EFFECT OF NEUROINFLAMMATION ON COGNITION AND POTENTIAL MECHANISMS INVOLVED

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LIST OF ABBREVIATIONS

Aβ	: Beta amyloid
AD	: Alzheimer's disease
AGEs	: Advanced glycaltion endproducts
AMPA	: Alpha-amino-3 hyroxyl-5 methylisoxazole-4-propionate
AMPAR	: Alpha-amino-3 hyroxyl-5 methylisoxazole-4-propionate receptor
ANOVA	: Analysis of variance
AP-1	: Activator protein- 1
APP	: Amyloid precursor protein
Arc	: Activity-regulated cytoskeleton-associated protein
BACE	: Beta-site of amyloid precursor protein cleaving enzyme / beta-secretase
BBB	: Blood brain barrier
BCA	: Bicinchoninic acid
BDNF	: Brain-derived neurotrophic factor
BID	: *Bis in die* (twice daily dosing)
BrdU	: 5-bromo-2-deoxyuridine
CA	: *Cornu Ammonis*
CD	: Cluster of differentiation
CNS	: Central nervous system
COX	: Cyclooxygenase
CR	: Complement receptor
CREB	: cAMP response element binding
CS	: Conditioned stimulus
CSF	: Cerebrospinal fluid
d2	: Discrimination index
ED	: Ectodermal dysplasia
ELISA	: Enzyme-linked immunosorbent assay
EPSC	: Excitatory post synaptic current
FC	: Fear conditioning
GLT1	: Glutamate transporter 1
GluR1	: Glutamate receptor 1
GTP	: Guanosine triphosphate
HPA	: Hypothalamic-pituitary-adrenal
ICV	: intracerebroventricular
IEG	: Immediate early genes
IFNγ	: Interferon gamma
IL	: Interleukin
iNOS	: Inducible nitric oxide synthase
IP	: Intraperitoneal
JNK	: Jun-N terminal kinase
LABORAS™	: Laboratory animal behaviour observation registration and analysis system
LPS	: Lipopolysaccharide
LTD	: Long term depression
LTP	: Long term potentiation

MAC1 : Macrophage antigen complex 1
MAPK : Mitogen activated protein kinase
MCI : Mild cognitive impairment
MHC : Major histocompatibility complex
MPO : Myeloperoxidase
mRNA : Micro ribonucleic acid
MWM : Morris water maze
nAChRα7 : Nicotinic acetylcholine receptor alpha seven
NADPH : Nicotinamide adenine dinucleotide phosphate
NC : Nitrocellulose
NFκB : Nuclear factor kappa B
NMDA : N-methyl-D-aspartate
NO : Nitric oxide
NOR : Novel object recognition
NSAID : Non steroidal anti inflammatory drug
PBS : Phosphate buffered saline
PD : Parkinson's disease
PG : Prostaglandin
PSD-95 : Post synaptic density -95
rpm : Rates per minute
RT : Room temperature
ROS : Reactive oxygen species
SEM : Standard error of mean
SGZ : Subgranular zone
solTNF : Soluble circulating trimer tumour necrosis factor
SC : Spatial cue
TCF : T-cells factors
TIR : Toll/IL-1 receptor
TLR : Toll-like receptor
TMB : Tetramethylbenzidine
TNFα : Tumour necrosis factor alpha
TRK B : Neurotrophic tyrosine kinase receptor type two
tmTNF : Type-2 transmembrane tumour necrosis factor
US : Unconditioned stimulus
VAChT : Vesicular acetylcholine transferase
VC : Visual cue

Summary

Chronic inflammation in the central nervous system (CNS) is thought to play a role in learning and memory deficits that are prevalent in neurodegenerative diseases such as Alzheimer's disease (AD) (Rosi et al. 2005). The association between neuroinflammation and learning and memory deficits were investigated. Below are a summary of the findings of the present work.

1. Acute peripheral administration of lipopolysaccharide (LPS), a bacteria cell wall proteoglycan, is unable to elicit spatial learning and object recognition deficits when tested 24 hours after administration. This contradicts what was previously reported where a single acute dose of LPS was sufficient to induce a cognitive deficit in rodents.

2. A spatial learning and object recognition memory deficits were observed in animals dosed with the increasing LPS dose regime. This is the first time that peripheral administration of LPS was shown to be able to elicit an object recognition deficits in rats. During the time of test, animals did not exhibit any sickness behaviour. This strengthens the hypothesis that the cognitive impairment observed were devoid of confounding factors such as sickness behaviour.

3. The increasing LPS dosing regime was shown to elicit a neuroinflammatory response where elevated tumour necrosis factor α (TNFα) and major histocompatibility complex II (MHCII) were observed in both hippocampus and

cortex even after the completion of the treatment. The continuous inflammatory response seen is specific only to the CNS as peripheral system TNFα expression was only shown to be elevated only after the first dose of LPS and returned to the basal level in subsequent doses.

4. The LPS treatment induced several changes that may serve to explain the cognitive deficits observed. In the hippocampus, an increase in amyloidogenesis, demonstrated by the increase in amyloid precursor protein (APP). Furthermore, LPS treatment may affect glutamatergic transmission, cholinergic innervations and also synaptic plasticity. The alteration of these properties in neural networks may be associated with the cognitive deficits observed and illustrate the role of neuroinflammation in AD.

5. The effect of the LPS treatment is not limited to an acute effect. When the animals were tested 8 to 12 weeks post LPS treatment, a similar spatial learning deficit. This suggests that there exist a critical window where a delayed cognitive impairment can be observed. This deficit could be due to the alteration in the neurogenesis processes in the dentate gyrus.

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) are examples of neurodegenerative diseases that are becoming more prevalent in today's population. While the etiology of each disease may differ, there is a common defining characteristic in which inflammation is present in most neurodegenerative diseases. For example, acute phase reactants proteins, cytokines, complement components and other inflammatory mediators that are associated with local inflammatory response are commonly found surrounding the characteristic β-amyloid deposits in AD patients (Akiyama et al. 2000). Elevated levels of proinflammatory cytokines, urpegulation of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and activated microglia were similarly observed in PD patients in the substantia nigra and striatum (Whitton 2007). However, neuroinflamation in these disorders were previously viewed as an epiphenomenon, where damaged neurons are able to induce proinflammatory response via glia cells (Skaper 2007).

Numerous data has challenged this idea and are indicative that neuroinflammation may play a more prominent role in the onset in addition to disease progression. In the CNS, glial cells, in addition to providing support to neuronal function, serve to respond to stress and insults by transiently upregulating inflammatory processes. Under normal circumstances, these responses are kept in check by other endogenous anti-inflammatory

and neuroprotective mechanisms (Skaper 2007). In the diseased brain however, the dysregulation of the glial cells, in a self perpetuating manner (Block et al. 2007), inevitably promotes severe and chronic neuroinflammation that could lead to degeneration of the neurons which is now widely touted as the neuroinflammation hypothesis (Griffin et al. 1998).

Hence, one of the key objectives of this project is to recapitulate the neuroinflammation component that is prevalent in most neurodegenerative diseases in a rodent model to study the effect of chronic inflammation on learning and memory as cognitive deficits are a key feature in most neurodegenerative diseases.

1.1 Cells involved in neuroinflammation

1.1.1 Microglia

Microglia is generally found throughout the CNS and plays an integral part of the immune defence. These cells account for approximately 20% of the total glial population (Kreutzberg 1995) and in the adult mice, they predominate in the grey matter with the highest concentrations being found in the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra (Block et al. 2007). They have a mesodermal origin and belong to the monocyte macrophage lineage. Under normal conditions, the resting microglia, with its ramified structure, is able to move and survey the environment to detect for any changes in the surrounding area, thus acting as the CNS first line of immune defence (Gao and Hong 2008). In the event of an immunogenic stimuli or injury, the microglia is activated and functions similar to a macrophage. It was postulated that

the activated microglia could be functionally discerned into two states, namely the phagocytic phenotype (innate activation) or an antigen presenting phenotype (adaptive activation) that could ultimately determine the range of cytokines that are produced (Town et al. 2005). The activation of the microglia are accompanied by a significant morphology change (ameboid shape where the cells undergo shortening of cellular processes and enlargement of the soma). These activated microglia are able to phagocytose cellular debris or foreign materials. At the same time, they produce chemokines to attract more microglia, cytokines and factors that promotes microglia proliferation (Gehmann 1995). Furthermore, the activated microglia also up-regulate a myriad of cell surface antigens such as MHC type I and II, cluster of differentiation (CD) 4 and ectodermal dysplasia (ED) 1 (Schoeter et al. 1999).

Tightly regulated neuroinflammation is beneficial for recovery under certain circumstances. For instance, microglia have been shown to stimulate myelin repair, eliminate toxic proteins and avert neurodegeneration (Gao and Hong 2008). However the problem arises when regulations of these inflammatory processes are derailed. Under such conditions, the activated microglia produce significantly large amount of cytotoxic factors such as superoxide ($O_2^{\cdot -}$), nitric oxide (NO) and tumour necrosis factor-α (TNFα) (Block et al. 2007). This excessive, uncontrolled inflammation, that induce an increase in cytotoxic factors, if left uncheck, could produce considerable bystander damage to neighbouring healthy tissue.

1.1.2 Astrocytes

Astrocytes were long believed to be structural cells as they make up to about 50% of human brain volume. However in recent years, astrocytes have been shown to serve many housekeeping functions, including maintenance of the extracellular environment and stabilization of cell-cell communications in the CNS. Characterised by its star-shaped cells, these cells are important for amino acid, nutrient and ion metabolism in the brain, coupling of neuronal activity and cerebral blood flow and modulation of excitatory synaptic transmission (Margakis and Rothstein 2006).

In the diseased state such as in multiple sclerosis and AD, activated astrocytes, are believed to facilitate leukocyte recruitment to the CNS by increasing leukocyte adhesion molecules and chemokine production (Moynagh 2005). It is difficult to tease out the contribution of astrocytes in inducing chronic neuroinflammation as it is functionally intertwined with other cell types. However, there are evidences from genetic mutations in astrocytes able to mimic certain neurodegenerative diseases. For instance, in cells expressing the familial AD persenilin 1 mutation, calcium oscillations were found to occur at lower ATP and glutamate concentrations than in wild-type astrocytes, supporting the idea that the change in calcium signalling between astrocytes could ultimately contribute to dysfunction of neurons in a diseased state (Margakis and Rothstein 2006).

More interestingly, similar to microglia, stimulation of γ-interferon (IFNγ) *in vitro* was shown to be able to increase the expression of MHC type I and II antigens in astrocytes. Furthermore, it has been shown that lipopolysaccharide (LPS) is able to stimulate

astrocytes to produce prostaglandins, complements C3 and factor B, and cytokines (Liebermann et al. 1989). These observations suggest that astrocytes may play an important role during immunological response as it shares many important functional characteristics with macrophages.

1.2 Neuroinflammation and cognition

1.2.1 Effect of cytokine on cognition

Excessive activation of the glial cells such as microglia and astrocytes induced a significantly higher production of cytokines such as interleukin (IL)-1β and TNFα (Block et al. 1997). Elevation of cytokines has been associated with cognitive deficits where in AD and mild cognitive impairment patients, a stage described as a preclinical stage of AD and is applied as a transitional period between normal aging and early AD, an increased in inflammatory cytokines were observed in blood samples (Magaki et al. 2007, Guerreiro et al. 2007). Furthermore, it was recently reported that an increase in TNFα induced by acute and chronic inflammation were associated to a decrease in the performance of AD patients in cognitive tasks (Holmes et al. 2009). In PD patients, elevated levels of IL-6 were also observed in the nigrostriatal region and cerebrospinal fluid (CSF) (Hofmann et al. 2009). In addition, transgenic animals that overexpressed IL-6 exhibit neuropathological changes that are closely correlated with the cognitive deficit seen (Akiyama et al. 2000), thus suggesting a possible correlation between inflammation and cognitive deficits.

Under normal physiological conditions however, these cytokines may play an important role in cognitive processes. In animal models using TNF knock out animals, it has been shown that TNFα is essential for normal functions of learning and memory. These animals under immunologically non-challenged conditions, performed significantly worse in cognition tasks (Baune et al. 2008). In addition, under specific conditions, TNFα may play a role against neuronal death where TNFα treatment can protect against focal cerebral ischemia (Nawashiro et al. 1997). *In vitro*, TNFα through the activation nuclear factor kappa B (NFκB) may protect neurons against metabolic, excitotoxic or oxidative insults by upholding maintenance of intracellular Ca^{2+} homeostatsis and inhibition of reactive oxygen species (ROS) (Pickering and O'Conner 2007). The dysregulation of microglia and astrocytes, leading to the excessive production of pro-inflammatory cytokines, has since been suggested to prevent the proper function of normal cognitive processes to the extent of dire consequences.

Many labs have tried to induce cognitive deficits in rodent model by increasing the levels of cytokines in the CNS. In rodents, Oitzl et al. (1993) had shown that direct intracerebroventricular (ICV) infusion of IL-1β was able to induce a transient deficit in rodent spatial learning and memory task such as the Morris water maze (MWM). Although animals treated with IL-1β did not show any deficit in acquiring the location of the platform, they were unable to recall the location of the hidden platform, when tested 24 hours later.

Not limited to centrally infused cytokine, peripheral administration of cytokine was also shown to be able to induce cognitive deficit. The intraperitoneal (IP) injection of 100ng IL-1β was shown to be effective in disrupting spatial learning and memory (Gilbertini et al. 1995). Mice treated with IL-1β showed a significantly higher latency in finding the hidden platform location. It was hypothesized that the administration of IL-1β significantly affected memory acquisition suggesting that centrally and peripherally administerions of IL-1β may have differing effect on learning and memory. IL-1β was also shown to induce a deficit on long-term memory in contextual fear (Pugh et al. 1998). These neuroinflammatory mediators have been shown to be able to induce cognitive deficit through several mechanisms that affect the cell survival and neuronal properties.

1.2.2 Effect of inflammation on long term potentiation

Long term potentiation, a form of synaptic plasticity that is widely touted as a model of learning and memory is characterized by a persistent enhancement of neurotransmission following an appropriate stimulus (Kerchner and Nicoll 2008). There is evidence to suggest that cytokines are able to abrogate the action of LTP where peripheral LPS injection is able to impair LTP in the hippocampus (Vereker et al. 2006). LPS has been shown to be able to impair LTP through IL-1β activated pathway by increasing the activity of the stress-activated kinases, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) by increasing the phosphorylation of these kinases, ultimately leading to the impairment in neuronal function (O'Donnell et al. 2000).

LPS was also shown to disrupt glutamate release by the activation of p38 and NFκB (Kelly et al. 2003). As glutamate is an important player in the propagation of LTP, disruption of glutamate release will inevitably lead to the impairment of LTP. By studying the glutamate release in synaptosomes of dentate gyrus from rats treated with IL-1β, it was shown that IL-1β reduces the amount of glutamate release after being tetanised. SB203580, a p38 inhibitor was able to fully reverse this effect (Kelly et al. 2003). In addition, peripheral administration of an immunogenic property such as LPS is sufficient to induce, not only neuroinflammation but also impairment in LTP that is reflected in the cognitive deficit observed in animal behaviour tests.

1.2.3 Effect of inflammation on neurite outgrowth

Activation of microglia has also been shown to induce cell death at high concentrations of endotoxins such as LPS and advanced glycation endproducts (AGEs) *in vitro* (Münch et al. et al. 2003). Albeit it is known that activated microglia is able to produce various factors that are cytotoxic. However, the exact mechanism through which these reactive glial cells induce neuronal death is not completely understood. At a sublethal dose of LPS or AGEs, it was reported that these immunogenic properties were able to induce activation of microglia that can lead to a reduction of neurite outgrowth (Münch et al. 2003). More specifically, TNFα has been shown to reduce neurite outgrowth and branching in the hippocampal neurons via small GTPase Rho proteins (Neumann et al. 2002). The reduction of neurite outgrowth during a mild inflammation (with an absence of T cell amplified systemic inflammation) with factors secreted by the activated microglia could interfere with the cytoskeleton reorganization. This change in synaptic

reorganization is sufficient to induce learning and memory deficits even in the absence of cell death (Gallagher et al. 1996). The reduction of neurite outgrowth has since then been linked to NO and NO-derived products. NO can directly regulate actin reorganization in the neurite, by inducing signaling cascades involved in growth cone collapse and through regulation of gene transcription (Münch et al. 2003).

1.2.4 Effect of inflammation on oxidative stress generation

Oxidative stress is a prevalent feature in numerous neurodegeneration diseases albeit the source of ROS is still debatable (Block et al. 2007). In the microglia, the ROS production is catalysed by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex that converts oxygen to superoxide. Distributed in both the cell membrane and membrane of organelles, the ROS generated under normal conditions has some beneficial functions as ROS generation plays a vital role in host defense. ROS are involved in cell defence against pathogens, but also in reversible regulatory processes in most cells and tissues (Bedard and Kraus 2007). Hence, like the proinflammatory cytokines as discussed previously, the beneficial or detrimental effect of ROS lies on a fine balance.

In normal aging humans, the level of ROS increases with age as predicted by the "free-radical theory of aging" (Harman 1956) and this increase in ROS levels is usually accompanied by a decline in cognitive and motor functions although not associated with a significant loss of neurons (Dröge and Shipper 2007). Furthermore, a decrease in antioxidant enzymes and concentrations of small-molecular-weight antioxidants in blood

and tissue cells, also induce an age-dependent elevation in the proportion of ROS and free radicals that are normally being "removed" (Wei and Lee 2002). The involvement of NADPH oxidases in aging has been linked to the increased level of ROS in the CNS (Krause 2006). More interestingly neural damage induced by extracellular secretion of ROS has been shown to be mediated by NADPH oxidase through the activation of microglia (Walder et al. 1997). These oxidative conditions are able to induce irreversible damage to proteins, lipids, carbohydrates and nucleic acids.

In AD and PD patients, NADPH oxidases were reported to be upregulated in the CNS (Block et al. 2007). In addition to the reduction in the concentrations of antioxidants present in the system, most patients suffering from AD and PD also experience an increase in ROS production, further uncoupling the redox balance in the CNS. The excessive ROS in the system could ultimately trigger the mitochondrial apoptosis pathway, inducing a mitochondrial dysfunction by the release of cytochrome C into the cytoplasm (Dean 2008). Thus, during chronic neuroinflammation, the increase in ROS production induced by the upregulation of ROS producing enzymes is able to induce cognitive deficits as the excessive ROS produced is able to trigger the apoptotic pathway that culminates with neuronal death.

The generation of ROS, is reported to act as a common signaling mechanism for phagocytes where the gangliosides activate microglia through protein kinase C and NADPH oxidase (Min et al. 2004). Furthermore, changes in the morphology and proliferation of microglia (microgliosis) are regulated by hydrogen peroxide produced

from NADPH oxidase (Block et al. 2007). In return, higher levels of ROS in the intracellular positively regulate the inflammatory response where an increase production of pro-inflammatory response is able to affect cell survival by increasing lipid peroxidation and protein nitration (Engelhardt et al. 2001). Hence, it seems that the catalytic events of NADPH oxidase in the activated microglia are essential contributors of oxidative stress and inflammation that in extreme conditions could lead to neuronal damage and ultimately affect cognitive ability.

1.2.5 Effect of inflammation on neurogenesis

Neuroinflammation has also been shown to induce a blocakade in neurogenesis (Monje et al. 2003). Neurogenesis refers to the birth of new neurons that occur within the CNS. In the hippocampus, the birth of these new neurons continues throughout life and the amount of neurogenesis correlates closely with the hippocampal functions of learning and memory (Monje et al. 2003). Any disruption to the environment of these proliferating neural stem or progenitor could lead to a disruption of neurogenesis and ultimately cognitive deficits. For example, in patients receiving therapeutic cranial radiation therapy a decline in cognitive function has been reported as the therapy is known to ablate any cell proliferation in the CNS (Monje and Palmer 2003). To illustrate the effect of an altered microenvironment using a rodent model, peripheral administration of LPS, inducing an increase in central pro-inflammatory cytokine production, was sufficient to induce a 35% decrease in hippocampal neurogenesis (Monje et al. 2003). This disruption of neurogenesis by LPS was also shown to be able to induce spatial learning and memory deficits task (Wu et al. 2007).

The direct mechanism as to how neuroinflammation is able to induce a disruption to neurogenesis has yet to be fully elucidated. However it is hypothesised that inflammatory cytokines such as IL-6 and TNFα were able to indirectly inhibit cell proliferation and neurogenesis in the dentate gyrus by increasing the levels of circulating glucocorticoids via centrally stimulating the hypothalamic-pituitary adrenal (HPA) stress axis (Vallières et al. 2002). It was suggested that glucocorticoids could affect cell proliferation by directly repressing the transcription of cyclin D1, a common cell-cycle regulator that controls G1-S phase transition, by binding to the promoter and affecting the β-catenin/TCF pathway (Boku et al. 2009).

In a separate study, it was also suggested that peripheral administration of LPS could induced cognitive deficits via COX-2. An increase in COX-2 expression in the granular cell layer and blood vessels, areas that are known to be neurogenic in the dentate gyrus was observed after LPS treatment. The involvement of COX-2 was associated with a decrease in newborn cell survival but not cell differentiation where the number of 5-bromo-2-deoxyuridine (BrdU) labelled cells decreased significantly after LPS treatment (Bastos et al. 2008). COX-2 may modulate neurogenesis in the dentate gyrus through the generation of prostaglandins such as prostaglandin (PG) E_2 and PGD_2 that are able to induce apoptosis in a variety of cell types (Bastos et al. 2008). However, the involvement in COX-2 in reducing cell proliferation is still under investigation as other studies have reported that the reduction of the number of newborn neurons were associated with neuronal differentiation rather than neuronal proliferation. Inflammatory mediators such

as IL-6, TNFα and IL-18 were reported to induce an increase in glial differentiation (Liu et al. 2005, Cacci et al. 2008). This suggests the complexity of the effect of neuroinflammation in neurogenesis in the dentate gyrus.

1.3 Neuroinflammation as a neurodegenerative disease model

Neuroinflammation is a common feature in most neurodegenerative diseases. Elevated levels of cytokines have been seen in most AD and PD patient and these cytokines have been shown to have an effect on cognition. Furthermore transgenic animals that overexpressed specific cytokines such as IL-6 and TNFα have been shown to perform worse in cognitive tasks (Akiyama et al. 2000).

Therefore, one of the main objectives of this project was to mimic this neuroinflammation in the rodent model, in order to recapitulate the cognitive deficits that are prevalent in neurodegenerative diseases. In order to do that, LPS was used to induce inflammation in the CNS. LPS is known to stimulate the immune system through the activation of macrophage-like cells in peripheral tissues (Takeda and Akashi 2004). LPS is recognised by the CD14/toll-like receptor (TLR) 4 signal transduction receptor complex expressed by microglia and astrocytes in the CNS (Rosi et al. 2006). $TLR4^{-/-}$ mice have shown reduced susceptibility to sepsis with systemic administration of LPS (Poltrorak et al. 1998).

The TLR family consists of 10 members (TLR1-TLR10) where the cytoplasmic portion of TLRs displayed similarity to the IL-1 receptor family which is now also known as

Toll/IL-1 receptor (TIR) domain. Unlike the IL-1 receptors, the TLRs bear leucine-rich repeats in the extracellular domain (Takeda and Akira 2004). The TLR4 upon activation by LPS triggers a signaling cascade that ultimately induces the transcription of inflammatory cytokines such as TNF-α and IL-6 via NF-κB as shown from the schematic diagram (figure 1.1).

Infusion of LPS into the fourth ventricle in young rats produced a chronic neuroinflammation with an activation of microglia and astrocytes within the hippocampus, piriform and entorhinal cortex. (Hauss-Wegrzyniak et al. 1998). Chronic infusion of LPS was also shown to induce the expression of IL-1β, TNF-α and β-amyloid precursor protein mRNA levels in the hippocampus. Furthermore, these animals displayed impaired hippocampal-dependent memory task such as the T-maze but not object recognition memory (Hauss-Wegrzyniak et al.1998).

Peripheral administration of LPS was also able to elicit similar cognitive deficits. In a study conducted by Arai et al. (2001), LPS, administered intraperitoneally (IP), was able to elicit a deficit on spatial learning performance in the water maze. The LPS treated animals had a higher escape latency and path length compared to the vehicle treated animals and at the same time performed much worse in the Y-maze test. Hence, this suggests that systemic administration of LPS could induce neuroinflammation in the CNS mediated by the activation of microglia. These activated microglia would in turn produce inflammatory mediators such as cytokines to drive the cognitive impairment as seen in centrally administered LPS.

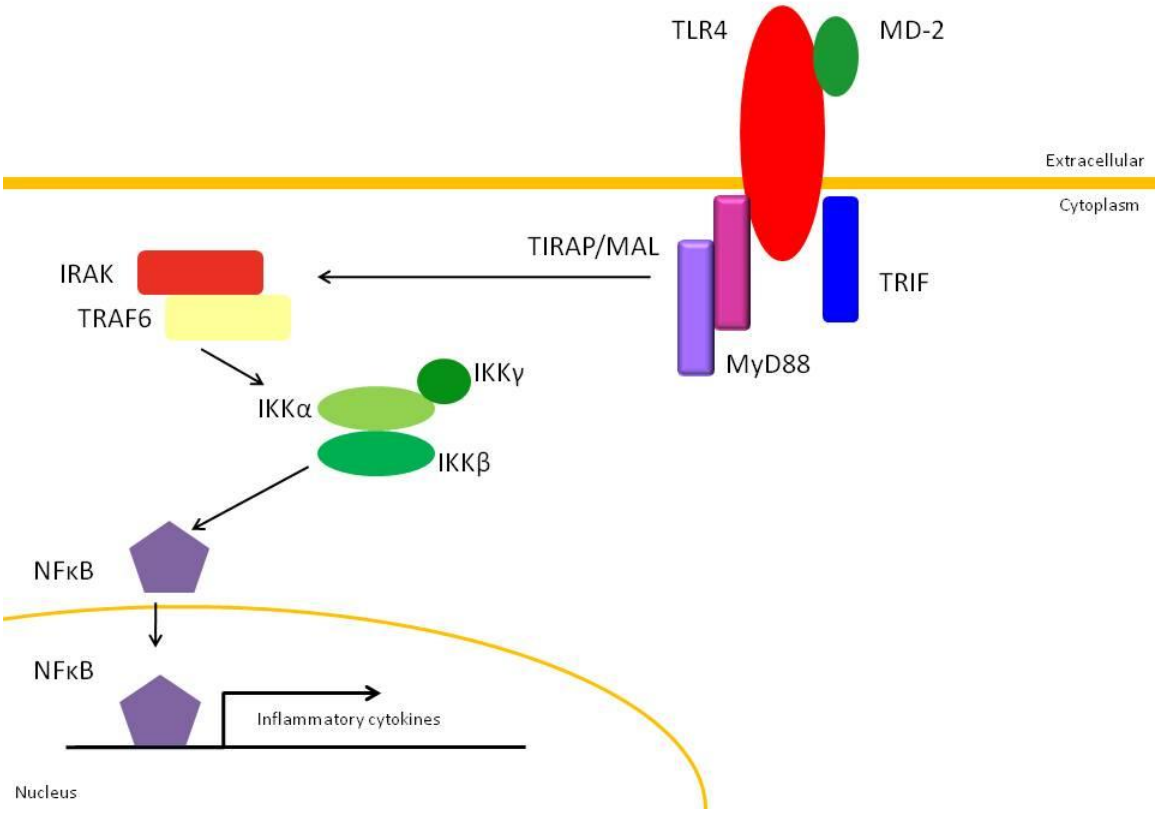


Figure 1.1 Schematic diagram of activation of TLR4 and its signalling cascade in inducing the transcription of inflammatory cytokines (Adapted from Takeda and Akashi 2004)

1.4 Objectives

Earlier experiments using LPS were conducted two to four hours after LPS injection (Arai et al. 2001, Gibertini et al. 1995, Sparkman et al. 2005) during which most animals treated with LPS were exhibiting sickness behaviour. Sickness behaviour is generally associated with a lack of motivation, an increased stress or anxiety response, decreased locomotor activity, decreased reward activities, anorexia and a marked activation of HPA stress axis (Cunningham and Sanderson 2008). All the behaviours stated are able to confound behavioural results. These studies have made contradictory claims on the effect of LPS in inducing cognitive deficits. The contradictory results reported could arise due to the misinterpretation of the sickness behaviour as a deficit in the behavioural test

This project tries to elucidate whether systemic infection induced by peripheral LPS administration is able to induce cognitive deficits in rodent learning and memory tasks that are devoid of any confounding factors such as sickness behaviour. LPS is chosen as an agent to induce neuroinflammation as it has been previously shown to be efficacious in activating the microglia in the CNS and inducing a host of inflammatory response that are similar to most neurodegenerative diseases. In addition, systemic induction of inflammation was chosen over a centrally induced inflammation as peripheral LPS administration is less invasive.

Furthermore, potential mechanisms in which a systemic infection is able to drive changes in the CNS were also investigated. The effects of inflammation on several key proteins that are involved in the learning and memory processes were investigated. This may thus

explain the possible mechanisms that neuroinflammation may induce cognitive deficits. As neuroinflammation normally precedes cognitive deficits in AD, this may thus offer certain enlightenment as what is occurring in the diseased brain.

Lastly, this project aims to examine whether the effect of the peripheral LPS administration is temporary and/or whether there is a delayed deficit that could be detected several weeks post treatment. A delayed deficit could suggest a potential link to the disruption of the neurogenesis process as newborn neurons were shown to be preferentially recruited in spatial learning and memory tasks (Kee et al. 2007). The alteration of learning and memory induced by neurogenesis may underlie the need to look for possible therapeutic treatment based on the complex nature of the memory deficits induced by neuroinflammation.

CHAPTER 2

MATERIAL AND METHODS

2.1 Animals

Male Lister Hooded rats were obtained from Harlan, UK. Animals were housed four to five rats per cage in a temperature (20 ± 1°C) and humidity (40 ± 2%) controlled environment on a twelve-hour light/dark cycle (lights on 7:30am), with *ad libitum* access to food and water. Prior to all experiments, rats were habituated to the testing rooms for a week. All experiments were carried out in accordance with the Singapore National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines for the use and care of animals for scientific purposes and GlaxoSmithKline animal research ethical standards.

2.2 Behavioural analysis

2.2.1 Morris water maze

The watermaze apparatus consists of a white fibreglass pool (diameter: 1.7m, height: 0.65m) housed in a custom built room. Extramaze cues such as screens, posters and other objects (e.g. lamps) were placed on the walls of the room, to help in the formation of a spatial map. The location of the objects remained unchanged throughout the experiments. The water was pre-heated so that the final water temperature was maintained at 26ºC ± 1ºC. A litre of opacifier (Syntran ® 5905, Yeochem Singapore) was added into the water prior to the start of the experiment to make the water opaque. The pool was divided into

four imaginary quadrants, namely North, South, East and West. The platform (diameter-20cm) was placed in the centre of one of the four quadrants and the location remains fixed throughout the training session for each rat. The platform, located 2cm beneath water surface, remains invisible to the rat while swimming. A video camera was positioned directly above the pool to directly feed data such as latency, pathlength and swim speed by using the Watermaze^{TM} software (Actimetrics Inc., USA)

In each study, ten animals were allocated into each group based on the visual cue performance. At the start of each trial, the animals were placed into the water maze tank facing the wall of the maze at a random location. A typical training consists of 1 day of visual cue (VC) training followed by 4 days of spatial cue (SC) training.

In the VC training (4 trials), a curtain is drawn around the pool, effectively hiding the extra maze spatial cues. A black cylindrical object (visual cue) is placed 40cm above the platform. The animals were allowed to use the visual cue to locate the platform location. Once the platform was located, the trial is stopped and the rat is left on the platform for thirty seconds.

In the SC training, the curtain is removed, hence revealing the extra-maze cues. SC training performance was assessed over four days (4 to 6 trials per day). The starting positions for each trial were randomized and each rat was allowed to search the platform for two minutes, after which it would be guided to the platform. When the platform was found, the trial was stopped and the rat was left on the platform for thirty seconds.

2.2.2 Novel Object Recognition: One hour temporal model

Animals were handled (8 to 10 minutes each) for two days before the T1 trial in the observation cage (Tecniplast, UK) to reduce novelty-induced stress. Objects used in these studies were custom made black acrylic cubes and cylinders (Labman Design, Singapore). A small magnet was embedded at the bottom of each object to prevent the animals from moving the objects during trials.

2.2.2.1 T1 trial

All animals were first habituated to an empty observation cage for two minutes. The animals were then briefly moved to an adjacent cage, while two identical objects were placed in the observation cage. The objects were placed at the front of the cage and at equal distances from both sides, in order to allow the rat to freely explore the objects, but yet at the same time allowing the experimenter to observe the rats carefully. The rats were then returned to the observation cage and were observed for another three minutes. The total time spent exploring each object was scored on-line by a trained observer and the video data was recorded.

2.2.2.2 T2 trial

The animals were again habituated in the observation cage for two minutes, one hour after the T1 trial. This is followed by the presentation of one novel and one familiar objects for three minutes. Objects were assigned using a randomized procedure to ensure treatment groups were balanced for novel object and position (left or right). Total exploration for each object was scored on-line and video data was recorded. The

discrimination index (d2) was calculated by subtracting the novel from familiar exploration divided by total exploration time (novel-familiar/total exploration). Object exploration was only scored when the animal's nose or mouth was in direct contact with either object. Climbing or resting on the objects was excluded.

2.2.3 Fear conditioning

All studies were performed using eight automated video-based fear conditioning (FC) systems (MED Associate, USA). Prior to training, all chambers were calibrated to ensure that the conditioning tone intensity (for cued FC) and shock currents (for both cued and contextual FC) were consistent across all chambers.

2.2.3.1 Hyperalgesia test

The rats were habituated to the FC test chambers for three minutes prior to the delivery of the shock to reduce novelty-induced stress. Thereafter, rats were given a 0.5mA foot shock. The shock responses of rats were scored by another observer who was unaware of the treatment groups: 1 - no response to the foot shock, 2 - the animal freezes for a moment, 3 - the animals were startle for a while, 4 – all four paws of the animals were lifted from the ground and jumps for a moment and 5 – all four paws of the animals were lifted from the ground and jumps vigorously for a while.

2.2.3.2 Cued fear conditioning

The experiment was conducted over a period of three days. On the first day, animals were habituated to the FC chambers for five minutes. On the second day, the animals were

trained to associate a mild foot shock (unconditioned stimulus -US) with an auditory tone (conditioned stimulus- CS). To create a novel olfactory context, the chambers were wiped with 3% acetic acid. Rats were given a five-minute habituation which is followed by a CS (2kHz, 90dB, 5s). The US (0.5mA foot shock, 1s duration) was then co-terminated with the CS. Animals were then left in the chambers for an additional thirty seconds before returning to their home cages. On the final day, the contextual environment was altered by wiping down the chambers with 3% ammonium hydroxide solution and changing the patternless panel to polka dotted panel. Animals were then returned to their respective chambers (same chamber on the second day) and were habituated for two minutes. This is followed by the presentation of 3 minutes CS tone, during which the total amount of time animals spent freezing were scored by the software. An animal is considered to have frozen when there is an absence of all movements, except those relating to respiration.

2.2.3.3 Contextual fear conditioning

Prior to the start of the experiment, the chambers were wiped down with 3% acetic acid. The animals were then habituated in the chambers for five minutes before being shocked (6 x 0.5s, 0.8mA, inter shock interval of 1 minute). After the completion of the shocks, the animals were left in the chamber for an additional 30 seconds before being returned to the home cage. Forty eight hours later, the animals were returned to the test chambers and were observed for ten minutes. Prior to this, the chambers were once again wiped down using 3% acetic acid to ensure that the context was similar to the previous day. The animals were then returned to the home cage before being re-tested in the same conditions 24 hours later to determine the extinction rate. As was done previously, the

total amount of time spent freezing during the ten minutes were extracted and analysed using Video freeze software (Med Associate, USA).

2.2.4 Laboratory animal behaviour observation registration and analysis (LABORAS™)

General behaviour such as locomotion, grooming, rearing and immobility can be monitored using the LABORAS™ (Metris™, Belgium) software. The LABORAS™ system catalogues these behaviours by using vibrations generated on the force transducers due to the movements of each animal. The signal recorded could then be analysed using the LABORAS™ software

Prior to test, all LABORAS™ kits were calibrated. The animals were placed into LABORAS™ kits and were observed for half an hour. Food and water were available *ad libitum*. After the completion of the experiment, the animals were then returned to the home cages.

2.2.5 Rotarod

The animals' motor-coordination were assessed using the rotarod apparatus (Linton Instrumentation, UK) by testing the animals' ability to stay on a rotating rod through successive five minutes trials at increasing speed. Before the actual test, the animals were pretrained on the rotarod at low speed (4 - 5rpm) for 120s. The animals were then returned to their home cages. During the actual test, the animals were placed on the rotarod with increasing speed (4 to 40rpm). The amount of time animals spent on the

rotarod was recorded. Each animal was tested three times in quick succession and the sum total of the time spent on the rotarod was used.

2.2.6 Body temperature monitoring

Body temperature was recorded using a rectal digital thermometer probe (Bioseb, France) at the same time each day (8-9am) to minimize any variation due to the circadian rhythm.

2.2.7 Body weight and food consumption

The animals were weighed at the same time everyday (8 – 9am). The amount of food consumed was monitored by measuring the food pellet that was left on the feeder each day at the same time (8 – 9am). The average amount of food consumed by each rat was calculated by taking the total amount of food consumed divided by the number of animals in that cage.

2.3 Biochemical analysis

2.3.1 Enzyme linked immunosorbent assay (ELISA)

Animals were euthanized using pentobarbital (300mg/ml/animal, IP, Age D'or, Singapore) two hours after the LPS/PBS treatment. In-house data demonstrated that the TNFα level reads its peak at two hours after LPS injection. Cardiac puncture were performed to collect blood samples in microtainer tube containing EDTA two hours after treatment. Plasma samples were separated by centrifuge at 10 000g at 4°C for 10 mins and stored at -20°C until use.

ELISA was conducted using Quantikine® kits (R&D Systems, USA) specific for rat TNFα/TNFSF1A according to manufacturer's instruction. In brief, all reagents were brought to room temperature before the experiment. The TNFα control and standards were prepared. 50µl diluent was added to each well followed by an equal amount of sample. The plate was then incubated for 2h at room temperature (RT). The plate was then aspirated and washed for 5 times with the wash buffer. 100µl of TNFα conjugate was then added to each well and was incubated for 2h at RT. This was followed by another five times wash with the wash buffer. The substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB) were added to the wells and incubated at RT for 30min. To quench the reaction, 100µl of stop solution was added and mixed thoroughly. The absorbance at 450nm and 570nm was then measured within 15min after the addition of the stop solution using the microplate reader thermo multiskan Ascent (Labsystems, USA). The amount of TNFα is determined by absorbance value (A_{570}-A_{450}) and compared to the standard curve to obtain the corresponding concentration value.

2.3.2 Myeloperoxidase activity

To determine microglia activity in the CNS (hippocampus) and neurotrophil activity in the peripheral tissue (spleen), the myeloperoxidase (MPO) activity assay was conducted as previously described with modification (Bhatia et al. 2003). In brief, animals were first perfused with saline before the respective tissues were harvested and stored at –80°C freezer. To extract the enzyme, the tissues were thawed at 4°C. For the spleen, due to its weight, these tissues were homogenised using a Polytron homogenizer in 20mM sodium phosphate buffer (pH 7.4). For the hippocampus however, the tissue was lysed using

tissue lyser (Qiagen, UK) in 20mM sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at 13000g at 4°C for 10min. The pellet obtained was resuspended in 50mM sodium phosphate buffer (pH 6.0) with 5% hexadeacylmethylammonium bromide (Sigma, USA). The resultant suspension was then subjected to four rapid freeze-thaw cycles. After that, the suspension was sonicated for a total time of 30s using the autogizer (Tomtec, USA). The suspension was then centrifuged again at 13000g for 10min at 4°C and the supernatant was used in the for the subsequent MPO assay. The reaction mixture consists of the extracted enzyme, 1.6mM TMB, 80mM sodium phosphate buffer (pH 5.4) and 0.3mM hydrogen peroxide (Sigma, USA). It is then incubated at 37°C for 2 min. To stop the reaction, 3% acetic acid of equal volume was added into the reaction mixture. The absorbance was then measured at 450nm using the multiskan Ascent microplate reader (Labsystems, USA). The results were expressed as fold increase over control group.

2.3.3 Western blot

2.3.3.1 Whole lysate preparation

Brain regions of interest were dissected and lysed in lysis buffer using the tissue lyser (Qiagen, UK). The lysate was then spun for 13000rpm at 4°C for 15min. The supernatant was collected for Western blot analysis.

2.3.3.2 Synaptosome preparation

Dissected brain regions of interest were homogenised in 0.32M sucrose in HEPES buffer using tissue lyser (Qiagen). The lysate was then centrifuged at 800g for 10min at 4°C.

The supernatant was collected and spun at 13000 rpm at 4°C for 30min. The pellet was then resuspended in ice cold RIPA buffer before western blot analysis.

2.3.3.3 Bicinchoninic acid (BCA) protein assay

Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, USA) according to manufacturer's instruction. In brief, the working reagent containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, cupric sulfate and sodium tartate was mixed with the homogenate. The mixture was incubated at 37°C for 30min. All samples were then measured for their absorbance using a microplate reader thermo multiskan Ascent (Labsystems, USA) at 560nm. The total amount of protein present was determined using a standard curve.

2.3.3.4 Western blot analysis

Loading buffer (4X) was added to each sample (3mg/mL) solution and denatured for 5 min at 95°C. Electrophoresis was conducted at 150V for 1.5h on 4-12% Bis-Tris Nupage gel (Invitrogen, USA). The gel was then transferred onto the nitrocellulose (NC) membrane at 20V for 1h. The NC membrane was then placed in blocking buffer (3% non-fat milk in phosphate buffered saline (PBS), (Sigma, USA) and was incubated at RT for 1h to prevent non-specific binding. This was followed by incubating the membrane with primary antibody (1:1000) in blocking buffer at RT for 1h. The membrane was then washed 6 x 5min. The infrared secondary antibody was diluted in the wash buffer (1:10000) and incubated again at RT for 1h in the dark. The membrane was given another 6 x 5min washes. The membrane was then imaged using Odyssey (LiCor, USA) system,

quantified and analysed using the Odyssey 3.0 software to obtain the luminosity and intensity of the bands.

2.4 Morphological analysis

2.4.1 Immunohistochemistry

Animals were euthanized using pentobarbital (300mg/ml/animal, IP, Age D'or, Singapore) and were perfused with 100ml of sterile saline followed by equal amount of freshly prepared 4% formaldehyde. Brains were then removed and post-fixed in 4% formaldehyde overnight followed by dehydration in 30% sucrose solution. Samples were kept in the 30% sucrose until use.

Prior to sectioning, the dehydrated samples were frozen in a slurry of ethanol and dry ice before being left at -20°C chamber for 30min. Thirty μm coronal sections were obtained using the cryostat (Leica, Germany). Sections were then transferred to poly-L-lysine coated slides for immunohistochemistry.

For immunohistochemistry, the primary antisera were prepared in PBS containing 0.01% triton-X100 and 5% serum albumin obtained from the secondary antibody host species. The secondary antisera were prepared in PBS containing 5% serum albumin.

The slides were first incubated in 0.3% triton-X100 at RT for 5min to unmask antigen of interest. The slides were then washed for 15min in PBS and incubated in blocking solution (10% serum albumin from the secondary antibody host diluted in PBS) for one

hour. This step prevents the binding of primary antibody to non-specific antigen. The primary antisera were then added and incubated overnight at RT.

The slides were then washed twice in PBS for 15min and then incubated for 1h in seconday antisera. The slides were washed in PBS for 4min and allowed to air dry at 37°C for 1h. Dry slides were mounted using Vectashield™ (Vector Laboratories, USA) mounting medium and viewed using an Olympus™ BX61 epifluorescence microscope.

2.5 Chemicals and compounds

2.5.1 LPS treatment

Bacterial lipopolysaccharide (LPS E.Coli 055:B5, Sigma) in PBS was administered via intraperitoneal injection at 1ml/kg. The dosing regime for each study is as followed

Single dose LPS : 1mg/kg, single dose

Three doses LPS : 3 doses of 1mg/kg LPS, once daily for 3 days

Sub chronic constant dose : 20 doses of 1mg/kg LPS, twice daily for 10 days

Sub chronic increasing dose : Twice daily for 10 days as shown below (adapted and modified from Chen et al. 2005)

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Dose	AM	0.25	0.25	0.5	0.5	1	1	2	4	8	16
	PM	0.25	0.5	0.5	1	1	2	4	8	16	16

2.5.2 Indomethacin administration

Indomethacin (Sigma, USA) was administered once daily 15min prior to the LPS injection for ten days (1mg/kg, IP, in 0.3% sodium bicarbonate, 10 days). Control animals were given 0.3% sodium bicarbonate.

2.5.3 Antibodies

The following antibodies and working concentrations were used; mouse anti-rat CD11b (1:200), mouse anti-rat synaptophysin (1:1000, Sigma, USA) Texas Red (1:200, Vector laboratories, USA), FITC conjugate (1:200, Jackson Laboratory, USA), Arc (1:200 Santa Cruz, Singapore), VAChT (1:200 Santa Cruz, Singapore), APP (1:200 Santa Cruz, Singapore), and TNFα (1:200 Santa Cruz, Singapore)

2.6 Statistical analysis

All graphs were prepared using GraphPad Prism^{TM} (version 5.0) and all data are expressed as means ± SEM. Statistical analysis was performed using StatSoft Statistica^{TM} (version 8.0) and all data was checked for normality prior to analysis.

For the water maze, body temperature, ELISA, the daily performance of the treatment groups was analysed using repeated-measures ANOVA followed by planned comparison. For NOR, a one-way ANOVA followed by Fisher LSD *post-hoc* analysis for group comparisons was used to compare treatment groups *versus* vehicle groups for the T1 trial. For the d2 index; a repeated-measures ANOVA followed by Fisher's LSD (plank) *post-hoc* comparison test was used to compare familiar *versus* novel object per treatment

group in the T2 trial. For the LABORAS, the individual five minutes time bins was analysed using repeated-measures ANOVA followed by panned comparison. For the rotarod and western blot, an independent T-test followed by Dunnet *post-hoc* analysis for group comparisons was used to compare the effect of the treatment groups *versus* vehicle groups.

CHAPTER 3

RESULTS

3.1 Effect of LPS treatment in inducing cognitive deficits in rodent learning and memory tasks

As previously described, peripheral LPS treatment has been associated with cognitive deficits that can be detected in rodent learning and memory tasks such as the MWM. Preliminary studies were conducted to identify a suitable dosing regime that is able to elicit a cognitive deficit.

3.1.1 Effect of single acute LPS (1mg/kg) treatment

Arai et al. (2001) and Sparkman et al. (2005) have previously shown that a single IP dose of LPS was sufficient to induce a deficit in the MWM and Y-maze task in mice. To recapitulate the results seen by Arai et al. (2001) in rats, animals were dosed with either LPS (1mg/kg) or vehicle and were subsequently tested in the MWM. However, LPS is known to be able to induce sickness behaviour that may confound the performance of the animals in behaviour tasks. Animals were rested for 24h before any behavioural tasks to ensure that the LPS treated animals were physiologically no different from vehicle treated animals instead of being tested 6h after LPS treatment as was done by Arai et al. (2001).

Peripheral administration of 1mg/kg of LPS did not affect the spatial learning and memory of rodents. When tested in the MWM, no significant effect of the LPS treatment

in latency (figure 3.1a), swim speed (figure 3.1b) and path length (figure 3.1c) was detected.

Object recognition memory was then investigated using the novel object recognition tasks. From figure 3.2 and 3.3, no significant object recognition memory was observed. Both treatment groups spent significantly more time exploring the novel object (figure 3.3), suggesting that the animals remembered the familiar objects and spend a significantly higher amount of time exploring the novel object. Animals displayed no significant difference in exploring the objects (figure 3.2b) suggesting that LPS treatment did not significantly affect the motivation of the animals from performing the task. A lack of sickness behaviour during the time of the test was also observed.

To ensure that the animals were devoid of any physiological changes induced by sickness behaviour, the overall behaviour of the animals were observed using the LABORAS™ system and core body temperature were monitored 2 and 24h after treatment. Based on the LABORAS™ data (figure 3.4 and 3.5), at two hours after treatment, the LPS treatment seemed to affect certain behaviours. The LPS treated animals spent a significantly higher percentage of time immobilised while reducing the amount of time spent on locomotion and rearing. A significantly lower body temperature was also observed in animals treated with 1mg/kg LPS (figure 3.6) when measured 2h after LPS treatment, indicating the aforementioned sickness behaviour. However when the animals were monitored 24h later at the time in which the cognitive tests were conducted, there was no difference in the physiological response of the animals when tested in the

LABORAS and body temperature. To further ensure that the motor coordination of the LPS treated animals were not compromised, animals were also tested using rotarod. When the animals were tested 24h after the LPS treatment, no significant difference was observed, suggesting that the LPS treatment did not compromise the motor coordination of the animals (figure 3.7).

3.1.2 Effect of three doses of LPS (1mg/kg, 3 days, once daily) treatment

As single 1mg/kg LPS was unable to induce a cognitive deficit, the lack of efficacy was hypothesised to be due to the insufficient levels of LPS. In subsequent experiments, the amount of LPS delivered was increased. The LPS treatment was increased to three daily doses of LPS (1mg/kg) over a period of three days.

Based on the water maze data, no spatial learning and memory deficit was observed in animals treated with LPS using this dosing regime (figure 3.8). However, judging on the results on SC day 1 and SC day 2 for latency and pathlength (figure 3.8a and c), the LPS treated animals performed slightly worse compared to the vehicle treated animals.

To ensure that the animals did not suffer from sickness behaviour at the time of test, the core body temperature was used as a surrogate measure to determine the effect of LPS treatment in general behaviour. Animals displayed a significantly lower body temperature when tested 2h after LPS treatment that subsided on subsequent treatment days (figure 3.9) similar to what was previously seen in the animals treated with an acute 1mg/kg of LPS.

Animals were also tested in the rotarod. Based on the results, no significant difference was observed between two groups, implying that during the time of test, the motor coordination of the rodents were unaffected by LPS treatment (figure 3.10).

3.1.3 Effect of twenty doses of LPS (1mg/kg, 10 days, twice daily) treatment

When tested in the MWM, the effect of LPS treatment was absent as no significant difference was observed in latency, swimspeed and pathlength (figure 3.11). The lack of treatment effect may arise from rats developing tolerance to the repeated dosing of LPS.

3.1.4 Effect of increasing dose of LPS (0.25 to 16 mg/kg, 10 days, twice daily) treatment

In order to prevent endotoxin tolerance from developing, an increasing dose of LPS was given to rats using a dosing regime by Chen et al. (2005). Due to an increase in dose concentration and duration, animals were given more time to recover from the side effect of LPS treatment to 40h. When tested in the MWM, LPS treated rats showed a spatial learning deficit on SC day 1 as demonstrated by the longer escape latency and pathlength while there was no significant difference in swim speed (figure 3.12). The vehicle treated animals had a steeper learning curve when compared to LPS treated animals as the LPS treated animals took much longer time to remember the location of the platform (figure 3.13). However on subsequent days, LPS treated animals performed as well as vehicle treated animals as there was no significant difference in the latency and pathlength on SC day 2 to SC day 4, suggesting that the spatial memory remains intact.

To determine whether spatial memory was the only memory domain that was affected by the increasing LPS dosing regime, animals were also tested in NOR. Based on the T1 trial results, both treatment groups spent an equal amount of time exploring the objects, suggesting that there was no difference in the motivation of the animals to explore the objects (figure 3.14a). When animals were tested one hour later in T2, the vehicle treated animals spent a significantly more time exploring the novel object as compared to the familiar object. The LPS treated animals however, spent an equal amount of time exploring both novel and familiar objects (figure 3.15a). Based on the D2 index that normalised the effect to the total time spent exploring both objects, the vehicle treated animals were able to remember which objects they had seen previously. However the LPS treated animals were unable to remember which objects they had seen previously and spent an equal amount of time exploring both familiar and novel objects (figure 3.15b). Therefore, the increasing dose of LPS treatment induced an object recognition deficit that has not been reported prior to this.

In addition to this, LPS treated animals were also tested in a fear conditioning paradigm. Previous study has shown that LPS is able to elicit allodynia in rodents (Danzter 2004). Hence a blind study to determine the fear responses to the electrical shock were conducted to ensure the increasing LPS dosing regime did not induce allodynia. The results demonstrated that there was no significant difference in the fear response to the electrical shock between LPS and vehicle treated animals (figure 3.16a).

In the cued fear conditioning, rats are required to learn the associations between the cue (CS) with the shock (US). When LPS treated animals were tested in the cued fear conditioning paradigm, there was essentially no difference in the response between the LPS and vehicle treated group (figure 3.16b). The LPS treatment failed to induce any cognitive deficits in the cued fear conditioning paradigm signifying that peripheral LPS treatment did not induce any disruption in the amygdala dependent fear learning (Philip and LeDoux 1992).

In the contextual fear conditioning, rats are required to associate the shock to their environment. Similarly, when the LPS treated animals were returned to the test chamber 24h later, the freezing was recorded over a period of 10 min. In the first four minutes, there were no significant difference between the LPS and vehicle animals, suggesting that both groups were able to associate the environment and the shock received the previous day (figure 3.16c). However, the vehicle animals demonstrated a steeper reduction in the percentage of freeze. By the end of the tenth minute, the percentage of freeze observed was similar to the beginning of the test. On the other hand, LPS animal spent more time freezing throughout the ten minutes of the recall trial. In total, the LPS treated animals froze more than 50% of the time compared to 20% in vehicle animals.

To ensure that the animals had fully recovered from the side effect of LPS treatment, a battery of tests were conducted to monitor the overall behaviour, motor coordination and core body temperature.

The overall general behaviour of the rodents was investigated using LABORAS™. The rats were monitored 2h after the first dose of LPS at 0.25mg/kg. Based on figure 3.17, at 2h after a low dose of 0.25mg/kg LPS, LPS treated animals showed significant difference in behaviours such as locomotion, immobilisation and rearing. Animals spent less time in locomotion and rearing. They also spent more time immobilised. Hence, even at a low dose of LPS, it was sufficient to induce sickness behaviour that was described previously.

At the end of the increasing LPS dosing regime, animals were examined once again. There was no significant difference observed in the general behaviours (locomotion, rearing and immobilisation) of LPS animals when compared to the vehicle treated animals, suggesting that the sickness behaviour component observed previously was tolerated by the end of the 10 days dosing regime (figure 3.18).

The core body temperatures were recorded throughout the ten days of dosing. Previously, it has been shown that the LPS treatment induced a significant reduction of body temperature after the first dose of LPS treatment at 1mg/kg. Similarly, in the increasing LPS dosing regime, the first dose of LPS treatment at 0.25mg/kg induced a hypothermic effect in the treated animals (2h after treatment). On subsequent treatment, the animals showed no difference in their core body temperature compared to the vehicle treated animals (figure 3.19). The reduction of body temperature after LPS treatment has also been previously observed where systemic injection of LPS could increase the possibility for development of a hypothermic response rather than fever at ambient temperatures

below 30°C as a regulated adaptive strategy against immunological challenge (Akarsu and Mamuk 2007).

Finally, the effect of the increasing LPS dosing regime was also tested in the rotarod to determine whether the LPS treatment had elicit a disruption in motor coordination. The LPS treated animals displayed similar motor coordination and motivation as vehicle treated animals with similar amount of time spent on the rotarod (figure 3.20).

Similar to results obtained from the single acute dose of LPS, the overall general behaviour suggest that at 40h after the completion of the dosing regime, the animals were devoid of any potential side effects that could confound the results obtained in the cognitive tests. Based on the results obtained from the previous results, the learning and memory deficits seen, could be attributed to the ability of the animals to tolerate the sickness behaviour component by repeatedly dosing the animals with LPS over a period of ten days (as seen from the 20 doses of 1mg/kg LPS over a period of 10 days) but was still sufficient to drive the cognitive deficits.

However, the increasing LPS dose regime, does has its caveats. LPS has previously been shown to produce an anorexic effect (Dantzer 2004). Therefore, the weight changes of animals were monitored during the ten days of treatment. At the beginning of the treatment, a significant weight loss that is reflected by a reduction in food consumption, indicative of the anorexia induced by sickness behaviour, was observed (figure 3.21). However, by day 4, the animals seemed to have recovered from the LPS treatment and

showed similar weight gain with the vehicle treated animals. However at the higher doses (e.g. 16mg/kg) the LPS animals showed a stabilised weight without further increase or decrease, while the vehicle animals continue to gain weight. The stabilisation of the weight during the last few days of treatment is also reflected in a reduction in the food consumption but to a lesser extend as to what was seen earlier in the treatment regime. However, as soon as LPS treatment was completed, the LPS treated animals shown an increase in the food consumption, suggesting that the effect was temporary.

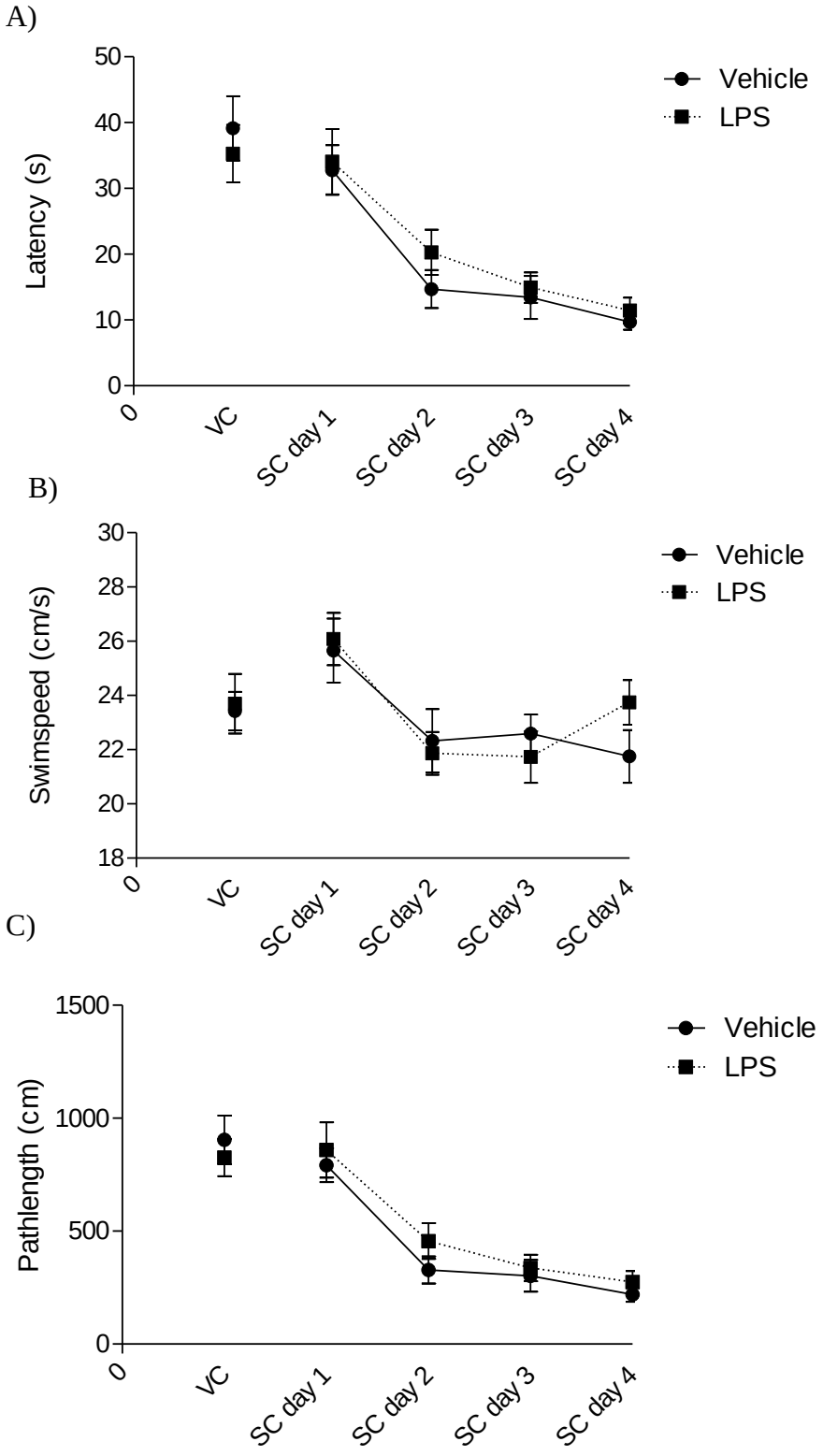


Figure 3.1 Effect of single dose of LPS (1mg/kg) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden platform in the Morris watermaze test. Data points represent mean ± SEM. n=10

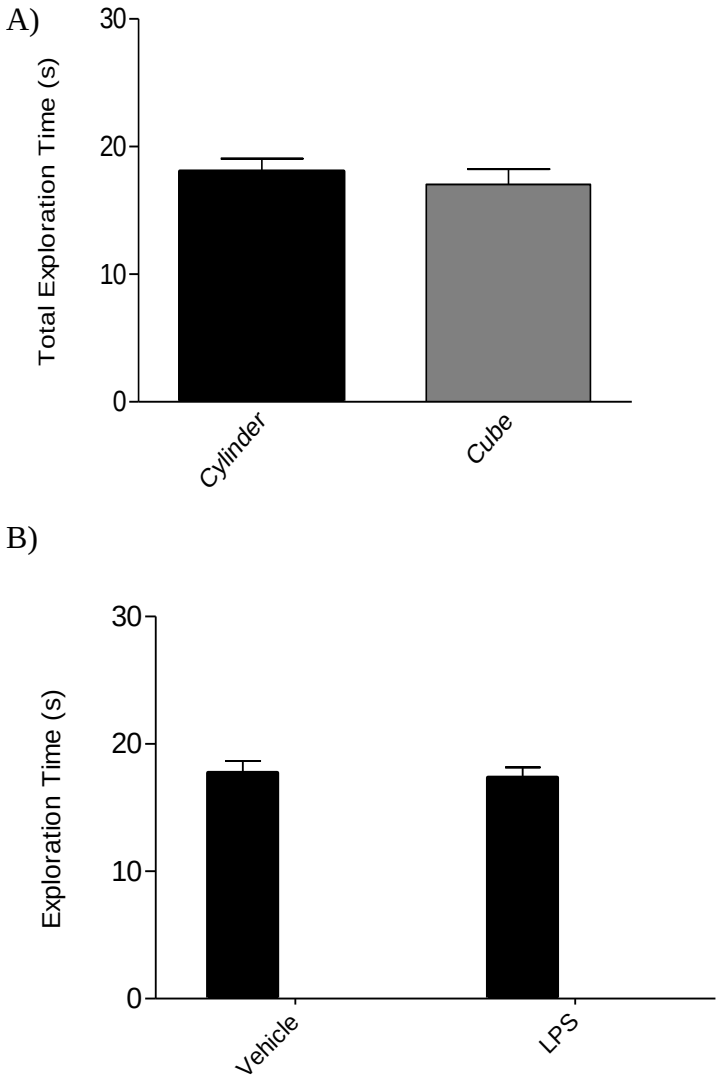


Figure 3.2 Effect of single dose of LPS (1mg/kg, IP) on NOR T1 (A) trial objects exploration time and (B) trial exploration time. Data points represent mean ± SEM. n=10

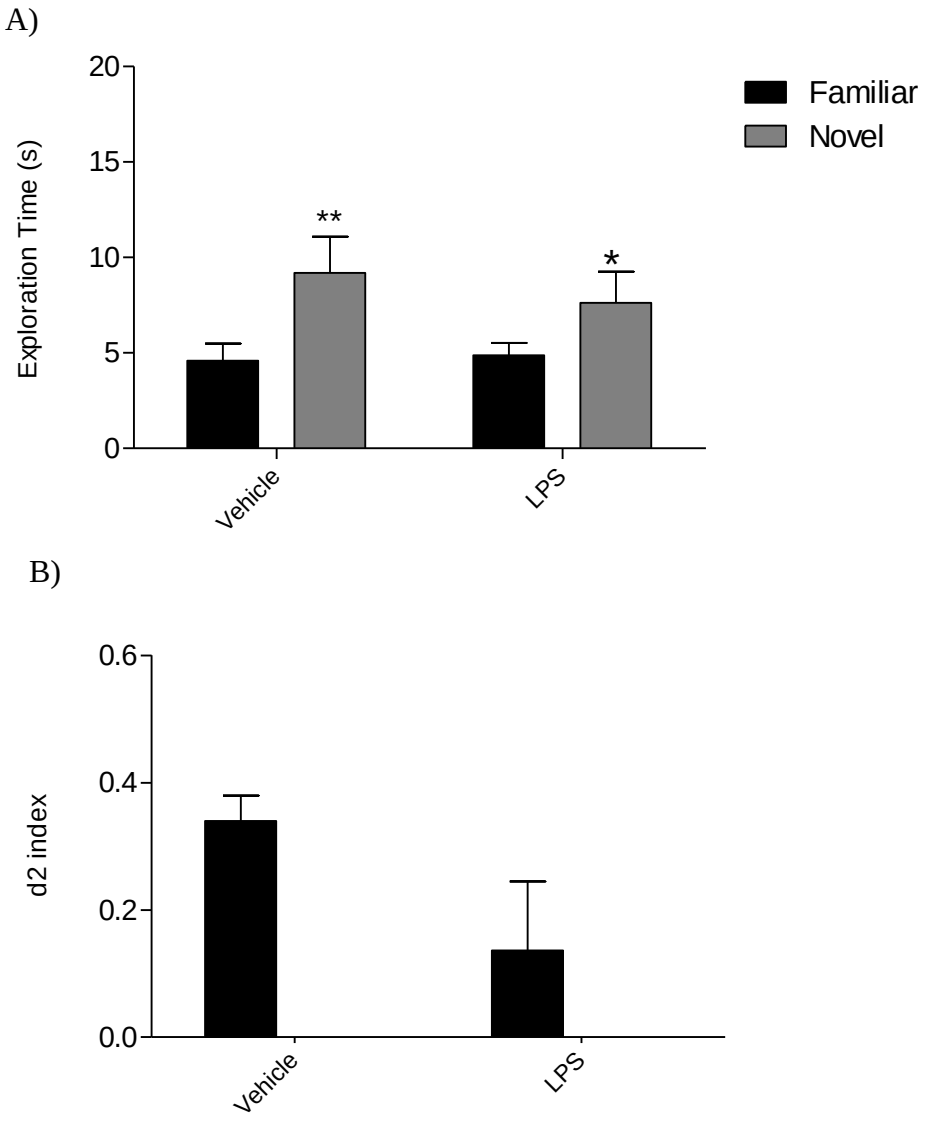


Figure 3.3 Effect of single dose of LPS (1mg/kg, IP) on NOR T2 trial novel and familiar objects (A) exploration time and (B) discrimination (d2) index. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$, **$p<0.01$). n=10

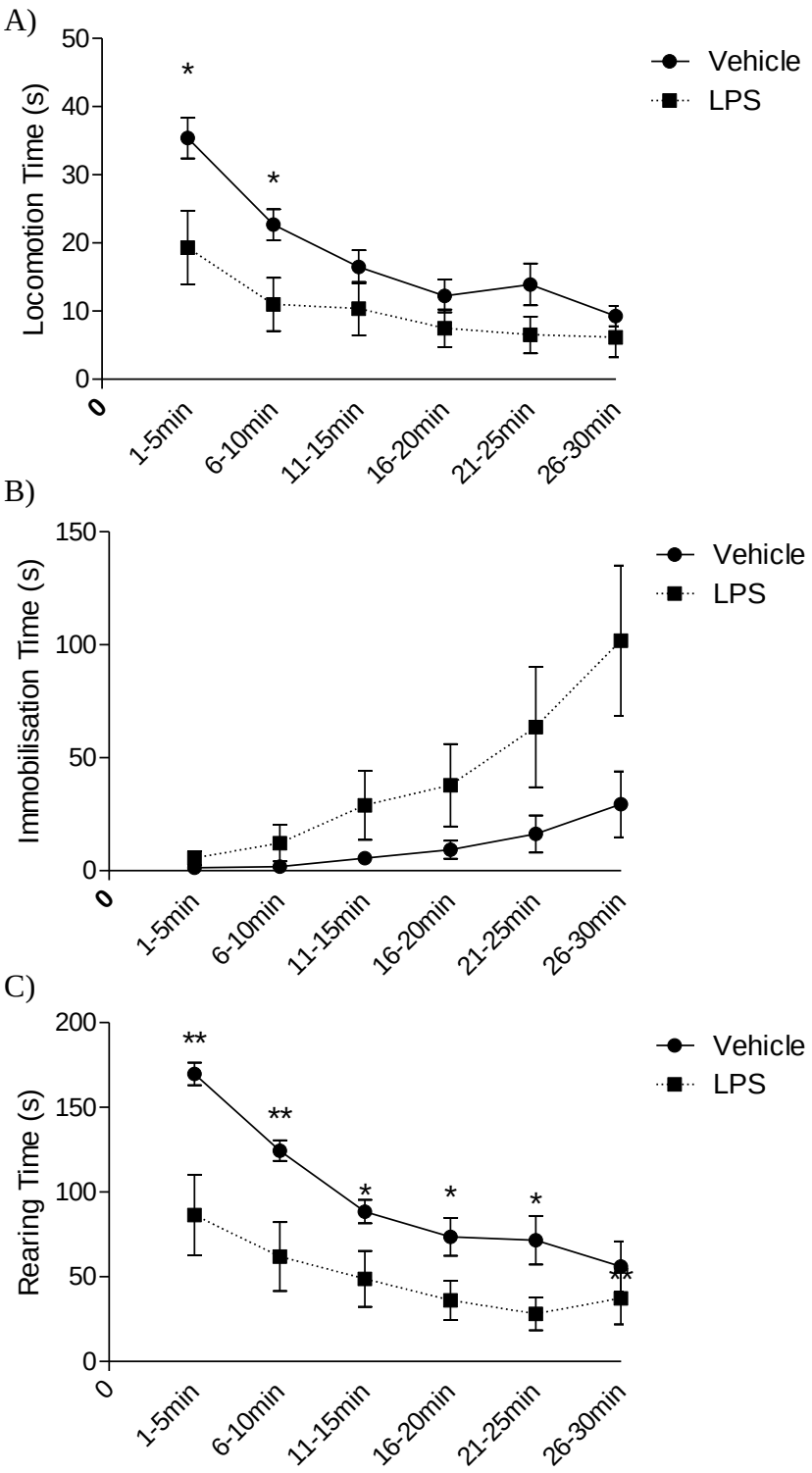


Figure 3.4 Effect of single dose (1mg/kg) of LPS on (A) locomotion (B) time spent immobilised and (C) rearing using the LABORAS™ at two hours after LPS treatment. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*p<0.05, **p<0.01). n=10.

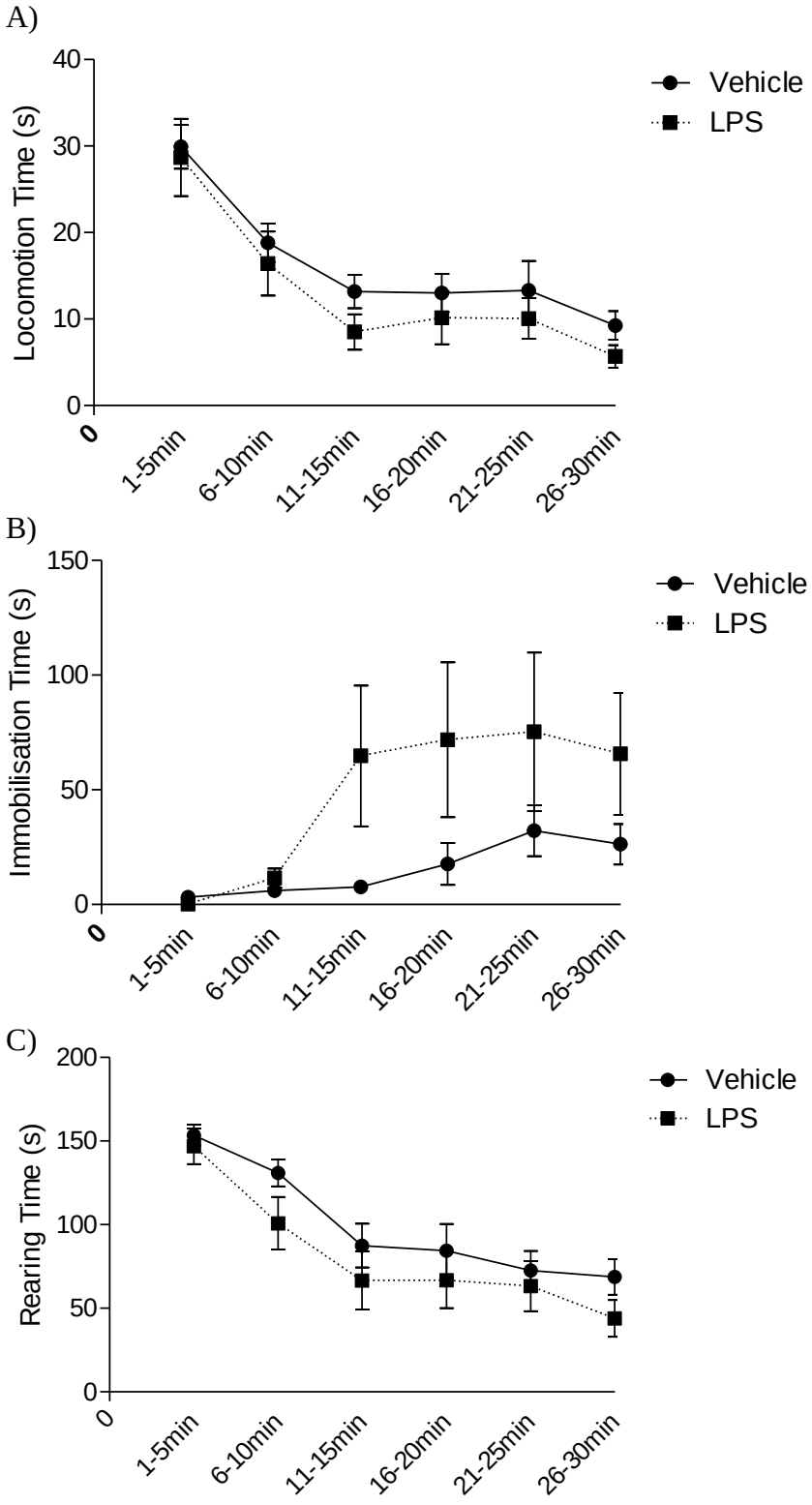


Figure 3.5 Effect of single dose (1mg/kg) of LPS on (A) locomotion (B) time spent immobilised and (C) rearing using the LABORAS™ at 24 hours after LPS treatment. Data points represent mean ± SEM. n=10.

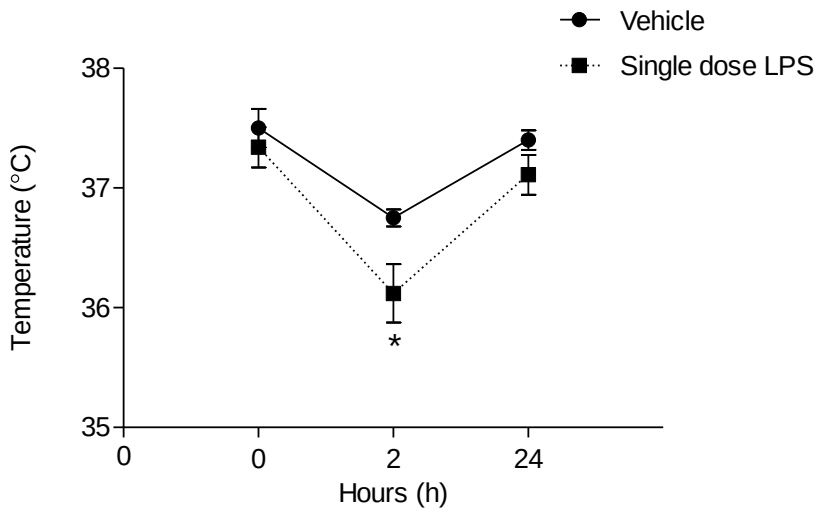


Figure 3.6 Effect of single dose of LPS (1mg/kg) on body temperature (°C). Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5

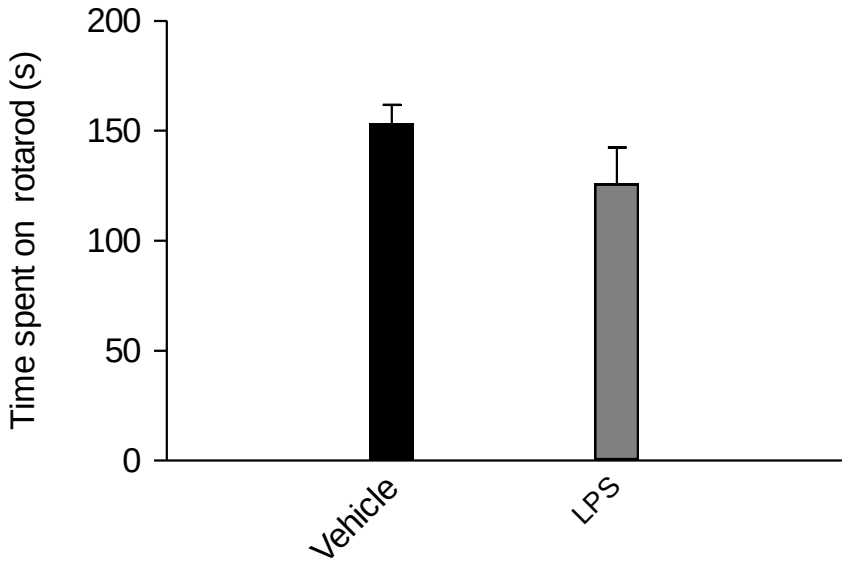


Figure 3.7 Effect of single dose of LPS (1mg/kg) on rotarod (s). Data points represent mean ± SEM. n=5

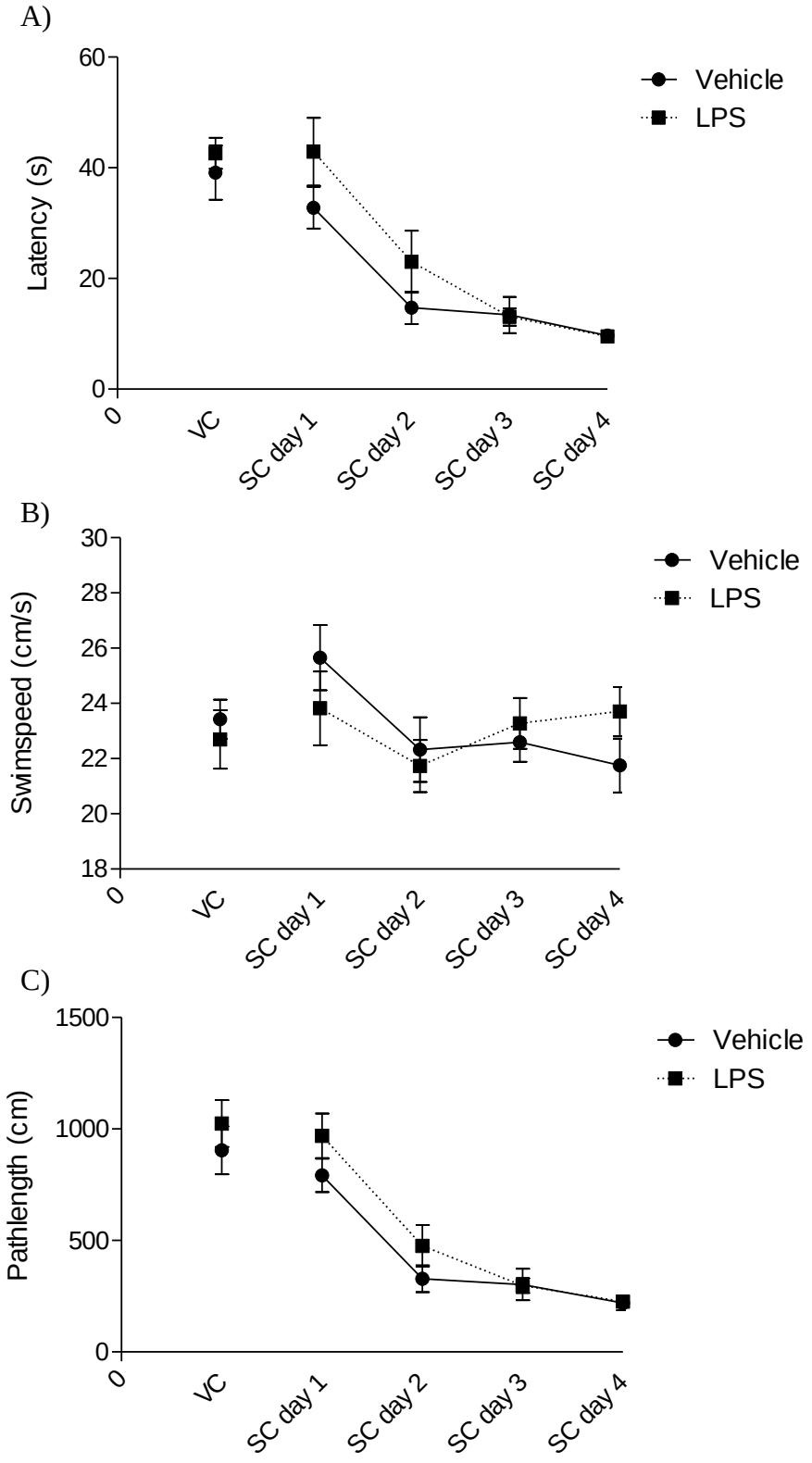


Figure 3.8 Effect of three doses of LPS (1mg/kg, once daily for three days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden platform in the Morris watermaze test. Data points represent mean ± SEM. n=10

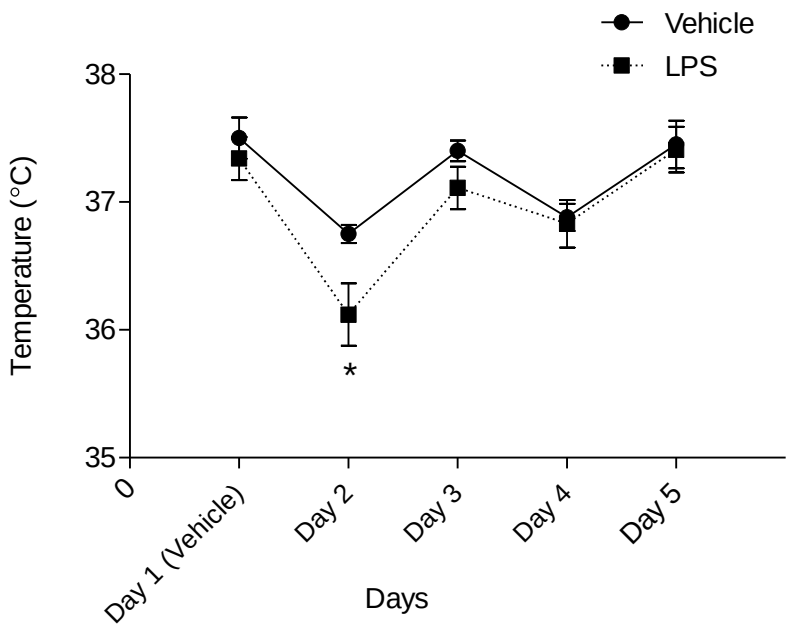


Figure 3.9 Effect of three doses of LPS (1mg/kg, once daily for three days) on body temperature. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*p<0.05). n=5

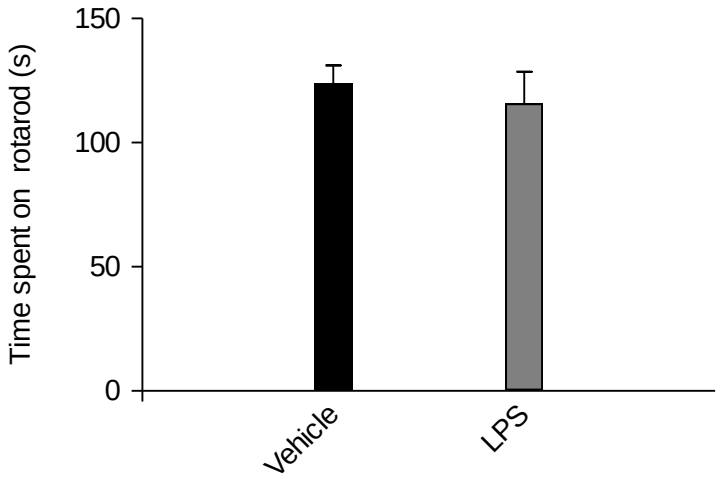


Figure 3.10 Effect of three doses of LPS (1mg/kg, 3 days) on rotarod (s). Data points represent mean ± SEM. n=5

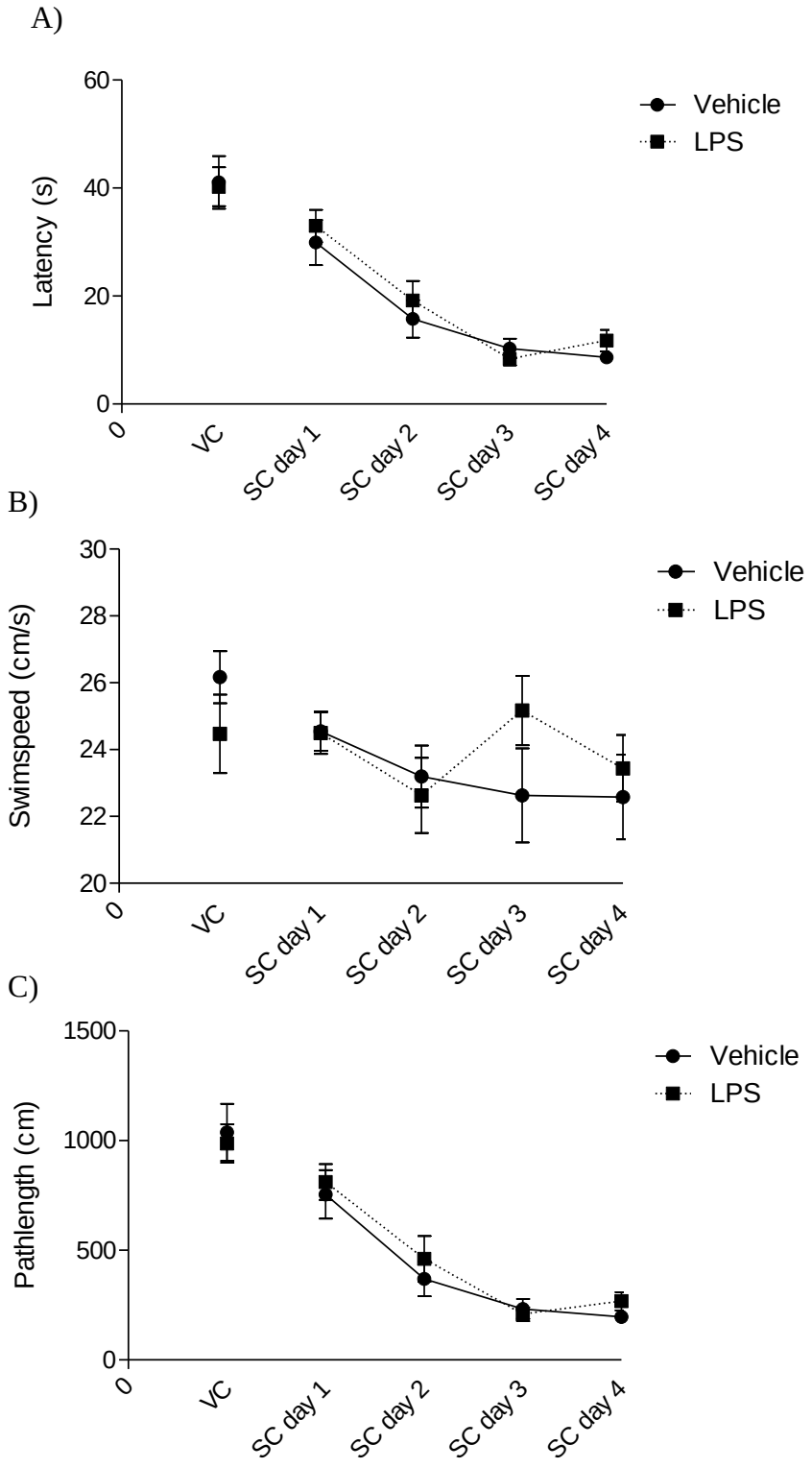


Figure 3.11 Effect of constant dose of LPS (1mg/kg, twice daily for ten days) on (A)escape latency (B) swimming speed and (C) pathlength to find the hidden platform in the Morris watermaze test. Data points represent mean ± SEM. n=10

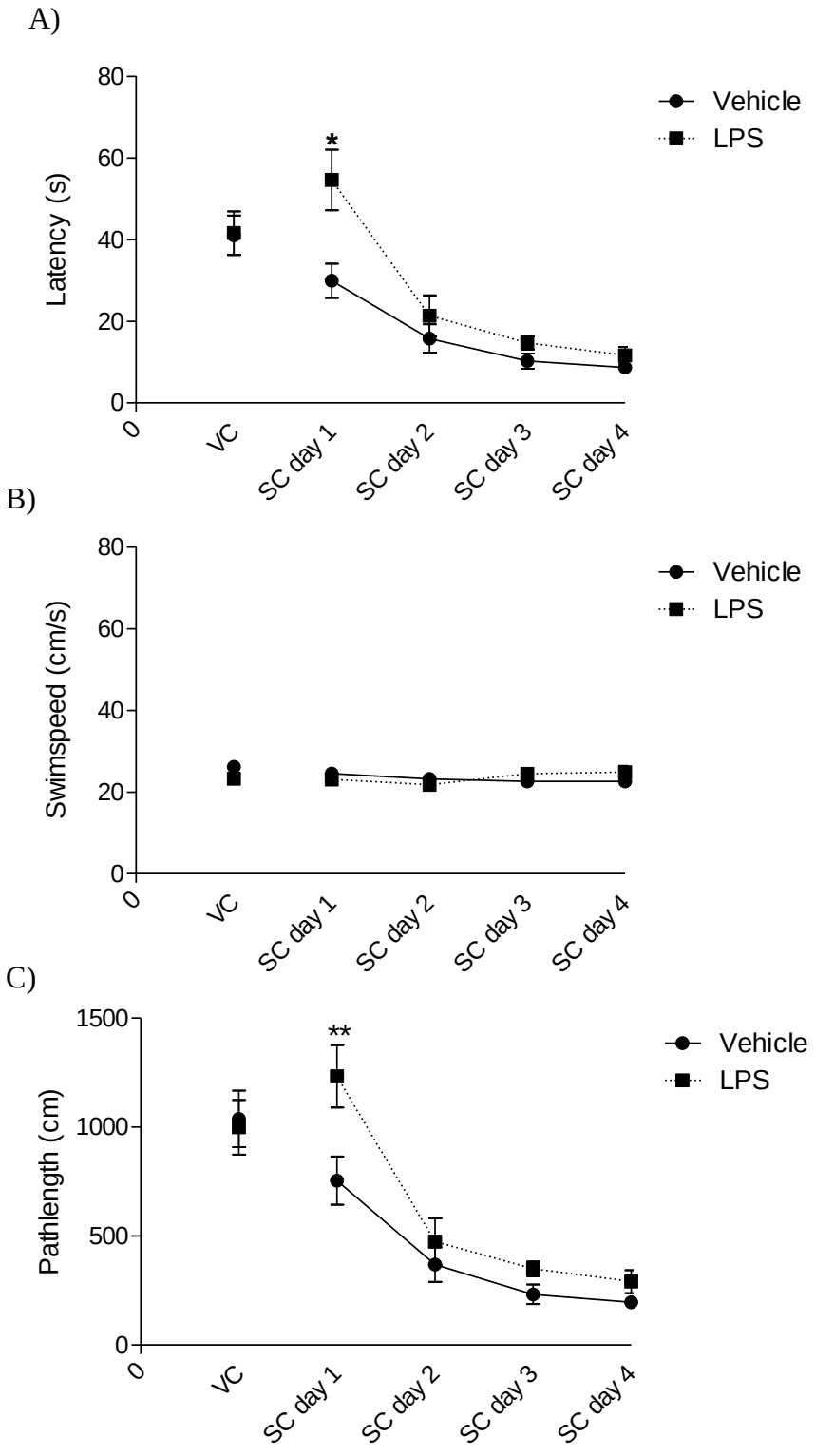


Figure 3.12 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden platform in the Morris watermaze test. Data points represent mean ± SEM. (* $p<0.05$, ** $p<0.01$). n=10.

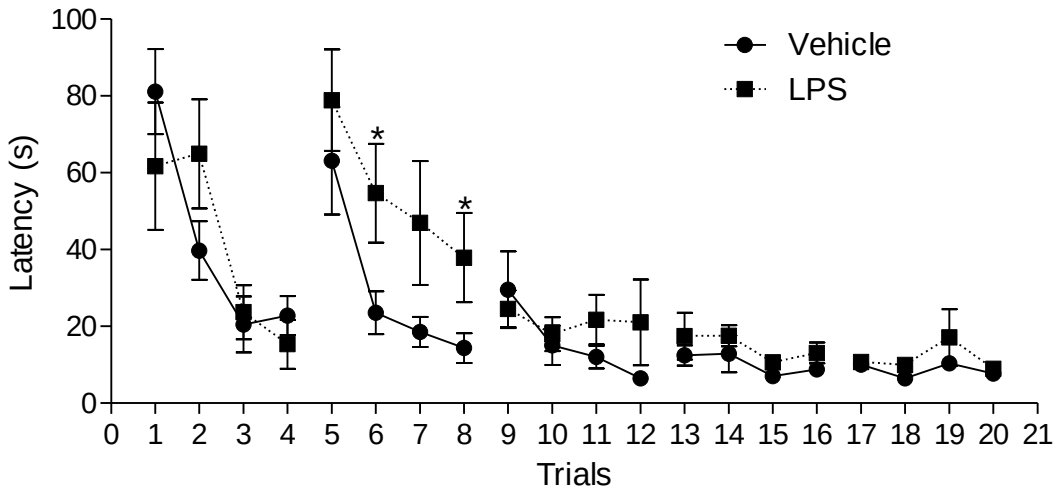


Figure 3.13 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on escape latency required to find the hidden platform in Morris watermaze test performance. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=10

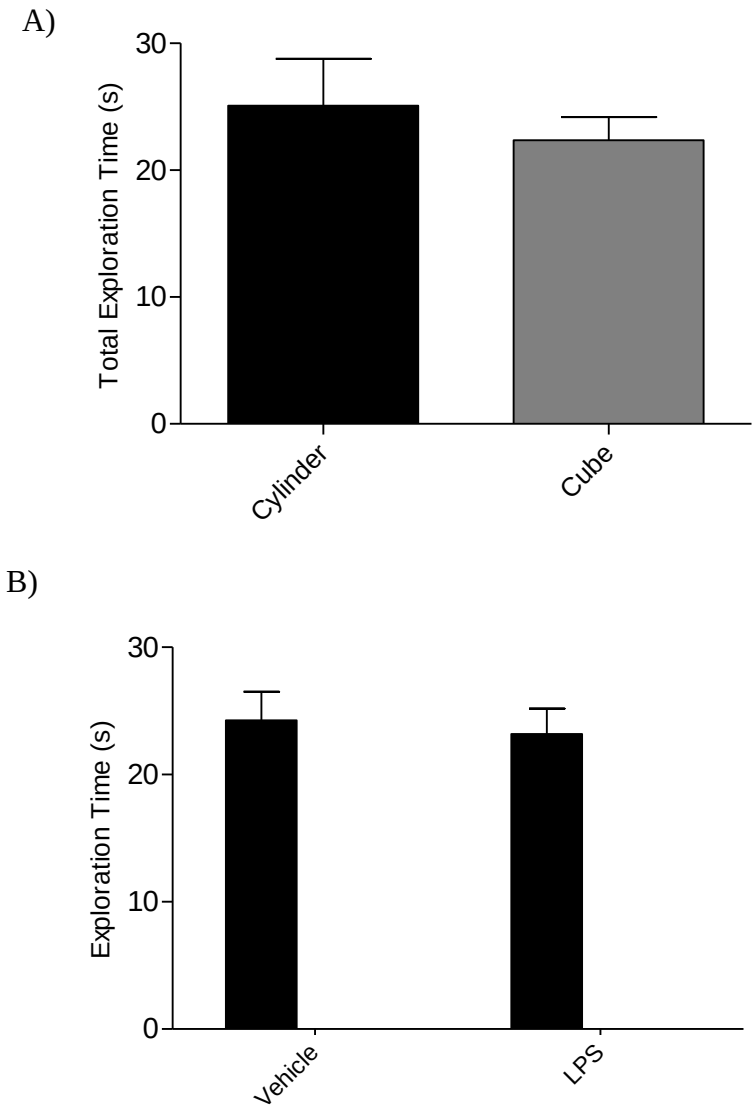


Figure 3.14 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on NOR T1 trial (A) objects exploration time (B) trial exploration time. Data points represent mean ± SEM. n=10.

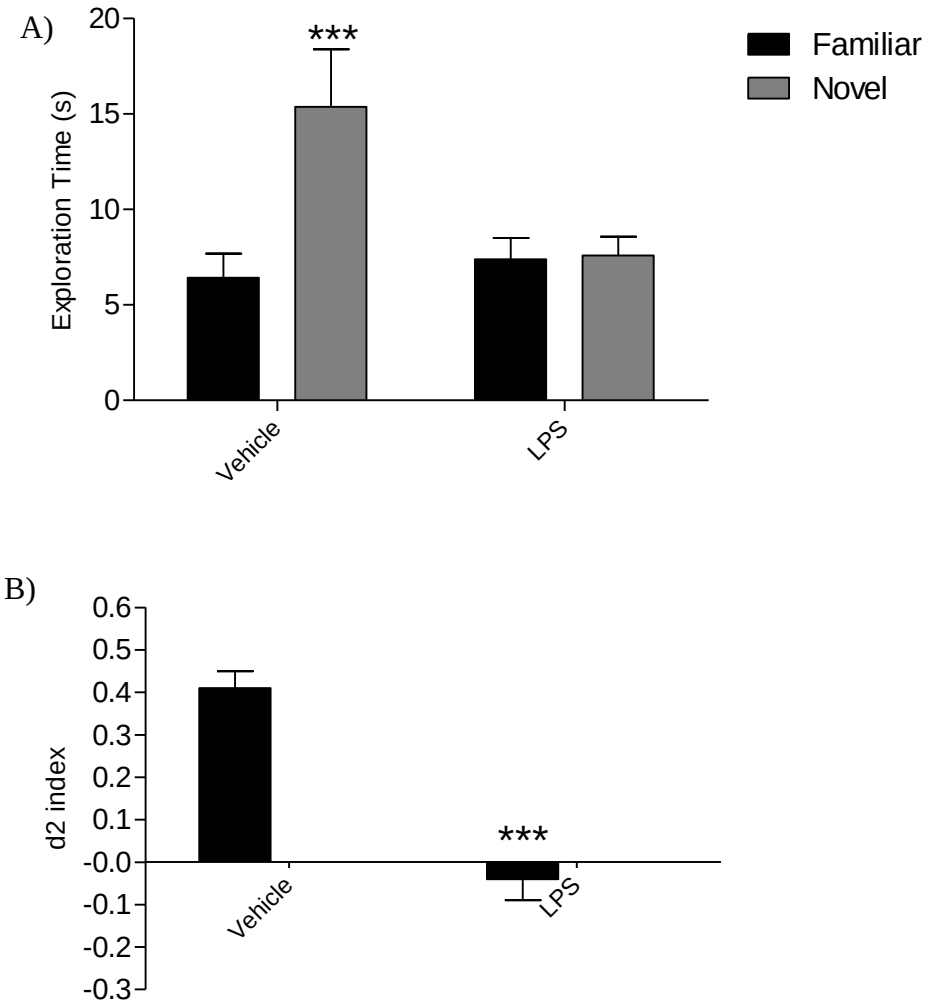


Figure 3.15 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on NOR T2 trial novel and familiar (A) objects exploration time (B) discrimination (d2) index. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (***$p<0.001$). n=10.

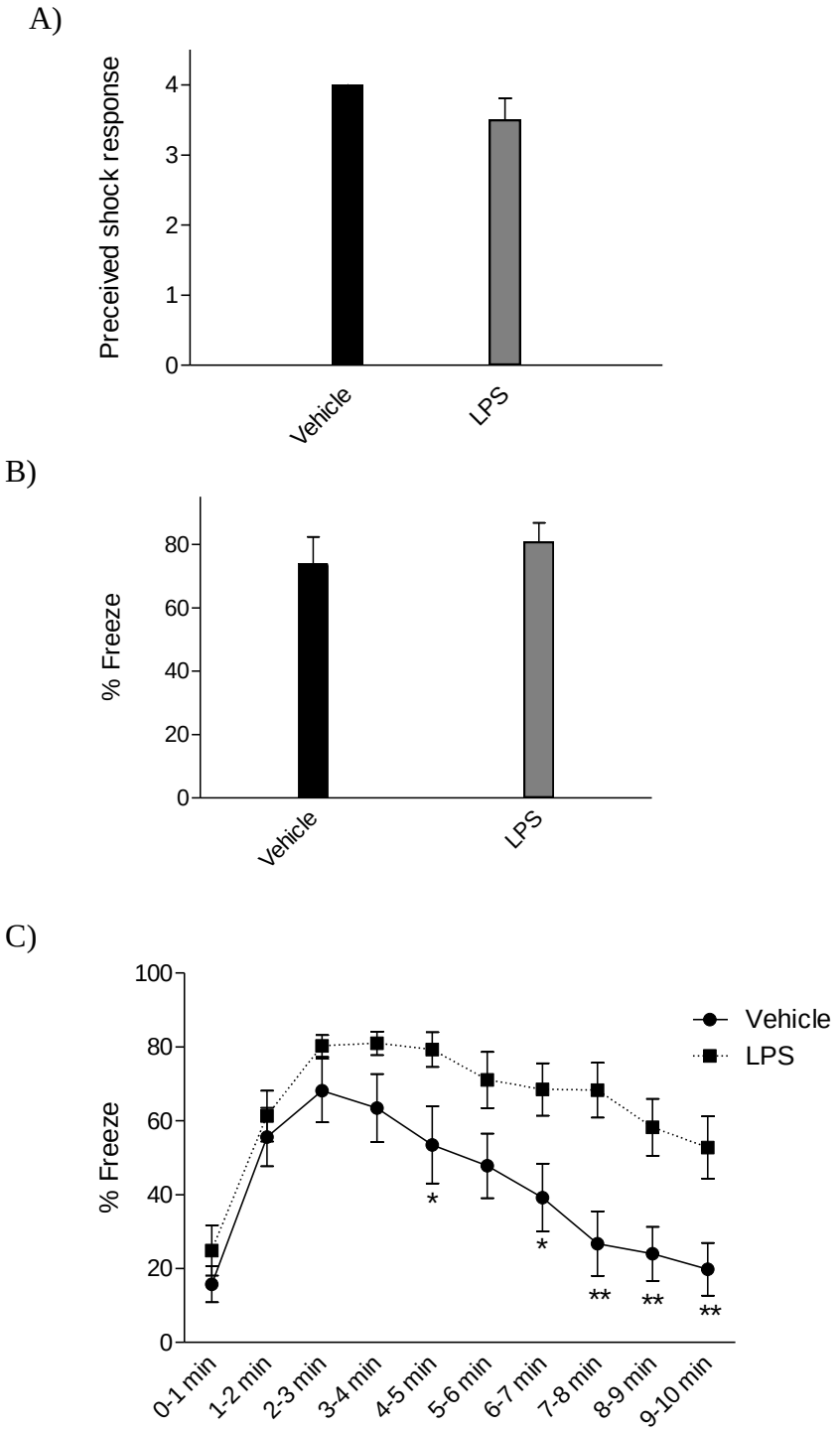


Figure 3.16 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) (A) on hyperalgesia. Animals were scored based on the shock response by an observer that was unaware of the treatment groups (B) cued fear conditioning (C) contextual fear conditioning. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$, ** $p<0.01$). n=10.

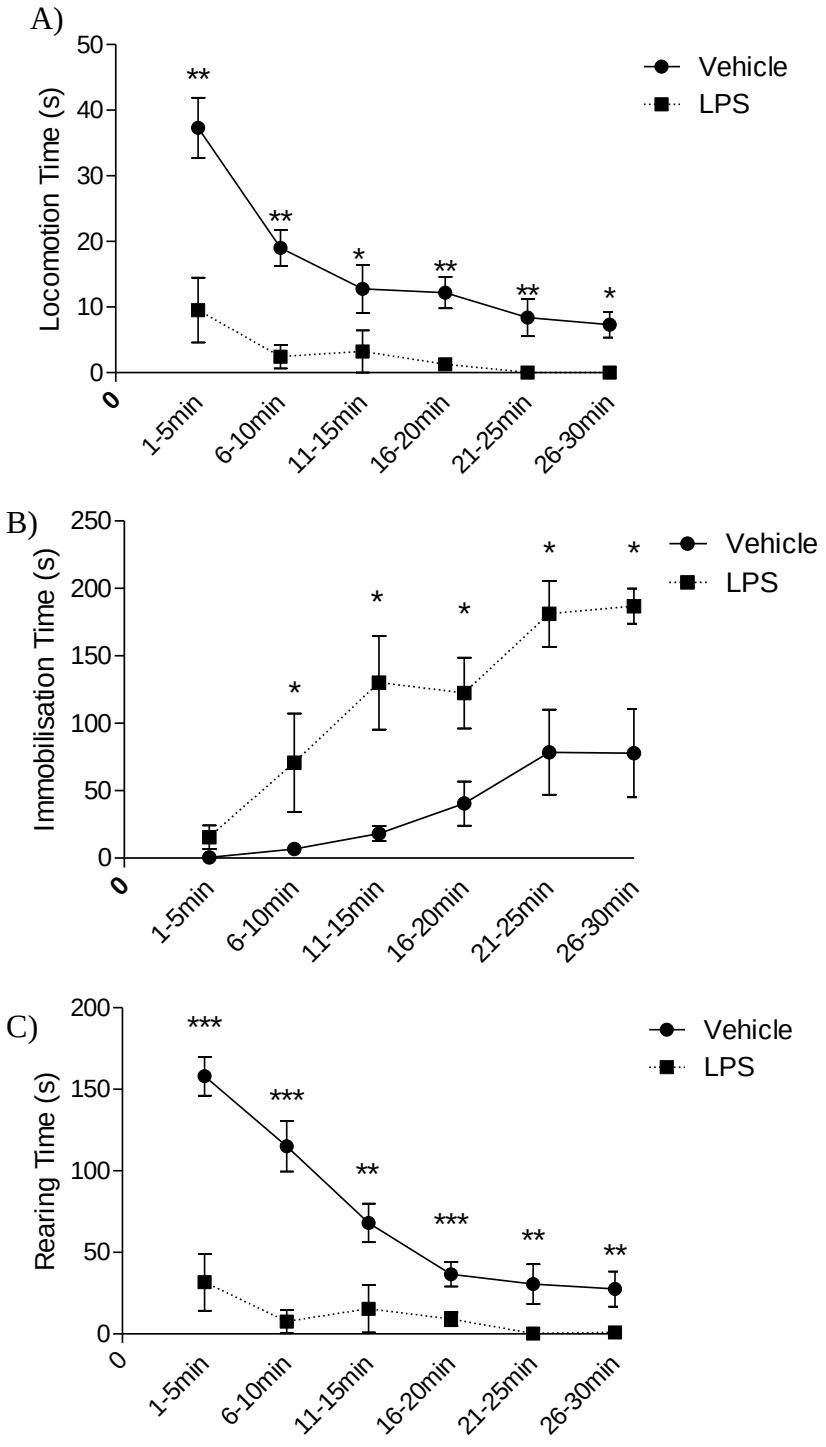


Figure 3.17 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) locomotion (B) time spent immobilised and (C) rearing using the LABORAS™ at two hours after the first LPS treatment (0.25mg/kg). Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). n=10.

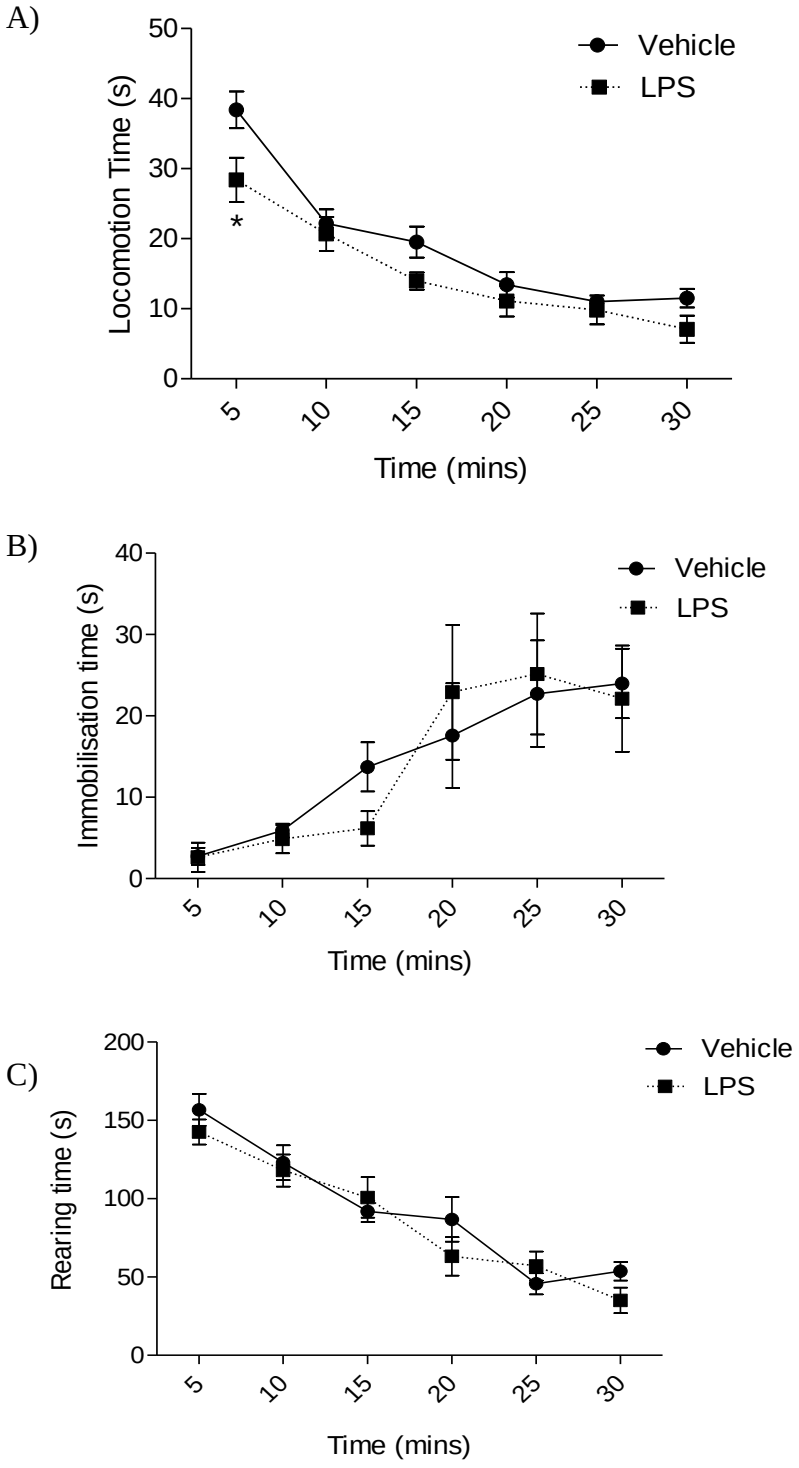


Figure 3.18 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) locomotion (B) time spent immobilised and (C) rearing using the LABORAS™ 40 hours after the last LPS treatment. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=10.

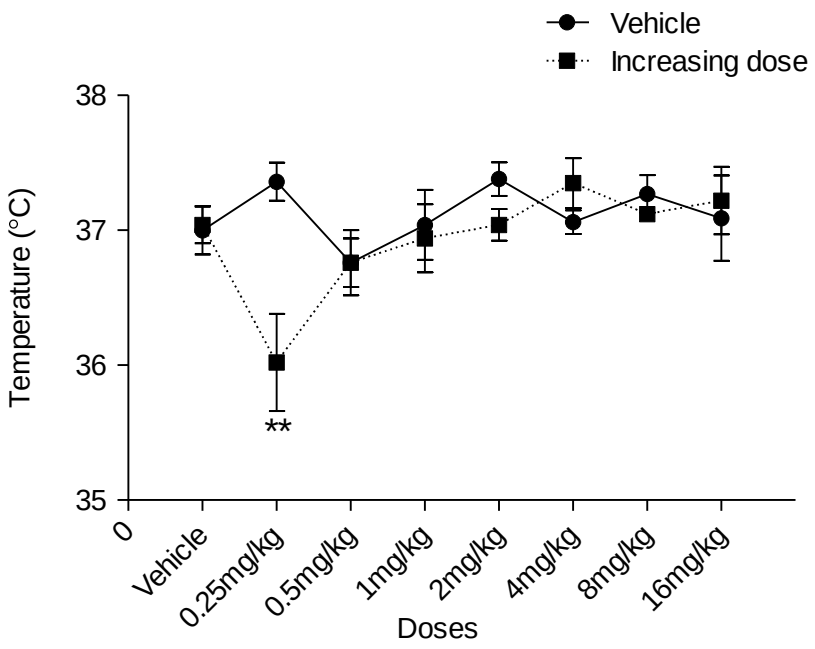


Figure 3.19 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on body temperature. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (**p<0.01). n=5.

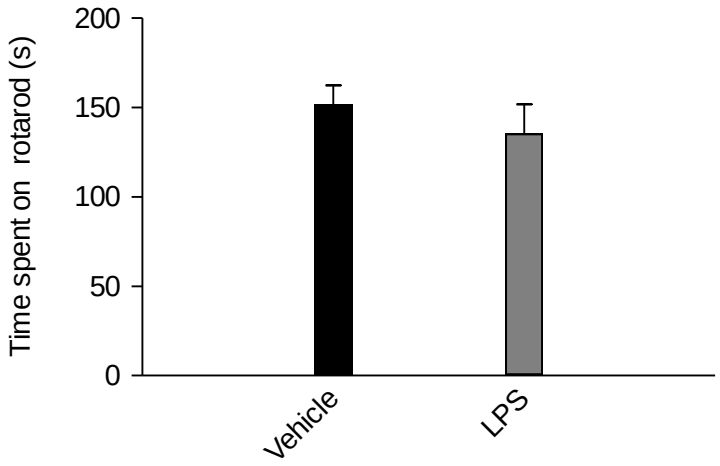


Figure 3.20. Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on rotarod (s). Data points represent mean ± SEM. n=5.

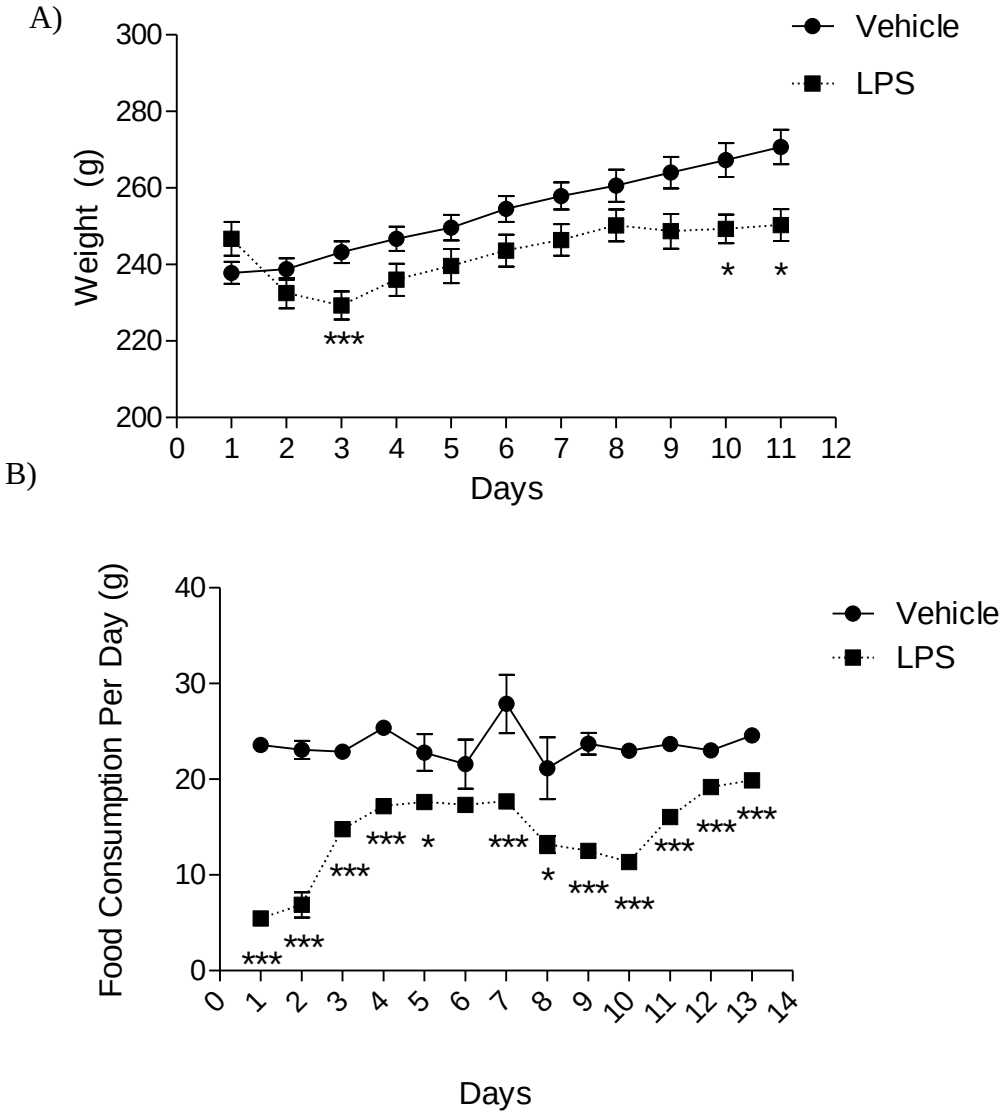


Figure3.21 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) body weight and (B) amount of food consumed (g/day/animal). Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$, ***$p<0.001$). n=10.

3.2 Effect of LPS treatment in inducing inflammation

3.2.1 Effect of LPS treatment in TNFα expression

Production of cytokines, such as TNFα, is elevated during inflammation. To study the effect of the increasing dose of LPS in inducing inflammation, a TNFα response profile was conducted.

The peripheral TNFα response profile was obtained from blood plasma in which the animals were culled two hours after each dose increment or after the final dose. The LPS treated animals showed elevated levels of TNFα after the first dose of LPS treatment at 0.25mg/kg. However, in subsequent treatments, similar TNFα levels were observed in both groups, suggesting that TNFα response was abolished by the time the animals received the 0.5mg/kg LPS dose (figure 3.22).

The protein level for TNFα in a peripheral organ such as the liver was also obtained. When liver samples were obtained 2 hours after the first LPS treatment at 0.25mg/kg, an elevated TNFα protein level was detected in the liver (figure 3.23). When liver samples were obtained 2 hours after the last LPS treatment at 16mg/kg, there was no significant difference in the protein level in both treatment groups (figure 3.24). Thus, both ELISA blood plasma and the protein level showed that only at the 0.25mg/kg and by the end of the treatment, the TNFα response was abolished.

To find out if the CNS is affected by LPS induced inflammation, the brain of animals treated with either LPS or vehicle were harvested 2 hours after the first dose of LPS at

0.25mg/kg and the last dose of LPS at 16mg/kg ten days later. The brain was subdivided into two different regions namely the hippocampus and cortex as both brain regions have been previously been shown to be involved in learning and memory. The western blot data from these brain samples suggest that after the first dose of LPS treatment at 0.25mg/kg, a significant elevation of TNFα protein was expressed in both the hippocampus and cortex (figure 3.25 and 3.27). Peripheral administration of LPS is able to elicit an inflammatory response in the CNS. More interestingly, brain samples from animals that have completed the ten days dosing regime showed a significant elevation in TNFα protein level in both brain regions (figure 3.26 and 3.28). This suggested that there may be a differential effect of LPS in CNS and peripheral system.

3.2.2 Effect of LPS treatment in microglia

Microglia are the prime component of the CNS immune response. Hence, to understand the effect of LPS in inducing neuroinflammation in the CNS, the effect of LPS on microglia was investigated.

To study the effect LPS treatment may have on microglia, the immunohistochemistry staining using CD11B/CD18 was selected as this protein is only expressed by the microglia in the CNS. CD11B/CD18 is part of the complement receptor (CR) 3 and is also known as the macrophage antigen complex 1 (MAC1). In orchestrating inflammation, the MAC1 performs diverse functions involved in adhesion, chemotaxis and phagocytosis. Furthermore, its expression has been shown to be elevated in numerous neurodegenerative diseases such as AD (Gao and Hong 2008). The hippocampus,

especially the dentate gyrus was examined. The immunohistochemistry staining suggests that there is an increase expression of CD11b/CD18 as seen from the intensity of the staining (figure 3.29).

In addition to immunohistochemistry, Western blot analysis was used to determine the CD11B/CD18 protein level in the CNS. Based on the data, it suggests that there is an increase expression of MHC II in the cortex and hippocampus (figure 3.30 and 3.31). There is evidence to suggest that the peripheral administration of LPS is able to induce changes in microglia in which more antigens that implicates the function of microglia as antigen presenting cells are expressed.

3.2.3 Effect of LPS on myeloperoxidase activity

To protect the body against invading microgorganisms, microglia produces free radical such as hypochlorite (OCl^-) and radicalized oxygen species (O_2^-, $ONOO^-$). The generation of free radicals require the conversion of hydrogen peroxide by the MPO enzyme and has been regarded as a key component of inflammation.

After the first treatment of LPS, there was an increase in the MPO activity in the spleen when compared to the naïve animal and in subsequent dose; a lower level of MPO activity was observed (figure 3.32). Albeit there is no significant difference observed in the data obtained, there is a similar trend observed in the periphery system with the TNFα cytokine level expression (figure 3.22)

When the activity of the MPO enzyme in the CNS was determine, there was no significant difference in the MPO activity in all treatment group, although the LPS treated animals did display a trend to an increase in MPO activity in the hippocampus (figure 3.32).

3.2.4 Effect of Indomethacin in reversing the LPS induced cognitive deficit

To confirm that the cognitive deficit seen was due to neuroinflammation induced by the peripheral LPS administration, animals treated with increasing dose of LPS were treated with indomethacin, an anti inflammatory agent.

Similar to results obtained previously (figure 3.12 and 3.13), animals treated with LPS performed significantly worse in SC day 1 and in this instance on SC day 2 as well as longer time was required to find the platform location (figure 3.33). However animals treated with 1mg/kg indomethacin 15 minutes prior to each morning dose of LPS treatments no learning and memory deficit was observed. No significant difference was observed in both swim speed and path length.

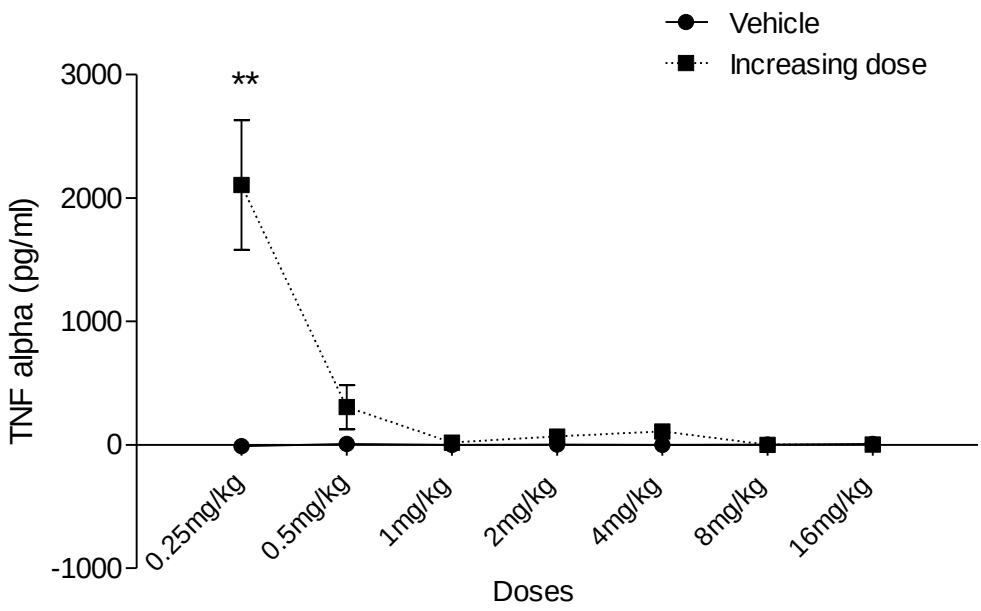


Figure 3.22 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on TNFα level. Plasma was collected 2 hours after each dose increment. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (**p<0.01). n=10.

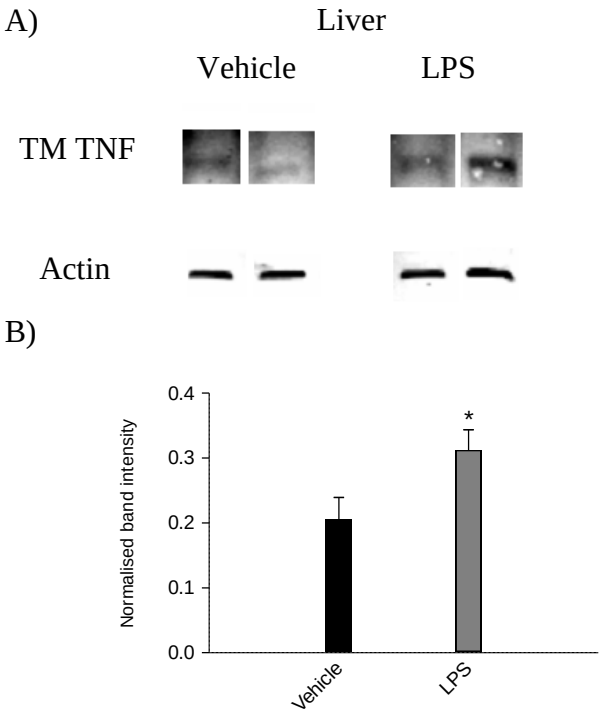


Figure 3.23 Effect of a single dose of 0.25mg/kg of LPS on transmembrane tumour necrosis factor (TM TNF) using whole cell lysate samples (3ug/lane) in the liver A) representative TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF to actin. The average intensity of the TM TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

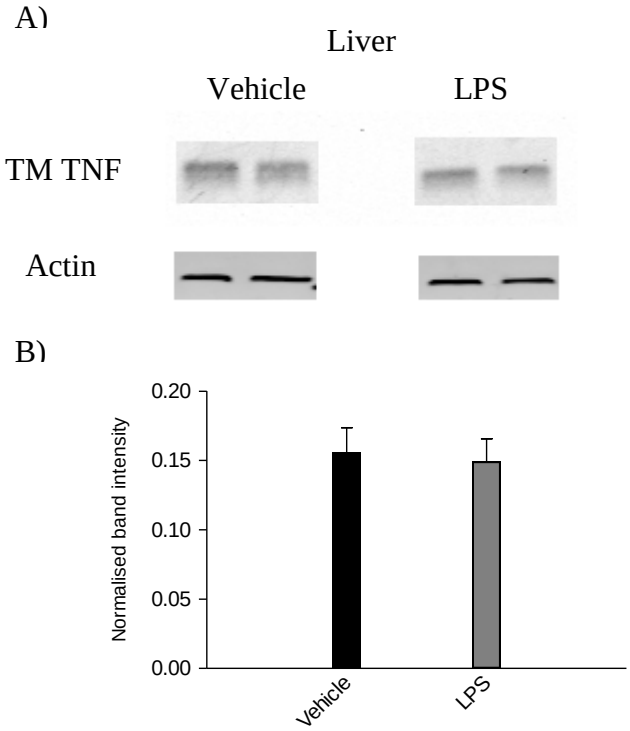


Figure 3.24 Effect of increasing dose of LPS on transmembrane tumour necrosis factor (TM TNF) using whole cell lysate samples (3ug/lane) in the liver A) representative TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF to actin. The average intensity of the TM TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. n=5.

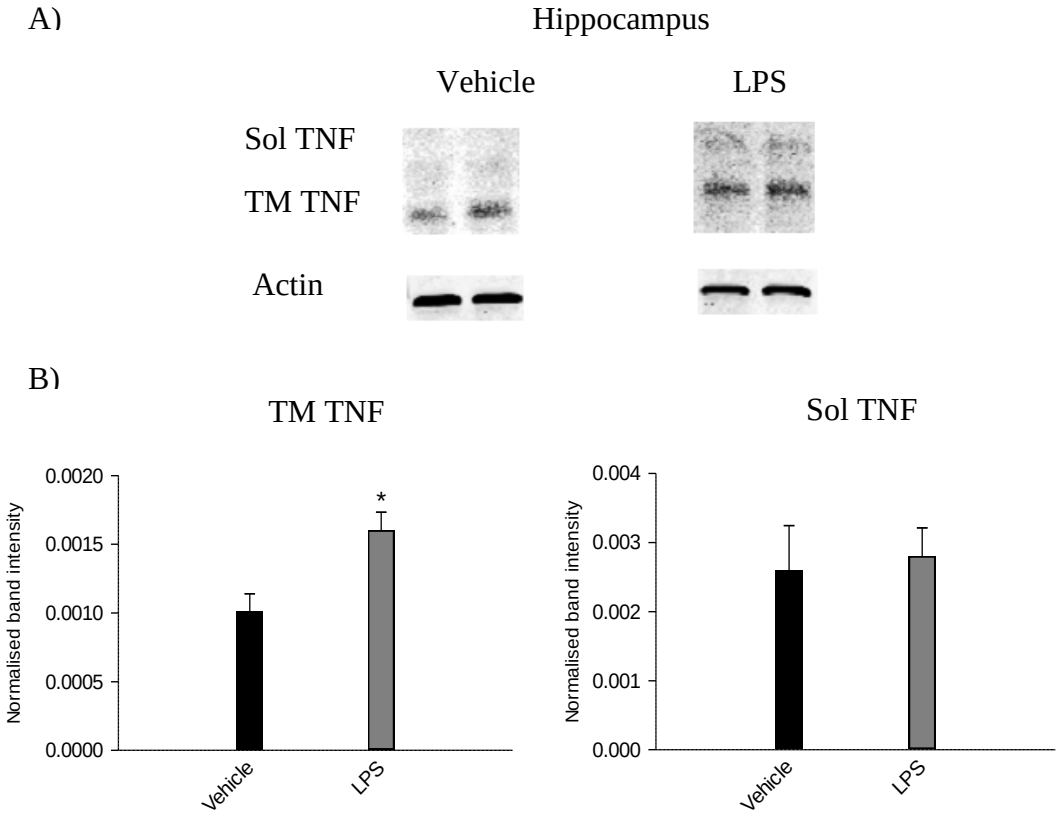


Figure 3.25 Effect of a single dose of 0.25mg/kg of LPS on soluble tumour necrosis factor (Sol TNF) and transmembrane (TM) TNF using whole cell lysate samples (3ug/lane) in the hippocampus A) representative Sol TNF, TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF and sol TNF to actin. The average intensity of the TM TNF and Sol TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

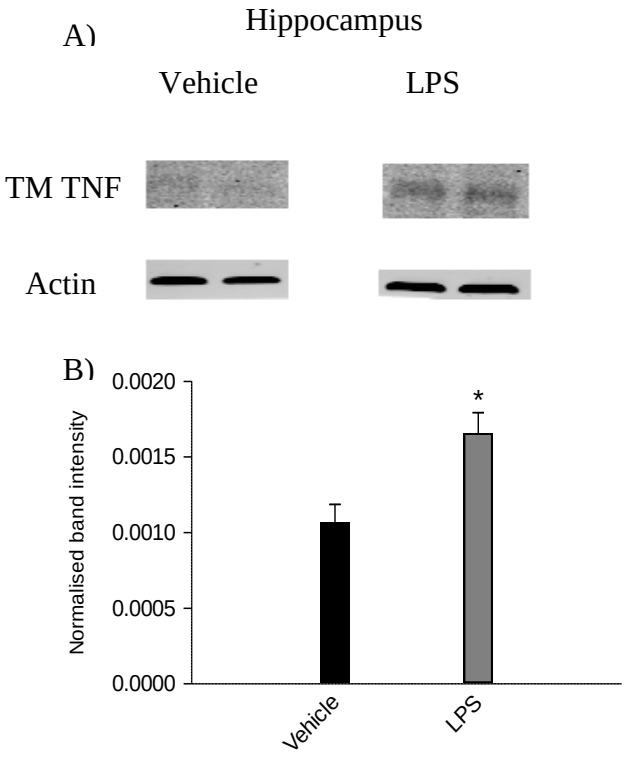


Figure 3.26 Effect of increasing dose of LPS on TM TNF using whole cell lysate samples (3ug/lane) in the hippocampus A) representative TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF to actin. The average intensity of the TM TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

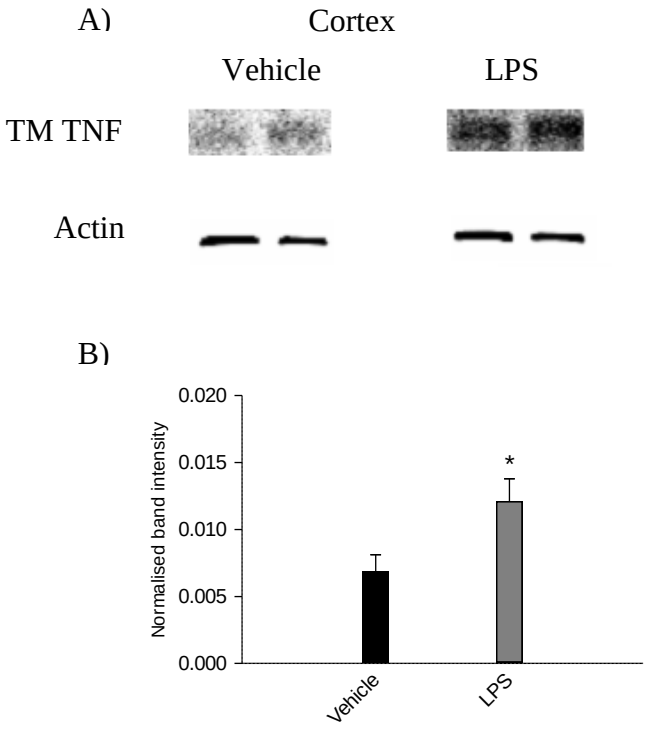


Figure 3.27 Effect of a single dose of 0.25mg/kg of LPS on TM TNF using whole cell lysate samples (3ug/lane) in the cortex A) representative TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF to actin. The average intensity of the TM TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

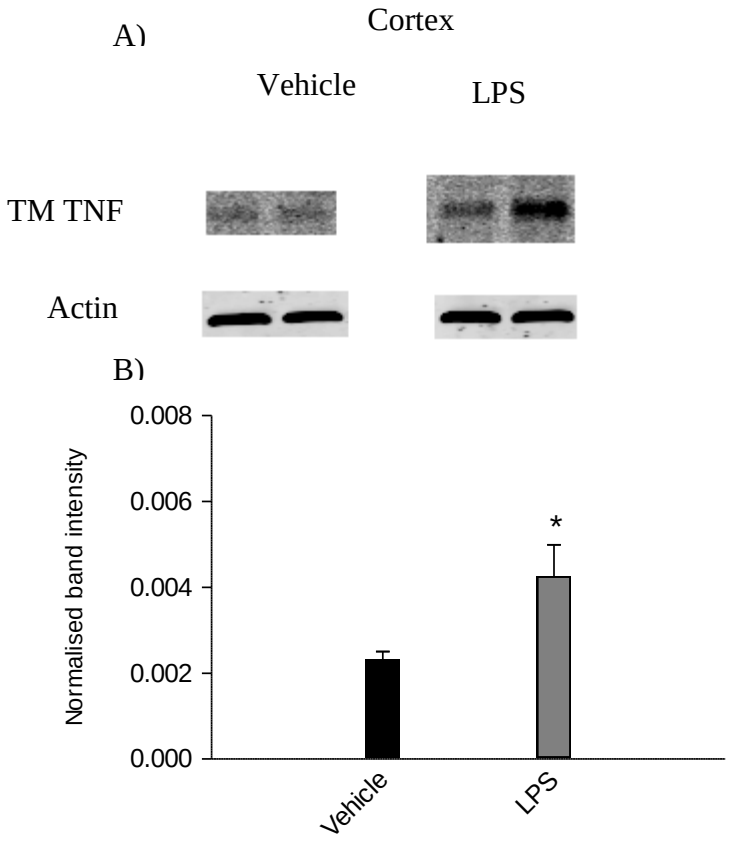


Figure 3.28 Effect of increasing dose of LPS on TM TNF using whole cell lysate samples (3ug/lane) in the cortex A) representative TM TNF and actin immunoblots B) bar graph showing the ratio of TM TNF to actin. The average intensity of the TM TNF was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

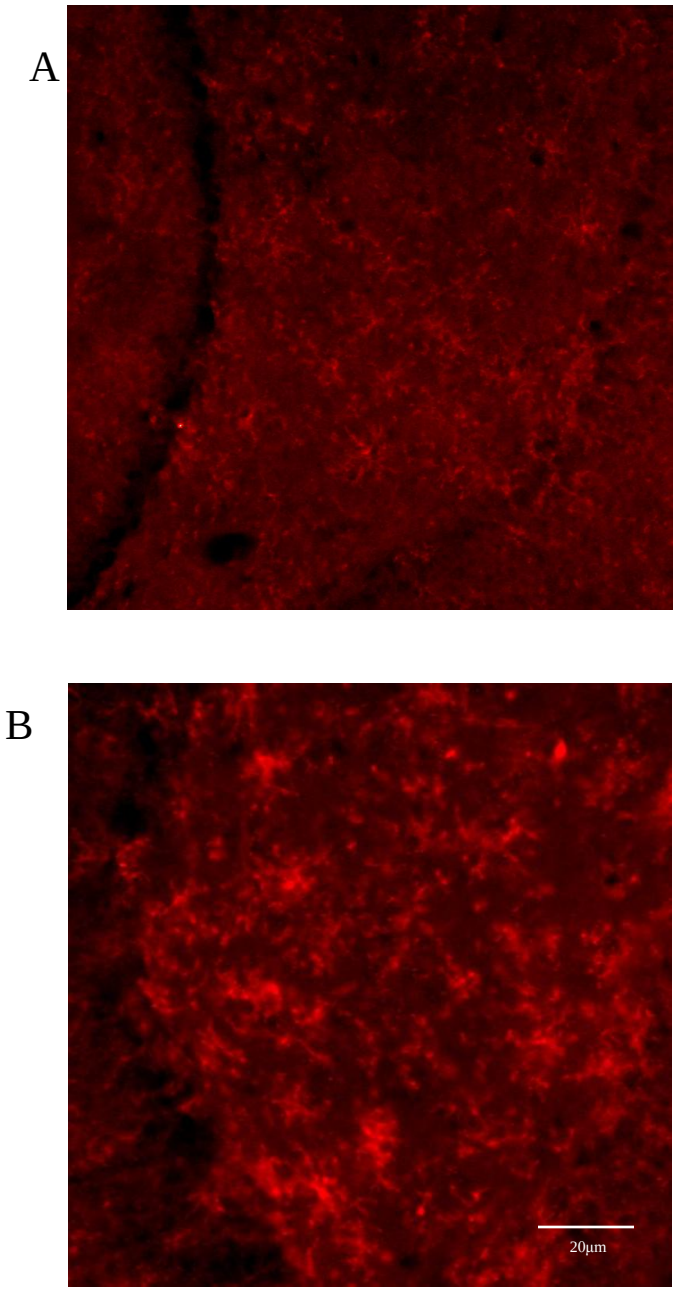


Figure 3.29 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on CD11b expression. Brain samples were collected 2 hours after each dose increment where **A)** vehicle **B)** 0.25mg/kg LPS **C)** 0.5mg/kg **D)** 1mg/kg **E)** 2mg/kg **F)** 4mg/kg **G)** 8mg/kg **H)** 16mg/kg. Pictures were taken under 20x magnification.

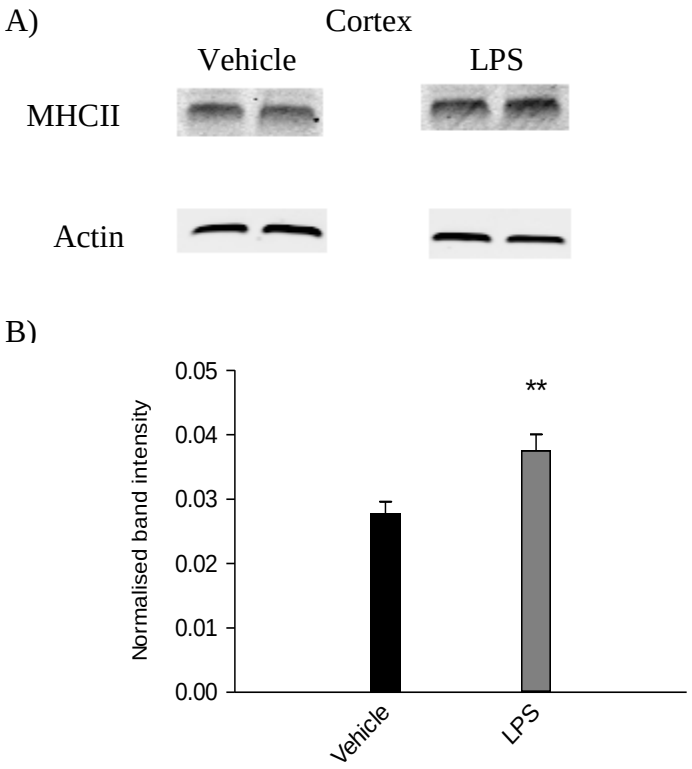


Figure 3.30 Effect of increasing dose of LPS on major histochemistry complex II using whole cell lysate samples (3ug/lane) in the cortex A) representative MHCII and actin immunoblots B) bar graph showing the ratio of MHCII to actin. The average intensity of the MHCII was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (**$p<0.01$). n=5.

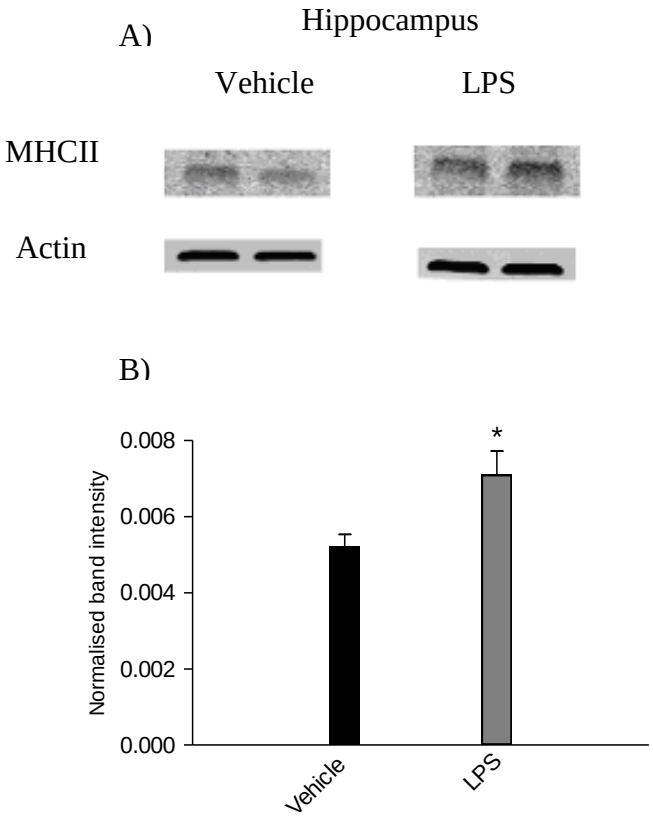


Figure 3.31 Effect of a single acute dose of 16mg/kg of LPS on major histochemistry complex II using whole cell lysate samples (3ug/lane) in the hippocampus A) representative MHCII and actin immunoblots B) bar graph showing the ratio of MHCII to actin. The average intensity of the MHCII was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (*$p<0.05$). n=5.

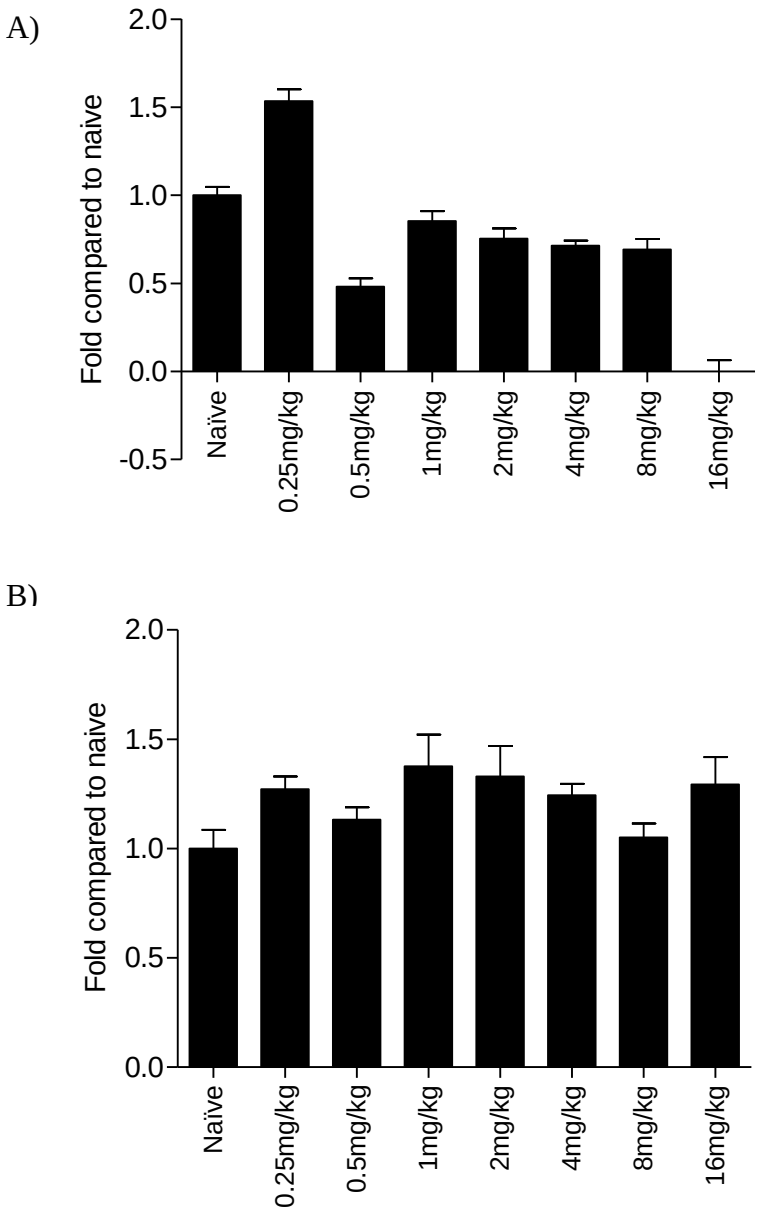


Figure 3.32 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on myeloperoxidase activity in the (A) spleen and (B) hippocampus. Tissue samples were collected 2 hours after each dose increment. Data points represent mean ± SEM. n=5.

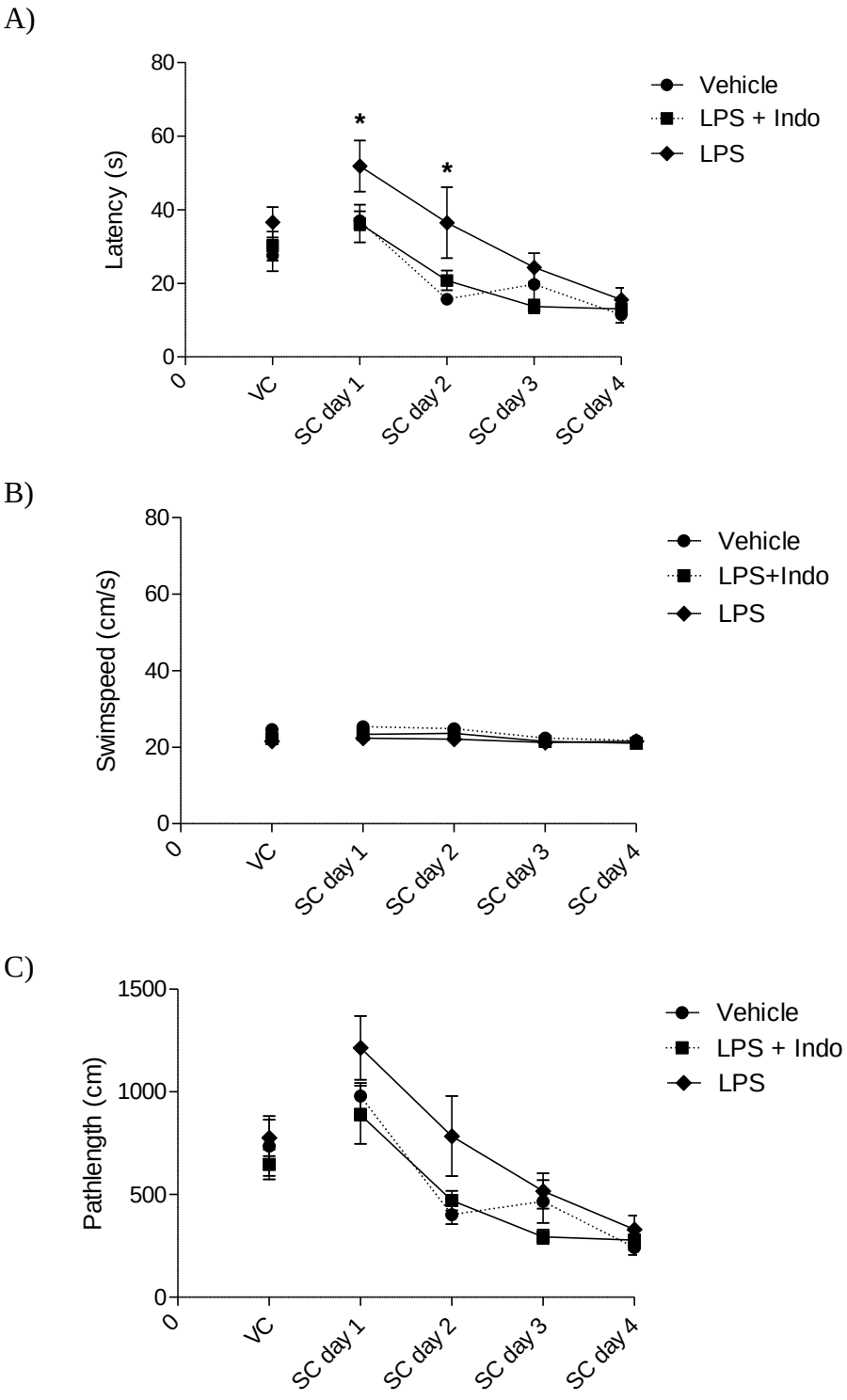


Figure 3.33 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) and indomethacin (1mg/kg) treatment on animals dosed with increasing dose of LPS on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden platform in the Morris watermaze test. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=10.

3.3 Neuroinflammation induced cognitive impairment: potential mechanisms

3.3.1 Activity-regulated cytoskeleton-associated protein (Arc)

The immunoblot demonstrated that there was no change in the protein expression of Arc protein in the hippocampus (figure 3.34). In the cortex however, a significant decrease in Arc protein expression was observed. To confirm that the decrease in protein expression observed in the cortex was not due to the reduction in the number of neurons, an immunoblot specific for synaptophysin was conducted to ensure that the total number of synapses present were similar in both vehicle and LPS treated group. The immunoblot for synaptophysin demonstrated that there was no significant difference in the number of synapses between the vehicle and LPS treated animals in both hippocampal and cortical regions (figure 3.35).

3.3.2 Amyloid precursor protein (APP)

There was a significant overexpression of APP protein in the hippocampus region. However, no significant difference was observed in the cortex (figure 3.36)

3.3.3 Acetylcholine expression

To determine the effect of LPS treatment on the cholinergic network, the expression of vesicular acetylcholine transporter (VAChT) was investigated. Western blotting analysis showed a significant increase in the VAChT in the hippocampus, and a decreased expression of VAChT in the cortex (figure 3.37).

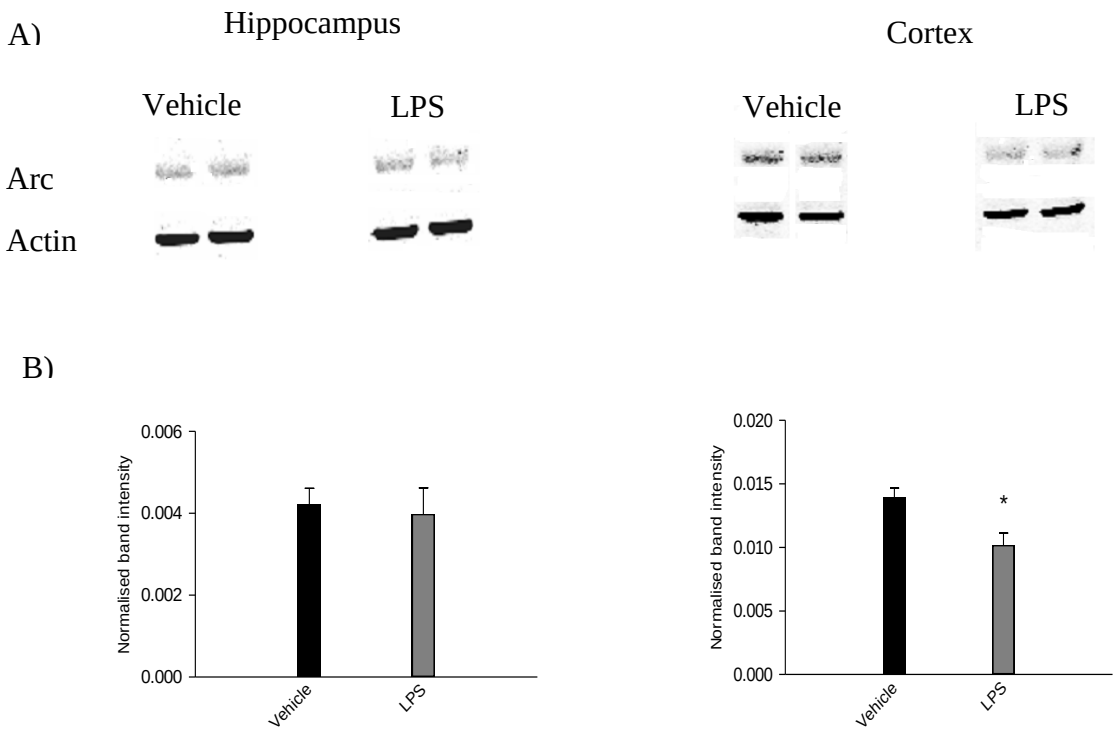


Figure 3.34 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on arc using whole cell lysate samples (3ug/lane) in the hippocampus and cortex A) representative arc and actin immunoblots B) bar graph showing the ratio of arc to actin. The average intensity of the arc was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=5.

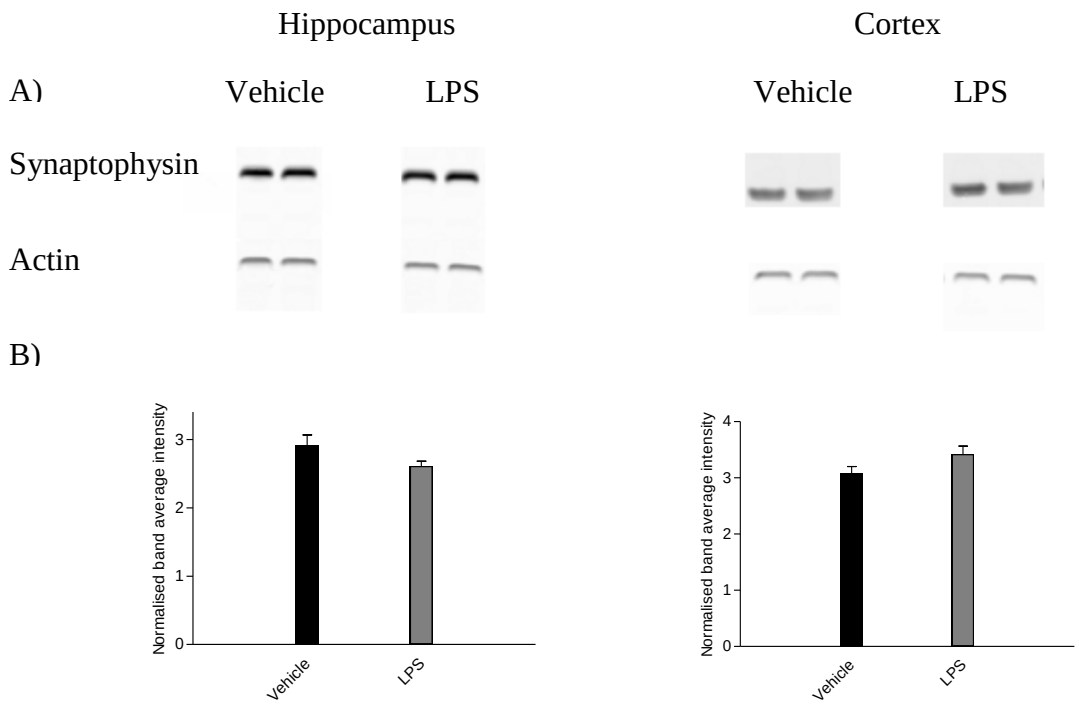


Figure 3.35 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on synaptophysin using synaptosome preparation samples (10ug/lane) in the hippocampus and cortex A) representative synaptophysin and actin immunoblots B) bar graph showing the ratio of synaptophysin to actin. The average intensity of the synaptophysin was normalized to the actin band from the same sample. Data points represent mean ± SEM. n=5.

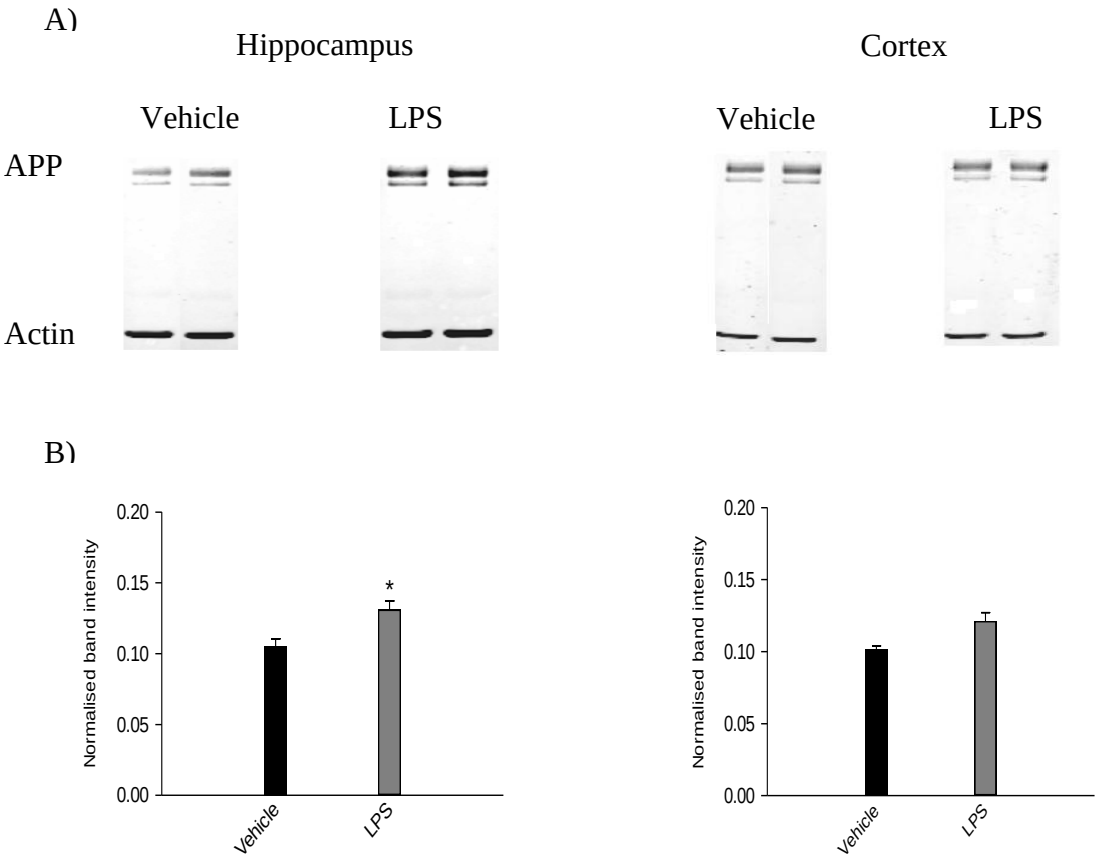


Figure 3.36 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on amyloid precurosor protein (APP) using whole cell lysate samples (3ug/lane) in the hippocampus and cortex A) representative APP and actin immunoblots B) bar graph showing the ratio of APP to actin. The average intensity of the APP was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=5.

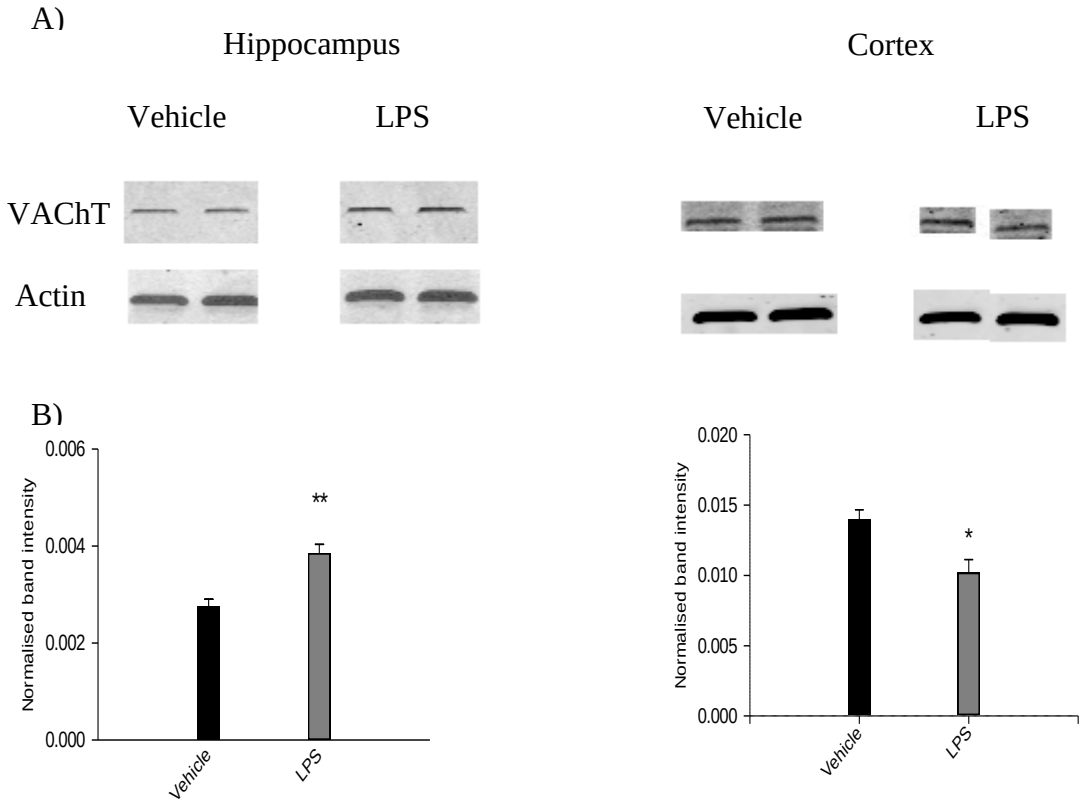


Figure 3.37 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on vesicular acetylcholine transporter (VAChT) using whole cell lysate samples (3ug/lane) in the hippocampus and cortex A) representative VAChT and actin immunoblots B) bar graph showing the ratio of VAChT to actin. The average intensity of the VAChT was normalized to the actin band from the same sample. Data points represent mean ± SEM. *Asterisks* indicate significantly different from the vehicle group (* $p<0.05$). n=5.

3.4 Delayed in cognitive impairment: Possible involvement of neurogenesis

Although a cognitive deficit was observed as seen in the MWM, NOR and FC 40 hours after the last LPS treatment, it was unsure whether the cognitive impairment was only limited to the acute state and whether such deficits could still be observed at a later point in time. Hence, a time course/protracted study was conducted to study the effect of increasing LPS dosing regime in spatial learning and memory task when the animals were tested at 2, 4, 6, 8, 12, 16, and 24 weeks after the last LPS treatment.

When the animals were tested 2, 4, and 6 weeks after the LPS treatment, no significant difference was observed in the animals in latency, swimspeed and pathlength (figure 3.38-3.40)

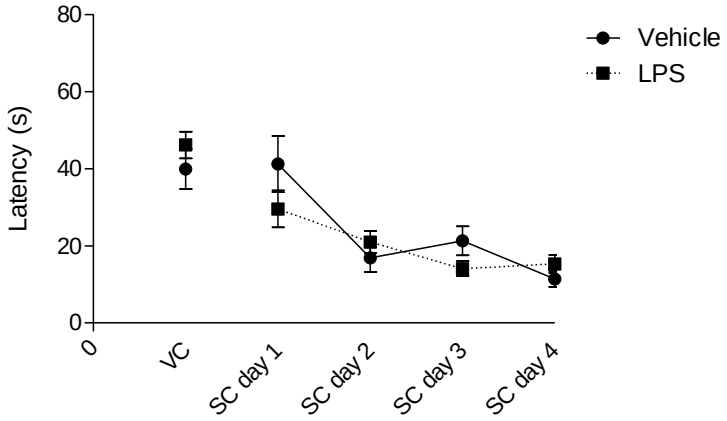
At the 8^{th} week after LPS treatment, LPS animals showed a significantly higher latency on SC day 1 compared to vehicle. No significant difference was observed in swimspeed and pathlength (figure 3.41).

By the 12^{th} week, a stronger deficit was observed in LPS animals. On SC day 1, LPS animals took a longer time and distance to find the platform. However, like the acute cognitive deficits described earlier, no significant deficit was observed in the subsequent days (figure 3.42).

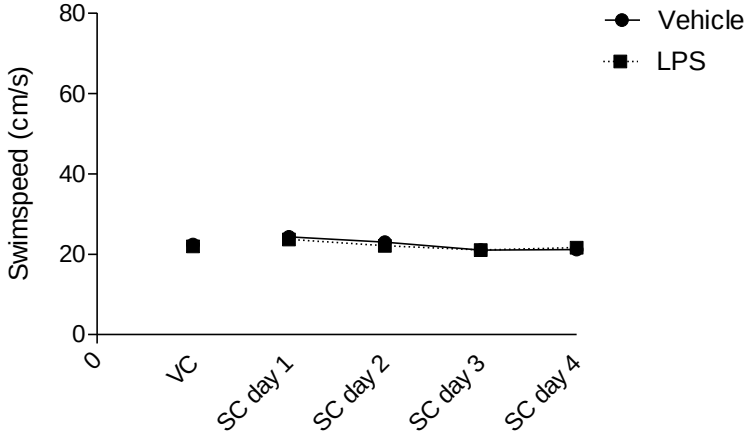
When the animals were tested 16 weeks after LPS treatment, although no significant difference was observed in latency and pathlength, the LPS treated animals did show a

slightly higher latency and pathlength on SC day 1. No significant difference was observed in swimspeed (figure 3.43). By the 24th week, no significant difference was observed in both treatment groups in all parameters (figure 3.44).

A)



B)



C)

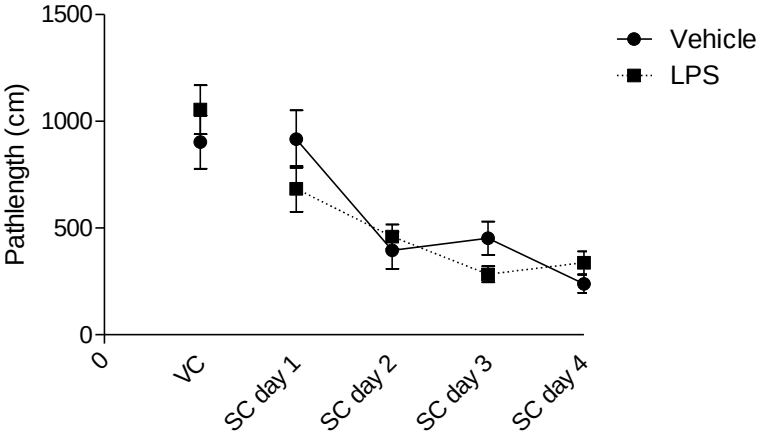


Figure 3.38 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 2 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

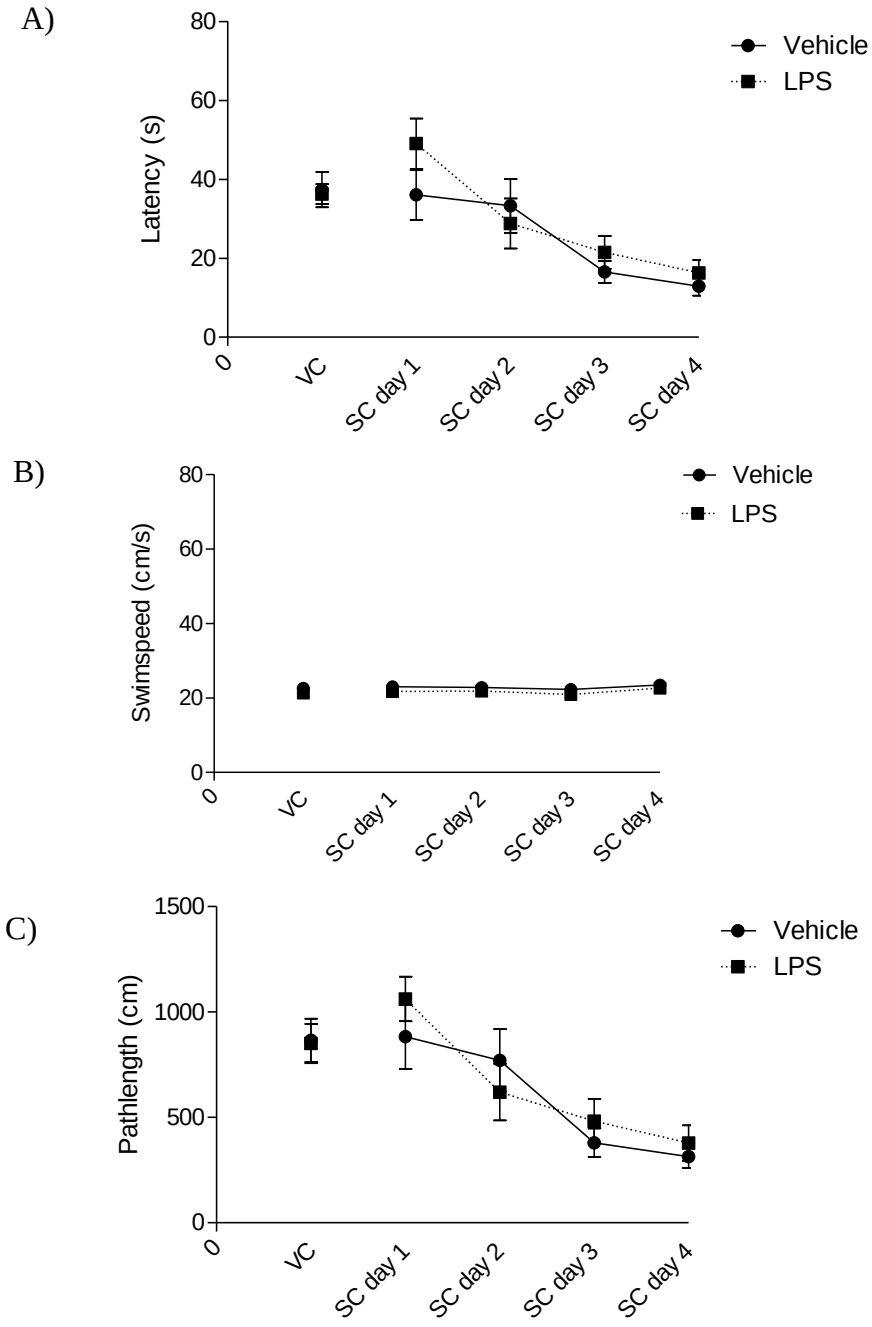


Figure 3.39 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 4 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

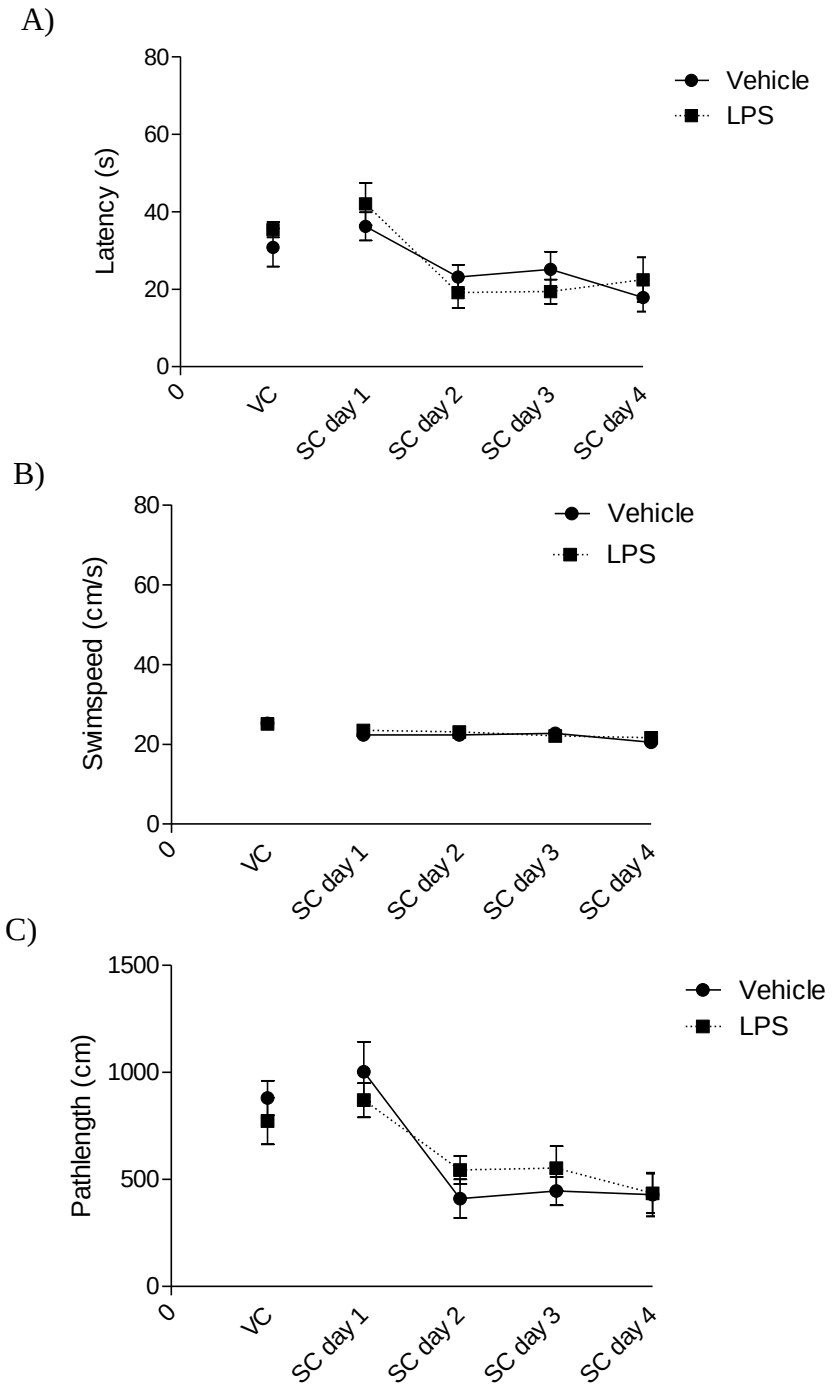


Figure 3.40 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 6 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

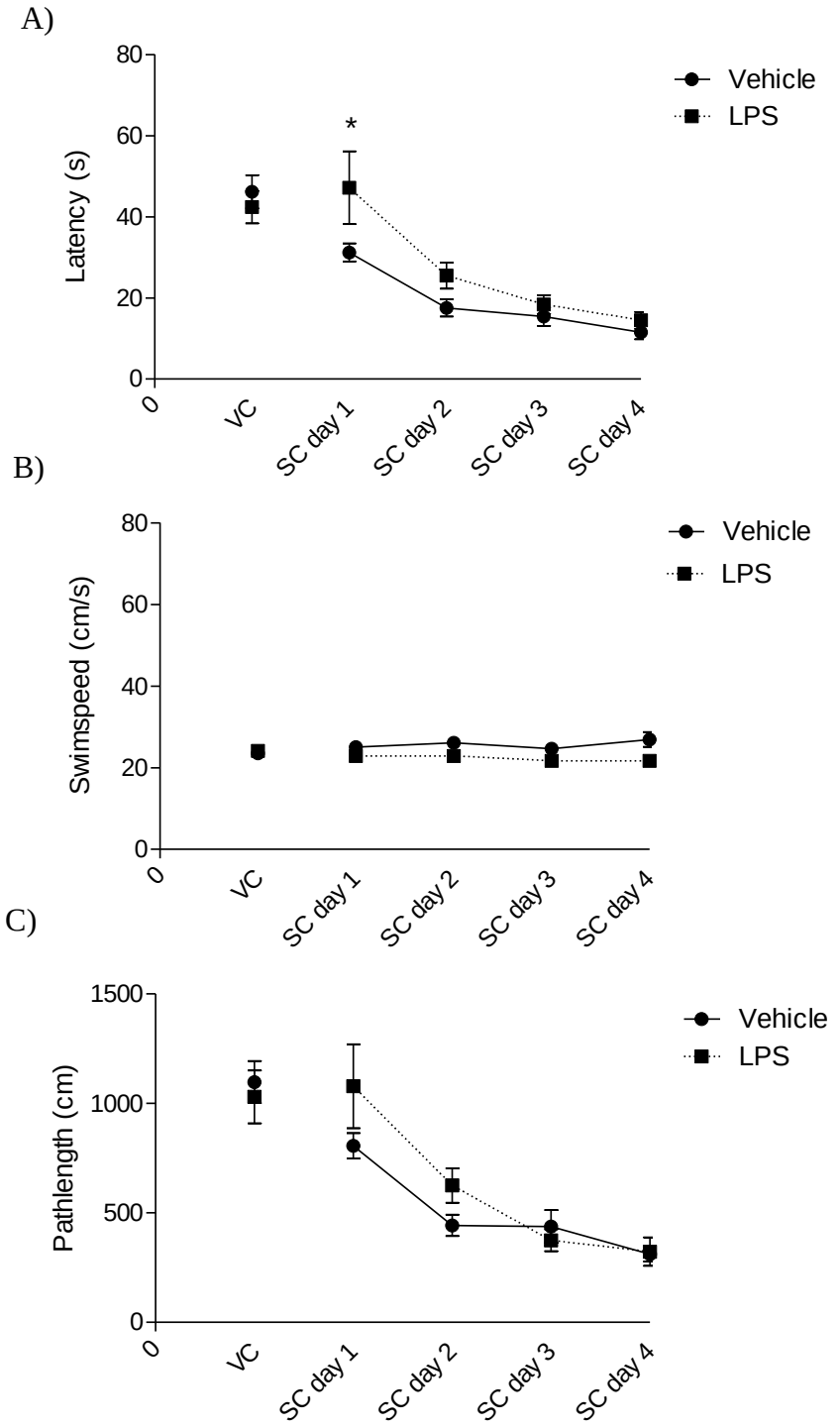


Figure 3.41 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 8 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

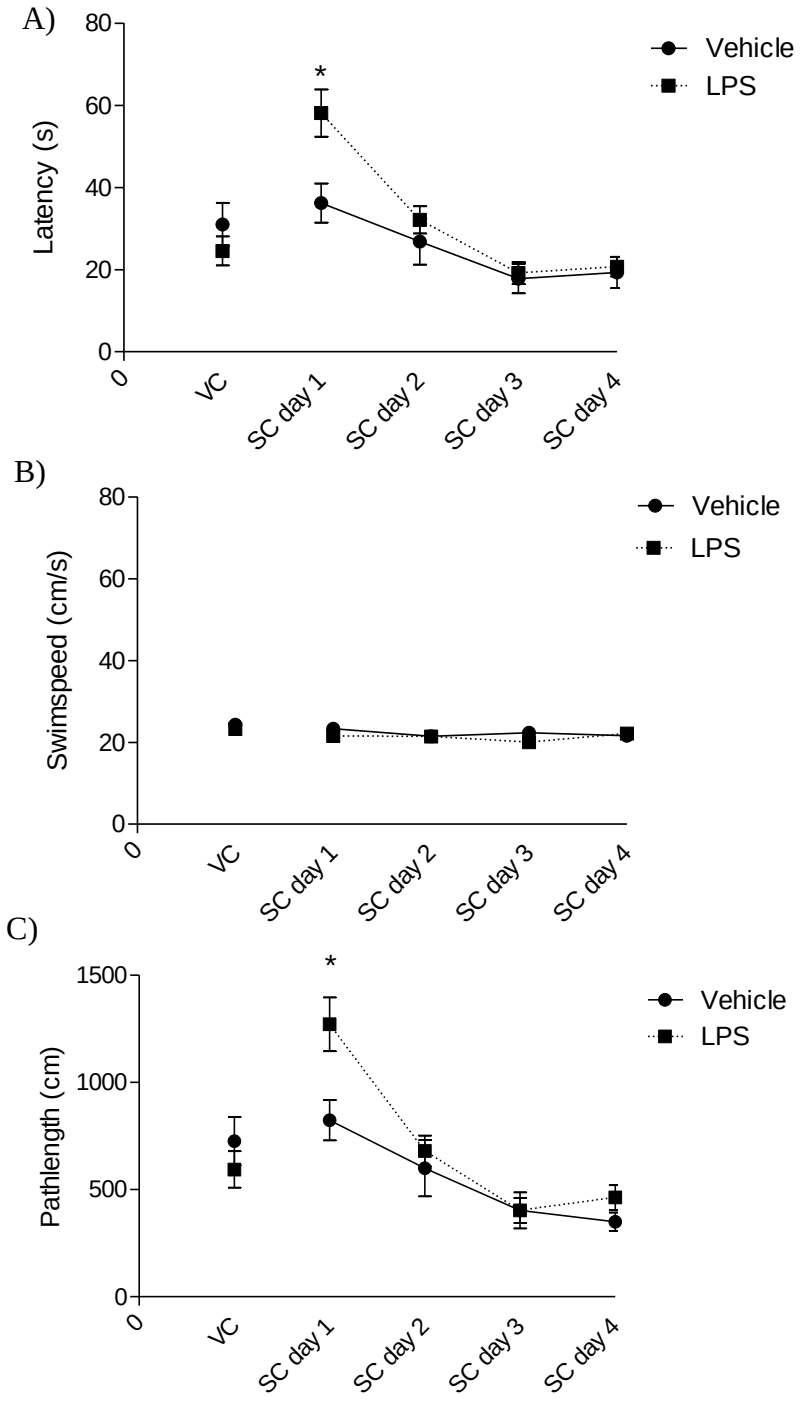


Figure 3.42 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 12 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

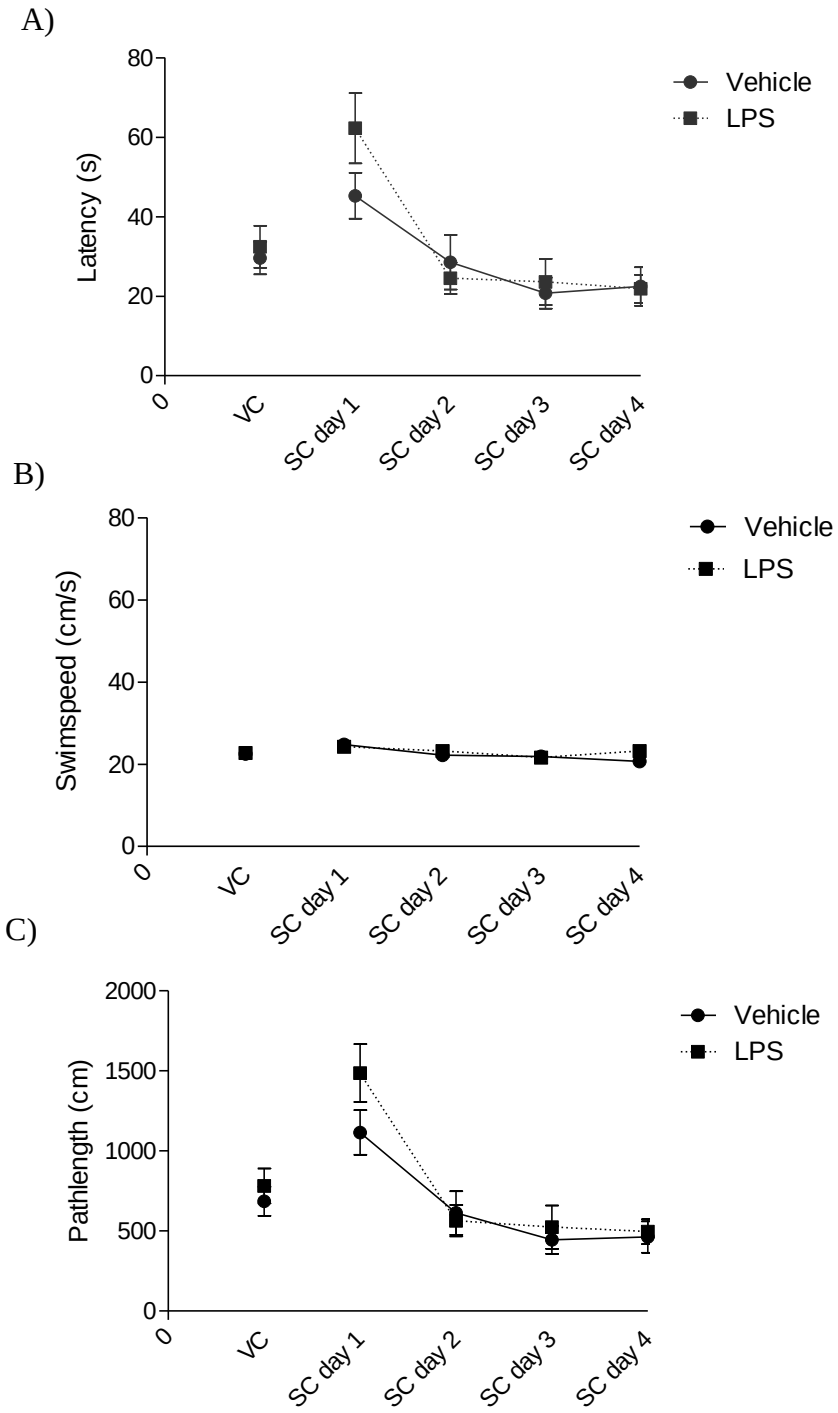


Figure 3.43 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 16 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

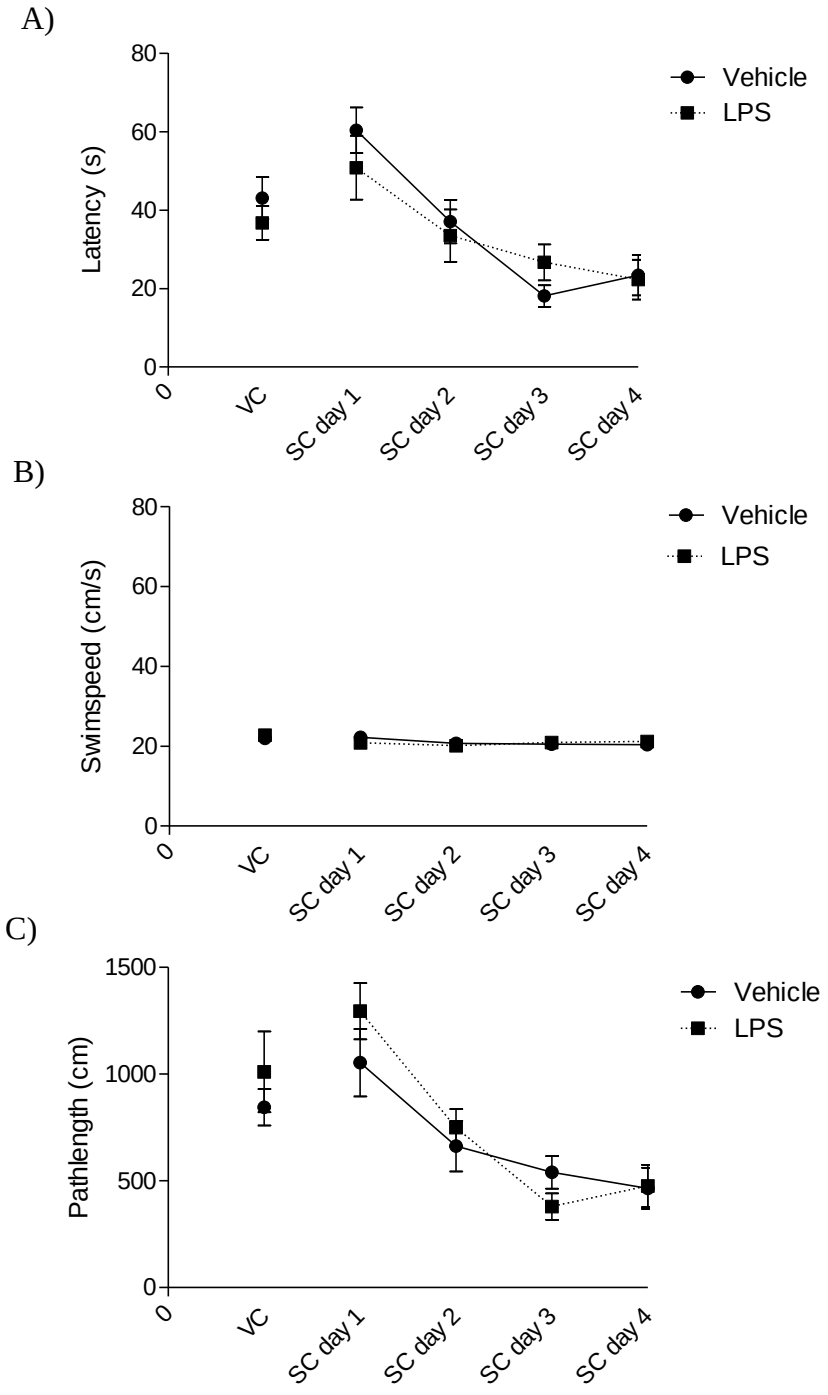


Figure 3.44 Effect of increasing dose of LPS (0.25-16mg/kg, twice daily for ten days) on (A) escape latency (B) swimming speed and (C) pathlength to find the hidden watermaze platform in the Morris watermaze test 24 weeks after LPS treatment. Data points represent mean ± SEM. n=10.

CHAPTER 4

DISCUSSION

4.1 Identification of suitable dosing regime to induce cognitive deficit

Earlier experiments conducted using the peripheral administration of LPS had shown inconclusive results as to whether peripheral treatment of LPS is able to elicit a cognitive deficit in rodents. One possible reason that may explain the varying outcomes is that the behavioural testings in the earlier experiments were conducted two to six hours after LPS treatment, a period when animals were exhibiting varying degrees of sickness behaviour. These behaviours only appear two to four hours after injection, last for four to six hours and dissipate by 24 hours (Danzter 2004). Hence, to investigate if systemic inflammation is able to induce cognitive deficits, behavioral tests should only be conducted when rats were devoid of any sickness behaviour. Preliminary studies have demonstrated that a single dose of LPS at 1mg/kg was insufficient to induce any changes in the performance of rodents in the MWM and NOR (figure 3.1 and 3.2) (Arai 2001, Sparkman et al. 2005). Therefore it is perhaps not unexpected that the current data indicating a single IP dose was insufficient to induce spatial cognitive impairment, seem to contradict previous findings where tests were conducted several hours after LPS treatment (2-6 hours) (Arai 2001, Sparkman et al. 2005). However, these earlier tests were conducted several hours after LPS treatment (2-6 hours) while animals were still suffering from the side effects of LPS treatment. Our LABORAS™ data has shown that rats were behaving differently at two hours after LPS treatment. The changes occurring during this period of time could

influence how these animals encode and form new memories. For instance, the reduction in motivation as a consequence of sickness behaviour, could easily affect the performance of the animals due to reduction in attention and motivation. The lack of motivation and attention could hence easily be misconstrued as a cognitive deficit. It is vital that when performing any cognitive tests, animals should be devoid of any potential side effects that could affect their normal behaviour.

Direct administration of LPS into the CNS has been shown to induce cognitive impairment (Hauss-Wegrzyniak et al.1998), suggesting that inflammation induced by LPS was able to elicit cognitive deficits. The lack of efficacy seen in peripherally administered LPS in inducing cognitive deficits suggests that there were insufficient LPS in the peripheral system to drive the neuroinflammation required in inducing changes in the CNS. In subsequent studies, multiple doses of LPS were given in order to increase the exposure of the immunogenic stimuli in rats. When the LPS treatment was increased to three and twenty doses of LPS at 1mg/kg, no cognitive deficits were observed in the water maze. The lack of cognitive impairment even with the increased LPS exposure suggests that by using a constant dose, the animals were able to develop a tolerance to the LPS. A reduction in pro-inflammatory cytokine release is normally observed when the body begins to tolerate the endotoxin (Broad et al. 2006). The reduction in pro-inflammatory cytokines, a potential driver in inducing cognitive deficits in rodents, may curtail any effect of neuroinflammation induced changes in the memory systems.

There arises a scenario in which to induce a cognitive deficit detectable using rodent models, the ideal dosing regime has to fulfil several basic requirements. Firstly, sufficient amount of LPS must be administered to drive the desired cognitive deficit. Secondly, endotoxin tolerance is known to be a consequence of repeated dosing. The new dosing regime would need to overcome this phenomenon in a manner where the tolerance developed to sickness behaviour could be maintained but at the same time the effect of LPS in inducing changes in learning and memory systems in the CNS could be potentiated.

With these requirements in mind, a subchronic increasing dose regime was identified and used (adapted and modified from Chen et al. 2005). In order to ensure sufficient exposure to LPS, the concentration of LPS administered was increased with time, thus increasing the amount of circulating endotoxin in the system that could ultimately induce pathological changes in the CNS without building tolerance in the CNS.

Under this dosing regime, the LPS treated animals showed spatial learning and object recognition deficits. This is the first report that demonstrated an object recognition memory deficit after peripheral administration of LPS. It was previously reported that a 10 day infusion of LPS into the fourth ventricle failed to induce an object recognition deficit (Hauss-Wegrzyniak et al. 2000). The disparities could be explained by the manner in which the experiments were conducted. In the current experiment, Lister-hooded rats were tested 1 hour after the T1 trial while Hauss Wegrzyniak et al. (2000) had tested the Sprague Dawley rats 24 hours after T1. However, based on in-house data (at GSK), most

normal and untreated Lister-hooded rats were unable to recall and discriminate the novel from the familiar objects 24 hours after T1. Hence the 1 hour delay was chosen over the 24 hours. The difference in rat strains and the time point in which the T2 trials were conducted may also have contributed to the observed disparities.

In a cued fear conditioning (FC) test, rodents are required to learn the association between CS (tone) to the aversive US (footshock). The amygdala, hippocampus and sensory neocortical areas are believed to be important in cued FC, with lesions of amygdala interfering both cued and contextual FC, while lesions in the hippocampus only affects the contextual FC (Philips and LeDoux 1992). The increasing dose of LPS treatment did not induce a significant deficit in cued FC as the LPS treated animals performed similarly to the vehicle treated animals. This suggests that the LPS treatment may not have an effect on the amygdala and other associated brain regions (figure 3.16).

When the animals were tested in contextual fear conditioning test, a test that is hippocampus-dependent (Philips and LeDoux 1992), both treatment groups spent similar amount of time freezing in the first four minutes during the recall trial. However, a schism between both treatment groups was observed from 4^{th} to 10^{th} minute. The vehicle treated animals showed a significantly lower percentage of time spent freezing compared to the LPS treated animals. As no reinforcing stimulus was presented to strengthen the fear-stimulus association, the vehicle group was able to reverse the association. The LPS treated animal however, froze for more than 50% of the time (figure 3.16). The persistently higher amount of freezing time could be due to the alteration in neural

networks due to LPS. LPS treatment had encoded the association between the environment and the shock strongly and more time is required to dissociate the learned association. Alternatively, the inability of these rats to forget the association suggests a potential reversal learning deficit. However, as reversal learning deficits are not routinely detected using the contextual FC paradigm, more experiments are needed to test this hypothesis.

No significant difference was observed in the overall behaviour and motor coordination during the time of the test, confirming the absence of sickness behaviour. The increasing dose of LPS treatment however did induce a significant change in body weight. The initial weight loss was attributed to the reduction in food consumption induced by an increase in sickness behaviour response after LPS treatment. At the higher dose of LPS, the weight of animals although did not show an increase as compared to the vehicle treated animals, there was no reduction in weight at the same time (figure 3.21). Response to an endotoxin insult is phasic in nature divided by the acute and chronic phases of the insult (Rudiger et al. 2008). The initial acute phase is normally accompanied by pro-inflammatory activity that leads to an increase in endocrine, thermogenic and metabolic activity, excessive catabolism and fuel utilisation. The subsequent stage, the anti-inflammatory phase, an evolutionary mechanism to prevent excessive damage induced by the pro-inflammatory agents, is normally associated with bioenergetic failure and a reduction of metabolic rate (Rudiger et al. 2008). Thus the reduction in food intake at higher doses of LPS could be reflective of the reduction in the metabolic rate.

4.2 Systemic inflammation induced inflammation in the CNS

4.2.1 TNFα expression in the CNS and the periphery

TNFα level in the liver demonstrated a classical bi-phasic inflammatory reaction to an endotoxin insults (Rudiger et al. 2008); an acute proinflammatory phase, as seen by the elevated levels of TNFα in plasma and liver, and a chronic anti inflammatory phase, as seen by the lack of TNFα production in subsequent dose. In the CNS however, both hippocampus and cortex showed elevated protein expressions even after the completion of the ten days dosing regime. The differential response suggests a divergent effect of chronic inflammation in the peripheral system and CNS. The reason of these differential responses is unknown but they could possibly be explained by the different levels of tolerance to the endotoxin insult (Ru et al. 2004). The elevated TNFα seen in the brain however suggests neuroinflammation was occurring in the brain at the time of testing.

Several mechanisms have been suggested to be involved in the regulation of the brain cytokine levels in peripheral inflammation (Turrin et al. 2001). A possible mechanism involves the transfer of intermediate soluble factors such as prostaglandins or cytokines across the blood brain barrier (BBB) and/or circumventricular organs where the BBB is sparse. A second possible mechanism involved the production of pro-inflammatory cytokine by the circumventricular organs directly activated by peripheral inflammatory factors. Finally, a third possible mechanism involves neural afferent signalling that leads to cytokine production in the CNS.

4.2.2 Changes in the microglia in the CNS

The dentate gyrus, part of the hippocampal formation, is believed to be important for normal functioning of the hippocampus as the dentate gyrus is the primary input into the hippocampal formation after receiving inputs from the entorhinal cortex (Li and Pleasure 2005). In addition, the dentate gyrus is also one of the two known regions in the CNS that undergoes neurogenesis. Recent experiments have shown that new born cells were vital in spatial and object recognition memory in adult rats (Jessberger et al. 2009).

In order to determine the effect of neuroinflammation on the hippocampus, in particular the dentate gyrus region, the microglia expression was examined using the CD11B/CD8 epitope. The CD11B/CD18, a CR 3 complex marker that is specific for microglia was chosen to examine the microglia distribution in the dentate gyrus. Based on the immunohistochemistry data, CD11B/CD18 expression seems to be upregulated in the dentate gyrus of the LPS treated animals as the intensity of the stain is stronger compared to the vehicle treated animals (figure 3.29). During inflammation, the CD11B is upregulated and an increase in intensity and number were observed when compared to normal healthy animals.

In addition to the elevated expression of CD11B/CD18 complex, the activation of microglia and astrocytes may also be characterized by the increased expression of MHC class I and II in addition to the cytokine production. In microglia, which acts as the main resident antigen presenting cells, MHC class I and II are upregulated to initiate immune response via T-cells (Dimayuga et al. 2005). Similar to the CD11B/CD18 expression

after the completion of the increasing LPS dose regime, the LPS treated animals showed a significantly higher amount of MHCII in the cortex and hippocampus, suggesting a role for the microglia to act as antigen presenting cells during chonic inflammation (figure 3.30 and 3.31). The increased expression of inflammatory markers such as CD11B/CD18 and MHCII suggests that the peripheral administration of LPS could trigger the upregulation of these pro-inflammatory molecules.

4.2.3 Myeloperoxidase activity

MPO is a glycosylated, heme enzyme found in the azurophilic granules of neutrophils and is commonly used as a histopathological marker for neutrophils (Breckwoldt et al. 2008). It is expressed in the myeloid line, including cells such as microglia. In recent years, the MPO has been used as a biomarker for inflammation as it is known to be upregulated in "diseased" brains but this enzyme is rarely expressed in normal brain microglia (Lefkowitz and Lefkowitz 2008).

The spleen MPO activity displayed a similar TNFα profile, again suggesting a biphasic reaction to the increasing LPS dosing regime. The insignificant level of MPO activity observed in the hippocampus in the LPS animals however was in parallel with the immunohistochemistry data. Elevated level of MPO is commonly produced by activated microglia. Although some microglia in the dentate gyrus exist in the ameboid shape, the majority of the microglia present were in the ramified state. The cognitive impairment observed in LPS treated rats could thus be induced by other mechanism beside damages induced by oxidative stress.

4.2.4 Effect of indomethacin on the increasing LPS dosed animals.

To ascertain whether the cognitive deficits seen in rodent learning and memory tasks were induced by neuroinflammation, the effect of anti-inflammatory drug, such as indomethacin, was investigated. Indomethacin, a non-steroidal anti-inflammatory drug (NSAID) that inhibits COX, is commonly used in treatment for inflammation and pain. COX, especially COX 2, has been shown to be a downstream protein produced by glial cells after inflammatory stimuli such as LPS and Aβ peptides (Melnikova et al. 2006). COX 2 is the inducible isoform of COX 2 and can be regulated by synaptic plasticity (Chen et al. 2002), whereas COX 1 is constitutively expressed in cells. In COX 2 transgenic mice, as the animal ages, an increase in cortical neuronal apoptosis and glial activation that goes hand-in-hand with cognitive impairment was observed (Andreasson et al. 2001). Therefore in order to determine whether the deficits were indeed related to inflammation, the inhibition of a downstream protein such as COX2, would demonstrate an amelioration of the deficits seen by using the increasing LPS dosing regime.

Rats that were treated with indomethacin prior to the LPS treatment showed a reversal of the cognitive deficits induced by the LPS treatment as they performed as well as the vehicle treated animals. Indomethacin penetrates the BBB readily (Parepally et al. 2006), thus allowing the anti-inflammatory agent to act directly in the peripheral system and the CNS. Current results which demonstrate the ability of indomethacin to reverse the neuroinflammatory process and abolish the learning and memory deficit, further strengthen the hypothesis that the cognitive deficits observed in LPS treated animals.

4.3 Inflammation induced cognitive deficit

4.3.1 TNFα induced cognitive deficit

TNFα is a member of the TNF superfamily of ligand that promotes inflammatory signalling (McCoy and Tansey 200). Existing as type-2 transmembrane protein (tmTNF) or soluble circulating trimer (solTNF), both types have been shown to be biological active and found to be released in the CNS by microglia, astrocytes and neurons (Morganti-Kossman et al. 1997). Both tmTNF and solTNF regulate cellular processes such as inflammation, differentiation and cell death through the activation of TNF receptor 1 or 2 via mitogen-activated protein kinases (MAPKs) and IκB kinase by modulating transcriptional factors such as activator protein-1 (AP-1) and NFκB that inevitably affects gene transcription (Medeiros et al. 2007).

Toxic concentration of TNFα can lead to excessive activation of NMDA receptors, which in turn results in excitotoxicity induced neuronal death. TNFα has also been reported to potentiate glutamate neurotoxictiy by inhibition of glutamate transport by predominantly inhibiting GLT1 via the NFκB signaling pathway transport in both neurons and astrocytes (Zou and Crews 2005). Glutamate transporter (GLT) 1 is predicted to contribute up to 90% of glutamate transport in certain brain regions. These transporters have been shown to contribute to glutamate synaptic signal termination, to recycle glutamate and maintain the glutamate concentrations below the excitotoxic threshold (Sims and Robinson 1999). As TNFα induces an inhibition in the glutamate transport, increased glutamate levels in the synaptic cleft may induce a spill over, allowing excess glutamate to bind to the extrasynaptic NMDA receptors. Activation of extrasynaptic NMDA receptors has been

suggested to lead to excitotoxicity (Hardingham et al. 2002). These receptors have an opposite effects on cAMP response element binding (CREB) function where CREB has been shown to be necessary for gene regulation and neuronal survival. The activation of these receptors ultimately leads to the loss of mitochondrial membrane potential and neuronal death (Hardingham et al. 2002).

One possible mechanism through which the elevation of TNFα may induce cognitive impairment is via the generation of excessive NO. The generation of NO by iNOS has been shown to involve the activator protein-1 (AP-1) pathway. AP-1 is a transcriptional factor composed of the protein products of c-jun and c-fos (Angel and Karin 1991). The sustained activation of the AP-1 and jun-N terminal kinase (JNK) pathway induced by TNFα signalling has been demonstrated to induce iNOS upregulation (Zou and Crews 2005). Furthermore, to strengthen the association between TNFα signalling and iNOS, molecular cloning and analysis, have reported that the promoter region of iNOS gene consists of binding sites to several transcription factors such as AP-1, NFκB and TNF response element. This strengthens a possible link between TNFα levels and iNOS transcription (Eberhardt et al. 1996). The predominant mechanism through which NO promotes neuronal toxicity is through the reaction of NO with superoxide anion to generate cytotoxic substance peroxynitrite (Eberhardt et al. 1996).). During inflammation, excessive accumulation of NO will lead to cellular dysfunctions and cell death. In fact, in AD patients where neuroinflammation is prevalent, increased expression of iNOS and peroxynitrite damage have been reported (Zou and Crews 2005).

TNFα has been shown to reduce neurite outgrowth and branching in hippocampal neurons via small guanosine triphosphatase (GTPase) Rho (Neumann et al. 2002). The Rho GTPases activity has been shown to be important for the signal transduction between membrane proteins and actin cytoskeleton (Hall 1998). The direct mechanism through which TNFα interfere the Rho GTPase activity is unknown. However there is evidence that suggest the involvement of NO and/or NO-derived products (Münch 2003). During neuroinflammation, the elevation of TNFα may affect neurite elongation, possibly serving as an anti-regenerative factor in addition to other growth inhibition factors such as Nogo and glial scars (Neumann et al. 2002). This would also prevent the repair of damaged neural networks that could be vital for learning and memory.

4.3.2 Activity-regulated cytoskeleton-associated protein (Arc)

Arc or Arg3.1, is part of the immediate early genes (IEG) family. Transcription of Arc correlates both temporally and spatially with activity induced stimulus (Rosi et al. 2008). This gene is highly conserved in vertebrates and biochemical studies have demonstrated that Arc protein coprecipitates with polymerized actin that is extensively found in dendritic spines (Bramham et al. 2008). Since then, Arc protein has been identified to play a vital role in the maintenance phase of LTP and spatial memory compression (Rosi et al. 2008). It regulates glutamate transmission mediated by controlling the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor trafficking via its interaction with components of the endocytic machinery such as dynamin and endophilin (Chowdhury et al. 2006). Overexpression of Arc induced a reduction in surface expression of GluR1-containing AMPA receptors, while Arc null primary neurons

showed an elevated surface expression of AMPA receptors (Centonze et al. 2009). More interestingly, in AD patients with pronounced deficits in hippocampus-dependent memory functions, an almost 3.5 decrease in the baseline level of Arc expression was observed when compared to non-AD patient (Rosi. et al. 2008).

Recently, it has been reported that centrally injected LPS in mice resulted in a lower expression of Arc in the cortex but not hippocampus (Bonow et al. 2009). These results are consistent with the current finding in which the increasing LPS dosing regime was able to elicit a reduction of Arc protein in the cortex but not hippocampus. This change was independent of synapse number as similar levels of synaptophysin were observed.

The exact mechanism that induced a reduction of the Arc protein level has not been fully elucidated. The reduction in the cortical level could be attributed to the decrease expression of the Arc mRNA or alterations in proteosome processing that could increase the rate of degradation of Arc proteins in dendritic spines. Arc protein level was not reduced in the hippocampus. This may suggest higher tolerance to LPS insult in the hippocampus or alternatively, other compensatory mechanisms were available to prevent the changes in the neural network.

Nonetheless, there exists a possible association between the change in Arc protein expression and the cognitive impairment observed. The two pathways that are involved in the induction of Arc are the activation of glutamatergic synapses and the release of brain-derived neurotrophic factor (BDNF) (Spulber et al. 2008). In the glutamatergic pathway,

the activation of the NMDA receptors upregulates Arc while stimulation of the AMPA glutamate receptor downregulates Arc (Rao et al. 2006, Shepherd et al. 2006). Furthermore, changes in Arc level are also dependent on the NMDA/AMPA receptor ratio that could in turn enhance negative feedback control of Arc expression, thus influencing the transcription of Arc (Rosi et al. 2008). TNFα has been shown to modulate the expression of AMPA. The application of TNFα increases the expression of the surface AMPA receptors (AMPAR) in the plasma membrane in hippocampal neuronal culture (Beattie et al. 2002). As demonstrated previously, TNFα was elevated in the cortex when animals were given the increasing doses of LPS, suggesting that the reduction in the Arc protein expression could be linked to the elevated TNFα expression seen in the cortex. During inflammation, TNFα could induce an increase in the expression in the AMPAR on the surface of dendritic spines that ultimately changes the NMDA/AMPA receptor ratio, suppressing the expression of Arc in the cortex.

On the other hand, the expression of Arc has also been shown to be stimulated by BDNF (Rosi et al. 2008). BDNF belongs to the neurotrophin family of growth factor which has been shown to regulate synaptic transmission in the brain such as LTP and long term depression (LTD) (Schnydrig et al. 2007). In rodents that were peripherally treated with LPS, a decreased level of BDNF was observed in the brain (Schynydrig et al. 2007). Physical activity is able to reverse this (Wu et al. 2007). Hence, the reduction in Arc expression could be attributed to the reduction of neurotrophic factors such as BDNF when animals were injected using the increasing LPS dose regime.

In addition, as a member of the IEG family, Arc can also serve as a marker for neural activity (Bonow et al. 2009). The reduction of Arc in cortex could be a consequence of diminished neural activity. In an electrophysiology study, Vereker et al. (2000) has demonstrated that when animals were dosed with LPS, there was a significantly drop in glutamate released, suggesting a suppression of the excitatory neurotransmission. The reduction in Arc level could be a reflection of diminishing neural activity as LPS has been shown to cause the reduction of glutamatergic transmission.

Arc has been demonstrated to play an important role in LTP and is normally expressed within minutes after being subjected to a cognitive task. It will be interesting to investigate the Arc expression level when the LPS treated animals were subjected to a cognitive task. The reduction in the basal level of Arc may explain the acute deficits seen in learning and memory tasks as an increased amount of Arc is needed to be produced to achieve levels required for learning and memory processes.

4.3.3 Amyloid precursor protein (APP)

It has been recently suggested that peripheral administration of LPS could induce a cognitive impairment in passive avoidance and water maze tests in mice through the enhancement of Aβ generation (Lee et al 2008). Consistent with the current data, an increase expression of APP in the hippocampus and not cortex were observed in animals treated with LPS. In addition, Lee et al. (2008) also demonstrated that accompanying the elevation of APP, an accumulation of Aβ1-42 was observed in the hippocampus together with the increase in β- and γ-secretase activities. This suggests that processes of

amyloidogenesis may also contribute to the cognitive impairment observed in LPS animals.

The exact mechanism through which peripheral LPS administration could lead to the elevation of APP and generation of Aβ in the hippocampus is still unknown. However it was postulated that amyloidogenesis induced by LPS treatment could involve the activation of AP-1 via TNFα, as a potential activator of AP-1 recognition site was detected in the gene (Quitschke and Goldgaber 1992). Furthermore, LPS has been shown to increase MAPK activity, a signalling pathway that has been implicated in AD (Lee et al. 2008).

Using whole cell recording and immunocytochemistry, APP has been shown to induce synaptic depression via a selective reduction of AMPAR (Ting et al. 2007). As described earlier, Arc is also involved in the endocytosis of AMPAR. However, as the immunoblot data showed no elevation of Arc protein in the hippocampus, it is likely that the endocytosis process can be induced by another mechanism. One possible mechanism of inducing an increase in the endocytosis of AMPAR is by PSD-95 which is a protein involved in recruiting and anchoring glutamate receptor subunits. A reduction in the PSD-95 expression has been linked to a reduction in the AMPAR in cell surface of APP mutant mice (Almeida et al. 2005). Conversely, an overexpression of PSD-95 led to an increase surface expression in AMPA *in vitro* (Bhattacharyya et al. 2009)

Not limiting to the post synaptic terminal, APP via the generation of Aβ induces a deficit in vesicle recycling during sustained stimulation. The disruption of vesicle recycling could be linked to the reduction in dynamin 1, a GTPase responsible for pinching off synaptic vesicles during endocytic retrieval from the plasma membrane (Ting et al. 2007). In AD brains, levels of dynamin 1 were reported to be significantly reduced as well (Yao et al. 2003) and Aβ application in cultured hippocampal neuron also significantly reduces dynamin level (Kelly et al. 2005).

More work is required to determine whether the overexpression of APP seen in the hippocampus would be translated to an increase in deposition of Aβ. However, it is interesting to note that the overexpression of APP was only observed in the hippocampus but not in the cortex; while reverse was true for the reduction of Arc in hippocampus. More interestingly, the overexpression of APP is associated with the increase internalisation of AMPAR while the reduction of Arc is linked to the overexpression of AMPAR in dendritic spines (Centzone et al. 2009). Albeit such differences in mechanisms, both ultimately could lead to the impairment in the learning and memory paradigm.

4.3.4 Vesicular acetylcholine transferase (VAChT)

In recent years, the cholinergic innervations in the CNS have been implicated as a potential mechanism to counteract excessive production of pro-inflammatory cytokines in addition to its role in attention and learning and memory (Nicolussi et al. 2009). The activation of nAChRα7 on non-neuronal cytokine producing cells has been shown to

inhibit the overproduction of inflammatory cytokines (Pavlov and Tracey 2006). Interestingly, when a high dose of LPS was administered peripherally to mimic sepsis, substantial loss of neurons and cholinergic innervations was observed in different brain regions (Semmler et al. 2007).

In order to determine the effect of the increasing concentration of LPS has on the cholinergic innervation in the cortex and the hippocampus, the VAChT was chosen to label cholinergic terminals. The VAChT is a more specific marker in revealing the cholinergic networks rather than the acetylcholinesterases since the latter has been found in cholinergic and cholinoceptive neurons whereas VAChT is exclusively found in the synaptic terminal of cholinergic neurons (Wong et al. 1999).

Interestingly, the Western blot analysis demonstrated differential effects in the results between the hippocampus and cerebral cortex. An increase in VAChT expression was observed in the hippocampus while a decrease was noticed in the cortex (figure 3.37). The reduction of the VAChT expression in the cortex is in agreement with the loss of cholinergic innervations as reported previously by Semmler et al. (2007). In AD patients where neuroinflammation is prevalent, it is normally defined by a massive loss of the cholinergic innervations especially in the basal forebrain. This strengthens the hypothesis that the neuroinflammation precedes the cholinergic loss in AD.

In transgenic animals that are overexpressing the mutated form of APP (APP_{C695T}), an increase in cholinergic synaptic density was also reported by elevated levels of VAChT

in affected brain regions (Wong et al. 1999). Although the physiological function of APP is unknown, it has been postulated to have trophic properties. Hence in brain regions that are overexpressing APP, there could be not only an increase in the production of potentially toxic Aβ peptides, but also the secretion of the neurotrophic APP region as well (Wong et al. 1999). Consequently an upregulation of cholinergic synapses could be observed. Alternatively, the upregulation of the VAChT could be linked to a sustained inhibition of the cholinergic function as Aβ peptides have been shown to potently inhibit the K^+-evoked acetylcholine release from hippocampal slices. The sustained inhibition could induce a transient compensatory changes or sprouting of the cholinergic network (Wong et al. 1999). The increase in the VAChT seen could hence be associated with the increased expression of APP in the hippocampus.

4.4 Effect of LPS on neurogenesis

Neurogenesis, a process of generating functionally integrated neurons from progenitor cells, occurs in regions such as the subgranular zone (SGZ) of the dentate gyrus (Ming and Song 2005). Within 3 weeks, most of these newborn neurons become integrated into existing dentate gyrus circuitry, receiving excitatory synaptic input from perforant path afferents and transmitting signals along the axons to CA3 (Kee et al. 2007).

The field of neurogenesis and its correlation with cognitive functions has remained controversial. However, there is evidence to suggest that neurogenesis could be related to learning and memory. Irradiation has been shown to block neurogenesis and lead to deficits in learning and memory (Wu et al 2007). It was recently reported that some of the new neurons were incorporated into the dentate gyrus circuits to support spatial memory function in an age-dependent manner. The recruitment of new neurons did not occur until they are at least 2 weeks old. By 4 weeks, new neurons were more likely than mature neurons to be recruited into the dentate gyrus circuits (Kee et al. 2007) as they showed decreased threshold plasticity (Schmidth-Heiber et al. 2004).

Chronic neuroinflammation may invariably have a negative effect on neurogenic processes. In adult transgenic mice with chronic astrocytic production of IL-6, it was reported that the overall neurogenesis in the hippocampal dentate gyrus decreased by 63%, where the proliferation, survival and differentiation of the neural progenitor cells were significantly reduced in the granule cell layer (Vallières et al. 2002). A single

1mg/kg LPS IP administration also demonstrated a 35% decrease in hippocampal neurogenesis and could be reversed by treatment using NSAID (Monje et al. 2003).

Thus, the cognitive impairment observed using the increasing LPS dose concentration seems to suggest that the delayed effect could be linked to the disruption of neurogenesis. As reported previously (Kee et al. 2007), there exist a critical period in which the disruption of neurogenesis by neuroinflammatory processes could induce a cognitive deficit. Based on current results, this critical period lies between 8 to 12 weeks post LPS treatment. When animals were tested beyond this time window, no cognitive impairment was observed. However, Kee et al. (2007), reported that the preferential recruitment of these newborn neurons in spatial memory begins at 2 weeks and peaks at 4 weeks. The cause of this discrepancy is still unclear.

There are several hypotheses on the relationship between neuroinflammation and neurogenesis. One of these is that the stimulation of the HPA stress axis with the elevation of glucocorticoids, leading to the alterations in the relationship between progenitor cells and cells of the neuro-vasculature (Monje et al. 2003). Glucocorticoids levels were previously measured by Chen et al. (2005) where the increasing LPS dosing regime was adapted and modified, had reported elevated glucocorticoids levels in animals treated with this dosing regime. The elevated glucocorticoids level may be the underlying reason to the disruption of neurogenesis.

Furthermore, proinflammatory cytokines especially IL-6, have been shown to induce a non-specific decrease in cell survival and a decrease in the accumulation of neurons specifically *in vitro*. The decrease in the number of neurons has been linked to the reduction in neuronal differentiation (Monje et al. 2003). The inflammatory mediators such as IL-6, TNFα and IL-18, in addition to preventing neuronal differentiation, have been reported to induce an increase in glial differentiation (Liu et al. 2005, Cacci et al. 2008). Thus, systemic administration may not only induce an overall decrease in cell proliferation in the dentate gyrus, but it may affect the differentiation of cells to the neuronal pathway. With more progenitor cells showing preference to commit to the glial pathway, the number of available new neurons to be preferentially recruited to form the spatial map is significantly reduced.

LPS administration has been reported to induce a reduction of BDNF (Wu et al. 2007). The BDNF signalling pathway includes the expression level of TrkB, has been shown to modulate cell survival (Sairanen et al. 2005). The reduction of cell survival signals could ultimately lead to the alteration in neurogenesis.

While neurogenesis may offer a possible explanation on the deficits seen when LPS animals were tested at 8 and 12 weeks after treatment, experimental evidence is required to support this hypothesis. Another possible mechanism that may induce the deficit seen could be due to the long-term consequences of sepsis induced by LPS treatment. Semmler et al. (2007) have shown that when rats injected with a single acute dose of 10mg/kg LPS displayed memory deficits in the radial maze three months after treatment.

The deficits seen by Semmler et al. (2007) were associated with a reduction of neurons in the prefrontal cortex and hippocampus in addition to a reduction by the cholinergic innervations in the parietal cortex. Hence, it would be interesting to investigate whether similar changes in the hippocampus and prefrontal cortex could be seen in rats that were dosed using the increasing LPS dosing regime.

CHAPTER 5

CONCLUSION

The purpose of the current study was to investigate the effect of neuroinflammation induced by peripheral administration of LPS on learning and memory. Initial studies were conducted to determine a suitable dosing regime to induce cognitive impairment. Peripheral administration of LPS using the increasing LPS dosing regime was able to induce deficits in spatial learning and object recognition tasks. Furthermore, impairment in reversal learning was also demonstrated using FC. As no significant difference was observed in the side-effect profiling tasks, these cognitive impairments were not induced by sickness behaviour.

Peripheral administration of LPS was shown to be able to induce an inflammatory response in the CNS as elevated TNFα and MHCII levels were observed. These proinflammatory mediators produced in response to LPS are hypothesised to affect cognition. Cytokines such as TNFα have been shown to be able to affect learning and memory processes such as LTP by affecting glutamatergic transmission in terms of receptor numbers (Vereker et al. 2006), transporters (Zou and Crews 2005) and glutamate release (Vereker et al. 2006). Furthermore, excessive glutamate present in the synaptic cleft due to the alteration in transmission is able to induce exitotoxicity (Hardingham et al. 2002). Not limiting to glutamatergic transmission, LPS treatment has also been shown to affect cholinergic innervations. The reduced expression of VAChT in the cortex

suggested a loss of cholinergic innervations. The loss of cholinergic innervations suggests that in AD, neuroinflammation precedes the occurrence of cognitive deficits and neuronal loss in the basal forebrain.

These proinflammatory mediators are also able to affect synaptic plasticity as the translation of proteins belonging to the IEG family such as Arc was shown to be decreased when tested 40 hours after the treatment in the hippocampus. As IEG are indicative of neuronal activity (Vereker et al. 2000), the reduction of Arc expression in basal levels suggests that there is an alteration in the neuronal activity after the LPS treatment. In addition, cytokines such as TNFα has been suggested able to affect neurite outgrowth as well (Neumann et al. 2003).

Deficits induced by the LPS treatment were also detected at 8 and 12 weeks post LPS treatment. The delayed impairment observed suggest a possible disruption in the neurogenesis processes induced by the LPS treatment. Although it remains to be controversial, new born neurons were hypothesised to be preferentially recruited during learning and memory processes (Kee et al. 2007). Unfortunately, inflammation has been shown to affect these new born neurons in terms of cell survival (Sairanen et al. 2005) and in cell differentiation where progenitor cells were more likely to enter the glial cell pathway (Monje et al. 2003). The alteration in these neurogenesis processes could be attributed to the excessive production of proinflammatory cytokines and glucocorticoids and the reduction of neurotrophic factors such as BDNF.

Neuroinflammation, hence could potentially have two phases on cognition. On one hand, immediate effect of the LPS treatment could be detected within 40 hours after the last treatment by affecting glutamatergic and cholinergic transmission as well as synaptic plasticity. On the other hand, the LPS treatment could induced a delayed cognitive impairment as demonstrated when the rats were tested 8 to 12 weeks after LPS treatment induced by an alteration in the neurogenesis process. Therefore, in patients suffering from neurodegenerative diseases with a prevalent neuroinflammation, the cognitive impairment could thus be a complex interaction between the immediate and delayed effects of neuroinflammation. Treatment of anti-inflammatory agents such as indomethacin, however, was shown to be effective in reversing the effect of the neuroinflammation induced cognitive deficits when tested 40 hours after treatment. This suggests that anti-inflammatory agents may potentially be effective in treating patients suffering from cognitive impairment such as in AD and MCI. In conclusion, the current study illustrates the detrimental effect of neuroinflammation on cognition and the potential mechanisms that are involved during chronic inflammation in diseases.

CHAPTER 6

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