (Cankaya et al., 2019).

The clinical signs of AD include behavioural, cognitive and psychiatric disorders. The patho-histologic features of AD include neuronal loss in the temporal and parietal lobe and amyloid accumulation together with neurofibrillary tangle formation. There is no curative treatment for neurodegeneration-related AD. The available treatments only alleviate and slow the course of symptoms by ameliorating neuroinflammation and amyloid formation and increasing acetylcholine (ACh) levels (Bermejo et al., 2008).

Indeed, neuroinflammation induced neurodegeneration, which causes further inflammation in brain tissue. The early pathogenic event in NDs has been postulated to be brain mitochondrial dysfunction and free radicals production. Another postulated mechanism is disruption of the blood-brain barrier (BBB), synaptic neurotransmission and hypometabolism. On the other hand, amyloid accumulation and phosphorylated tau protein formation have been identified as one of the underlying mechanisms (Kempuraj et al., 2016).

Moreover, it is well-recognised that neuroinflammation and oxidative stress have negative consequences on neural tissues. Neuroinflammation and oxidative stress have been shown to play an essential role in the onset and progression of NDs s (Andersen, 2004; Dugger and Dickson, 2017; Guo et al., 2020; Motaghinejad et al., 2020; Hansson, 2021; Lin et al., 2022).

2.3 Neuroinflammation

Neuroinflammation is an inflammatory response in the CNS that has become a major concern in the last few decades. It is mediated via various inflammatory and immune cells such as microglia, neutrophils, T-cells, macrophages and mast cells. In general, acute mild neuroinflammatory reactions have neuroprotective effects since they promote tissue repair and diminish injury (Rogers, 2008). Acute inflammation ceases after the elimination of the threat and this is followed by restoration of homeostasis (Glass et al., 2010).

On the other hand, the chronic neuroinflammatory response is self-perpetuating, persists for a long time after initial noxious stimulus exposure, and promotes detrimental effects on neurologic functions (Shabab et al., 2017). Chronic neuroinflammation induces glial cell hyperactivity, which in turn mediates inflammatory pathways and the release of inflammatory and neurotoxic factors. Many of these factors contribute to the initiation and progression of pathologic neurologic dysfunction and damage found in a variety of NDs (Glass et al., 2010; Shabab et al., 2017).

In general, accumulating evidence suggests that neuroinflammation plays a role in the pathophysiology of NDs (AD, PD, HD & MS). Glial cells are activated in these diseases and the factors and components activating these cells differ in each of these diseases (Kempuraj et al., 2016). The most prevalent ND is AD. It is chronic progressive NDs characterised by amyloid plaque formation and neurofibrillary tangle (NFT) formation. Amyloid plaque is made up of amyloid precursor protein (APP) and is found extracellularly, while NFT is composed of tau protein and is found

intracellularly. The inflammatory mediators implicated in AD pathophysiology involve microglia, astrocytes, classic and alternate pathways of the complement system, neurons, cytokines, chemokines and other neurotoxic factors (Bagyinszky et al., 2017).

The initial triggering factors are amyloid beta (Aβ) deposition and hyperphosphorylated tau protein formation that activates glial cells (microglia and astrocytes), which in turn generate more Aβ and hyperphosphorylated tau protein, triggering neuroinflammatory pathway in AD. In AD brain tissue, Aβ activates and then attracts glial cells, which stimulate the TLR-4/NF-kB signalling pathway and the release of several cytokines and chemokines. Neurons also participate in neuroinflammation by releasing various complement proteins, cytokines, chemokines, c-reactive proteins (CRP) and Aβ (Bagyinszky et al., 2017).

Chronic persistent neuroinflammation is essential in the pathogenesis of neurodegeneration. In fact, most animal models in various experimental studies do not express extensive neuroinflammation and neurodegeneration compared to human diseases. Several experimental studies elucidated the pathogenesis of NDs using animal models of AD, PD, HD and MS such as transgenic, genetic and toxic substances. These experimental studies have their limitation. Interestingly, experimental studies that mimic neuroinflammation in humans have used neuroinflammation-based models for AD and PD, such as the LPS model of AD and PD and the glial fibrillary acidic protein (GFAP)-IL-6 model of AD (Andersen, 2004; Dugger and Dickson, 2017; Guo et al., 2020; Motaghinejad et al., 2020; Hansson, 2021; Lin et al., 2022).

The role of neuroinflammation in NDs has been extensively studied over the last few decades. Emerging evidence implicated the role of neuroinflammation in the onset and progression of NDs as well as neuronal death. Mounting evidence demonstrated that in the physio-pathologic state, there is bidirectional influence and communication between peripheral immune response and neuronal tissue. A previous study reported that systemic inflammation, as measured by an increase in the peripheral level of TNF-α, is closely associated with glial-mediated neuroinflammation and cognitive impairment (Andersen, 2004; Dugger and Dickson, 2017; Guo et al., 2020; Motaghinejad et al., 2020; Hansson, 2021; Lin et al., 2022).

Furthermore, peripheral inflammation promotes neuroinflammation by increasing BBB permeability and activation of microglial cells. Furthermore, peripheral inflammatory and immune cells pass through defective BBB and migrate into the brain. Then, they proliferate in neuroinflammatory sites of neuronal tissue, causing neuroinflammation either directly or indirectly through glial and neuronal cells that release inflammatory mediators. Thus, suppression of neuroinflammation decreases the extent of neurodegeneration and ameliorates ND's symptoms (Kempuraj et al., 2016).

2.4 Lipopolysaccharide

Lipopolysaccharides (LPS)-induced inflammation is widely used worldwide in *in-vitro* and *in-vivo* models of neuroinflammation such as AD, PD, HD and ALS (Miklossy, 2008). Surprisingly, the dose used in animal models is 10^3 to 10^4 times higher than the dose used in humans to induce severe disease. Coinciding with neuroinflammatory disease-induced cognitive impairment, LPS-induced neuroinflammation causes a variety of cognitive and neuro-behavioural impairments. LPS is a potent endotoxin found in the outer cell membrane of Gram-negative bacteria, such as *Escherichia coli* (*E.coli*). It is a potent long-lasting inflammatory stimulus that is highly resistant to degradation by mammalian enzymes (Zakaria et al., 2017).

It stimulates various neuroinflammatory pathways, including the TLR-4/NF-kB signalling pathway as well as the release of proinflammatory cytokines and chemokines such as TNF-α, COX-2 and interleukins (ILs). It also triggers the oxidative stress pathway, Aβ accumulation and phosphorylated tau protein formation. LPS-induced neuroinflammation stimulates the neuroendocrine and neuroimmune systems, resulting in a deterioration of cognitive function (Miklossy, 2008). LPS-induced sickness behaviour, which is characterised by non-specific behavioural deficits. The behavioural deficit is manifested by a reduction in exploration, activity, social interaction and food intake as well as fever, hypersomnia and stimulation of the hypothalamic-pituitary-adrenal (HPA) axis that activates the sympathetic nervous system (Bassi et al., 2012). A previous study has revealed its direct mechanism of action in the CNS. LPS can enter neural tissue through peripheral nerve transduction, the postrema area, the circumventricular organ or the hypothalamus (Zakaria et al., 2017).

LPS binds to TLR-4 receptors that are mainly presented on microglial cells forming a complex. This complex then activates the NF-kB signalling pathway and signal transduction cascades, resulting in the transcription and release of various cytokines and chemokines such as TNF-α, IL-β, IL-1, IL-6, IL-10, IL-12, 1L-18, inducible nitric oxide synthase (iNOS) and COX-2. It has been reported that the level of inflammatory mediators was elevated in the hippocampus after LPS exposure (Sheppard et al., 2019). The activation of proinflammatory mediators stimulates the immune system. The inability of neuroinflammation to be reserved results in increased ROS and RNS generation, which further prolongs chronic inflammation and stimulates amyloid accumulation (Noworyta-Sokolowska et al., 2013).

In fact, LPS-induced chronic persistent neuroinflammation evokes mitochondrial dysfunction as well as oxidative stress and cellular damage. The damaging effects involve systemic vascular endothelium and are associated with mitochondrial respiratory dysfunction and redox-glutathione depletion, resulting in decreased O_2 consumption and adenosine triphosphate (ATP) levels. Neuroinflammation has been shown to promote amyloid precursor protein (APP) expression and Aβ formation. Similarly, accumulating evidence showed that cytokines upregulate the β-secretase enzyme, which is the key enzyme that initiates Aβ formation. Moreover, LPS alters Aβ transport across the BBB and decreases its clearance. This alteration results in increased influx and decreased efflux of Aβ across the BBB (Erickson et al., 2012). Thus, neuroinflammation, oxidative stress and amyloid accumulation are interwind and implicated in the pathogenesis of NDs (Kovacs, 2019).

LPS exposure interrupts the memory consolidation processes. A previous study reported that acute LPS exposure impaired contextual-cue fear conditioning in young and adult hooded Long Evans and albino SD rats, which is a hippocampus-dependent learning paradigm (Pugh et al., 1998). Impairment of spatial memory has been observed after a single LPS injection for 5 days as shown by increased escape latency in the acquisition and retention phases of the MWM test (Shaw et al., 2001).

Chronic LPS infusion-induced learning and memory impairment is similar to that seen in cognitive decline observed in AD (Hauss-Wegrzyniak et al., 1998). Single-dose exposure of Wistar rats to the LPS induced spatial learning and memory impairment (Amraie et al., 2020). LPS exposure caused sickness and anxiety-like behaviour in CD-1 mice as well as memory dysfunction and stress reactivity (Murray et al., 2020). Wistar rats exposed to LPS showed blocked long-term potentiation (LTP), reduced hippocampal synaptic activity and impaired recognition memory as shown by a significant reduction in the exploration time of novel objects using the Novel Object Recognition Task (NORT) (Hennigan et al., 2007).

2.5 Glial cells activation and cytokines production

The first sign of neuroinflammation is the activation of microglial cells. Microglia is a type of heterogeneous cell presented in the brain. It has different functional phenotypes. Resting microglia cells convert into active M1 or M2 phenotypes. Inflammatory stimuli such as bacterial debris and cytokines stimulate the conversion of resting microglia to the M1 phenotype, whereas parasitic debris, IL-4 and IL-13 promote the conversion of resting microglia to the M2 phenotype. The M1 phenotype has a proinflammatory function, while the M2 phenotype has anti-inflammatory, immunosuppressive and neuroprotective functions. Thus, at the site of neuronal injury, the M1 phenotype predominates over the M2 phenotype (Kempuraj et al., 2016).

Microglia activation is generally classified into classical and alternative activation based on the nature of activation. Classical microglial activation (M1 phenotype) results in the release of protease, ROS and proinflammatory factors such as NO, TNF-α, IL-1β, and IL-6. Alternative microglia activation (M2) leads to the release of anti-inflammatory cytokines such as IL-4, IL-13 and IL-10, which antagonise M1-meditated neuroinflammation to re-establish M1/M2 balance (Kempuraj et al., 2016). Microglia is resident brain macrophages that play a pivotal role in defensive mechanism and tissue repair. Exposure to pathogenic stimuli activates microglia. Chronic persistent microglial overactivation induces the release of neuroinflammatory and neurotoxic factors and detrimental neurologic damage. Microgliosis (overactivated microglia) damages neurons directly through phagocytosis or apoptosis (Graeber and Streit, 2010; Bernhardi et al., 2015).

Therefore, microglial cells are the principal cells that mediate the neuroinflammation seen in NDs. In general, microglial overaction stimulates the TLR-4/NF-kB signalling pathway and promotes several oxidants as well as genes and proteins such as TNF-α, COX-2, IL-1β, IL-6, iNOS, complements and ROS, which all together induces neurodegeneration and cognitive decline. The microglial overactivation persists for a long period during which they release inflammatory and neurotoxic factors that aggravate long-term neurodegeneration (Rojo et al., 2014; Bernhardi et al., 2015; Rauf et al., 2022).

Increased levels of cytokines and chemokines such as TNF-α, COX-2, IL-1β, IL-6, IL-8, macrophage inflammatory proteins-1α and transforming growth factor-beta (TGF-β) in the brain of AD patients have been reported (Brosseron et al., 2018; Shen et al., 2019). In addition, increased plasma levels of inflammatory markers such as TGF-β, IL-12, IL-16 and IL-18 have been reported in AD patients and have been used as predictors of mild cognitive impairment (MCI) (Bermejo et al., 2008). A previous report showed increased levels of TNF-α and interferon-gamma (IFN-γ) in Swedish mutant APP transgenic mice that stimulates Aβ deposition via beta-site APP-cleaving enzyme (BACE1) upregulation, Aβ clearance reduction and glial cells overactivation (Yamamoto et al., 2007).

Similarly, the production of ROS and the release of proinflammatory cytokines may alter kinase/phosphatase activity and substrate selectivity, resulting in tau hyperphosphorylation and insoluble tau accumulation with subsequent memory impairment in the 3xTg-AD mouse model (Sy et al., 2011). A study on microglia cell culture obtained from rodent and human brains revealed that Aβ deposit promotes

neuroinflammation as indicated by elevated levels of proinflammatory factors, ROS and RNS and neurotoxic molecules (Sondag et al., 2009; Goshi et al., 2020).

These findings highlight the correlation between neuroinflammation, oxidative stress and amyloid accumulation with hyperphosphorylated tau protein formation, all of which contribute to activated microglia migration. Activated microglia stimulates the production of proinflammatory cytokines and ROS and RNS, which aggravates the neurodegenerative changes.

2.6 Toll-like receptors (TLR-4) and nuclear factor kappa B (NF-kB) in neuroinflammation

Toll-like receptors (TLRs) are one of the PRR's largest families. There are almost 13 TLRs discovered in mammals. Among them, only TLRs 1 untill 10 are reported to function in the human body. TLR family members can recognise a variety of ligands. TLRs play an essential role in the immune system and their remarkable potential warrants several studies to focus on them as a pharmacological target. TLRs are activated by activation occurs via specific pathogen-associated molecular patterns (PAMPs) and other proteins such as lipopolysaccharide-binding protein (LBP), cluster differentiation antigen 14 (CD14) and myeloid differentiation protein 2 (MD2). Activation of TLR-4 is a key factor triggering neuroinflammation and requires the formation of the TLR4–MD2–LPS complex. Its signalling transduction pathway in microglia is well established (Li et al., 2016).

TLR-4 is the first mammalian toll-like receptor to be activated, resulting in the infiltration of resident glia and blood-derived immune cells, leading to