ELUCIDATING THE TOLL-LIKE RECEPTOR 4 INVOLVEMENT IN STRIATUM AND CEREBELLUM OF SWISS ALBINO ADULT MICE ON MOTOR AND SICKNESS BEHAVIOURS

DAYANG YASMIN
BINTI ABANG ABDUL WAHAB

UNIVERSITI SAINS MALAYSIA

2018

ELUCIDATING THE TOLL-LIKE RECEPTOR 4 INVOLVEMENT IN STRIATUM AND CEREBELLUM OF SWISS ALBINO ADULT MICE ON MOTOR AND SICKNESS BEHAVIOURS

By

DAYANG YASMIN BINTI ABANG ABDUL WAHAB

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Neuroscience

April 2018

MEMPERJELASKAN PENGLIBATAN *TOLL-LIKE RECEPTOR 4* DI DALAM *STRIATUM* DAN *CEREBELLUM* TIKUS DEWASA *SWISS ALBINO* MENGENAI PERGERAKAN DAN *SICKNESS BEHAVIOURS*

Oleh

DAYANG YASMIN BINTI ABANG ABDUL WAHAB

Tesis diserahkan untuk memenuhi sebahagian keperluan
bagi Ijazah Sarjana Neurosains

April 2018

ACKNOWLEDGEMENTS

First and foremost, praises and thanks to Allah, the Almighty for His blessings, for giving me the opportunity as well as the strength, knowledge, ability and patience to persevere throughout my research and successfully completing my dissertation.

My deepest and sincere gratitude to my supervisor, Dr. Sangu Muthuraju for his prudent guidance, words of encouragement and advice as well as his endless patience and support throughout the duration of the research. Without his able guidance and knowledge, this thesis would not have come to be and I am thankful for his continuous assistance. I would also like to extend my appreciation to my co supervisor, Associate Prof Dr. Muzaimi Mustapha and Dr. Mohd. Zulkifli Mustafa, their valuable insights and constructive comments have been a great contribution in the completion of this dissertation.

I am forever thankful to my parents and my brothers who have provided me with infinite love and prayers, for their unrelenting support and motivation, pushing me to strive harder and do better. Not to be forgotten, my friends who have been my source of laughter and fun, who have made all the weekends spent in the lab bearable and who have always been around to provide me with constant motivation, reminders and sound advices. I am grateful and thankful to them for all the good times that flowed. Also, I dedicate my sincere appreciation to fellow research colleagues, Nur Naznee Hirni Bt Abd Aziz, Chong Huei Gau and others for this research would have not been complete without their help, guidance and valuable insights.

Last but not least, a special acknowledgement to the Neuroscience department staffs and lecturers, staffs of the Central Research Laboratory (CRL) and personnel from the Animal Research and Service Centre (ARASC). Thank you to everyone who have contributed to the completion of this thesis directly or indirectly.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ARASC	Animal Research and Service Centre
ATP	Adenine triphosphate
bp	Base pairs
BBB	Blood brain barrier
JNK	c-Jun N-terminal kinase
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DALYs	Disability-adjusted life-years
DAMPs	Damage associated molecules patterns
DNA	Deoxyribonucleic acid
DRs	Dopamine receptors
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
EAAT1	Excitatory Amino Acid Transporter 1
EAAT4	Excitatory Amino Acid Transporter 4
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
g	Gram
GABA	Gamma amino-butyric acid

GLAST	Glutamate Aspartate Transporter
h	Hours
HTR1A	5-Hydroxytryptamine Receptor 1A
HTR2A	5-Hydroxytryptamine Receptor 2A
HD	Huntington's disease
kg	Kilogram
OFT	Open field test
IKKε	IκB kinase ε
iNOS	Inducible nitric oxide synthase
IKK	Inhibitor of nuclear factor-κB kinase
IRF3	Interferon regulatory factor 3
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IRAKs	Interleukin-1 receptor-associated kinases
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRAK4	Interleukin-1 receptor-associated kinase 4
GPi	Internal globus pallidus
GPe	External globus pallidus
LPS	Lipopolysaccharide
MSNs	Medium spiny neurons
mg/kg	Milligram/Kilogram
µg	Microgram
µl	Microliter

µm	Micrometer
MAPKs	Mitogen-activated protein kinases
M	Molar
MRI	Magnetic Resonance Imaging
MS	Multiple sclerosis
MSA	Multiple systemic atrophy
MYD88	Myeloid differentiation primary response 88
ng	Nano gram
NMDARs	N-methyl-D-aspartate glutamate receptors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
P38	P38 mitogen-activated protein kinases
PAMPs	Pattern-associated molecular patterns
PNS	Peripheral nervous system
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PFA	Paraformaldehyde
PRRs	Pattern recognition receptors
PCs	Purkinje cells
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RT-PCR	Reverse transcription Polymerase chain reaction
rpm	Revolutions per minute
5-HT	Serotonin
5-HT2A	Serotonin 2A

5-HT2C	Serotonin 2C
SEM	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
STN	Subthalamic nucleus
TAK-242	Resatorvid
TBK1	TANK Binding Kinase 1
TRIF	TIR-domain-containing adapter-inducing interferon-β
TIR	Toll-IL-1 receptor
TLRs	Toll-like receptors
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TRAF3	Tumor necrosis factor receptor-associated factor 3
TRAF6	Tumor necrosis factor receptor associated factor 6
TRAM	TRIF-related adaptor molecule
TAK1	Transforming growth factor beta-activated kinase 1
IFNs	Type I interferons
VTA	Ventral tegmental area

ABSTRAK

Ketagihan alkohol merupakan salah satu faktor yang boleh merangsang pengaktifan microglia otak dan membawa kepada inflamasi otak melalui *toll-like receptors* (TLR) yang terdapat di dalam microglia. Ketagihan alkohol menyebabkan defisit motor melalui inflamasi otak. Walau bagaimanapun, dasar mekanisme inflamasi otak dalam mendorong tingkah laku motor melalui pengaktifan TLR4 masih belum dijelaskan. TLR sentiasa didapati berkaitan atau terlibat dalam induksi inflamasi otak dalam penyakit degenerasi otak. TLR4 dirangsang oleh TLR4 agonis, *Lipopolysaccharide* (LPS), dan interaksi TLR4-LPS telah didapati menyebabkan perubahan fisiologi dan tingkah laku termasuk kecacatan aktiviti motor dalam model tikus. Oleh itu, kajian ini bertujuan untuk menyiasat tingkah laku lokomotor, gen reseptor serotonin (HTR1A dan HTR2A), reseptor dopamin (reseptor Dopamin D1 dan reseptor Dopamin D2) dan pengangkut glutamat (EAAT1 dan EAAT4) dalam striatum dan serebelum berikutan rawatan dengan TLR4 agonis. Haiwan telah dibahagikan kepada tiga kumpulan; (1) Kawalan (n = 12), (2) rawatan LPS (0.83mg / kg) (n = 12) 6 jam dan (3) rawatan LPS (0.83mg / kg) (n = 12) 24 jam. Selepas rawatan, tingkah laku lokomotor dianalisis dalam ujian *Open field*, *wooden beam* dan *hanging wire* pada 6 dan 24 jam selepas suntikan LPS. Mengikuti ujian tingkah laku, otak haiwan dikeluarkan dan bahagian striatum dan serebelum diasingkan untuk kajian gen. Hasil kajian menunjukkan bahawa terdapat defisit lokomotor pada 6 jam tetapi tidak dalam 24 jam. Kajian gen mencadangkan bahawa terdapat perubahan ketara dalam reseptor serotonin (HTR1A dan HTR2A), reseptor dopamin (reseptor Dopamin D1 dan reseptor Dopamin D2) dan pengangkut glutamat (EAAT1 dan EAAT4) dalam striatum dan serebelum bersama-sama dengan defisit motor. Kesimpulannya, TLR4 mungkin menyebabkan defisit

motor melalui pengawalaturan pengangkut glutamat EAAT1 dalam striatum dan serebelum.

Kata kunci: Inflamasi otak, *Toll-like receptor 4*, deficit motor, striatum dan serebelum.

ABSTRACT

Alcohol addiction is one of the possible factors in stimulating brain microglia activation and leading to neuroinflammation through toll-like receptors (TLR) which are present in microglia. In fact, alcohol addiction ultimately causes motor deficits through neuroinflammation. However, the underlying mechanisms of neuroinflammation inducing motor behaviour through activation of TLR4 receptors have not yet been elucidated. TLR are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. TLR4 is stimulated by TLR4 Agonist, Lipopolysaccharide (LPS), and the TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity in the mouse model. Therefore, the present study aimed to investigate the locomotor behaviour, gene expression of serotonin receptors (HTR1A and HTR2A), dopamine receptors (Dopamine D1 receptor and Dopamine D2 receptor) and glutamate transporters (EAAT1 and EAAT4) in the striatum and cerebellum following treatment with TLR4 agonist. The animals were divided into three groups; (1) Control (n=12), (2) LPS treatment (0.83mg/kg) (n=12) 6 h and (3) LPS treatment (0.83mg/kg) (n=12) 24 h. After treatment, locomotor behaviour was analysed in open field test, wooden beam test and hanging test at 6 and 24 h post-LPS administration. Following behaviour test, animal's brains were harvested and striatum and cerebellum isolated for gene expression studies. Results showed that there were locomotor deficits at 6 h but not in 24 h. The gene expression studies suggested that there were significant changes in serotonin receptors (HTR1A and HTR2A), dopamine receptors (Dopamine D1 receptor and Dopamine D2 receptor) and glutamate (EAAT1 and EAAT4) transporters in the striatum and cerebellum along with motor deficits. In conclusion,

TLR4 possibly causes motor deficits through regulation of glutamate transporter EAAT1 in striatum and cerebellum.

Key words: Neuroinflammation, Toll-like receptor 4, motor deficits, striatum and cerebellum.

CHAPTER 1

INTRODUCTION

Neurological diseases particularly Alzheimer disease (AD), Parkinson disease (PD), strokes and epilepsy are on the rise all around the world. As the second leading cause of morbidity and mortality globally, it has become one of the greatest threats to public health (Feigin et al., 2017; World Health Organization, 2006). All the aforementioned diseases share a common symptom of gradual loss or impairment of motor behaviour. Based on the Global Burden of Disease Study 2015, neurological diseases were listed and emerged as the top disease to cause 250.692 million disability-adjusted life-years (DALYs), comprising 10.2% of global DALYs, and 9.399 million deaths, comprising 16.8% of global deaths, the second highest in terms of global deaths. Therefore, from the statistics, it is evident how critical it is to research on ways to alleviate the distress, the physical constraint that is affecting the people (Feigin et al., 2017).

It has been noted that the motor loss associated with neurological diseases are possibly resulted from neuroinflammation induced by the neuroimmune system (Glass et al., 2010). Many studies had been performed involving animal models, proving the causal relationship between neuroinflammation and motor deficits. Neuroinflammation itself is considered a critical hallmark in the pathogenesis of neurological diseases, making it one of the major focus of this study. The immune system is one of the major functional component of the body that is responsible for the

occurrence of neuroinflammation. One of the major, active components of the neuroimmune systems is the toll-like receptors (TLRs).

TLRs are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. TLRs are a family of transmembrane pattern recognition receptors (PRR) that sense, recognize and identify pronounced structures in microbes, known as pathogen associated molecular patterns or PAMPs as part of the signal transduction of the innate immune system. TLRs are present in the central nervous system (CNS), specifically and primarily expressed in microglia and is also found on neurons and astrocytes, functioning mainly in the regulation of pro-inflammatory cytokines production that subsequently contribute to neuronal damage (Shmuel-Galia et al., 2017). For example, deficiency of toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) in mice exhibit reduced levels of pro-inflammatory cytokines resulting in milder clinical disease following traumatic brain injury. Subsequently, increased expression of TLR4 are also found in PD, AD and amyotrophic lateral sclerosis (ALS) patients as well as in animal models (Molteni et al., 2016).

TLR4 particularly has been demonstrated in various studies to have a significant causal relationship with motor dysfunction in neurodegenerative conditions. Activation of TLR4 through TLR4 agonist, lipopolysaccharide (LPS) stimulation initiate a signalling cascade whereby the TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity in the mouse model (Bison et al., 2009; Cunningham et al., 2009; Tar et al., 2012). LPS is a component of gram-negative bacteria known to trigger

inflammation, specifically known to activate TLR4. A number of studies reported the suppression of neuroinflammation through TLR4 inhibition which consequently minimized motor deficit in animal models involving neurodegenerative diseases as well as traumatic brain injury (Feng et al., 2017; Feng et al., 2016; Lee et al., 2015; Hines et al., 2013; Ahmad et al., 2013). Numerous studies had demonstrated a significant relationship between TLR4 and motor impairments in neurodegenerative disorders. A recent study demonstrated an increase in motor impairments in a mice model as a result of TLR4 activation using monophosphoryl lipid A (Venezia et al., 2017). The recent study done by Fellner and colleagues (2017) using ALS mouse model and TLR4 inhibitor TAK-242 (resatorvid), the inhibitory effect on TLR4 was reflected in the reduction of the spinal cord pathology along with the motor impairments. The role of TLR4 in the initiation of neuroinflammation is very much well-grounded and it is similar in the case of TLR4 causal effect on motor function.

Although many studies had demonstrated the correlation between TLR4 and motor deficits, the functional molecular mechanisms of TLR4 in contributing to motor impairments in neurodegenerative disorders however, still clearly has not been defined. Therefore, this study attempts to investigate the functional mechanism of TLR4 in contributing to motor impairments by activating the TLR4 using TLR4 agonist, LPS using a mouse model. This study proposed the use of LPS to stimulate the activation of TLR4 mainly due to the physiological and behavioural changes following the TLR4-LPS interactions. This is further supported by a study done by Sharma and colleagues (2016) which demonstrated the suppression of LPS induced motor deficits using Apocyanin in rat model. Previous studies also demonstrated that LPS induced neuroinflammation which later contributed to physiological and

neurobehavioral deficits (Tien et al., 2017). However, the underlying functional molecular mechanisms of TLR4 involved in causing motor deficit were not clearly mentioned.

Striatum which is a component of the basal ganglia, is involved in facilitating voluntary movement while the cerebellum is involved in the maintenance of balance and coordination of voluntary movements (Leisman & Melilo, 2013). Both these structures work together with the cerebral cortex in mediating movements and various neurotransmitters are involved in the circuitries involved in the process. Dopamine, serotonin, gamma-aminobutyric acid (GABA) and glutamate, to name a few, interact in regulating the excitation and inhibition of motor neurons. Studies had long demonstrated the involvement of such neurotransmitters in the proper functioning of motor neurons in striatum and cerebellum (Wankhar et al., 2017; Hernandez-Rabaza et al., 2016; Karamanolis et al., 2016). In the motor system, serotonin (5-hydroxytryptamine, 5-HT) is found to either enhance or depress glutamate-mediated transmission as well as GABA mediated transmission in structures controlling movement (Ciranna, 2006). Additionally, TLR4 activation by LPS is noted to release pro-inflammatory cytokines, interleukin 1 beta (IL-1β) from microglia that subsequently suppresses GABA receptor activities at the postsynaptic site and reduces GABA synthesis at the presynaptic site. Glial glutamate transporter activities are also found to be suppressed, showing the association between TLR4 activation and the related neurotransmitters and corresponding receptors and transporters in the event of neuroinflammation (Yan, Jiang & Weng, 2015). The lack of research on the underlying transmission of TLR4 induced motor deficit involving changes in neurotransmitters

and corresponding receptors and transporters create gaps that needs to be filled. This contributes to our initiative in pursuing this research project.

Loss of motor function disrupts and affects the quality of life, burdening people suffering from the associated diseases. Although relationship between neuroinflammation and TLR4 had been widely studied and its functional role in the pathogenesis of motor deficits among neurological disorders was demonstrated, the functional mechanism and pathway involved that led to the deficit however, has yet to be clearly defined. Findings constituting the underlying implication and pathway of TLR4 on motor functions can be used to provide alternatives in treating the effects of neurodegenerative diseases. Therefore, in this project, we investigate the relevant neuroinflammation mechanism of LPS-induced TLR4 on motor functions by assessing motor behaviours of LPS induced adult male mice and examining associated neuronal damage and changes in genes of neurotransmitter's receptors and transporters located in the striatum and cerebellum region that are involved in motor behaviour.

CHAPTER 2

LITERATURE REVIEW

2.1 Microglia

Microglia are the innate immune cells in the CNS whereby it monitors and regulates the brain homeostasis, maintaining it under normal physiological conditions by purging pathogens, as well as clearing dead cells through phagocytosis. Most notably, the microglia are critically involved in the neuroinflammatory response, serving as the initial indication of neuroinflammation when activated. The presence of pathogens, tissue damage, abnormal stimulation, neurotoxins, infection, injury or any threats to the microenvironment activates microglia and thereafter, the complex neuroinflammatory pathway (Shabab, 2016).

Macrophages can be activated into several distinct activation states and the microglia functions differently according to the different activation states. The classical M1 type activation is the response to micro-organism threats and is associated with cytotoxicity and inflammatory responses including the upregulation of pro-inflammatory cytokines expression. On the other hand, the M2 type activation is associated with immunoregulatory functions, tissue repair as well as wound healing and regeneration (Subramaniam & Federoff, 2017; Kaminska, 2016; Boche et al., 2013). In response to an extensive and diverse array of microbial stimuli, the differential activation of microglia regulates neuroinflammation by inducing the release of pro-inflammatory mediators that favour the permeabilization of the blood

brain barrier (BBB), which results in either neurotoxicity or neuroprotection (Chen et al., 2016). Such stimuli are recognized by an array of receptors on microglia. Then, the event follows the release of signals termed as PAMPs as well as damage associated molecules patterns (DAMPs) which are transmitted by the damaged cells. PAMPs and DAMPs in turn are recognized and bound by PRRs (Molteni, 2016). One of the highly conserved PRRs involved in the local immune response is the TLRs family.

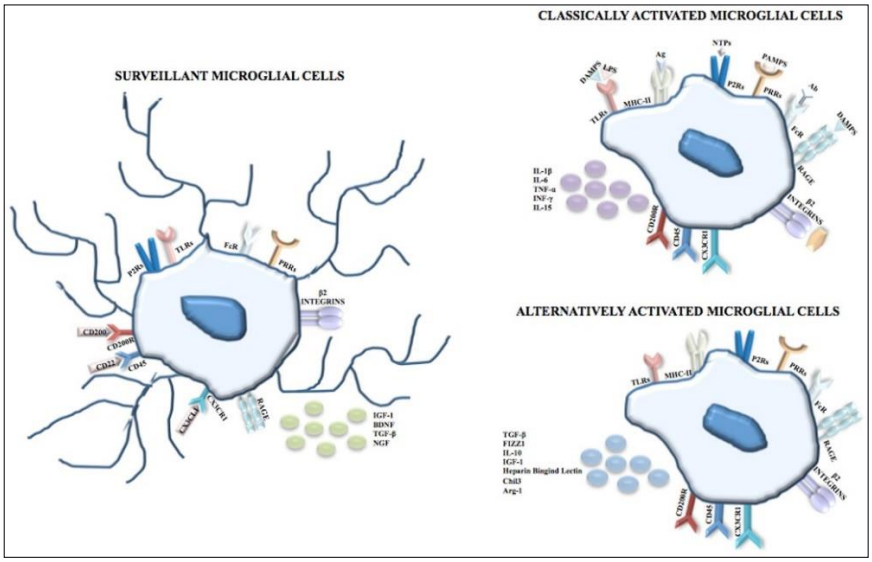

Fig. 2.1 Activation states of microglia (Arcuri et al., 2017)

2.2 Neuroinflammation

Motor deficit emerged as a prominent feature or symptom in neurodegenerative diseases such as PD, AD, stroke and etc. Motor deficits linked to said diseases usually shows in the form of motor slowing (bradykinesia), gait and posture disturbances, rigidity and resting tremor (Buchman & Bennett, 2011; Wirths & Bayer, 2008). It has been noted that the motor loss associated with neurodegenerative diseases are possibly resulted from neuroinflammation induced by the neuroimmune system (Glass et al., 2010). In researches involving PD and AD,

neuroinflammation has been reported to play the central role in the pathogenesis of these diseases. Neuroinflammation is regarded as an important feature of many neurodegenerative diseases such as multiple sclerosis (MS), narcolepsy and autism (Shabab, 2016).

Neuroinflammation stems from the immune system of the CNS and comprises of a complex series of local immune processes constituting CNS cells such as neuron and glia, cytokines, PRRs, and peripheral immune cells in response to threats such as pathogens, tissue damage, abnormal stimulation, neurotoxins, infection or injury. The initiation, progression and termination of neuroinflammation are mediated through the activation of glial cells such as microglia, macroglia and astrocytes and is subsequently followed by the release of pro-inflammatory cytokines and chemokines as well as reactive oxygen and nitrogen species (Saavedra-López et al., 2017). Neuroinflammation can assume a neuro-protective role or it can be counterproductive, causing damage to the nervous tissues. A persistent acute neuroinflammation can turn to a chronic neuroinflammation as it accumulates damage, bringing about neuronal degeneration. The effects or outcome of neuroinflammation has been indicated to be dependent on the time span of the inflammatory response and the activation state of microglia (Stone at al., 2016; Milatovic et al., 2014). Subsequently, one of the outcomes of neuroinflammation is the alteration of neurotransmission which consequently results in the modification of neurotransmitter receptors, dopamine, serotonin, glutamate and GABA, thus, causing a hindrance towards spatial learning, cognitive and motor functions (Leite et al., 2017; Ihara et al., 2016).

2.3 Toll-like receptor on motor and sickness behaviour

PRRs are employed as sensors in the signal transduction of the innate immune system for the initial detection of microbial threats. Activated PRRs effectuate downstream signaling pathways which induces the innate immune responses by producing pro-inflammatory mediators, resulting in inflammation. One out of the several distinct classes of PRRs include the TLRs family. TLRs are always found to be associated or involved in the induction of neuroinflammation in neurodegenerative diseases. TLRs are known to regulate the production of pro-inflammatory cytokines, which may contribute to further neuronal damage (Shmuel-Galia et al., 2017). There are a total of 10 members of the TLRs family in human; TLR1–TLR10 and 12 members in mice; TLR1–TLR9, TLR11–TLR13. TLRs are expressed either on the exterior of microglia cells or to intracellular compartments such as the ER, endosome, lysosome, or endolysosome. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 (Kawasaki & Kawai, 2014).

TLR4 particularly has been demonstrated in various studies to have a significant causal relationship with motor dysfunction in neurodegenerative conditions. TLR4 and other cell surface TLRs mainly detect and identify microbial membrane components, for example lipids, lipoproteins, and proteins (Kawasaki & Kawai, 2014). TLR4 activates upon stimulation of the gram-negative LPS which are known to trigger inflammation. The TLR4-LPS interaction has been found to result in physiological and behavioural changes including retardation of motor activity, loss of interest or pleasure, impaired cognitive function, social withdrawal, as well as reduced food and water intake (Lim et al., 2012; Bison et al., 2009). Additionally, pro-

inflammatory cytokines produced by TLR4-activated microglia has shown to induce sickness behaviour characterized by reduced food intake and locomotor activity (Felger & Miller, 2012). A study by Venezia and colleagues (2017), activation of TLR4 using monophosphoryl lipid A resulted in increased motor impairments. To further confirm TLR4 role in motor behaviour, the effect of completely blocking LPS-induced activation of TLR4 was investigated. TLR4 blockage with Tat-TLR4 interfering peptides injection was reported to suppress the event of sickness behaviour and exhibited absence of motoric and motivational effects of LPS-induced sickness (Hines et al., 2013). Additionally, morphological changes in microglia and cytokine production that are typically induced by LPS were also blocked. Inhibition of TLR4 signaling prevents changes in behaviour and motivation caused by inflammatory stimulation, further suggesting the role and contribution of TLR4 in motor deficit.

Another study by Fellner and colleagues (2017) demonstrated reduced motor impairments as well as spinal cord pathology in ALS mouse model by using the inhibitor TAK-242 to impede TLR4 activation. This was also supported by Lee et al. (2015) which demonstrated increased motor function in relation to a decrease in microglial activation in the absence of TLR4 in ALS mouse model. Furthermore, suppression of TLR4 was also observed to reduce motor deficits conditions in neurodegenerative disorders and traumatic brain injury animal model (Akbar et al., 2013). Feng et al., in 2016 and 2017, administered resatorvid, the TLR4 inhibitor TAK-242, in a rat subjected to controlled cortical impact injury. The result showed a neuroprotective effect through the inhibition of the TLR4-mediated pathway whereby the expression of TLR4 and its downstream signaling molecules, including MyD88, TRIF, NF-κB, TNF-α, and IL-1β, was found to be significantly downregulated.

However, a study by Zhu and colleagues (2016) revealed a morphological-based analysis that linked TLR4 deficiency with thinning of the molecular layer of the cerebellum. The loss of TLR4 reduced the number of Purkinje cells (PCs) which are the sole output neurons of the cerebellar cortex, thus impairing motor function as PCs are responsible in regulating the function of cerebellum which plays an essential role in balance and motor coordination (Zhu et al., 2016).

2.4 Toll-like Receptor 4 (TLR4) Neuroinflammatory Signaling Pathway

Activation of TLRs initiate two signal transduction pathways namely the MyD88-dependent pathway and the MyD88-independent pathway. TLRs except TLR3 initiate intracellular signaling through ligand-induced dimerization of intracellular Toll-IL-1 receptor (TIR) domain (Kaminska, 2016) (Fig. 2.2). TIR domains of TLR4 recruit TIR domain-containing adaptor proteins MyD88 and MAL of the MyD88-dependent pathway or TRIF and TRAM of the MyD88-independent pathway. The MyD88-dependent pathway activates IRAKs (IRAK1, IRAK2, and IRAK4) and TRAF6 that in turn activates TAK1. Subsequently, this leads to the activation of MAPKs (p38, JNK, and ERK1/2) and IKK pathways, resulting in NF-κB activation which then induce the production of pro-inflammatory cytokines. The MyD88-independent pathway on the other hand activates TRIF and TRAM adaptor proteins which then recruits TBK1/IKKε through the activation of TRAF3. This then follows the activation the transcription factor IRF3 in the nucleus leading to the production of type I interferons (IFNs) (Molteni, 2016; Kawasaki, 2014) (Fig. 2.3). Once LPS binds to TLR4 on the microglia surface, the signal transduction pathway is activated which in the end leads to NF-κB activation. Activated NF-κB functions to

control DNA transcription, mediating the production of pro-inflammatory cytokines, chemokines and inducible enzymes, namely, inducible nitric oxide synthase (iNOS) and COX-2 which are released from the microglia whereby all result in neuroinflammation (Rietdijk et al., 2016; Shabab et al., 2016).

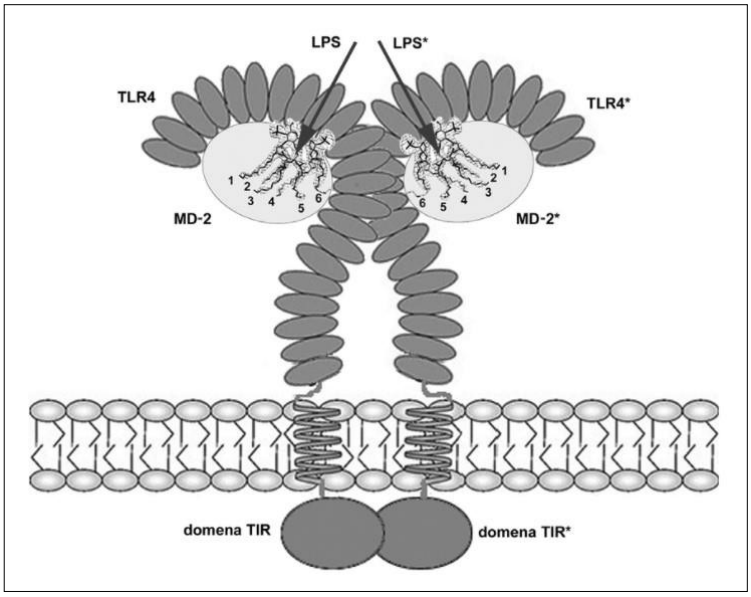

Fig. 2.2 Structure of Toll-like receptor 4 (Czerkies & Kwiatkowska, 2014)

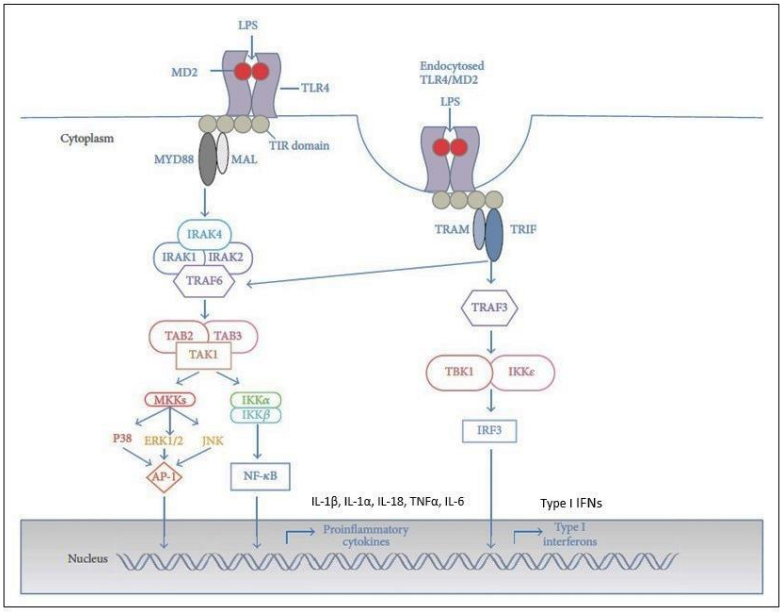

Fig. 2.3 TLR4 signaling pathway (Molteni et a., 2016)

Previous studies had demonstrated that microglia in the brain region comprise an expression of TLR4 (Crews & Vetreno, 2016) and that the TLR4 activation activates microglia which in turn produces more pro-inflammatory factors such as TNFα, IL-1β and IL6 (Sulakhiya et al., 2016; Fan et al., 2011; Bison et al., 2009), resulting in a self-propelling and vicious cycle of neuroinflammation and neurodegeneration of dopamine neurons (Qin et al., 2007). The production of pro-inflammatory cytokines is shown to be associated with reduced muscle mass and strength as well as affecting brain areas involved in motor coordination and fatigue (Krzyszton et al., 2008). To counter such reaction, IL-10, an anti-inflammatory cytokine, is produced by macrophages to suppress excess production of inflammatory cytokines and excessive inflammation (Iyer et al., 2012). Both NF-κB and IL-10 plays a functional role in the production and regulation of such pro-inflammatory cytokines respectively. Pro-inflammatory cytokines produced as a result of TLR4 activation and NF-κB triggering could affect the expression and regulation of neurotransmitters and receptors in the striatum and cerebellum in a way that possibly results in impaired motor functions.

2.5 Striatum and Cerebellum

The striatum is one of the main component of the basal ganglia which is involved in processes related to voluntary motor control. The striatum can be further divided into the dorsal striatum which consists of the caudate nucleus and putamen, and the ventral striatum which comprise of the nucleus accumbens and the olfactory tubercle. The striatum acts as the central glutamatergic and dopaminergic input receiving station and subsequently transmits these inputs to the rest of the basal

ganglia. Within the striatum, the received inputs are projected onto two distinct classes of medium spiny neurons (MSNs) specified as the direct (striatonigral) and indirect pathway (striatopallidal) MSNs (Hutton et al., 2017; Kravitz & Kreitzer, 2012).

These two pathways differ whereby the direct pathway MSNs directly transmits inputs from the cortex and thalamus to the internal globus pallidus (GPi) and substantia nigra pars reticulata (SNr) while the indirect pathway MSNs receives input from the cortex and thalamus and indirectly transmits the outputs to SNr through the external globus pallidus (GPe) and subthalamic nucleus (STN) (Fig. 2.4). Moreover, the direct pathway MSNs express high levels of D1 dopamine while the indirect pathway MSNs has a high expression of D2 dopamine. Additionally, projections from the direct pathway MSNs is reported to mediate motor output, whereas projections from the indirect pathway MSNs impede motor output. The opposing activity of the two pathways is what regulates motor control (Do et al., 2013).

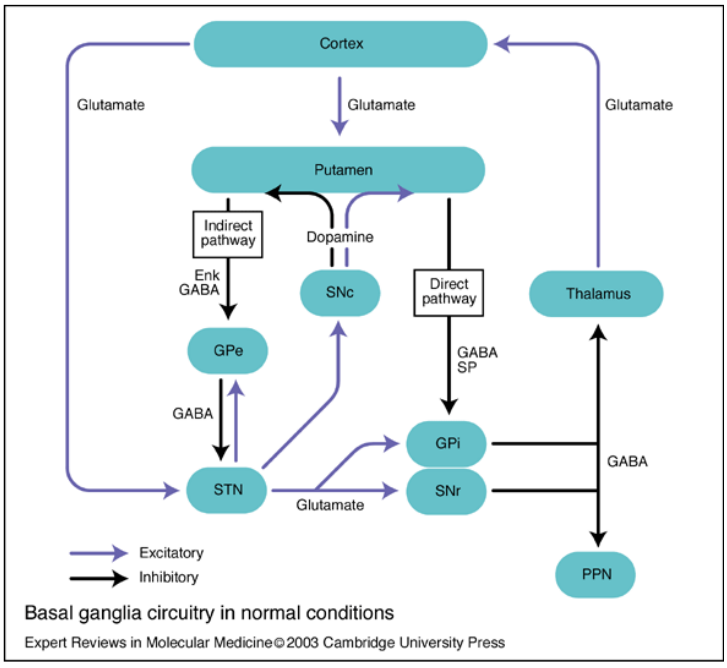

Fig. 2.4 Direct and indirect pathways of motor circuit in the striatum (Lewis et al., 2003)

Dysfunction of the connectivity or projections of the striatum is recognized as a notable cellular pathology in a number of motor and neurodegenerative diseases such as PD and Huntington's disease (HD). PD is associated with a progressive decline in motor control. The causal circumstance of such decline is due to a dysfunction of the motor circuits within the striatum which is resulted from dopamine denervation in the dorsal striatum ascribable to the death of dopaminergic neurons in the SNr (Simioni et al., 2016; Kravitz, 2012). The role of striatum in the pathogenesis of PD has been reiterated and supported by various past studies and articles (Singh et al., 2016; Hanganu et al., 2015; Maurice at al., 2015).

As like PD, HD is a neurodegenerative disease clinically characterized motor impairment. Equally, degeneration of striatal neurons is attributed as one of the underlying contributing cause of this disease. Guo and colleagues (2012) in their study reported a significant causal relationship between size and volume reduction as well as neuronal loss in both STN and putamen of post-mortem brains of HD patients with increase clinical motor impairment. Such losses and atrophy in both structure disrupts motor circuits within the striatum, thus leading to motor impairments in HD patients. This is supported by a recent study by Chan and colleagues (2015) on transgenic HD monkey model using magnetic resonance imaging (MRI) and reverse transcription and quantitative PCR (qPCR). Similarly, results showed degeneration distinguished by significant neuronal loss in both caudate nucleus and putamen suggests a progressive regional degeneration and significant volume reduction in the striatum. Consequently, these losses affect the fronto-striatal pathways and causes erratic striatal outputs, impeding the motor functions in HD monkeys.

The cerebellum, also known as the “little brain”, is the major folded structure of the hindbrain. It consists of two cerebellar hemispheres whereby the cerebellar cortex comprises of three layers, which are the internal granular layer with granule cells, the middle PC layer consisting of single row of PCs, and the external molecular layer which is made up of primary glial cells. Axons of granule cells and the dendrites of PCs stretch out all the way into the molecular layer. Inputs from the cerebral cortex is transmitted to the cerebellum by mossy fibres which then excites the granule cells of the granular layer. The granule cells then specialized into parallel fibres which synapse onto PCs dendrites, transmitting excitatory signals. At the same time, PCs also receive regulatory input through its axons from climbing fibres that stems from the inferior olive. PCs then sends an inhibitory signal to the deep cerebellar nucleus neurons that proceed toward the motor cortex. Concurrently, both mossy fibres and climbing fibres excites the deep cerebellar nucleus neurons. The output from deep cerebellar nucleus neurons thus depends on the overall inhibitory and excitatory stimulation (Salman & Tsai, 2016; Kandel et al., 2013) (Fig. 2.5).

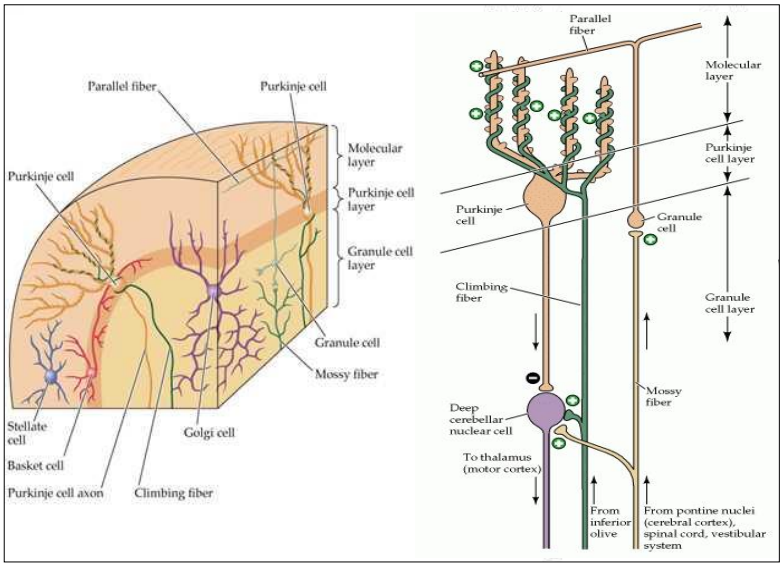

Fig. 2.5 Layers of the cerebellum and the transmission pathway

The cerebellum is critically involved in modulating various networks including voluntary motor control and cognition. Studies have showed a causal role of cerebellum dysfunction in motor impairment in a number of diseases such as PD, and neurological movement disorders such as dystonia and multiple systemic atrophy (MSA). Mormina and colleagues (2017) discussed changes in cerebellum in neurodegenerative diseases through the use of MRI. All the aforementioned diseases are characterized with distinguished motor impairments and cerebellum dysfunction as their pathological hallmark. Loss in cerebellar volume was reported in PD patients with tremor due to cerebellar atrophy. Additionally, cerebellar hyperactivity was shown to be higher in PD patients. Similarly, atrophy of the middle cerebellar peduncles and volume loss of the middle and inferior cerebellar peduncles were also observed in MSA patients. Cerebellar atrophy and increased cerebellum activation together with the presence of cerebellar lesions and morphological cerebellar anomaly were observed in dystonia patients with hand stiffness. Dystonia is associated with continuous, unusual muscle contractions.

Another MRI study on the involvement of cerebellum in the pathogenesis of ALS were conducted by Tan et al. (2014). ALS is a neurodegenerative disorder involving the motor neuron system in which it affects muscle contractions and progressively impact normal movement abilities. Motor impairments in ALS patients were linked with atrophy in the inferior cerebellum specifically the inferior lobules and vermis. Both the basal ganglia and the cerebellum interact with the cerebral cortex whereby the neuronal activity between the three structures are involved with parameters of movement (Leisman & Melilo, 2013). In addition, past literatures reported that the primary brain regions most affected by inflammatory response

include the basal ganglia, particularly the ventral striatum (Miller et al., 2013). Both striatum and cerebellum are selected as the areas of interest due to their involvement in motor control.

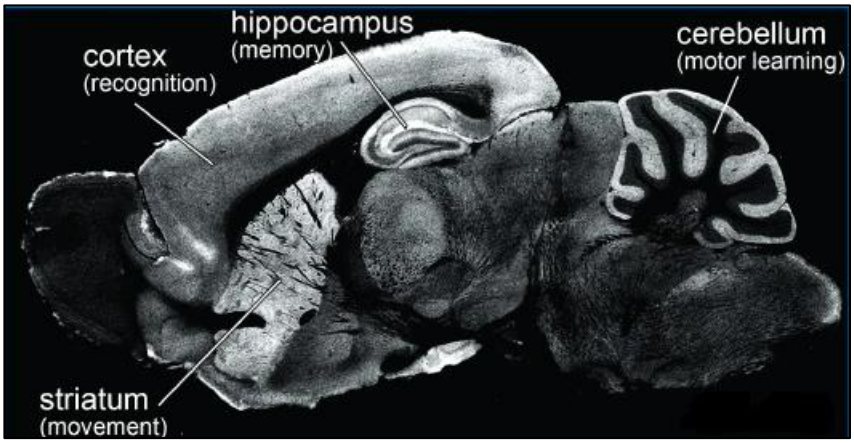

Fig. 2.6 Location of the striatum and cerebellum in an adult mouse brain (Adapted from Konno et al., 2014)

2.6 Neuroinflammation on Neurotransmitter's receptors and transporters

Neurotransmitters are a diverse group of chemical compounds that are involved in the transmission of information in chemical synapses from the presynaptic site of one neuron to postsynaptic site of the adjacent neuron. Neurotransmitters from the presynaptic neuron diffuses into the synaptic cleft where they bind accordingly to their specific receptors to activate the respective signalling cascades. The neurotransmitters then either undergoes the reuptake process by presynaptic transporter proteins and astrocytes or be degraded by specific enzymes that are present in the synaptic cleft. The resulting signalling cascade can elicit either an excitatory or inhibitory signal. Thus, neurotransmitters can be of either excitatory or inhibitory in

nature and are grouped accordingly based on structure and function (Leite et al., 2017). Some of the neurotransmitters group are as follows; acetylcholine, amino acids (glycine, glutamate and GABA (gamma amino-butyric acid)), amino acid derived amines (epinephrine, norepinephrine, dopamine and serotonin), peptides (substance P and endorphins), purines (ATP) and gases (nitric oxide). Excitatory neurotransmitters include serotonin, acetylcholine, epinephrine and norepinephrine whereas inhibitory neurotransmitters include glycine and GABA. Studies had long demonstrated the involvement of various neurotransmitters in the proper functioning of motor neurons in the striatum and cerebellum (Wankhar et al., 2017; Hernandez-Rabaza et al., 2016; Karamanolis et al., 2016). These studies involved the investigation of the functional relationship between neurotransmitters such as serotonin, GABA, dopamine and glutamate with motor functioning.

Dopamine, unlike other neurotransmitters, can act as both inhibitory and excitatory neurotransmitter depending upon its location in the brain and which receptor it binds to. Dopamine receptors (DRs), Dopamine Receptor D1 (DRD1) mediates excitatory signal while Dopamine Receptor D2 (DRD2) mediates inhibitory signals. DRD1 is highly distributed in the striatum, nucleus accumbens, olfactory tubercle, cerebral cortex and amygdala. Additionally, DRD2 is also highly communicated in the striatum, olfactory tubercle, nucleus accumbens as well as in the SNc and ventral tegmental area (VTA). The striatum acts as one of the main target region for dopamine involving the regulation of motor functions. Dopamine is critically involved in numerous brain circuits in the nervous systems associated with mediating motor control, feeding behavior, cognitive functions, emotion, motivation and reward (Nakamura et al., 2014).

Dopamine, is generally known to be involved in the modulation of motor functions and this has been stated and reiterated in numerous studies and articles. Neurotransmission and projections of dopamine from the substantia nigra (SN) to the striatum, and to the cerebellum from the VTA has been noted to influence the fine tuning of movements (Triarhou, 2013; Wu & Hallet, 2013). Nuclei in both SNc and the VTA is reported to make up the major dopaminergic tracts (Nakamura et al., 2014). The corticostriatal circuit expresses high levels of both DRD1 and DRD2, demonstrating the involvement of such receptors in controlling movement, thus justifying the selection of DRD1 and DRD2 in this study. Additionally, varied connection strength between striatum–cortical, striatum–cerebellar and cortico-cerebellar motor influenced by imbalanced neurotransmission of dopamine were observed in Parkinson's patients with akinesia (Wu & Hallet, 2013). Subsequently, the production of cytokine during neuroinflammation is found to be involved in the alterations in dopamine neurotransmission whereby cytokines ultimately lead to decreased dopamine synthesis, thus decreasing dopamine function which could lead to neurodegeneration.

Serotonin acts upon excitatory transmission and operates as a mediator in inflammatory processes. 5-HT neurons is widely dispersed in the raphe nuclei of the brain stem such as the pons and medulla oblongata and additionally other brain regions for example, the striatum, hippocampus, amygdala, cerebral cortex, thalamus, hypothalamus and spinal cord (Ohno et al., 2015). Besides governing the regulation of critical physiological processes such as motor activity, sleep, body temperature and pain, 5-HT is also significant in mediating endocrine and autonomic systems as well

as emotional behaviour and cognitive function (Strac et al., 2016; Ohno et al., 2015). 5-HT has been reported to enhance and/or depress glutamate-mediated transmission as well as GABA-mediated transmission in structures controlling movement (Ciranna, 2006). 5-HT receptors, sorted into 7 families consisting of 5-HT1, 5-HT2, 5-HT3, 5-HT4, 5-HT6 and 5-HT7, mediate the serotonergic signal transduction. These 7 families are further broken down into 14 subtypes which are 5-HT1A, 1B, 1D, 1E, 1F, 5-HT2A, 2B, 2C, 5-HT3, 5-HT4, 5-HT5A, 5B, 5-HT6 and 5-HT7. Found at both pre and post-synaptic membrane, the 5-HT receptors with the exception of 5-HT3 receptors which are ligand-gated ion channels, are G protein-coupled receptors (Ohno et al., 2015; 2013).

Various 5-HT receptors mediating 5-HT neurotransmission were reported in the regulation of extrapyramidal motor functions which are implicated in the pathophysiology of various neurological disorders. This is supported by the findings of the ameliorating effect of 5-HT1A receptors activation on antipsychotic-induced extrapyramidal side effects (EPS) and motor disabilities in animal models of PD in a study by Ohno et al. (2011). Specifically, 5-HT1A and 5-HT2A/2C receptors are amongst the multiple receptors that is of great significance in the modulation of motor disabilities in animal models of PD (Ohno et al., 2015). Past study of such findings include an experiment that demonstrated the weakening of L-dopa–induced dyskinesia in 6-hydroxydopamine-lesioned rats through in administration of mixed 5-HT1A/1B receptor agonist, eltoprazine (Bezard et al., 2013). Another study showed a decrement in tacrine-induced tremulous jaw movements in rats which is considered a primary motor symptom of tremor, through the administration of 5-HT2A receptor inverse agonist and antagonist, ACP-103 (Vanover et al., 2008). Additionally, both 5-HT1A

receptor agonist and 5-HT2A receptor inverse agonist and antagonist were shown to reduced l-dopa-induced dyskinesia in MPTP-treated macaques.

Glutamate is the most prevalent excitatory neurotransmitter in the CNS, having an extensive functional contribution in both the CNS and peripheral nervous system (PNS) processes as it is involved in various metabolic pathways. Present on glutamatergic neurons, glutamate execute glutamatergic signal transduction by binding to and hence, activating both ionotropic and metabotropic glutamate receptors located on postsynaptic neurons. Regulation of glutamate is critical as unsuppressed glutamate release will result in glutamate dysregulation which poses excitotoxicity within the CNS. Such occurrence leads to neuronal damage and even neuronal death. Glutamate dysregulation has been well characterized in certain psychiatric, neurodevelopmental, and neurodegenerative disorders. The excitatory amino acid transporters (EAATs) holds the responsibility in preventing glutamate dysregulation by governing the release and reuptake of glutamate. Furthermore, glutamate transporters also contribute to learning, memory, and motor behaviour regulation. There are a total of five EAAT subtypes which are EAAT1 or GLAST (glutamate/aspartate transporter), EAAT2, EAAT3 or EAAC1, EAAT4 and EAAT5 (Zhang et al., 2016).

Neurological disorders such as stroke, epilepsy, ALS, AD and PD exhibits alterations in the function or expression of EAATs in their pathogenesis (Benarroch, 2010). Zhang and colleagues (2016) in their study showed that PD animal models exhibit a decreased expression and function of EAATs. EAATs especially EAAT1 is important in the maintenance of extracellular glutamate concentrations below

glutamate excitotoxic levels where if exceeded results in glutamate neurotoxicity and subsequent dopamine neuronal death, movement disorder, and cognitive impairment (Chen et al., 2016; Sominsky et al., 2015). Concentration of extracellular glutamate increases in the early stages of neuroinflammation due to microglia activation. A study by Tsai et al. (2012) demonstrated that fluctuation in neuronal glutamate transporter EAAT4 expression levels can alter the extrasynaptic glutamate signaling. Furthermore, both direct and indirect pathway of the corticostriatal circuit receive glutamatergic inputs whereby Do et al. (2013) suggested that alteration of glutamatergic transmission in the dorsal striatum through a form of NMDARs blockade may perhaps contribute to hyperactivity of motor function.

While the above studies provide valuable information regarding the potential associative mechanism between TLR4, neuroinflammation signaling and motor behaviour, there are still gaps in between such as the involvement and potential changes in functional neurotransmitter receptors and transporters that needs to be investigated in order to know the complete mechanism of TLR4 activation in affecting motor behaviour. The known underlying pathway can provide alternative therapeutic treatment for existing neurological and motor neuron diseases. Therefore, in this study the major focus point was placed on the implication of TLR4 towards the motor behaviour and associated neurotransmitter receptors and transporters using mouse model.

2.7 Rationale

Gradual loss or impairment of motor behavior in neurological and motor neuron diseases disrupts and lowers the quality of life of affected people. Neuroinflammation has been reported to play the prominent role in the pathogenesis of these diseases with various researches reporting the involvement of TLR family, specifically TLR4 in resulting physiological and behavioral changes involving retardation of motor activity in the mouse model. However, the overall pathway and mechanism of how TLR4 is involved in motor behavior has not been known yet. The known underlying pathway can provide alternative treatment for existing neurological and motor neuron diseases. Therefore, in this project, we focused on the implication of TLR4 on motor behavior and associated genes and transporter in a mouse model.

2.8 Hypothesis

Toll-like receptor 4 may regulate motor and sickness behavior through dopamine, serotonin receptors and glutamate transporters in striatum and cerebellum of mice**.**