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# An Examination of the Long-lasting Effects of Lipopolysaccharide-induced Sepsis on Cognitive and Affective Behaviour in Mice

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## Publications Arising from this Thesis

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# Abstract

Post-septic encephalopathy is a poorly understood condition in survivors of sepsis that is characterised by cognitive and affective impairments. This thesis sought to understand this condition by undertaking a comprehensive behavioural and cognitive assessment of mice who had previously survived sepsis. Mice were treated with a septic dose of lipopolysaccharide and one month after this assessed on a battery of tests. Post-septic animals were found to display significant levels of depressive-like and anxiety-like behaviour but no cognitive impairments were found. The hippocampus and amygdala, two key brain areas for affective and cognitive functioning, were also examined in post-septic animals. Significant microglial activation was found in the hippocampus 24 hours post-sepsis and this remained upregulated at two months post-treatment. Other neuroimmune factors were also affected in the post-septic hippocampus and amygdala such as neural precursor cell production and the production of proteins important to long-term potentiation.

In order to investigate whether pharmacological intervention could ameliorate these post-septic changes NF-κB inhibitor pyrrolidine dithiocarbamate was administered prior to sepsis induction which significantly improved outcomes in post-septic mice. To examine whether any intervention after sepsis onset could improve outcomes another experiment was conducted investigating the effects of chronic fluoxetine administration to post-septic mice. This treatment significantly improved affective behaviours and had anti-inflammatory effects in post-septic mice. In order to further examine what may be leading to the behavioural and neuroimmune changes in post-septic mice it was also examined whether these mice were "primed" for excessive inflammatory responses to secondary insults and whether aspects of hippocampal circadian rhythmicity were altered in these mice. Mice were not found to be primed,

although circadian parameters were disrupted post-sepsis. In summary it is demonstrated that LPS-induced sepsis produces long-lasting, but ameliorable, affective changes in mice, accompanied by significant inflammation in the hippocampus. These findings contribute new knowledge which may aid the comprehension of this poorly understood condition.

# Chapter 1. General Introduction

## *1.1 Systemic Inflammatory Response Syndrome, Sepsis, and Sepsis Associated Encephalopathy*

Systemic inflammatory response syndrome (SIRS) refers to a severe inflammatory response co-occurring with alterations in at least two of the following parameters - body temperature, heart rate, respiratory rate and white blood cell count (Robertson & Coopersmith, 2006). Where this occurs as a result of infection patients are said to have sepsis. During sepsis, a complication known as septic encephalopathy (or sepsis-associated encephalopathy) develops in up to 70% of patients (Papadopoulos, Davies, Moss, Tighe, & Bennett, 2000). While altered mental state is the key diagnostic criterion of septic encephalopathy, those who develop this encephalopathy may experience a full spectrum of cognitive symptoms, from confusion to delirium and coma (Jacob, Brorson, & Alexander, 2011). The pathogenesis of septic encephalopathy, although poorly understood, is thought to be multi-factorial – involving blood-brain barrier disruption, reduced cerebral blood flow, an upregulation of inflammatory cells and cytokines, edema, altered synaptic transmission and of particular importance, inflammation (Jacob, et al., 2011). These structural and neuroinflammatory alterations often lead to impaired cognitive and emotional function, and are hypothesised to lead to an increased susceptibility to the development of neurodegenerative diseases (such as Alzheimer's disease) in survivors for many years following recovery (Hopkins, Ely, & Jackson, 2007; Lazosky, Young, Zirul, & Philips, 2010; Semmler, et al., 2013; Widmann & Heneka, 2014; Winters, et al., 2010; Ziaja, 2013), which is often referred to as post-septic encephalopathy.

Though some research has been done on the neurological pathology present in sepsis associated encephalothy and post-septic encephalopathy the aetiology of neither is currently well understood, and there are no successful treatments available. During systemic sepsis, sepsis-associated encephalopathy can manifest as a relatively mild symptomatology, or may develop into irreversible brain damage (Wilson & Young, 2003). Given the heterogeneous nature of sepsis itself, categorising the neurological and cognitive problems which follow during, and after, recovery is a difficult task. A pathology involving ischemic lesions in the cerebral cortex is common in sepsis-associated encephalopathy (Chelazzi, Consales, & De Gaudio, 2008), and imaging studies have provided evidence that blood-brain barrier permeability, influxes of inflammatory cells, neuronal death and subsequent neurotransmitter level alterations and endothelial activation are also likely to play a role (Stubbs, Yamamoto, & Menon, 2013). To what extent this damage is resolved or unresolved in the post-septic brain is not yet known, although the persistent neurological symptoms present in post-septic encephalopathy can include reduced hippocampal volume and altered EEG frequency (Semmler, et al., 2013). This imaging and neuropsychological data implicates the hippocampus as an important area in the pathology of post-septic encephalopathy – although it is not affected in isolation.

### *1.1.1 Cognitive Symptoms in Post-Septic Patients*

The cognitive and affective symptoms which affect post-septic patients are not surprising given the severe brain pathology they experience. Cognitive deficits appear to be the key symptoms which occur due to the persistent neurological damage present in post-septic encephalopathy, and importantly, these are newly acquired after sepsis (Widmann & Heneka, 2014). Although only a small number of assessments of post-septic encephalopathy in survivors exist, the majority support cognitive dysfunction as a

core symptom at various timepoints following hospital discharge. In comparison to survivors of severe cardiac illness, more sepsis survivors reported cognitive problems such as difficulty with problem solving, concentration and memory 1-4 years after hospital discharge (Lazosky, et al., 2010). This suggests that these problems are specific to sepsis and not just a product of an ICU stay. Likewise, six to twenty four months after hospital discharge sepsis survivors show significant verbal learning and memory, working memory, attention and phonetic verbal fluency deficits compared to other ICU survivors (Semmler, et al., 2013). Older patients also show increased cognitive dysfunction following sepsis; with a three year follow-up showing post septic patients were three times more likely to develop cognitive impairment than other age matched hospital survivors (Iwashyna, Ely, Smith, & Langa, 2010). Similarly, paediatric patients who survive sepsis also show significantly impaired cognition compared with population norms several years after hospital discharge (Bronner, et al., 2009).

Survivors of sepsis may also be at an increased risk for developing later neurocognitive diseases. A study over ten years which included survivors of severe sepsis showed that they had an increased hazard ratio for the later development of dementia (1.38-3.77) while 12% experienced severe cognitive decline and 25.3% experienced minimal cognitive decline (Shah, et al., 2013). In a study of American ICU survivors 21.7% of those who developed severe sepsis subsequently developed dementia within 3 years of hospital discharge, with an adjusted hazard ratio of 1.28-1.53 for those with severe sepsis as compared with other patients who received intensive care (Guera, Linde-Zwirble, & Wunsch, 2012). The neuroinflammatory aspect of post-septic encephalopathy may predispose survivors to the development of dementia – of which inflammation is often an important, although not necessarily causal, aspect (Enciu & Popescu, 2013).

*1.1.2 Affective Disorders in Post-Septic Patients*

There have been a number of studies examining affective changes in post-septic patients in the last decade with mixed findings. Survivors of intensive care units (ICUs) are already a high risk group for depression with one longitudinal study showing over 30% of patients reporting at least mild depression 12 months after ICU discharge (Jackson, et al., 2014). Survivors of sepsis are prone to developing a cluster of affective problems including anxiety, depression and post-traumatic stress disorder – in addition to the cognitive and somatic issues associated with post-septic encephalopathy (Jones & Griffiths, 2013; Ziaja, 2013). Indeed hospitalisation for any infection, including sepsis, increases one's relative risk of subsequent mood disorder diagnosis by 62%, with sepsis patients actually showing twice the incidence rate ratio for mood disorders relative to people without a hospital contact with infection (Benros, et al., 2013). Approximately two thirds of patients who survived severe sepsis subsequently reported post-traumatic symptoms, while close to 40% reported depressive and anxious symptoms in a study of German sepsis survivors (Jaenichen, Brunkhorst, Strauss, & Rosendahl, 2012). In another study, 44% of patients reported being at least moderately anxious or depressed at 6 months after sepsis, although this was actually lower than other ICU survivors (Granja, Dias, Costa-Pereira, & Sarmento, 2004). In a Dutch cohort one year following abdominal sepsis 22% were classified as mildly depressed, 7% as moderately depressed and 5% as severely depressed while 28% displayed moderate PTSD and 10% displayed severe PTSD symptoms (Boer, et al., 2008). Survivors of sepsis reported greater difficulty with emotional behaviour and psychosocial functioning relative to survivors of myocardial infarction suggesting that in some cases survivors of sepsis may suffer worse neuropsychological problems than other ICU survivors (Lazosky, et al., 2010), but only when analysing those who had not yet retired from work. This raises the possibility that affective changes may be magnified when post-septic patients are

confronted, and have difficulty, with more complex tasks such as those found in the workplace.

In other studies, however, post septic patients have shown only slightly lowered mental functioning relative to population norms (Cuthbertson, et al., 2013). Using the same measurement Hofhuis et al. (2008) showed that while mental component score showed a decline just after ICU discharge it had recovered by 6 months post-discharge, although the role emotional and mental health components remained significantly lower than population norms at this timepoint. although depressive symptoms may be higher in survivors of sepsis it has also been suggested that this depression, in contrast to cognitive diffciulties, may be present before sepsis onset in older populations and simply mainted after recovery from sepsis (Davydow, Hough, Langa, & Iwashyna, 2013). Thus, although cognitive and physical impairment seem to be common following sepsis, affective changes may only be present at certain timepoints or when survivors experience certain difficulties such as returning to work. It may be that the neuroinflammatory changes present post-sepsis can serve as a risk factor which predisposes individuals towards developing depression following stressful life experiences.

### *1.1.3 Animal Models of Sepsis and Post-Septic Encephalopathy*

The aetiology of sepsis associated encephalopathy and post-septic encephalopathy can be difficult to study in human populations. During septic incidents patients require intensive care and are treated with a variety of drugs which affect neurological and inflammatory measures such as norepinephrine, corticosteroids and vasopressin (Verceles, et al., 2012). A significant portion (~35%) of septic patients do not survive hospitalization (Daniels, 2011) and significantly elevated mortality levels are present in those who survive their hospital stay for years after discharge (Winters, et

al., 2010). This means that post-mortem studies may be influenced by bias towards those suffering from the most severe sepsis, while long-term studies of survivors may include those who suffered more moderate septic incidents. Another problem is that sepsis in humans is a heterogeneous condition which can originate due to a wide variety of infectious challenges and as such the subsequent neurological issues present in survivors are likely to differ significantly. In an effort to categorise the physiological and behavioural sequelae of sepsis a number of animal models have therefore been developed.

Sepsis is commonly induced via cecal ligation and puncture (Imamura, et al., 2011), specifically termed polymicrobial sepsis, which leads to a potent systemic inflammatory response, dysfunction in multiple organs, and often death (Rittirsch, Huber-Lang, Flierl, & Ward, 2008). Another common model involves the intraperitoneal or intravenous injection of a high dose of lipopolysaccharide (LPS) (Doi, Leelahavanichkul, Yuen, & Star, 2009). This stimulates the release of numerous pro-inflammatory cytokines such as tumour necrosis factor-α (TNF- α), interleukin-1 (IL-1) and interleukin-6 (IL-6) in addition to septic shock. In our model, mice are treated with 5mg/kg LPS from *escherichia coli* which induces systemic shock within hours of treatment, followed by a recovery period of several days, with a morbidity rate of around 10% (O'Callaghan, Anderson, Moynagh, & Coogan, 2012). This treatment has previously shown to upregulate pro-inflammatory factor TNF- α and increase the number of microglia active throughout the whole brain for up to 10-months post-treatment (Qin, et al., 2007). As with human studies of post-septic encephalopathy, in terms of animal models there has been significant differences in the time left between sepsis induction and behavioural testing – with measurements occurring anywhere from 10 days (Cassol-Jr, et al., 2011) to several months (Semmler, et al., 2007).

*1.1.4 The Present Model – Lipopolysaccharide Induced Sepsis*

While sepsis in humans is a heterogeneous condition, for the present research the intraperitoneal injection of a high dose of the bacterial endotoxin lipopolysaccharide (LPS) was selected due to its common use in modelling sepsis (Buras, Holzmann, & Sitkovsky, 2005; Doi, et al., 2009). Peripheral administration of high-dose LPS leads to a potent inflammatory response, via Toll-like receptor-4 (TLR-4) binding and subsequent Nuclear Factor-κB (NF- κB) pathway activation. This septic dose of LPS mimics many of the clinical features of sepsis and results in a hyper-inflammatory response, accompanied by depressed EEG rhythmicity, low blood pressure, oxidative stress, multiple organ failure and a significant mortality rate (Chang, Tsai, Sheu, Hsieh, & Chiang, 2013; Lin, Chen, Lee, Chen, & Yang, 2010; Okazaki, Tachibana, Koga-Ogawa, & Takeshita, 2013). Following recovery from the acute effects of LPS-induced sepsis, an increased level of microglial activation persists in the CNS, accompanied by prolonged over-expression of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) as well with time and dose dependent neuronal degeneration (Bossú, et al., 2012; Qin, et al, 2007; Semmler, et al., 2007; Weberpals, et al., 2009).

In addition to persistent neuroimmunological changes, there have been a number of studies examining cognitive and affective parameters following recovery from both LPS-induced, and CLP-induced sepsis. There is considerable variance in the behavioural changes present in post-septic animals depending on both the method of sepsis induction and the latency to test behaviour following recovery from sepsis. Within the domain of learning and memory, post-septic animals have shown cognitive impairments across tasks such as the step-down passive avoidance task, the radial arm maze, and novel object recognition tasks (Barichello, et al., 2007; Cassol-Jr, et al., 2010; Cassol-Jr, et al., 2011; Semmler, et al., 2007; Weberpals, et al., 2009). Ten

months after LPS-induced sepsis rats show no differences in the elevated plus maze or Morris watermaze, although they exhibit reduced exploration and recognition of a novel object in the open field test (Bossú, et al., 2012). Post-septic animals have also been shown to display increased levels of depressive-like behaviour including both anhedonia and behavioural despair (Comim, et al., 2010; Tuon, et al., 2008) as well as anxiety-like behaviour in the elevated plus maze ten days after CLP (Calsavara, et al., 2013). These affective changes may be linked to the growing evidence for a neuroimmune basis to mood disorders such as major depressive disorder (Berk, et al., 2013; Miller, Maletic, & Raison, 2009). The wide variation in methodology used to study sepsis and its behavioural consequences illustrate the need for a comprehensive examination of these effects in a single model.

## *1.2 Neuroimmune Signalling in the CNS*

### *1.2.1 LPS from the Periphery to the Brain*

In order to understand how a peripheral dose of LPS may affect brain function in this model it is first necessary to describe how peripherally administered LPS can exert effects on the central nervous system (CNS). Minimal amounts of peripherally injected LPS may cross the blood-brain barrier and the CNS does contain many cells which recognise and respond to LPS. Despite this, evidence suggests that peripherally administered LPS does not, except at the highest doses, directly affect the CNS but rather mediates its effects indirectly via a number of mechanism (Banks & Robinson, 2010; Singh & Jiang, 2004). Therefore, while Banks and Robinson (2010) suggest that a 5mg/kg dose of LPS may be enough to affect the most LPS-sensitive parameters of the brain via brain endothelial cells, it is also necessary to consider the other mechanisms through which peripheral LPS administration exerts such potent effects on the CNS. These are illustrated in Figure 1.1.

It is known that cytokines in the periphery, which are upregulated in response to LPS administration (Juskewitch, et al., 2012; Qin, et al., 2007), can affect brain cytokine levels via a number of different mechanisms (Dantzer, 2001). Firstly, LPS is known to stimulate the sensory pathways of the vagus nerve, representing one pathway through which immune signals can be transmitted from the periphery to the brain (Wan, Wetmore, Sorensen, Greenberg, & Nance, 1994). Afferent vagal fibers transmit information to the nucleus tractus solitaries which projects to sites such as the paraventricular nucleus of the hypothalamus (Arkrout, Sharshar, & Annane, 2009; Bailey, Hermes, Whittier, Aicher, & Andresen, 2007). While signalling via this pathway is important for peripheral LPS to induce neuroinflammation, vagotomy fails to block increased levels of IL-1β in the rodent brain following high doses of LPS (Van Dam, et al., 2000), indicating the importance of other pathways in eliciting the CNS response to systemic LPS.

Secondly, cytokines themselves, while too large to passively cross the blood-brain barrier, can enter the brain via the circumventricular organs which lack a blood-brain barrier (Arkrout et al., 2009). Both toll-like receptors and cytokine receptors are present within the circumventricular organs (Dantzer, 2004). From the circumventricular organs, cytokines may then move towards deeper structures such as the hippocampus.

Apart from entering passively via the circumventricular organs, cytokines may also be actively transported across the blood-brain barrier (McCusker & Kelley, 2013). While specific transporter systems for all cytokines have not yet been identified, blood to CNS transporter systems have been isolated for TNF-α, IL-6 and IL-1β (Banks, Kastin, & Broadwell, 1995; Pan & Kastin, 2002; Quan & Banks, 2007). LPS itself induces cell death in the endothelial cells which make up the blood-brain barrier by

activating microglia (Kacimi, Giffard, & Yenari, 2011), disrupting blood-brain barrier stability and increasing its permeability.

The fourth mechanism involves cytokines and LPS itself interacting with brain endothelial cells, causing them to produce their own cytokines (including IL-1, IL-6 and TNF) which can then activate microglia within deeper brain structures (Verma, Nakaoke, Dohgu, & Banks, 2006; Quan & Banks, 2007). Once activated, microglia within the CNS actively produce pro-inflammatory factors. This pathway is thought to primarily be mediated via the synthesis of prostaglandins, particularly prostaglandin E2, driven by cyclooxygenase (COX)-2 (Engblom, et al., 2002).

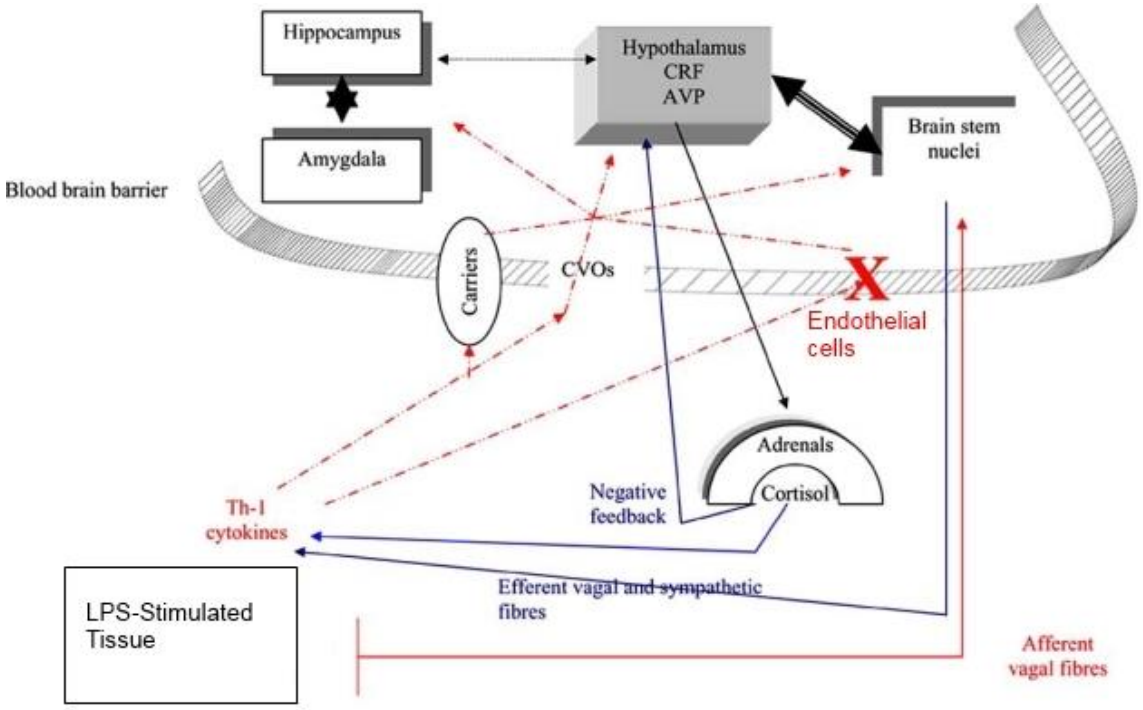



***Figure 1.1 Pathways via which peripheral LPS may act on the CNS.*** *CVO = circumventricular organs, CRF = Corticotrophin Releasing Factor, AVP = Vassopressin. Red represents activating signals while blue represents immunosuppressive signals. Adapted from Arkrout,et al., (2009).*

*1.2.2 LPS Signalling Cascade*

LPS is a component of all gram-negative bacteria. Located on the outer membrane of the cell, LPS elicits a potent innate immune response – which can lead to endotoxic shock (Bryan, Spring, Gangloff, & Gay, 2010). The primary mammalian receptor responsible for the recognition of LPS is TLR-4 (Poltorak, et al., 1998). As illustrated in Figure 1.2, there are several proteins necessary for TLR-4's recognition of LPS however. Firstly, plasma protein LPS binding protein (LBP) binds directly to the lipid A domain of LPS forming a complex which can then bind to the surface antigen CD14, which is present on monocytes and macrophages of the innate immune system (Schumann, et al., 1990; Wright, Ramos, Tobias, Ulevitch, & Mathison, 1990). Another protein involved is myeloid differentiation factor (MD)-2. Soluble MD-2 non-covalently associates with TLR-4 and is necessary for the proper recognition of LPS (Nagai, et al., 2002). Once bound to LPS via LBP, CD14 then allows for the transfer of LPS to a hydrophobic pocket of the MD-2 component of the TLR4/MD-2 complex (Lu, Yeh, & Ohashi, 2008; Tissières, et al., 2008).

Once LPS has bound to the TLR-4-MD-2 complex two different pathways may become activated involving four separate adaptor proteins - TIR-domain-containing adapter-inducing interferon-β (TRIF), translocating chain-associating membrane (TRAM), myelin and lymphocyte (MAL) and myeloid differentiation primary response 88 (MyD88) (O'Neill & Bowie, 2007). One pathway involves the adaptor protein pair TRAM-TRIF, while the other involves the MAL-MyD88 pair. The TRAM and MAL proteins have been referred to as bridging adaptors - associating with the MD2-TLR4 complex and recruiting TRIF and MYD88 respectively (O'Neill & Bowie, 2007). It has been suggested that the MAL-MyD88 pathway induces proinflammatory cytokine

expression while the TRAM-TRIF pathway activates Type I interferons and interferon-inducible genes (Lu et al, 2008).

The primary function of MyD88 is to induce rapid NF-κB activation. Once recruited by MAL, MyD88 subsequently activates IL-1R-associated kinase (IRAK)-4 via a death domain-death domain interaction which in turn can phosphorylate IRAK-1 (Li, Strelow, Fontana, & Wesche, 2002). Downstream tumor-necrosis-factor-receptor-associated factor 6 (TRAF6) is then activated – recruiting transforming-growth-factor-β-activated kinase 1(TAK1) and TAK1-binding protein 2(TAB2), as well as ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13) (O'Neill & Bowie, 2007). It is this complex which then activates IκB kinase (IKK) and mitogen-activaed protein kinase (MAPK) pathways which lead to the induction of p38 and JUN N-terminal kinase (JNK). Phosphorylated by IKKα, IKKβ and IKKγ, inhibitor of κ light chain gene enhancer in B cells (IκB) subsequently degrades allowing for the translocation of NF-κB to the nucleus, which is typically bound in a inactive state with IκB in the cytosol, stimulating the production of several proinflammatory cytokines (Lu, Yeh, & Ohashi, 2008). In addition to this, MAPK pathway activation induces the activation of the transcription factor activator-protein (AP)-1 which is involved in a wide range of functions including cellular proliferation, death and differentiation (Shaulian & Karin, 2002). MyD88s – a spliced variant of MyD88 may inhibit NF-κB, but not JNK, activation by interfering with IRAK4 recruitment (O'Neill & Bowie, 2007).

Unlike the MAL-MyD88 heterodimer, the TRAM-TRIF protein pair induce later phase NF-κB induction following TLR-4 activation, in addition to type I interferon activation via interferon regulatory factor(IRF)-3 and IRF-7 (Fitzgerald, et al., 2003). Upon phosphorylation of TRAM, TRIF is recruited which in turn activates TRAF-

family-member-associated NF-κB-activator-binding kinase 1(TKB1) leading to IRF3 and IRF7 activation. TRAF6 is also activated inducing NF-κB production via IKK phosphorylation (O'Neill & Bowie, 2007). Type I interferons induced by IRF3 and IRF7 are important primarily for responding to viral infections, but are also modulators of the immune response to bacterial infection (González-Navajas, Lee, David, & Raz, 2012). Importantly, TRAM-TRIF may also induce apoptosis by activating receptor interacting protein (RIP)1, a key regulator of cell survival and death (Festjens, Vanden Berghe, Cornelis, & Vandenabeele, 2007).

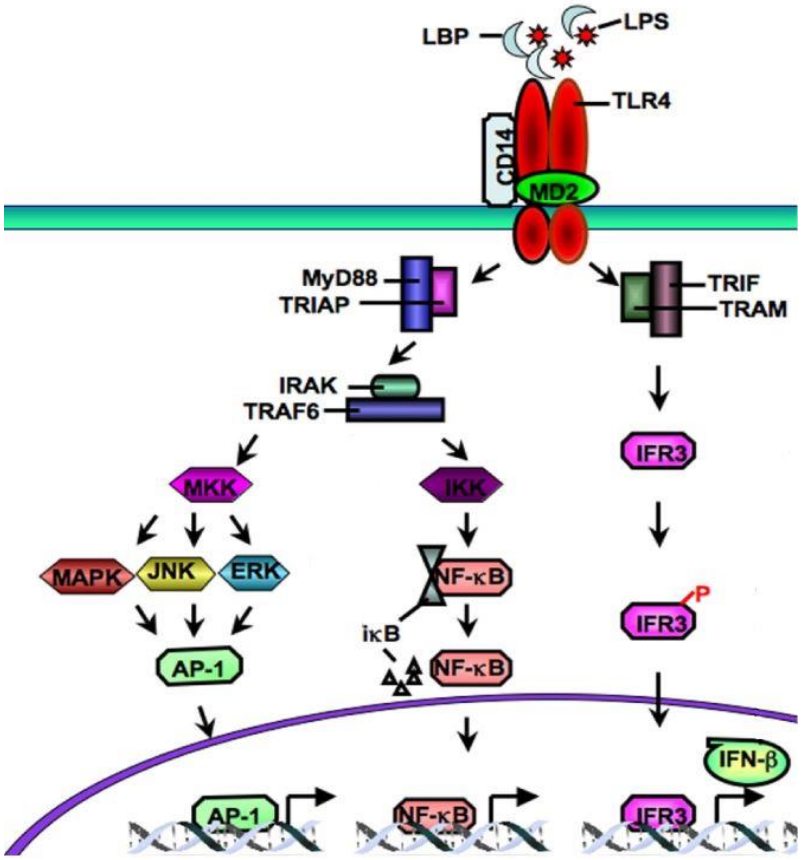



***Figure 1.2 Illustration of the key stages in LPS recognition by TLR-4 and subsequent NF-κB induction.*** *Adapted from Guo & Friedman (2010)*

*1.2.3 The NF-κB Pathway*

NF-κB is a transcription factor composed of a family of five proteins – NF-κB1 (p50), NF-κB2 (p52), c-Rel, RelA (p65) and RelB. Depending on the stimulus and context in which NF-κB is activated each of these five factors forms either hetero- or

homodimers (Hoesel & Schmid, 2013). Once liberated from IκB, NF-κB translocated from the cytoplasm to the nucleus binds with to DNA and can begin to regulate transcription of hundreds of target genes (Karin & Ben-Neriah, 2000). Depending on which NF-κB family members compose a hetero- or homodimer different genes will be regulated by NF-κB activation (Cao, Zhang, Edwards, & Mosser, 2006). LPS induces NF- κB activation via the canonical pathway (as described above) and this is typically thought to be associated with heterodimers containing RelA (p65) or c-Rel (Lawrence, 2009). NF-κB target genes are involved in a wide range of functions relating to inflammation and immune responses. These include cytokines, chemokines, cell adhesion molecules, interferons, IEGs and others. In addition to this, many of the genes which are upregulated by NF-κB lead to the activation of NF-κB themselves (Barnes & Karin, 1997).

Though the genes upregulated following the binding of NF-κB vary depending on the context of activation and cell type in which it is active, among the relevant genes regulated by NF-κB are the chemokines monocyte chemoattractant protein (MCP)-1, chemokine (C-C) motif ligand (CCL)5 (Jang & Lee, 2000) and IL-8 (Lokensgard, Hu, van Fenema, Sheng, & Peretson, 2000; Martin, Cardarelli, Parry, Felts, & Cobb, 1997); cytokines such as IL-1β (Greten, et al., 2007; Hiscott, et al., 1993), IL-6 (Libermann & Baltimore, 1990), IL-10 (Cao, et al., 2006), TNFα (Shakhob, Collart, Vassalli, Nedospasov, & Jongeneel, 1990; Qiutang & Verma, 2002); adhesion molecules such as intercellular adhesion molecule (ICAM)1 (Bunting, et al., 2007); enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase(COX)-2 (Arias-Salvatierra, Silbergeld, Acosta-Saavedra, & Calderon-Aranda, 2011; Kim, et al., 2007; Nadjar, et al., 2005); both pro-apoptotic factors such as Fas ligand (FasL) (Kasibhatla, et al., 1998) and inhibitor of apoptosis family members (xiap, hiap1, and hiap2) (Stehlik, et al.,

1998) and immediate early genes such as EGR-1 (Chapman & Perkins, 2000; Choi, Park, Do, Yang, & Moon, 2012). Some of these factors also positively (e.g. IL1β, TNFα) or negatively (e.g. EGR-1) regulate NF-κB transcription themselves. As this pathway represents the primary route through which LPS activates an immune response in sepsis an understanding of its mechanisms and the consequences of its activation and disruption are important for modelling and understanding septic pathologies.

*1.3 The Hippocampus*

The hippocampus was chosen as the primary brain region for investigation in the present study due to its susceptibility to damage during sepsis (Semmler, et al., 2013) and its importance in both cognition and emotion (Fanselow & Dong, 2010). The rodent hippocampus is a large brain structure that stretches rostro-caudally and dorso-ventrally from the septal nuclei within the basal forebrain (dorsal) to the temporal lobe (ventral) (Tanti & Belzung, 2012). Within humans, the hippocampus has rotated and shifted in location close to the base of the brain due to the extensive development of neocortical regions (Kempermann, 2012). When comparing human and rodent hippocampal areas the septal pole of the rodent hippocampus corresponds to the posterior section of the human hippocampus, while the temporal pole corresponds to the anterior hippocampus of humans (Tanti & Belzung, 2012). The hippocampus itself is an anatomically heterogeneous region and it has long been thought that this heterogeneity gives rise to functional dissociation between hippocampal structures (Grant & Jarrard, 1968; Moser & Moser, 1998). Following decades of work, this dissociation of function is now thought to exist between dorsal, intermediate and ventral regions, and also among the various hippocampal subfields encompassing the dentate gyrus and Cornus Ammonus (CA) regions 1 to 3 (Bast, Wilson, Witter, & Morris, 2009; Fanselow & Dong, 2010).

Here the functional and anatomical characteristics of each hippocampal area and the contribution of the region to both cognition and affective behaviour will be reviewed.

### *1.3.1 Dentate Gyrus*

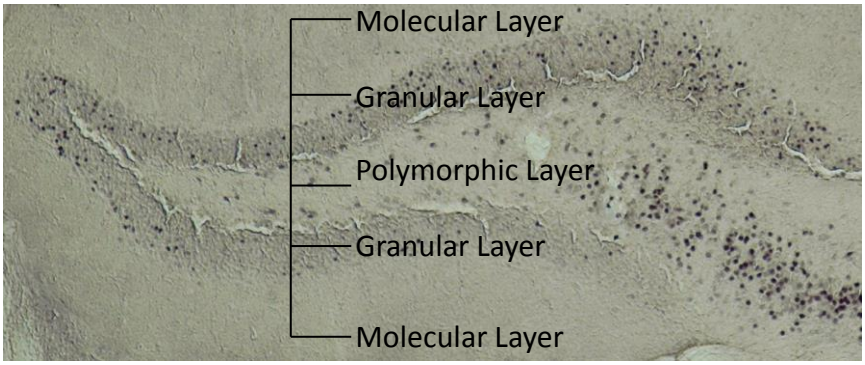
One of the most basic divisions within the hippocampus is between the dentate gyrus and CA1-3 regions. Anatomically, the dentate gyrus consists of several distinct layers, shown in Figure 1.3. Two blades of densely packed granule cell somata make up the granular layer, with axonal projections, known as mossy fibers, expanding into the hilus (polymorphic cell layer) and innervating the mossy cells there, while their dendrites expand to the molecular cell layer (Amaral, Scharfman, & Lavenex, 2007; Scharfman & Myers, 2013; Scorcioni, Bouteiller, & Ascoli, 2002). Sensory input is primarily transmitted to the dentate gyrus via the entorhinal cortex through the perforant pathway, with granule cells of the dentate then transmitting signals exclusively, in rodents, to the CA3 (Neves, Cooke, & Bliss, 2008). The molecular cell layer, granule cell layer and polymorphic cell layer each contain a variety of different cell types and each serve a different function within the dentate gyrus and hippocampus as a whole.

#### *1.3.1.1 Molecular Cell Layer*

The molecular cell layer, comprised of three sub-layers, is primarily composed of dendrites from granule cells as well as pyramidal basket cells and polymorphic layer cells, and its outer layer receives the principal sensory input to the hippocampus from layer II of the entorhinal cortex (Amaral, Scharfman, & Lavenex, 2007). It also receives other input originating outside the entorhinal cortex – for example cholinergic input from the septum (Guthrie, Tran, Baratta, Yu, & Robertson, 2005) and hypothalamic projections (Dent, Galvin, Stanfield, & Cowan, 1983). The area is also sparsely populated by a number of other neurons, for example GABAergic interneurons which signal to the subiculum (Ceranik, et al., 1997) as well as other cells which may control

granule cell activity such as molecular layer perforant pathway (MOPP) cells which have recently been shown to play a role in functionally incorporating newborn granule cells via feed forward inhibition (Li, et al., 2013). This control of granular and subsequent mossy cell activation has been referred to as "gating" – such that only specific signals are sent beyond the dentate gyrus (Walker, Pavlov, & Kullmann, 2010).

A)




B)

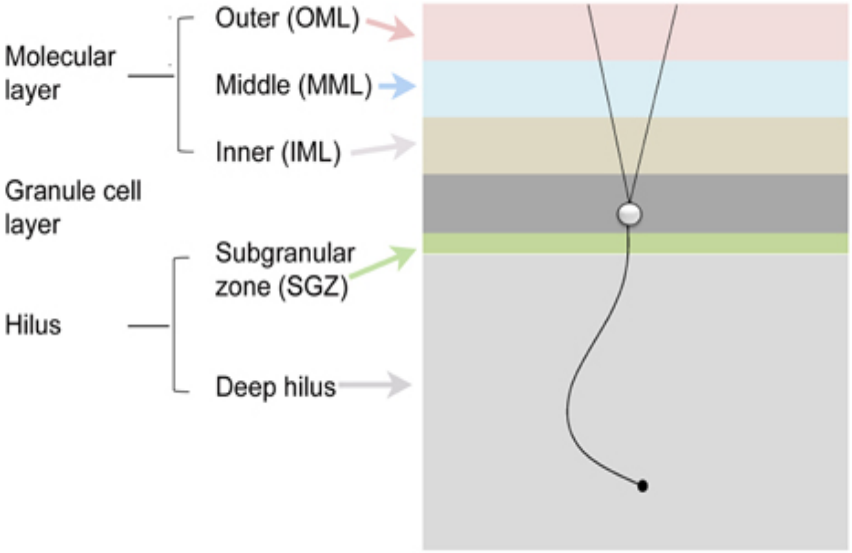



***Figure 1.3 Dentate gyrus neuroanatomy*** *A) Photomicrograph showing each layer of the mouse dentate gyrus, B) Diagram adapted from Scharfman & Myers, 2013 showing a granule cell and its dendritic projections from the granular layer through the three sections of the molecular layer as well as mossy fibre expansion to the hilus/polymorphic cell layer.*

*1.3.1.2 Granule Cell Layer*

The granule cell layer of the dentate gyrus contains the tightly packed cell bodies of a large number of granule cells and is divided into two "blades" – the

suprapyramidal and infrapyramidal blades (Amaral, et al., 2007). These granule cells are excitatory in nature and project both to the polymorphic cell layer (Jinde, Zsiros, & Nakazawa, 2013) and the CA3 (Szabadics & Soltesz, 2009) via specialised axons known as "mossy fibers". Granule cells transmit spatial information to the CA3, showing environmentally selective firing (Jung & McNaughton, 1993) and *Arc* expression (Chawla, et al., 2005). Brief spatial learning alone is sufficient to promote survival of newborn granule cells – highlighting the specialised nature of these cells (Feng, et al., 2010). Aside from spatial information, granule cells can also be affected by emotional experiences. Maternal deprivation has been shown to subtly affect granule cells, significantly lowering the cell density in the granule cell layer as well as altering cell morphology (Oomen, et al., 2011).

The granule cell layer becomes home to a large number of newborn neurons throughout adult life. In rats for example, roughly 9,000 cells are created each day (Cameron & McKay, 2001), and by the late stages of aging adult-born granule cells make up about 40% of the population of the granule cell layer (Snyder & Cameron, 2012). As only a small number of granule cells fire in response to a given situation, there has been speculation that mature granule cells may in fact be "retired" – with the more excitable newer neurons assuming their function (Alme, et al., 2010). It has been shown however that specific ablation of mature adult-born granule cells only interferes with memory recall when it takes place after training, rather than beforehand (Arruda-Carvalho, Sakaguchi, Akers, Josselyn, & Frankland, 2011). This indicates that while newborn granule cells are heavily involved in memory consolidation, mature granule cells are likely not retired despite their sparse activity. It has also been suggested that these older granule cells may be differentially activated in situations involving high

emotional salience, given the differential stimulation of these two types of granule cell by the amygdala (Wosiski-Kuhn & Stranahan, 2013).

*1.3.1.3 Polymorphic Cell Layer*

Positioned between both blades of the granule cell layer is the polymorphic cell layer of the hippocampus. While a variety of cell types inhabit this area, the principal neuron is the mossy cell. These cells feature large cell bodies and dendrites which project extensively throughout the polymorphic layer and sometimes to the molecular layer through the granule cell layer (Amaral, Scharfman, & Lavenex, 2007). They are primarily excitatory and can activate both granule cells and inhibitory interneurons in the dentate gyrus. (Scharfman, 1995). These mossy cells are characterised by large, complex spines known as thorny excrescences which are present on their dendrites (Buckmaster, Strowbridge, & Schwartzkroin, 1993) and receive input from the mossy fibers of granule cells (Henze & Buzsáki, 2007). Morphofunctional differences exist between dorsal and ventral mossy cells – dorsal mossy cells show more compact dendritic fields and more complex and numerous thorny excrescences while more ventral cells show a wider dendritic spread but smaller thorny excrescences (Fujise, Liu, Hori, & Kosaka, 1998). Mossy cells can also be stimulated via perforant pathway signals through their dendrites extended to the molecular layer. (Scharfman, 1991). It has been suggested that these mossy cells, given their ability to project to septotemporally distant dentate gyrus neurons, may function to pass on information from granule cells at one level of the hippocampus to those in distal areas (Amaral, et al., 2007).

Aside from mossy cells, the polymorphic layer is also home to a variety of interneurons. although many of these are not yet well understood, two of the best characterised are the hilar perforant path-associated cell (HIPP) and the hilar

commissural-associational pathway-related cell (HICAP) (Amaral & Lavenex, 2007). Sik, Penttonen, & Buzsáki (1997) conducted detailed physiological characterization of these interneurons using electrophysiology and electronmicroscopy. They described HIPP cells as responding to perforant pathway stimulation via granule cell activation, with their axons in turn extending both to the outer two thirds of the molecular layer and both blades of the granular layer. These spiny neurons expand extensively through the septotemporal axis of the hippocampus, as well as to the contralateral hippocampus, with a single cell making as many as 1355 synaptic contacts. While HIPP cells may modulate perforant pathway input by innervating the outer areas of the molecular layer, HICAP cells have axons located primarily in the inner third of the molecular layer, with roughly one quarter projecting to the granular layer and a small number also remaining within the polymorphic layer (Sik, Penttonen, & Buzsáki, 1997). These cells are GABAergic and their axonal projections are thought to be involved in feed forward inhibition of commissural and associational pathways.

Inhabiting the border between the granule cell layer and the polymorphic layer are pyramidal basket cells. These are the chief inhibitory neurons of the dentate gyrus (Amaral, et al, 2007). Primarily GABAergic, these cells receive input from mossy fibres although they also extend dendrites throughout the dentate, and most commonly signal to granule cells (Ribak & Seress, 1983). They are therefore thought to form an integral part of the dentate gyrus' inhibitory feedback loop, although they may also be involved in feed forward inhibition via their connections with the perforant path (Ribak, 1992). Basket cells vary in number across the extent of the dentate gyrus, with a higher ratio at the temporal pole relative to septal levels (Seress & Pokorny, 1981).

It is here in the subgranular zone that adult neurogenesis takes place (Kaplan & Hinds, 1977). Here, neural precursor cells (NPCs) become mature granule cells and may

then be incorporated into existing hippocampal networks – a process key for both cognitive and affective functioning (Ransome, Renoir, & Hannan, 2012). Multipotent neural stem cells exist within both the subgranular zone of the hippocampus and subventricular zone (Guo, Patzlaff, Jobe, & Zhao, 2012) and have the potential to develop into oligodendrocytes, astrocytes or granule neurons (Duan, Kang, Liu, Ming, & Song, 2008). These neural stem cells (NSCs) can be categorised as either quiescent or active – with some suggesting that active stem cells, which are highly proliferative and responsive to external input, may act as an intermediary between quiescent NSCs and more developed progenitors (Wang, Plane, Jiang, Zhou, & Deng, 2012). The process of neuronal differentiation, survival and incorporation is highly susceptible to pathological and environmental stimuli (Lugbert, et al., 2010) regulated through a variety of pathways and is spread across several developmental stages with a number of critical periods (Ming & Song, 2011).

The first stage of neurogenesis involves the proliferation of quiescent and active radial and horizontal neural stem (type 1) cells which leads to the generation of transit-amplifying progenitors (type 2A/B), also known as intermediate progenitor cells, which may then produce neuroblasts that can differentiate to form either granule cells or astrocytes in the dentate gyrus (Hsieh, 2012). Fate determination (neuronal vs. astrocytic) appears to occur in type 2 cells (Duan et al., 2008), and Type 2B cells are identifiable as the first stage to express doublecortin (DCX), a marker for immature neurons (Hsieh, 2012). The transition from type 2B cells to neuroblasts (type3) is the first critical period during which the majority of newborn cells undergo apoptosis followed by phagocytosis by nearby microglia (Sierra, et al., 2010). Interestingly, it has recently been shown that mature astrocytes, at least in the SVZ, can actually be reprogrammed to become neuroblasts – and subsequently develop into neurons, by the

administration of the transcription factor SOX2 (Niu, et al., 2013). Neuroblasts then go on to develop into immature granule neurons which, over the course of about 4 weeks, migrate to the granule cell layer and develop first GABAergic synaptic inputs, then glutamatergic inputs and finally a mature phenotype expressing NeuN, and exhibiting an axon projecting to the polymorphic cell layer, dendrites within the molecular layer and fast perisomatic GABAergic contacts (Espósito, et al., 2005). During this immature neuronal developmental a second critical survival period occurs regulated by input to the cells' NMDA receptors (Tashiro, Sandler, Toni, Zhao, & Gage, 2006).

In terms of overall functionality, the dentate gyrus has long been suggested to be responsible for pattern separation of entorhinal cortex input and transmitting this information to the CA3 region of the hippocampus (Newman & Hasselmo, 2014). Pattern separation in this context refers to the dentate's involvement in reducing the complexity and potential for overlap, via sparse activation patterns, in signals passed on from the entorhinal cortex which must then be stored by the CA3 (Myers & Scharfman, 2011). The dentate gyrus therefore responds to even moderate environmental change with significant shifts in activity patterns – even though these may involve some overlapping entorhinal input (Neunuebel & Knierim, 2014). As with the dentate gyrus anatomy however, functionality of this area is also believed to vary along the dorsoventral axis. Using optogenetic manipulation of granule cells at varying levels of the dentate gyrus Kheirbek and colleagues (2013) demonstrated that activation of dorsal granule cells led to large increases in exploration of novel environments while inhibition of these cells led to a disruption in the encoding of novel contexts. Activation of ventral granule cells on the other hand led to suppressed anxiety with inhibition failing to alter context encoding – interestingly, stimulation and inhibition of intermediate areas appeared to produce a hybrid effect. The ventral hippocampus may

however be important in some forms of memory processesing with Kesner, Hunsaker, & Ziegler (2011) showing that the ventral hippocampus exclusively contributes to olfactory working memory.

### *1.3.2 CA3*

The primary output of the dentate gyrus is to the CA3 region via mossy fibers – and unlike the pattern separation which occurs in the dentate, the CA3 is thought to be responsible for pattern storage and pattern completion by recreating stored outputs following the transmission of, at least partially, similar patterns from the dentate (Neunuebel & Knierim, 2014). The CA3 does not only receive "pre-processed" signals from the dentate gyrus however, also receiving direct input from layer II of the entorhinal cortex (Neves, Cooke, & Bliss, 2008). As with the dentate gyrus, the CA3 is organised in a laminar fashion, inhabited by numerous neuronal types and can be separated into a number of sub-regions based on their heterogeneous anatomy and function (Hunsaker, Rosenberg, & Kesner, 2008). The laminar organisation of the hippocampus varies only slightly between areas CA1, CA2 and CA3 – moving from the outermost layer the alveus, to the stratum oriens, followed by the principal layer the pyramidal layer, then unique to the CA3 is the stratum lucidum, the stratum radiatum is again common to all three divisions and is followed by the innermost stratum lacunosum-moleculare (Paxinos & Watson, 2007). Beginning proximally to CA2 the CA3 area has also been subdivided into area CA3a which encompasses the initial curved section, CA3b which extends up to the beginning of the granular cell blades of the dentate, and finally CA3c which refers to the area which extends into the polymorphic cell layer (Hunsaker, Rosenberg, & Kesner, 2008). These laminar and transverse distinctions are illustrated below in Figure 1.4.

The most well studied neuron type within the CA3 region are the pyramidal neurons which inhabit the pyramidal cell layer. Receiving input from both the perforant pathway as well as DG mossy fibers, these cells have a pyramidal shaped soma, with dendrites from the base extending into the stratum oriens, while the apex extends dendrites to the stratum radium and stratum lacunosum-moleculare, but not stratum lucidum, in CA3a/b and to the stratum radium alone in CA3c (Amaral & Lavenex, 2007). At ventral levels, additional segregation is possible based on the neuron's location either close to the stratum radium (inner) or close to the stratum oriens (outer) – with each subpopulation expressing different gene profiles (Thompson, et al., 2008). The most striking feature of these neurons although is their ability to project axons through as much as two thirds of the septotemporal axis of both the ipsilateral and contralateral hippocampus (Amaral & Lavenex, 2007). Characterised in vivo in the early 90s (Li, Somogyi, Ylinen, & Buzsáki, 1994), these projections form the Schaffer collateral pathway to the CA1 region, the associational commissural pathway to other neighbouring CA3 pyramidal neurons, and also more specified output to mossy cells and the septal nuclei.

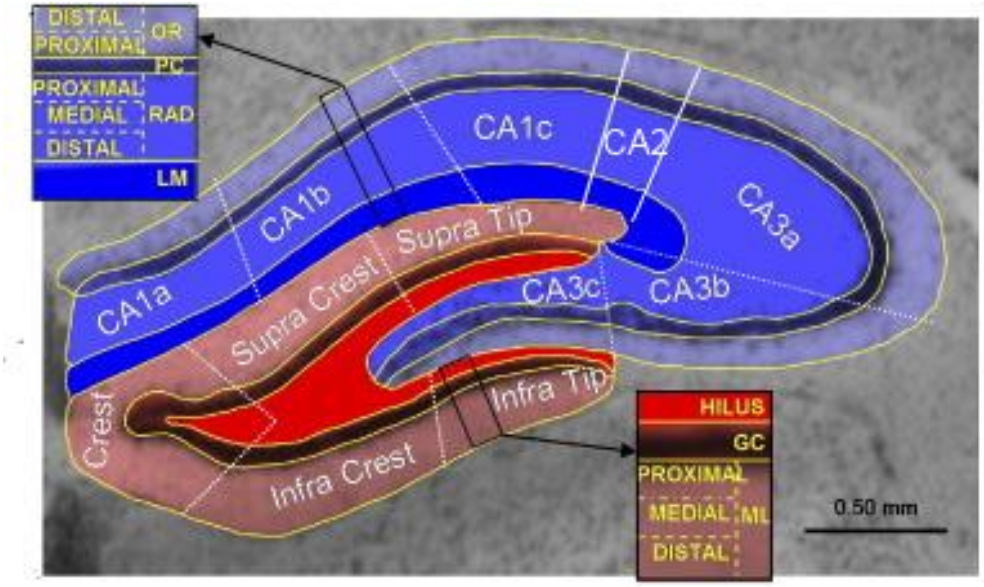



***Figure 1.4 Organisation of the hippocampal subregions*** *Microphotograph coloured to illustrate the laminar and transverse subdivisions of the dorsal hippocampus in rodents. Adapted from (Ropireddy, Bachus, & Ascoli, 2012).*

*1.3.2.1 Schaffer Collaterals*

The schaffer collateral connection to the CA1 forms the final part of the classical DG-CA3-CA1 trisynaptic circuit. CA3 pyramidal neurons extend their axons to both the stratum radiatum and stratum oriens of the CA1 where they innervate CA1 pyramidal cell dendrites via glutamatergic transmission (Sun & Kapur, 2012). These projections are commissural, innervating both the ipsilateral and contralateral CA1 region (Witter, 2007). Although all CA3 cells project both septally and temporally, these projections exhibit topographical organisation with CA3c (proximal) neurons projecting mainly to more septal CA1 neurons and CA3a/b (distal) neurons projecting more to temporal CA1 neurons (Brivanlou, Dantzker, Stevens, & Callaway, 2004; Ishizuka, Weber, & Amaral, 1990). The origin of the CA3 pyramidal neuron also dictate how deeply the axon tends to innervate the CA1 – with CA3c neurons tending to project axons to more distal CA1 regions and innervating mainly superficial levels of the stratum radiatum, while CA3b neurons project axons which terminate deeper in the stratum radiatum and penetrate the stratum oriens while CA3a neurons project to CA1 neurons closer to the CA2 and

innervate the deep stratum radiatum and stratum oriens dendrites. Although a single CA3 neuron can project a significant distance along the length of the hippocampus, making potentially tens of thousands of synapses (Li, Somogyi, Ylinen, & Buzsáki, 1994), it is still unknown how many CA1 neurons a single CA3 cell typically innervates.

*1.3.2.2 Associational Connections*

One of the key aspects to CA3 episodic memory storage is thought to be its autoassociative nature. This is facilitated by the extensive interconnections between CA3 pyramidal neurons, and allows for the recall of an episodic memory by the presentation of any part of that memory – for example a place or object (Rolls & Kesner, 2006). This pathway is known as the commissural-associational pathway (Hagenal & Manahan-Vaughan, 2011) and, involving similar topographical complexity to the Schaffer collaterals, features both ipsilateral and contralateral projections from proximal CA3c pyramidal neurons to other proximal CA3c pyramidal neurons, while projections from mid and distal CA3 pyramidal neurons innervate a larger expanse along both the septotemporal and transverse axes of the CA3 (Amaral & Lavenex, 2007). Projections from proximal CA3 project more towards cells dorsal from the origin of the cell, while projections from distal CA3 project more towards ventral areas, while mid CA3 neurons project to both areas equally (Witter, 2007). These pyramidal neurons also form synapses on CA3 stratum lucidum interneurons (Banke & McBain, 2006) which in turn inhibit pyramidal neurons, allowing for further modulation of synaptic strength in CA3 circuits. This auto-associative network is thought to be particularly important for learning associative memories. Gilbert and Kesner (2003), for example, showed that, relative to rats with CA1 and DG lesions, CA3 lesioned rats

could not perform a stop-go learning task involving the reinforcement of either paired-associate learning of either object-place or odor-place couplings.

#### *1.3.2.3 Other CA3 Outputs*

Aside from the Schaffer collaterals and associational pathway the CA3 also outputs to the dentate gyrus (Scharfman, 2007; Shi, Ikrar, Olivas, & Xu, 2014) as well as to the septal nuclei (Risold & Swanson, 1997). The "backprojection" to the dentate gyrus involves output from pyramidal neurons to dorsal polymorphic layer mossy cells as well as dentate gyrus interneurons, while ventrally these axons reach the granule layer itself and even the inner molecular layer (Myers & Scharfman, 2011). CA3 pyramidal cells may therefore inhibit their own firing not only by innervating CA3 interneurons, but also via inhibition of the granular cells which activate them originally. Output to the lateral septal nuclei may also eventually influence hippocampal functioning by signalling from the lateral septum to the medial septum which innervates the subiculum which may subsequently signal to the entorhinal cortex (Gilbert & Brushfield, 2009). CA3 output signals to the lateral septum may also then travel to the ventral tegmental area forming a circuit which has been shown to be responsible for context-reward associations – illustrating a potential role for the CA3 in processing context and subsequent motivational behaviours (Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011).

#### *1.3.2.4 CA3 Interneurons*

While the pyramidal cells are the most studied neurons of the CA3, the region is also home to a host of heterogeneous, and far less characterised, interneurons. Already noted are the stratum lucidum interneurons which receive mossy fibre and CA3 pyramidal cell input and in turn inhibit both pyramidal neurons and themselves (Banke & McBain, 2006). Other interneurons are generally found throughout all CA1/2/3

areas, although they may vary slightly in function or morphology according to the specific subfield they inhabit (Amaral & Lavenex, 2007). A second kind of GABAergic CA3 interneuron is the oriens lacunosum-moleclare cell (Katona, Acsády, & Freund, 1999) which project to pyramidal cell neurons in the distal stratum lacunosum-moleclare while their soma and dendrites may occupy any other stratum in the CA3 region (Amaral & Lavenex, 2007). Other important interneuron types in the CA3 region include the basket cells – GABAergic cells which have their cell bodies close to the pyramidal layer and extend dendrites both to the stratum lacunosum-moleclare and the stratum oriens receiving input from mossy fibers, pyramidal cells and the entorhinal cortex, while innervating pyramidal cells themselves (Tukker, et al., 2013). Also present in the CA3 region are axo-axonic interneurons which receive unique septal input and function by targeting the axon initial segment of CA3 pyramidal neurons (Viney, et al., 2013) and interneuron selective neurons which are unique in that their axons only innervate other interneurons (Amaral & Lavenex, 2007). These complex cellular circuits allows for finely tuned responses to CA3 input and facilitate its role in pattern completion and the transmission of information to the CA1.

### *1.3.3 CA2*

The CA2 is a small area which lies between CA3 and CA1 and has received relatively little attention compared to other hippocampal regions. Although it is similarly structured to the neighbouring CA3 and CA1 regions, CA2 is known to show a unique expression pattern of various cellular markers, is the sole hippocampal region to receive input from the supramammillary body, and its principal pyramidal cells display many characteristics which differentiate it from its neighbours (Caruana, Alexander, & Dudek, 2012; Mercer, Trigg, & Thomson, 2007). Traditionally, one of the main distinctions between CA2 and CA3 pyramidal cells was thought to be the absence of

DG mossy fibre input to the former (Amaral, et al, 2007). The CA2 was also found to receive entorhinal layer II & III input via distal dendrites and input from neighbouring CA3 pyramidal neurons (Chevaleyre & Siegelbaum, 2010). CA2 axons in turn project preferentially to the stratum oriens, but also stratum radiatum, of the CA1 (Tamamaki, Abe, & Nojyo, 1988) and also engages in reciprocal inhibition with CA3 pyramidal neurons, forming inhibitory synapses with proximal CA3 neurons (Kohara, et al., 2014). Very recent optogenetics work however has challenged these ideas, showing that granule cells actually can project to the CA2, while entorhinal cortex layer III does not (Kohara, et al., 2014). These authors propose that the conceptualization of a novel trisynaptic circuit from entorhinal cortex layer II to the deep CA1, via the granule cells and the CA2 which may regulate "overlearning" by the traditional trisynaptic circuit.

Aside from differences in pyramidal neurons, CA2 is also unique in aspects of its interneuron population. First, the CA2 region contains a higher density of interneuron than its neighbouring CA3 and CA1 (Piskorowski & Chevaleyre, 2013). As in the CA3 region, basket cells are present here with dendrites extending to the stratum oriens/alveus and via the stratum radiatum to the stratum lacunosum moleclare (therefore receiving entorhinal cortex input) with axons innervating the pyramidal layer – although two functional subtypes exist. The first type are more similar to CA1 basket cells – aspiny and extending only within the CA2 region, while the second subtype show a small number of spines and extend axons and dendrites throughout CA1, CA2 and CA3 (Mercer, Trigg, & Thomson, 2007). Another interneuron type identified by Mercer et al (2007) were bistratisfied cells – whose cell bodies occupied the pyramidal cell layer with dendrites extended to the stratum radiatum and stratum oriens (thereby receiving schaffer collateral but not entorhinal input) primarily of the CA2 and CA1. Interneurons in the CA2 region therefore have the potential for both feedforward

inhibition in CA1 and feedback inhibition in CA3 which may again contribute to the suggestion of CA2's involvement in the management of "overlearning" by other hippocampal regions (Kohara, et al., 2014).

In functional terms, recent studies have shown that the CA2 likely plays a role in social learning and contextual change. The CA2 has been shown to be especially involved in perceiving even minor changes to context and environment – often showing a complete remapping of activity following small changes in environmental context and may compare the context being experienced with one's internal representation (Wintzer, Boehringer, Polygalov, & McHugh, 2014). Mice knocked out for vasopressin 1b receptor (Avpr1b), which is preferentially expressed in the CA2 region, show reduced sociability and impaired social recognition – as well as impaired object recognition and temporal memory (DeVito, et al., 2009). Optogenetic inactivation of dorsal CA2 pyramidal neurons does not lead to impairments in spatial or fear memory, but it does prevent mice from forming social memories – without affecting sociability or olfactory function (Hitti & Siegelbaum, 2014). The CA2 is also thought to be particularly susceptible to dysfunction in schizophrenic and bipolar patients (Jones & McHugh, 2011). Several studies have shown that there is a loss of inter neurons in the CA2 region of schizophrenic and bipolar patients (Benes, Kwok, Vincent, & Todtenkopf, 1998; Knable, Barci, Webster, & Torrey, 2004) – with people suffering from schizophrenia typically showing significant social dysfunction (Goldberg & Schmidt, 2001). Although relatively small, CA2 clearly deserves consideration as a functionally and anatomically distinct region of the hippocampus rather than simply an intermediate between CA3 and CA1. One area which has not yet been researched however is whether the dorsal and ventral CA2 are functionally different as is seen in other hippocampal subregions.

### *1.3.4 CA1*

The CA1 region forms the final part of the traditional trisynaptic DG-CA3-CA1 circuit. As previously mentioned it is innervated by CA3 via the Schaffer collaterals, as well as by CA2 pyramidal cells and interneurons. The CA1 also receives strong excitatory innervations from layer III of the entorhinal cortex (Brun, et al., 2008). As with the other hippocampal sub-regions, CA1 is laminar and contains principal pyramidal neurons as well as at least sixteen types of GABAergic interneurons (Somogyi & Klausberger, 2005). The CA1 can also be subdivided into regions CA1a/b/c in a similar fashion to the CA3 – Although here CA1a (distal) refers to the area closest to the subiculum, with CA1c (proximal) closest to the CA2, and CA1b referring to the intermediate area (Ropireddy, Scorcioni, Lasher, Buzsáki, & Ascoli, 2011). Additionally, functional and anatomical differences exist along the dorsoventral axis of the CA1 in a similar manner to other hippocampal regions (Marcelin, et al., 2012).

#### *1.3.4.1 CA1 Principal Pyramidal Neurons*

As with the other CA regions, pyramidal cells are considered the principal cells of the CA1 region. Similar to the CA3, pyramidal neurons may be segregated into different lamina in which they show varying anatomical and functional features – which are somewhat more characterised than in the CA3 region. Although some morphological differences were identified in early anatomical studies (Bannister & Larkman, 1995), gene mapping originally delineated a number of strata of pyramidal neurons, with Dong, Swanson, Chen, Fanselow and Toga (2009) dividing the pyramidal layer into the superficial, deep, and middle pyramidal sublayers – although there is variation along the dorsoventral extent of the hippocampus. Further electrophysiological and anatomical characterization of CA1 pyramidal layers then

revealed that although they had many similarities deep (close to stratum oriens) pyramidal cells contain more place fields, have higher firing rates, and show alternate theta and gamma phase preference during maze exploration and REM sleep (Mizuseki, Diba, Pastalkova, & Buzsáki, 2011). As with the CA3 pyramidal subdivision, the behavioural consequences of these distinct populations is as of yet unknown.

CA1 pyramidal cells are typically smaller than CA2/3 neurons, with dendrites that extend both to the stratum oriens and also downwards towards the stratum radiatum and eventually the stratum lacunosum-moleclare (Amaral & Lavenex, 2007). As previously discussed the CA1 can be divided along a transverse axis, and entorhinal input is separated along this axis with distal CA1 neurons receiving lateral entorhinal cortex input and proximal neurons receiving input from medial entorhinal cortex neurons via the stratum lacunosum-moleclare (Amaral & Lavenex, 2007). As the final part of the trisynaptic circuit, CA1 neurons in turn project hippocampal information to a variety of extra-hippocampal areas. The primary output of the dorsal CA1 is to the subiculum – which in turn activates the entorhinal cortex layers V and VI (MacDougall & Howland, 2013). Study of axonal projections from the ventral CA1 reveal three major routes – the primary connection to the subiculum, another projection to the amygdaloid complex, amygdalopiriform-transition area and parahippocampal regions, and a third projection which travels to the septum, hypothalamus, ventral striatum and olfactory regions (Arszovski, Borhegyi, & Klausberger, 2014). These projections make sense given what is known about the ventral hippocampus' preferential role in odour memory and emotional processing.

Aside from its function as an output to extra-hippocampal areas, the CA1 also fulfils other roles. While pattern separation and completion are attributed to the DG and CA3 respectively, it has been hypothesised that CA1 may bring together these CA3

representations leading to the recall of an episodic memory (Rolls, 2013). Temporal aspects of episodic memory recall have also been attributed to CA1 with dorsal CA1 lesioned animals unable to recalling the order in which objects have been presented to them (Hoge & Kesner, 2007). Dorsal CA1 electrophysiology and lesion studies also implicate the area in the acquisition and consolidation of contextual fear memory (Daumas, Halley, Francés, & Lassalle, 2005; Ognjanovski, Maruyama, Lashner, Zochowski, & Aton, 2014; Wang, et al., 2012). This indicates that the dorsal CA1 does not only encode spatial information but is also involved in processing emotional memories. Ventral CA1 is also highly involved in spatial memory performance as well as object recognition – despite a growing trend for the attribution of emotional rather than spatial processing to the ventral hippocampus (Beer, Chwiesko, & Sauvage, 2014). In terms of the transverse axis it has been suggested that proximal CA1 neurons are tasked with processing large spatial information while distal neurons deal with olfactory and detailed object representations (Igarashi, Ito, Moser, & Moser, 2014).

*1.3.4.2 CA1 Interneurons*

As in CA3 and CA2, interneurons play an important role in CA1 functioning. although many interneurons have similar properties across the CA1/2/3 region, there are also many discrete differences (Amaral & Lavenex, 2007). Oriens lacunosum-moleclare interneurons are also present in the CA1 region, although unlike in the CA3 region, here their soma and dendrites occupy only the stratum oriens (Amaral & Lavenex, 2007). Basket cells are present as well within the CA1 region but display some differences to the CA3 counterparts – namely their dendritic trees are located much more in the stratum radiatum than the stratum lacunosum-moleclare and receive much less entorhinal input (Tukker, et al., 2013). Parvalbumin-positive CA1 inhibitory interneurons, which are compromised in diseases such as schizophrenia, have been

shown to be essential for spatial working, but not reference, memory (Murray, et al., 2011). CA1 interneurons appear to innervate all strata and have also been found which backproject to the CA3 (Sik, Penttonen, Ylinen, & Buzsáki, 1995).

### *1.3.5 Hippocampal Place cells*

Place cells, originally discovered in 1971 (O'Keefe & Dostrovsky, 1971), are present within all hippocampal subregions, are neurons which fire in isolation when an animal enters a certain spatial location – referred to as that cell's "place field" (Park, Dvorak, & Fenton, 2011). These neurons fire again in the same patterns after spatial exploration is completed – during sleep or immobility, thereby facilitating consolidation of this information (O'Neill, Senior, & Csicsvari, 2006). Place cells gain directional specificity as animals become more familiar with an environment (Navratilova, Hoang, Schwindel, Tatsuno, & McNaughton, 2012), and can be remapped following both changes to spatial context (Solstad, Yousif, & Sejnowski, 2014), or emotional context (Moita, Rosis, Zhou, LeDoux, & Blair, 2004) of an environment. Place cell field sise has been shown to vary along the dorsoventral axis of the CA1 region, as occurs in CA3 (Keinath, et al., 2014). These authors also show that despite spatial memory typically being attributed to the dorsal hippocampus, ventral CA1 neurons do show detailed place fields.

### *1.3.6 Long-term Potentiation – Molecular Mechanisms of Memory Formation and Consolidation*

While appreciating the gross anatomy of the hippocampus is essential to understanding its function, the cellular processes which contribute to this functioning must also be considered. The most important concept to consider in the cellular mechanisms for memory formation and consolidation is that of synaptic plasticity. Synaptic plasticity refers to the brain's ability to modify the strength of a synaptic

connection either by brief, high frequency stimulation – long-term potentiation (LTP), or by prolonged low frequency stimulation – long-term depression (LTD) (Purves, et al., 2001). LTP and LTD typically occurs in glutamatergic neurons, and takes place at many important connections within the hippocampus (e.g. Schaffer Collaterals, perforant pathway) (Collingridge, Peineau, Howland, & Wang, 2010). The best characterised forms of LTP and LTD depend on N-methyl-D-aspartate (NMDA) glutamate receptors (Lüscher & Malenka, 2012) – although some forms of NMDA-independent LTP and LTD can also occur. LTP is now known to take place in the hippocampus as a result of many different forms of behavioural learning – inhibitory avoidance (Whitlock, Heynen, Shuler, & Bear, 2006), spatial learning (Liu, et al., 2014) and emotional learning (Schulz & Korz, 2010). Although LTD involves the weakening of synaptic strength, it is not simply functionally opposite to LTP, and is also required during learning (Sacai, et al., 2014) and behavioural flexibility (Nicholls, et al., 2008). Like LTP, LTD has also been implicated in emotional memory (Diamond, Park, Campnell, & Woodson, 2005). Despite decades of research however a comprehensive mechanism by which LTP/LTD actually form memories within the hippocampus is still elusive.

### *1.4 Contribution of non-neuronal cells to CNS functions*

Though hippocampal neuroanatomy has primarily been discussed here in terms of neuronal populations and circuits – other cell types are also key to proper functioning. Many of the cognitive impairments observed in post-septic encephalopathy necessitate the hippocampus, and indeed significant neurological alterations are seen in this area in post-septic patients (Semmler, et al., 2013). It is therefore pertinent to consider the hippocampus not just in terms of its neuronal population, but also in terms

of the immune cells which reside there and are essential both to normal functioning, as well as orchestrating the response to infectious events such as sepsis.

### *1.4.1 Microglia, Astrocytes and immunity within the brain*

Astrocytes and microglia are two types of glial cells which are involved both in brain immune function as well as in the maintenance and regulation of synaptic plasticity – a key process in learning and memory. Microglia are macrophages which reside within the CNS and are involved in phagocytosis of both pathogens and unnecessary synapses (Kaushik & Basu, 2013; Marin & Kipnis, 2013). Astrocytes also react to brain insult, releasing inflammatory mediators, and when necessary forming glial scars which surround injured tissue isolating it from healthy tissue (Wanner., et al., 2013). Astrocytes are involved in synaptic plasticity during both development and normal adult functioning, releasing both glutamate and ATP to alter activity at presynaptic glutamatergic terminals (Stipursky, Romão, Tortelli, Neto, & Gomes, 2011). Given astrocytes close proximity to the synapse and their ability to both act as receptors for, and release, neurotransmitters it is thought that they regulate synaptic functioning via bidirectional signalling with neurons (Allen & Barres, 2009). Due to the importance of perisynaptic astrocytes the concept of a "tripartite" synapse is now replacing the traditional idea of communication existing only between a presynaptic and postsynaptic neuron, with perisynaptic astrocytes present at the majority of rat hippocampal synapses (Ota, Zanetti, & Hallock, 2013; Witcher, Kirov, & Harris, 2007).

While both microglia and astrocytes are crucial for host response to both injury and pathogens, there is growing evidence to suggest that their prolonged activation can be detrimental to many aspects of normal CNS functioning. Microglia undergo various phenotypic and morphological changes in response to pro- or anti-inflammatory stimuli in their environment (Michelucci, Heurtaux, Grandbarbe, Morga, & Heuschling, 2009).

These are represented in Figure 1.5. Upon the detection of activating signals or the cessasation of "deactivating" signals such as CD200 (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011) microglia move from a ramified state (also referred to as a "resting" or "surveilent" state) where they monitor and support normal synaptic functioning (Morris, Clark, Zinn, & Vissel, 2013) to a deramified or "active" state accompanied by an increase in pro-inflammatory cytokine release. As described above, in sepsis this changes is primarily driven by NF-κB activation following LPS recognition by TLR-4. This response is necessary to deal with infection and/or injury, but microglia may also stay chronically activated and subsequently neurotoxic by a self-propelling mechanism if this active state is not properly resolved. It is thought inducible reactive oxide species, nitric oxide and cytokines released by activated microglia may maintain microglia themselves in an activated state which ultimately becomes harmful (Lijia, Zhao, Wang, Wu, & Yang, 2012).

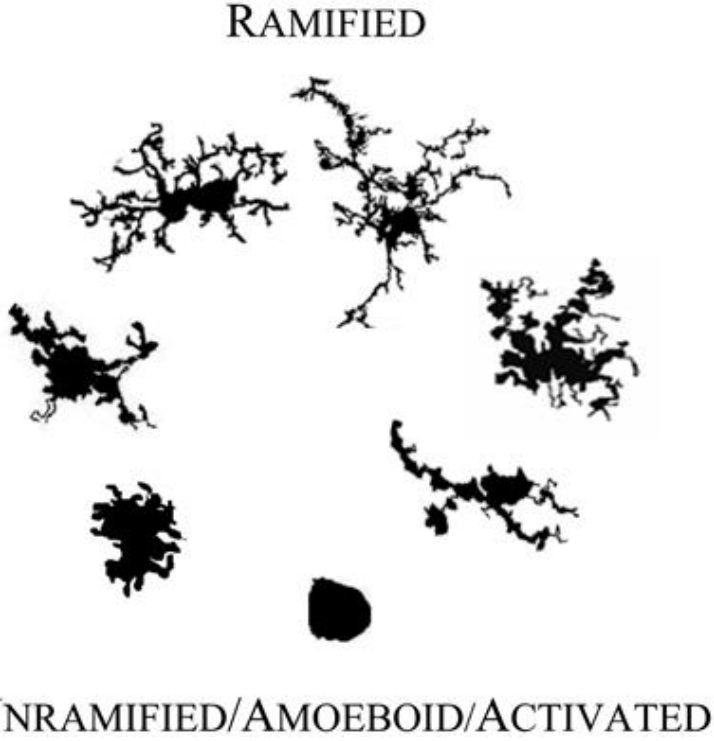



***Figure 1.5 Microglial morphology in the CNS*** *Illustration of the morphological changes microglia undergo when shifting to an activated state. Adapted from Karperien, Ahammer, & Jelinek, 2013.*

Given their requirement during normal functioning, if microglia remain activated within the brain then deleterious changes are likely to occur. It is now becoming accepted that a neuron-microglia-astrocyte triad is necessary for normal brain function (Cerbai, et al., 2012). Indeed neuroinflammation and cytokine signalling are implicated in the pathogenesis of several neuropsychiatric disorders such as major depression (Miller, Maletic, & Raison, 2009) and schizophrenia (Na, Jung, & Kim, 2012) as well as other neurological disorders such as Parkinson's disease (Collins, Toulouse, Connor, & Nolan, 2012). It is therefore probable that activated microglia play a deleterious role in the post-septic brain where they may affect proper neuronal function.

*1.4.2 Cytokines and synaptic transmission*

Cytokines, produced by immune cells both in the periphery and the CNS, function as messenger proteins which are essential for an appropriate response to injury/pathogenic invasion (Stow & Murray, 2013) in addition to being necessary for normal synaptic function. Synaptic scaling, a homeostatic process through which cells in a system alter their firing rate by uniformly revising the strength of all their synapses is dependent on glial produced TNF-α (Stellwagen & Malenka, 2006). Apart from homeostatic synaptic regulation, cytokines also show specific and regional effects on LTP and LTD (Pribaiag & Stellwagen, 2014). Recently, Il-1β, IL-6 and IL-18 (but not TNFα) have been shown to be induced by LTP in the rat hippocampus (Del Rey, Balschun, Wetzel, Randolf, & Besedovsky, 2013). The authors also showed that IL-1β, IL-6 and IL-18 were upregulated following spatial training, with a difference between ventral and dorsal as well left and right hippocampi. In other experiments Il-1β has been shown to be induced by, and necessary for LTP maintenance in the hippocampus (Yirmiya & Goshen, 2011). IL-6 on the other hand may be necessary for the termination

of LTP – with the blockade of IL-6 inducing prolonged LTP and improved hippocampal dependant long-term memory (Balschun, et al., 2004). TNFα also appears to have an inhibitory effect on long-term potentiation in the hippocampus (Butler, O'Connor, & Moynagh, 2004).

Examination of hippocampal inflammation in the context of experimental autoimmune encephalomyelitis, an experimental multiple sclerosis, has shown that impairments of GABAergic transmission by activated microglia *in vitro* depends on IL-1β, being rescued by treatment with IL-1β receptor antagonist (Nisticò, et al., 2013). The application of IL-1β (but not TNFα or IL-10) to cell cultures containing hippocampal primary neurons significantly reduces the phosphorylation and surface expression of AMPA receptor subunit GluR1 (Lai, Swayze, El-Husseini, & Song, 2006). Interestingly, researchers investigating the effects of peripheral inflammation induced via treatment with Freund's adjuvant and mycobacteria found increased hippocampal microglia expression and impaired LTP in the absence of an increase in either hippocampal TNFα or IL-1β levels (Di Filippo, et al., 2013).

Given their necessity for normal synaptic functioning, it follows that over- or under-expression of these cytokines can affect normal neurotransmission. Psychiatric illnesses are increasingly being recognised not only as brain disorders, but as involving neuroimmune components in their pathophysiology (Müller, 2009). Cytokines such as IL-1β and TNF-α are known to influence both serotonergic (Baganz & Blakely, 2013) and dopaminergic signalling (Felger & Miller, 2012), two neurotransmitters strongly implicated in affective disorders. The examination of the effects of glia and cytokines on synaptic plasticity, neurogenesis and apoptosis therefore represent a valuable target for understanding how neurobehavioural deficits in post-septic encephalopathy, and other neuroimmune disorders, may develop and persist.

### *1.5 Hippocampal Neuroimmune Interactions and Affective Disorders*

#### *1.5.1 Adult Neurogenesis in Immune and Affective Disorders*

Though cognitive impairment may be more demonstrable in post-septic patients, it has also been outlined that affective changes may also be present in some sepsis survivors. While the hippocampus has long been studied in terms of its contribution to memory and learning, newer research has also been assessing its role in depression and anxiety. Impaired neurogenesis has been one of the most important factors implicated in many of the main animal models of depression such as olfactory bulbectomy in mice (Islam, Moriguchi, Tagashira, & Fukunaga, 2014) and rats (Jaako-Movits, Zharkovsky, Pedersen, & Zharkovsky, 2006), maternal immune activation (Lin & Wang, 2014; Meyer, et al., 2006), and unpredictable chronic mild stress (Tanti, Rainer, Minier, Surget, & Belzung, 2012; Nollet, et al., 2012). Indeed, human studies have also shown that hippocampal volume is reduced in sufferers of unipolar depression (Videbech & Ravnkilde, 2004). In fact, hippocampi from unmedicated depressed patients show reduced granule neurons in the dentate gyrus compared with controls and medicated depressed patients (Boldrini, et al., 2013). Importantly however, while anti-depressants increase NPC proliferation in patients with depression, untreated depressed patients do not show significantly lower NPC levels relative to controls (Boldrini, et al., 2009)

Early research suggested that hippocampal neurogenesis may actually be required for the behavioural effects of antidepressants such as fluoxetine (Santarelli, et al., 2003). Increased adult neurogenesis is not always a requirement for fluoxetine to exert its anti-depressant effects however. For example, in cyclin D2 (CD2) knock-out (KO) mice which show almost no adult neurogenesis, fluoxetine reversed depressive-like behaviour following stress despite having no effect on adult neurogenesis in KO mice (Jedynak, Kos, Sandi, Kaczmarek, & Filipkowski, 2014). It has also been

suggested that neurogenesis may be required for anti-depressant effects only in certain models/paradigms such as the novelty suppressed feeding test, but not others such as the forced swim test & open field behaviour (David, et al., 2009).

### *1.5.2 Brain Derived Neurotrophic Factor in Immune and Affective Disorders*

Aside from adult hippocampal neurogenesis, the neurotrophin brain derived neurotrophic factor (BDNF) has also been implicated both in depression and the mechanism of action of anti-depressants such as fluoxetine (Jiang & Salton, 2013). BDNF binds to the receptor TrkB and is involved in a number of key processes within the hippocampus including both synaptic transmission and long-term potentiation (Murray & Holmes, 2011), as well as promoting neurogenesis (Scharfman, Goodman, Macleod, Antonelli, & Croll, 2005) and the differentiation to (Babu, Ramirez-Rodriguez, Fabel, Bischofberger, & Kempermann, 2009) and survival of newborn neurons (Sossin & Barker, 2007). Intact BDNF-TrkB signalling is also required for the integration of these adult-born neurons into existing hippocampal networks (Bergami, et al., 2008).

Given the important role of BDNF in synaptic plasticity and neuronal survival, and the potential for these processes to be disrupted in affective disorders a number of studies have investigated a possible role of BDNF in the neurobiology of depression. Reduced mRNA and protein expression of BDNF as well as protein levels of TrkB have been shown to be present in post-mortem hippocampus and prefrontal cortex of suicide victims (Banjerjee, Ghosh, Ghosh, Bhattacharyya, & Mondal, 2013; Dwivedi, et al., 2003). A later study showed that, in suicide victims, hippocampal BDNF was significantly lower in people who had an antemortem diagnosis of major depressive disorders who were not receiving medication, and that this effect was absent in those who had a diagnosis of depression but were taking antidepressant medication at the time

(Karege, Vaudan, Schwald, Perroud, & La Harpe, 2005). BDNF gene and protein levels are also reported to be significantly lower in the lymphocytes and platelets of drug-free sufferers of major depression (Pandey, et al., 2010). Interestingly, serum BDNF levels are decreased in bipolar patients and unipolar major depressed patients during both depressive and manic episodes, with levels negatively correlating with the severity of symptoms in both cases (Cunha, et al., 2006; Karege, et al., 2002).

Anti-depressant medications have been shown to upregulate BDNF levels relative to untreated patients in post mortem hippocampal tissue (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001). Importantly, anti-depressants have actually been shown to increase serum BDNF levels in patients after chronic treatment to drug-free baseline levels (Aydemir, Deveci, & Taneli, 2005; Gonul, et al., 2005), although this effect may only be present in responders to anti-depressant treatment (Yoshimura, et al., 2007). There is also evidence that other treatments such as electroconvulsive therapy may lead to increased serum BDNF in patients who do not respond well to pharmacological antidepressants (Bocchio-Chiavetto, et al., 2006). In animal models BDNF administration itself leads to anti-depressive effects (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002), while physical exercise which is known to have anti-depressant effects also increases hippocampal BDNF (Baj, et al., 2012).

As with neurogenesis however, the restoration of altered BDNF levels may not always be essential to anti-depressant activity. Ninan, Shelton, Bao and & Guico-Pabia (2014) for example found that a 12 week treatment with desvenlafaxine (SNRI) did not significantly increase serum BDNF levels despite eliciting a significant anti-depressant effect, although a larger increase was present in severely depressed patients than those with less severe symptoms. Interestingly 28-day administration of fluoxetine at a 5mg/kg dose did not increase hypothalamic BDNF mRNA despite attenuating pro-

inflammatory cytokine production (Alboni, et al., 2013). Most likely, changes in BDNF levels among those treated with anti-depressants depend on the type of treatment received and the severity of as well as specific symptoms present (Deuschle, et al., 2013; Hellweg, Ziegenhorn, Heuser, & Deuschle, 2008).

### *1.5.3 Cognitive Disruptions in Mood Disorders*

Further evidence for the involvement of the hippocampus in depression comes from studies which involve the measurement of functions known to require the hippocampus in patients suffering from affective disorders. Evidence from human studies of patients with both unipolar and bipolar depression suggested hippocampal dysfunction, with inferior spatial navigation in virtual reality tasks as compared with healthy controls (Cornwell, et al., 2010; Gould, et al., 2007). Therefore rather than framing depression as a consequence or symptom of hippocampal dysfunction it may be wise to consider how affective disorders can impact on other aspects of hippocampal functioning. As has been discussed, although emotional functioning is more commonly attributed to the ventral than dorsal hippocampus there is significant interconnectivity between these two areas. Therefore mnemonic and emotional information may be transmitted across hippocampal axes and it would not be surprising if disorders which involve disruption to the physiology or function of one hippocampal subregion affect that of other subregions. In a stress-induced model of depression, behavioural despair has for example been shown to involve glutamatergic DG to CA3 signalling along the entire dorsoventral axis of the hippocampus (Wang, Zhang, & Lu, 2014). Given the complex circuitry within the hippocampus it can be seen how disturbances to even one layer or type of cell may have follow on effects throughout the whole system. Disruption to interneurons for example may cause over stimulation of primary neurons – while a lack of newborn granule neurons may lead to a heightened threshold for CA3

activation via mossy fibers and subsequent CA1 activation disrupting hippocampal output to other areas.

*1.5.4 Other Key Affective Areas – Amygdala and Prefrontal Cortex*

Though the hippocampus is an important area in processing emotion the contributions of areas such as the amygdala and medial prefrontal cortex (mPFC) cannot be ignored. The amygdala, shown in Figure 1.6, is actually a collection of distinct nuclei which can be broadly divided into five areas – the basolateral, lateral and basomedial/accessory basal nucleus as well as the central nucleus and the medial nucleus (Soma, et al., 2009).

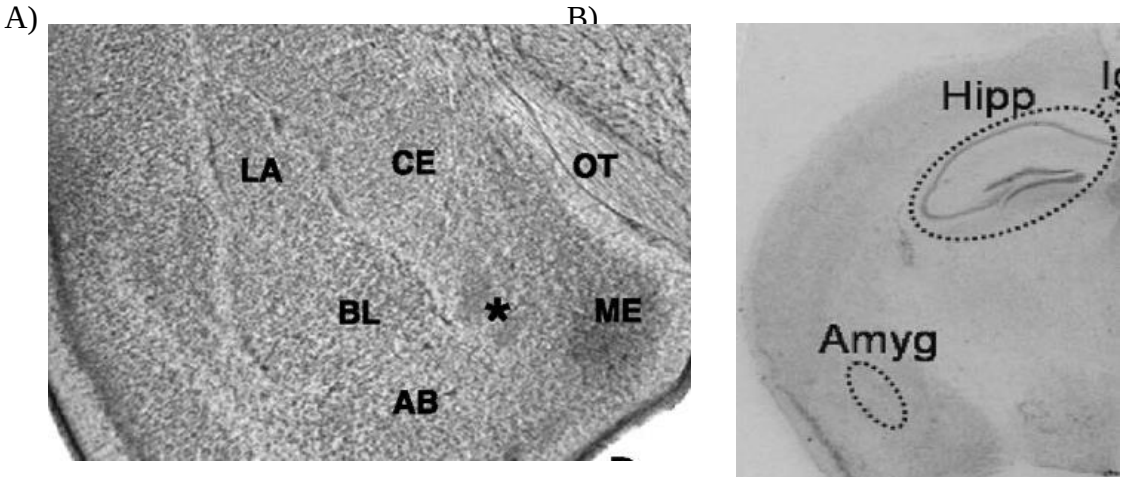



***Figure 1.6 Location and anatomy of the amygdala*** *Photomicrographs showing A) the macroscopic organization of the rodent amygdala and B) its position in relation to the hippocampus - * represents the intercalated cell clusters of the amygdala. Adapted from (Pape & Pare, 2010) (A) and (Brinks, van der Mark, de Kloet, & Oitzl, 2007) (B). LA – Lateral Amygdala, BL – Basolateral Amygdala, AB – Accessory Basal Amygdala/Basomedial Nucleus, CE – central amygdala, ME – medial amygdala.*

This region is particularly important for mediating social and emotional behaviour, as well as pairing neutral stimuli with emotionally-salient information (for example during fear conditioning paradigms) (Manning, Martin, & Meng, 2003). The amygdala, most significantly, the basolateral nucleus, is known to input to the ventral hippocampus CA3 and CA1 stratum oriens and radiatum thereby glutamatergically

innervating pyramidal neurons (Pikkarainen, Rönkkö, Savander, Insausti, & Pitkänen, 1999) with recent optogenetic work illustrating the control this connection exerts over anxiety (Felix-Ortiz, et al., 2013) and social behaviour (Felix-Ortiz & Tye, 2014). Electrophysiological work has also shown that this nucleus can induce LTP in the dentate gyrus (Vouimba & Richter-Levin, 2013). Studies in humans reveal functional and morphological abnormalities in anxiety disorders – for example under-activation of the amygdala in generalised anxiety disorder (Yassa, Hazlett, Stark, & Hoehn-Saric, 2012) amygdala hyperactivity in obsessive-compulsive disorder (Simon, Adler, Kaufmann, & Kathmann, 2014), and hyperactivity with impaired functional connectivity in social anxiety disorder (Hahn, et al., 2011) and hyper-activation. One fMRI study in unipolar depression patients has shown that the amygdala shows reduced functional connectivity with many other areas – an effect the authors suggest may be relative to the anhedonia and dulling of emotions experienced in depression (Benson, et al., 2014).

Though the amygdala is often referred to as a unitary structure, the five nuclei mentioned above appear to serve distinct roles and relate to fear processes in different ways. The basolateral and lateral nuclei receive input both from sensory regions as well as the hippocampus and prefrontral cortex, and it is suggested that these regions are involved in assessing the emotional significance of these inputs (Rosen & Donley, 2006). The central amygdala in turn receives input from the lateral and basolateral nuclei (Wilensky, Schafe, Kristensen & LeDoux, 2006), and is key both for the consolidation and expression of fear. Due to its projections to areas such as the brainstem and hypothalamus, the central nuclei are thought to be important in driving our behavioural and physiological responses to fear (Kalin, Shelton & Richardson, 2004). The medial amygdalar nucleus is functionally connected to olfactory areas in

rodents, though it is suggested that this area is also important for more unconditioned emotional responses (Keshavarzi, Sullivan, Ianno & Sah, 2014). While most studies delineating the differential functioning of amygdala nuclei have been done in rodents, there is also evidence that similar divisions exist within humans – though these are far less studied (Ball, Rahm, Eickhoff, Schulze-Bonhage, Speck & Mutschler, 2007).

The medial prefrontal cortex and its neighbouring anterior cingulate cortex also play a key role in regulating our emotions and, as with the hippocampus, are often divided into dorsal and ventral portions (Etkin, Egner, & Kalisch, 2011). The rodent medial prefrontal cortex is actually a point of convergence for axons of the hippocampus and amygdala, receiving input from both the BLA and CA1 pyramidal neurons (Ishikawa & Nakamura, 2003) and in turn projects to the BLA (Sesack, Deutch, Roth, & Bunney, 1989). Apart from its role in emotional functioning, the prefrontal cortex also works synergistically with the hippocampus during memory-based tasks. In particular, the prefrontal cortex is important for working-memory based tasks (Yoon, Okada, Jung & Kim, 2008). Inputs from the ventral hippocampus to the medial prefrontal cortex are suggested to be essential not just for emotional behaviour but also for cognition (Parent, Wang, Su, Netof & Yuan, 2010).

While the amygdala is usually studied in the context of fear and anxiety, the mPFC may be more involved in positive affect – controlling positive emotional learning in a rat model (Burgdorf, et al., 2011). In humans the medial prefrontal cortex is involved in high level emotional processing and the recall of emotional autobiographical memories (Oddo, et al., 2010). Patients with damage to this area suffer from an inability to correctly regulate their emotional expression (Anderson, Barrash, Bechara, & Tranel, 2006) and also to interpret the intent (harmful or otherwise) behind the actions of others (Young, et al., 2010). Damage to the human prefrontal

cortex also affects the autonomic response to stressful situations such as cortisol release and heart rate (Buchanan, et al., 2010). These patients also show dysregulation of amygdala activity, with increased reactivity in the area to aversive stimuli implicating the mPFC in the pathogenesis of anxiety and mood disorders (Motzkin, Philippi, Wolf, Baskaya, & Koenigs, 2014).

Aside from its connections with the hippocampus, the amygdala is also functionally connected to the prefrontral cortex, and it has been suggested that hippocampal-prefrontal-amygdalar connectivity is key to the consolidation of conditioned fear (Moustaga, Gilbertoson, Orr, Herzallah, Servatius & Myers, 2013). During fear conditioning, the amygdala outputs from the BLA to the hippocampus, which in turns signals from ventral hippocampal neuros to the medial prefrontal cortex (Marek, Strobel, Bredy & Sah, 2013). In humans, the strength of amygdala to prefrontal connections, assessed using diffusion tensor imaging, has been shown to predict trait anxiety levels – with higher connectivity predicting lower anxiety (Kim & Whalen, 2009). Post-traumatic stress disorder patients also show excessive amygdala activity but reduced medial prefrontal cortex size and responsivity highlighting the importance of these pathways not just in learning fear, but also in fear extinction (Shin, Rauch & Pitman, 2006). Untreated patients suffering from MDD also show dampened amygdala to medial prefrontal cortex signalling, when asked to process emotionally-salient information, illustrating how the importance of these connections in emotions other than fear (Kong et al, 2013).

### *1.6 Outstanding Questions*

Despite a handful of good studies which have described some of the long-lasting neurological and behavioural effects of a septic dose of LPS (Bossù, et al., 2012; Fan, et

al., 2012; Qin, et al., 2007; Semmler, et al., 2007; Weberpals, et al., 2009) comprehensive examination of the long-term effects of LPS on a wide variety of cognitive and affective behaviours in a single model has yet to be carried out. Given that the hippocampus is of central importance in both cognitive and affective functioning, and that sepsis survivors show neuroinflammatory (Lemstra, et al., 2007) and hippocampal morphometry (Semmler, et al., 2013) changes it was also decided to examine the state of the hippocampus both shortly following LPS administration and during the chronic state of post-septic encephalopathy. In this regard a wide range of cytokines, glial markers, apoptotic markers, immediate early genes and investigated cell proliferation within the hippocampus were examined to build a profile of the neurological changes which accompany behavioural impairments. Although recent research has critiqued both CLP and LPS-administration models of sepsis (Seok, et al., 2013; Zofaghari, Pinto, Dyson, & Singer, 2013) and their relevance to the human condition there are obvious limitations to research in human post-septic encephalopathy and as of yet the entire scope of effects produced during LPS-induced sepsis remains undescribed.

In light of the poor understanding at present of the aetiology of post-septic encephalopathy and the lack of available treatments, following the characterisation of the neuroimmunological and behavioural profile of this mouse model of post-septic encephalopathy, the aim was to answer questions about the mechanisms by which long-term alterations may be elicited by systemic LPS administration and whether there are any treatments which might be useful. Since the hippocampus of post-septic mice displays elevated levels of microglial markers without over-expression of pro-inflammatory cytokines it was hypothesised that microglia may be “primed”. This was investigated by examining the response of post-septic mice to another small dose of

LPS, mimicking a secondary inflammatory insult following recovery from sepsis. As NF-κB is known to be a key driver of the immune response to LPS I investigated whether inhibition of this pathway might rescue animals from some of the long-lasting effects of LPS administration. Although NF-κB has previously been investigated in relation to its effects on acute LPS treatments, no long-term investigation of its inhibition has been carried out. I also sought to investigate the potential of the SSRI fluoxetine to alleviate some of the changes present in post-septic mice given its utility in affective disorders and anti-inflammatory properties.

## Chapter 2. Cognitive and Affective Behaviour in post-septic mice

*Abstract*

The aim of this chapter was to characterise the behavioural alterations present in post-septic mice. Mice were administered a septic dose of LPS and then left to recover for one month before testing. Behavioural tests were then conducted within both the affective and cognitive domain. Unlike previous research which showed cognitive problems in post-septic mice, here no deficits were present in tasks assessing either long-term or short-term memory – although there are some changes to novel object exploration. Within the affective domain post-septic mice show increased anxiety-like and depressive-like behaviours in some taks (elevated plus maze, tail suspension test, sucrose preference test), but no changes in others. It is also shown that an acute treatment with fluoxetine can reduce this depressive-like behaviour in the tail suspension test. Finally it is also demonstrated that single versus group housing does not affect behaviours in the marble burying or forced swim test. It is concluded that although these post-septic mice do not show cognitive deficits in the tests selected, they do show some affective changes which are also a common symptom in patients recovering from sepsis.

## *2.1 Introduction*

### *2.1.1 Memory and Learning Deficits in Sepsis survivor rodents*

Analagous to patients who have recovered from sepsis, sepsis-survivor rodents have been shown to exhibit distinct cognitive impairment and behavioral changes following recovery from the acute effects of sepsis. Ten days after the induction of sepsis via cecal ligation and puncture (CLP), a time point at which animals are known to be free from infection (Comim, et al., 2010) rats have been shown to have impaired memory in the step down passive avoidance task (Cassol-Jr, et al., 2010). In addition to altered open field test exploration patterns, memory impairment has also been shown both 10 and 30 days after CLP in rats in a continuous multiple-trials step-down inhibitory avoidance task (Barichello, et al., 2007). Short-term and long-term novel object recognition have also been shown to be impaired in sepsis survivor rats 10 days after CLP, indicating non-spatial and non-aversive memory disruption (Cassol-Jr, et al., 2011). 15 days post-CLP rats have shown impaired fear memory and Morris watermaze performance, which was protected against by genetic and pharmacological inhibition of oxidative stress (Hernandes, et al., 2014)

Cognitive impairments have also been shown in sepsis models using an i.p. administration of LPS rather than CLP. Male Wistar rats administered a 10mg/kg dose of LPS have been found to have impaired performance in an 8-arm radial maze following recovery, as well as altered open field exploration patterns (Semmler, et al., 2007). These animals spent longer in the corner squares and the wall corridor, as well as a reduced time in the centre square - behaviour which is thought to be indicative of anxiety. Interestingly however, unlike rats who underwent CLP in other studies, these rats did not display impaired learning in a passive avoidance test, although the apparatus used was different. A 5mg/kg dose of LPS given to C57BL/6 mice was found not to

produce any locomotor or behavioural alterations in an open-field test 2 months post-treatment, but signficantly increased both working and reference memory errors on an eight-arm radial maze test, as well as significantly increasing the number of trials necessary to complete the task (Weberpals, et al., 2009). These results indicate that while there are some differences between the models in terms of the specific memory deficits which follow recovery, cognitive processes are negatively affected to a large degree in the absence of sickness behaviour or locomotor effects in both models. The amount of time which has passed since the induction of sepsis may also factor in the results obtained in these cognitive tests.

*2.1.2 Memory and Learning in the Presence of Chronic Neuroinflammation*

Neuroinflammation is one of the most central pathophysiological aspects of post-septic encephalopathy, and as such, models involving chronic neuroinflammation are particularly relevant to the understanding of cognitive deficits which follow sepsis recovery. Chronic neuroinflammation has previously been shown to affect multiple forms of memory accross several models of central nervous system (CNS) disorders. For example, in the Morris water maze (Morris, 1981), a task which requires animals to learn the location of an invisible platform based on the position of external spatial cues sustained interleukin-1β over-expression for 14 days in a transgenic mouse line has been found to disrupt acquisition and long-term memory retention in mice in a Morris water maze task (Moore, Wu, Shafter, Graham, & O'Banion, 2009). Chronic infusion of LPS into the $4^{th}$ ventricle has also been shown to significantly impair rats' performance in the watermaze (Cui, et al., 2008; Rosi, et al., 2006) and induce alterations in the T-maze, but not novel object recognition (Hauss-Wegrzyniak, Vannucchi, & Wenk, 2000). In addition to long-term spatial memory disruption, chronic infusion of LPS (0.25 µl/hr) into the $4^{th}$ ventricle of Wistar rats has been found to disrupt spatial

working memory, an effect which was reversed by daily administration of the non-steroidal anti-inflammatory drug dexibuprofen (Jin, et al., 2008).

Non-spatial memory is also known to be affected by chronic neuroinflammation. In a contextual fear-conditioning paradigm, mice who over express the pro-inflammatory cytokine 1L-1β display significantly impaired long-term contextual memory (Hein, et al., 2010). Auditory conditioning was not impaired however, indicating an effect on hippocampus-dependent, but not independent learning. Injections of IL-1β itself have also been shown to impair contextual fear learning in Sprague-Dawley rats (Hein, et al., 2007). Peripheral LPS injection has also been shown to disrupt contextual but not auditory fear conditioning when given after the conditioning session had finished, suggesting it disrupts consolidation processes which occur after the learning event has taken place (Pugh, et al., 1998). Microglial activation appears to be the key factor in producing long-term cognitive deficitis following CLP, as its inhibition with minocycline protects sepsis-survivor rats from oxidative stress in the hippocampus and rescues cognitive ability in the step-down inhibitory avoidance task (Michels, et al., 2014)

Further evidence for a role of the immune system in learning and memory comes from the fact that severe combined immune deficitent (scid) mice display impaired cognitive performance relative to wild-type controls (Brynskikh, Warren, Zhu, & Kipnis, 2008). These mice are deficient in both T cells and B cells, and have been shown to display impaired learning in the Morris water maze, the radial arm water maze test, and the Barnes maze (Brynskikh, et al., 2008; Kipnis, Cohen, Cardon, Ziv, & Schwartz, 2004). These impairments can then be saved by T cell vacination with glatiramer acetate. This provides evidence that while inflammatory factors may adversely affect learning in some contexts, they are also required for learning in others.

although heterogeneous in terms of the models used, this literature illustrates clearly the necessity of a functioning immune system for several phases and types of memory processessing, as well as the deficits which follow where the immune system becomes dysregulated.

### *2.1.3 Affective Changes in Post-septic Animal Models*

Affective changes are also present in a number of studies involving animal models of post-septic encephalopathy. Ten months after LPS-induced sepsis rats show no differences in the elevated plus maze or Morris watermaze, although they exhibit reduced exploration and recognition of a novel object in the open field test (Bossú, et al., 2012). Post-septic animals have also been shown to display increased levels of depressive-like behaviour including both anhedonia and behavioural despair (Comim, et al., 2010; Petronilho, et al., 2012; Tuon, et al., 2008) as well as anxiety-like behaviour in the elevated plus maze ten days after CLP (Calsavara, et al., 2013). Similarly to post-septic affective changes in humans, affective behavioural change here seems to be affected by the time since sepsis onset as well as the precise construct being measured.

### *2.1.4 Affective States in Populations with Chronic Neuroinflammation*

A large comorbidity exists between mood disorders and anxiety disorders. Those who are diagnosed with an anxiety disorder are 12 times more likely to suffer with depression than those who do not, while those diagnosed with depression are eight times more likely to be diagnosed with generalised anxiety disorder in the next 12 months (Ross, 2009). Studies in both clinical, and non-clinical, populations have revealed a link between the inflammatory state and affective disorders. As previously noted, sepsis survivors sometimes display more emotional problems than patients who were chronically hospitalised for other serious conditions. Studying the interaction between the inflammatory system and affective states in other populations may provide

clues as to how post-septic encephalopathy contributes to an altered affective state above and beyond that experienced following life-threatening illnesses in general.

In a study involving 453 men, and 400 women anxiety levels as rated by the Spielberger State Anxiety Questionnaire higher anxiety levels were found to correlate significantly with C-reactive protein (CRP), TNF-α and IL-6 levels in men, as well as CRP and IL-6 levels in women (Pitsavos, et al., 2006), all of which are upregulated during neuroinflammation. A randomised-control trial of omega-3 and omega-6 polyunsaturated fatty acids in a non-clinical population revealed that a reduced omega-3:omega-6 ratio led to lower LPS-stimulated IL-6 and TNF-$\alpha$ levels along with reduced anxiety symptoms (Kiecolt-Glaser, Belury, Andridge, Malarkey, & Glaser, 2011). The relationship between pro-inflammatory factors and anxiety does not appear to be universal however. One study of elderly, non-demented participants found no significant relationship between 10 markers of systemic inflammation – including CRP, IL-6 and TNF-α, and anxiety levels, although a significant correlation was found between between IL-6 and IL-8 and depressive symptoms at baseline, increasing IL-8 and depressive symptoms after 2 years, and an association between plasminogen activator inhibitor-1 and remitting depressive symptoms (Baune, et al., 2012).

Further support for this association comes from a birth cohort study of 2688 males and 2837 females in Finland which found that anxiety symptoms alone doubled the probability of elevated CRP levels in males, while comorbid anxiety and depressive symptoms predicted a 1.7-fold increase in probability of elevated CRP levels (Liukkonen, et al., 2011). An Irish study of both clinically anxious and non-clinical participants found significantly higher levels of IL-6, and significantly lower morning levels of the stress hormone cortisol, with no difference between sexes, ages or depressive symptoms (O'Donovan, et al., 2010). Interestingly, there was no between

groups difference in CRP levels. These results indicate an increase in low-grade inflammation among males with anxiety and comorbid anxiety with depression but not females, indicating that there may be sex differences, as well as inter-region differences in the relationship between inflammatory factors and anxiety levels.

Interferon-α is a key component of the immune system, employed as a treatment for a variety of illnesses. Treatment with interferon-α however commonly leads to the development of depression, following which patients are often required to cease treatment. Treatment with interferon-α has been shown to correlate with depressive and anxious symptoms in numerous human populations, such as chronic hepatitis C patients (Bonaccorso, et al., 2001), multiple sclerosis (Loftis & Hauser, 2004) and systemic mastocytosis (Casassus, et al., 2002). Many of these studies do not employ a control treatment group however making it difficult to discern whether the increased depressive and anxious symptoms were caused soley by treatment or whether the progression of illness may play a role.

Although depression is most likely a result of an interaction between the environment and many genes (Wong & Licinio, 2001), there have been some links between particular immune genes and the disorder. In Caucasian patients with major depression, diminished efficacy of antidepressant treatment and as well as altered amygdala and anterior cingulate cortex response during emotional processing has been shown to associated with a genotype of two single nucleotide polymorphisms of the IL-1β gene (Baune, et al., 2010). Single nucleotide polymorphisms in two genes necessary for T-cell function have been shown to be predictive of Major Depressive Disorder among Mexican Americans (Wong, Dong, Maestre-Mesa, & Licinio, 2008). An association was found to exist between patients' response to antidepressant and multiple

SNPs which support the T-cell functioning (Wong, Dong, Maestre-Mesa &Licinio, 2008).

*2.1.5 Affective Behaviour in Rodent Models of Neuroinflammation*

Research in rodents has generally pointed towards increased anxiety- and depressive-like behaviours in the presence of elevated immune activity. A 4-week administration of the pro-inflammatory cytokine interferon-α (IFN-α) led to increased anxiety like behaviour in the elevated plus maze in otherwise healthy male Wistar rats, accompanied by increased anhedonia in the sucrose preference test and behavioural despair in the forced swim test (Fahey, Hickey, Kelleher, O'Dwyer, & O'Mara, 2007). The acute administration of LPS and IL -1β have also been shown to induce anxiety like behaviour in a dose-dependent manner in male CD-1, virus antibody free mice (Swiergiel & Dunn, 2007). Importantly however, the dose required to induce these changes led to decreased locomotor activity – a common problem in the case of studies of acutely induced neuroinflammation. Relatively few studies however examine the chronic effects of neuroinflammation on anxiety. In another model of anxiety-like behaviour in Sprague-Dawley rats, repeated IL-1β (5μg/kg) administration for 7 days was found to increase latency to escape shocks in the shuttle box test (Bonaccorso, Maier, Meltzer, & Maes, 2003).

Other studies however have shown an opposite effect. IL-1β administration has been shown to reduce or increase time spent in the open bars of an elevated plus-maze in a dose dependant fashion 20 minutes following ICV injection to adult Wistar rats (Montkowski, Landgraf, Yassouridis, Holsboer, & Schöbitz, 1997). The authors found that a 0.1ng dose increased the amount of time spent in an open arm, while a 100ng dose significantly reduced it, although other doses ranging from 0.001 to 10.0 had no effect. These doses both significantly reduced locomotor activity under basal conditions

0-6 hours post-treatment, but not in animals who had previously been exposed to a novel environment until 6-12 hours post-injection.

As in human populations, a link between depression and neuroinflammation has been uncovered in many animal models. Chronic unpredictable mild stress (CUMS) for example, although not a model of neuroinflammation, increases depressive-like behaviours as well as serum IL-1β levels relative to controls (Wang, et al., 2011). Rats undergoing CUMS have also been found to have significantly higher levels of interleukin-1β and TNF-α mRNAs in the cortex, as well as significantly higher levels of TNF-α and IL-18 in the hippocampus. (You, et al., 2011). An 830μg/kg dose of LPS has been shown to significantly increase time spent immobile in the forced swim test, as well as to decrease sucrose preference, but not affect the tail suspension test, in the absence of locomotor impairment in mice 24 hours after injection, indicating test-specific effects (Frenois, et al., 2007).

Other studies however have found that chronic activation of the immune system does not always lead to increased behavioural despair. Five days of IL-1β (2μg) administration for example has been found not to decrease immobility time in the forced swim test in rats, despite a loss of weight which may have been indicative of anorexia (Wilhelm, Murphy-Crews, Menasco, Huckans, & Loftis, 2012). Overall, although limited work has been done on affective behaviour in rodents with chronic neuroinflammation in the absence of sickness behaviour, there seems to be an interaction between the two systems. This is clearly influenced by a number of factors however, including the test being used, how the inflammation is produced, and the amount of time that has passed since the induction of inflammation.

*2.1.6 Drug Treatments*

Adding support to the idea of an interaction between factors which contribute to affective disorders and the inflammatory system is a body of evidence indicating that drugs which act on inflammatory pathways may ameliorate some affective behaviour, while several anti-depressants exert anti-inflammatory properties. Experimentally induced neuroinflammation by daily administration of a low dose of LPS (100mg/kg) has also been shown to block the effects of selective serotonin reuptake-inhibitor fluoxetine (10mg/kg) in reducing depressive behaviour (forced swim, novelty suppressed feeding and sucrose preference) in rats undergoing an unpredictable chronic mild stress paradigm (Wang, et al., 2011). The authors also found that neuroinflammation blocked an increase in hippocampal neurogenesis produced by fluoxetine treatment in non-neuroinflammed rats. In fact, fluoxetine was found to decrease the levels of serum IL1-β in animals administered LPS, suggesting an interplay between the anti-depressant effects of fluoxetine and the inflammatory system.

In an experiment involving an acute treatment with 100µg/kg LPS, 4 hours post-LPS treatment, there was a reduction in line crossing and rearing in the open field test compared with saline-treated animals. Line crossings were not rescued by fluoxetine administration, while fluoxetine administration abolished the difference between saline and LPS-treated mice in terms of rearing. The fluoxetine treatment also attenuated the reduction in food consumption over the 24 hours following LPS treatment, indicating an effect on LPS induced anorexia (Yirmiya, et al., 2001). The effects of acute LPS treatment (20µg/kg) on reward behaviour – an effect the authors attribute to anhedonia rather than anorexia, have also been shown to be reversed by a treatment with the non-steroidal anti-inflammatory drug diclofenac (De La Garza II, Fabrizio, Radoi, Tudor, & Asnis, 2004). This treatment also reduced the levels of corticosterone produced.

In patients receiving interferon-α for the treatment of malignant melanomas, pre-treatment with the SSRI paroxetine was found to significantly reduce the likelihood of patients developing depressive symptoms compared to a control group receiving interferon-α and a placebo (Musselman, et al., 2001). Treatment with paroxetine is effective in reducing symptoms of depression, anxiety, cognitive impairment and pain while patients do not respond as well in terms of fatigue and anorexia (Capuron, et al., 2002). Another study of 8131 adults however has found that treatment with anti-depressants, in particular tricyclic anti-depressants, correlates with increased C-reactive protein levels independent of mental state (Hamer, Batty, Marmot, Singh-Manoux, & Kivimäki, 2011).

Relatively little work has been done on the effects of drug treatments on chronic models of neuroinflammation. In rats which have undergone CLP ten days prior to behavioural testing, a single i.p. injection of the tricyclic antidepressant imipramine has been shown to reverse an increase in time spent immobile during a forced swim test (Tuon, et al., 2007). A 10mg/kg dose of the Alzheimer's drug memantine, an NMDA-receptor antagonist has been found to protect spatial learning and the induction of the learning-related immediate early gene *Arc* from disruption during the chronic infusion of LPS into the 4$^{th}$ ventricle over for 28 days (0.25µl/hr). These results indicate that both the chronic and acute effects of neuroinflammation on cognitive and affective behaviour are treatable with a variety of drugs.

### *2.1.7 Aims*

Given the lack of research into the behavioural effects of LPS-induced sepsis, the aim of these experiments is to categorise the behavioural and cognitive deficits present post-sepsis in mice administered 5mg/kg doses of LPS. In certain experiments the effects of single and group housing were examined, as housing condition is thought

to contribute to behavioural alterations. Also examined is whether the serotonin reuptake inhibitor fluoxetine could reverse behavioural changes induced in post-septic mice.

In order to investigate the effects of our treatment on cognition and behaviour a total of 12 different behavioural tasks were carried out investigating a diverse range of functioning. Constructs targeted included working memory, long-term spatial memory, behavioural despair and anxiety. Memory tasks were chosen as it is known that immune factors are heavily involved in the neural processes of learning and memory, and while the immune system is affected by physiological/psychological events (Yirmiya & Goshen, 2011) such as during post-septic encephalopathy. Thus long-term spatial memory was investigated using the Morris water maze (Vorhees & Williams, 2006), working spatial memory using the Morris watermaze and the radial arm maze (Levin, et al., 2009). Non-spatial working memory was also tested using a 5 trial novel object task. As there are has been mounting evidence of a possible role of neuroinflammation in affective disorders such as major depression (Dowlati, et al., 2010; Mössner, et al., 2007), and anxiety (Pitsavos, et al., 2006) several tasks aimed at measuring depressive- and anxiety-like behaviour were also conducted.

*2.2 Materials and Methods*

*2.2.1 Animals*

Male C57BL/6 mice between 8 and 12 weeks old were used for the purpose of all experiments. Group-housed animals were housed two to four per group in plastic cages (12cm x 9cm x 9 cm) with wire lids containing wood-chip bedding and shredded paper for environmental enrichment. Singly-housed animals were housed in the same conditions. All animals had *ad libitum* access to water at all times, and *ad libitum* access to food except during food restriction protocols. Animals were kept in a 12:12 light:dark cycle at 200lux and 21±1°C and 50±5% relative humidity. Unless otherwise stated, all animals were group housed. Where an animal was moribund and deemed unlikely to recover he was removed from the sample and euthanised. No animals was used for more than 3 experiments, and no more than one experiment deemed to be stressful (Tail Suspension Test, Forced Swim Test, Watermaze) to the animal. Experiments were conducted in order from least stressful to most stressful.

*2.2.2 Drug Treatments*

Mice were allowed to acclimatise to the housing facility for at least two weeks prior to injection. All saline and LPS injections were made up fresh on the treatment day, and given intraperitoneally in a final injection volume of 0.125ml. 0.9% sterile saline was made up fresh for control injections, while lipopolysaccharide (Sigma Aldrich, *Escherichia Coli 0114b*) was made up to a 5mg/kg dose in sterile saline. Injections were given between zeitgeber time (ZT) 05 and 06. Animals were allowed to recover for at least 1 month prior to behavioural testing.

Treatments of 20mg/kg fluoxetine were made up in sterile saline on the day of treatment. 20mg/kg fluoxetine and corresponding control injections were given in a

final volume of 0.25ml – a dose which has previously been shown to be effective in C57bl/6 mice (Holmes, et al., 2005).

*2.2.3 Marble Burying Task*

Animals were placed in an isolated testing arena (26cm length x 20cm width x 27 cm high) filled with roughly 6cm of wood chip bedding for thirty minutes with marbles arranged in four rows of three, evenly spaced from each other and the sides of the test cage (Deacon, 2006). After thirty minutes mice were removed and marbles were considered buried where at least 2/3 of their surface was covered with woodchip. As mice may bury novel objects which they find anxiety-inducing the number of marbles buried during the test functioned as the dependent measure of anxiety.

*2.2.4 Open Field Test*

Open field testing occurred in a circular arena of diameter 100cm. Trial length was 300 seconds during which distance moved, velocity in cm/sec and time spent moving as well as time in the inner and outer 50% were automatically tracked with Ethovision 3.1 software (Ethovision 3.1; Noldus Information Technology, Leesburg, VA). Here, as animals tend to avoid open spaces (Ennaceur, Michalikova, van Rensburg, & Chazot, 2006) time spent in the inner corridor versus the outer corridor of the arena is a measure of anxiety-like behaviour while distance, velocity and time spent moving are indexes of general locomotor behaviour.

*2.2.5 Hyponeophagia Test*

Testing occurred in a large transparent plexiglass container (26 length x 20 width x 13.5 cm high) placed on a white surface using existing methodology with chocolate as the novel food (Deacon, 2011). Animals, which were not subject to food deprivation, were placed in the testing arena where the novel food covered the floor and were given up to three trials of four minutes with latency to eat the dependent measure

of anxiety. Data shown represents the sum of these trials. After three trials testing for that animal stopped and they were given a score of 720 seconds if they had not yet eaten.

*2.2.6 Forced Swim Test*

Animals were placed singly in a 2 litre clear plexiglass beaker (12.5cm diameter x 23.5cm height) filled to a height of 12cm with 1.25l water (25°C) such that their tails could not touch the bottom of the beaker. Following the method of Porsolt et al. (1997) each test lasted six minutes, with the immobility (absence of movement except leg kicks to stay afloat) scored in the final four minutes only. This test measures depressive-like behaviour with immobility taken as the dependent measure of behavioural despair.

*2.2.7 Sucrose Preference Test*

Following a method set out in Strekalova et al (2004) bottles were filled with either animals' standard drinking water or 1% sucrose in the animals standard drinking water, lids securely taped in place to minimise spillage and weighed. Cages were modified to accommodate two bottles, and food was placed in both sides. Mice were then given a free choice between either tap water, or a 1% sucrose in tap water solution for 24 hours. After 12 hours had passed, the position of the two bottles were switched, in order to control for a side preference in drinking behaviour. After 24 hours had passed, the bottles were then weighed to measure how much liquid was consumed, and sucrose preference was calculated as [sucrose consumed / (water consumed + sucrose consumed)] for each mouse. This sucrose preference functions as the dependent measure of anhedonia. The data of animals were removed if they were found to have liquid in their cages.

*2.2.8 Tail Suspension Test*

According to a previously used protocol (Steru et al, 1985) mice were attached to a support raised to a height of 121cm using tape placed 1cm from the tip of their tales for six minutes. Mice were suspended for six minutes each, with immobility (complete absence of movement) being recorded throughout the entire six minutes. This is a test of depressive-like behaviour with immobility taken as the dependent measure of behavioural despair.

*2.2.9 Tail Suspension Test (Fluoxetine)*

On the experimental day 1 animals were injected with 0.9% sterile saline 90 minutes before undergoing the tail suspension test. Two weeks later on experimental day 2 animals were injected with 20mg/kg fluoxetine dissolved in sterile saline 90 minutes prior to repeating the test. Mice were removed from the home cage and singly attached to a support raised 121cm above a cage containing woodchip bedding by tape placed 1cm from the tip of their tales for six minutes. Mice were suspended for six minutes each, with immobility being recorded throughout the entire six minutes. Immobility was defined as the complete absence of movement.

*2.2.11 Novel Object Recognition Test*

Animals were first habituated to a circular arena of 50cm. Each animal underwent 5 trials lasting two minutes each. During the first four trials, animals were habituated to two objects. Between each trial the animal was removed while the objects and arena were cleaned with 70% ethanol solution. On the fifth trial one familiar object was replaced with a novel object. The objects had previously been examined for preference with no differences being observed. These objects were pseudo-randomised so that each object could function as either a familiar object or the novel object in any given session. This randomization was balanced across each group. Animals were

measured on their time touching, and number of nose touches for each object during each trial. This is typically a test of working memory with novel object recognition, the dependent variable, calculated as [time exploring the novel object / (time exploring the novel object + time exploring the familiar object)] or [number of touches on the novel object / (number of touches on the novel object + number of touches on the familiar object)] (Anderson, Commins, Moynagh, & Coogan, 2014). In this experiment time touching and number of touches on the familiar and novel objects were also taken as measures of exploratory behaviour.

*2.2.12 Radial 8-arm Maze*

Animals were placed on an 80% restricted diet for five days before the first day of experimental testing followed by a 60% restricted diet throughout the duration of the experiment. The maze consisted of a centre area of diameter 13.5cm, from which 8 arms extended of length 34.5cm, width 5cm and height 13.5cm. Similar to previously used methodology (Janitzky et al., 2011), all eight arms of the maze were baited with a food reward (chocolate). Trials were of five minutes length, unless the animals successfully consumed all of the bait. The apparatus was wiped down with 70% ethanol between all trials to remove the previous animals' odour. Each animal underwent one trial per day, for fourteen days. Entrances to an arm were counted when all four of the mouse's paws were within the arm. The trial was ended once the animal either consumed all rewards or once five minutes had passed. The dependent variables were latency to complete the maze, a measure of learning, and the number of repeat entries to arms where bait was already consumed, taken as a measure of working memory errors.

*2.2.13 Working Memory Watermaze*

Testing followed the protocol of Vorhees & Williams (2006) in a circular pool of diameter 100cm, with water 21±1°C. A rectangular white card (55x81cm) to the west

of the pool was visible to the mouse, and there were lights in the northeast and southeast. Each animal underwent twenty eight sixty-second trials over seven sessions (4 trials/session). Two sessions took place per day such that there was four hours between the first and second session, then twenty hours between the second and third session and so on. Each mouse was placed into the pool at the northeast, northwest, southeast and southwest positions in a pseudo-random order. In session one an invisible platform of diameter 9cm was submerged 0.5cm below water-level in the north-western quadrant of the pool. This platform was moved clockwise from quadrant to quadrant on each subsequent session. The mouse was returned to a cage for 10 seconds between trials. Escape latency was defined as the time it took the mouse to mount the hidden platform. All experiments were tracked using a video camera and Ethovision 3.1 software (Ethovision 3.1; Noldus Information Technology, Leesburg, VA). Escape latency, distance travelled and velocity (cm/second) were measured throughout the trials. Escape latency was the primary dependent variable of interest functioning as a measure of working memory.

*2.2.14 Long-term memory Watermaze*

Testing occurred in the same maze conditions previously described in the section above. Each animal underwent 4 trials per session for 6 sessions. Two sessions took place per day such that there was four hours between the first and second session, then twenty hours between the second and third session and so on. Each mouse was placed into the pool at the northeast, northwest, southeast and southwest positions in a pseudo-random order. In all sessions an invisible platform of diameter 9cm was submerged 0.5cm below water-level in the North-Eastern quadrant of the pool where it remained throughout testing. The mouse was returned to a cage for 10 seconds between trials. Escape latency, distance travelled and velocity (cm/second) were measured

throughout the trials. Escape latency was the primary dependent variable of interest functioning as a measure of spatial memory acquisition. During the retention trial, conducted 24 hours after the final trial, each animal was allowed to swim for sixty seconds in the pool with no platform present.

*2.2.15 Elevated Plus Maze*

The elevated plus maze was conducted in accordance with the protocol set out by (Walf & Frye, 2007). The maze consisted of a centre area of diameter 13.5cm, from which four arms extended of length 34.5cm, width 5cm. The maze was raised 20cm from the ground. Two arms were open without walls, while the other two were enclosed by high walls. Entrance to an arm was counted where all four of an animals' paws were within the arm. Animals underwent one five-minute testing session each. This test assesses anxiety-like behaviour by measuring time spent in the open arms and number of entries to the open arms as dependent variables.

*2.2.16 Statistical Analyses*

All results were analysed in IBM SPSS Statistics version 20. The type of test used is indicated in the results section for each experiment, with the appropriate test chosen based on fulfilling normality criteria. P levels below 0.05 were considered significant for all tests. Where data violated statistical assumptions adjusted values are presented. All data are presented as means ± standard error of the mean (SEM). * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$.

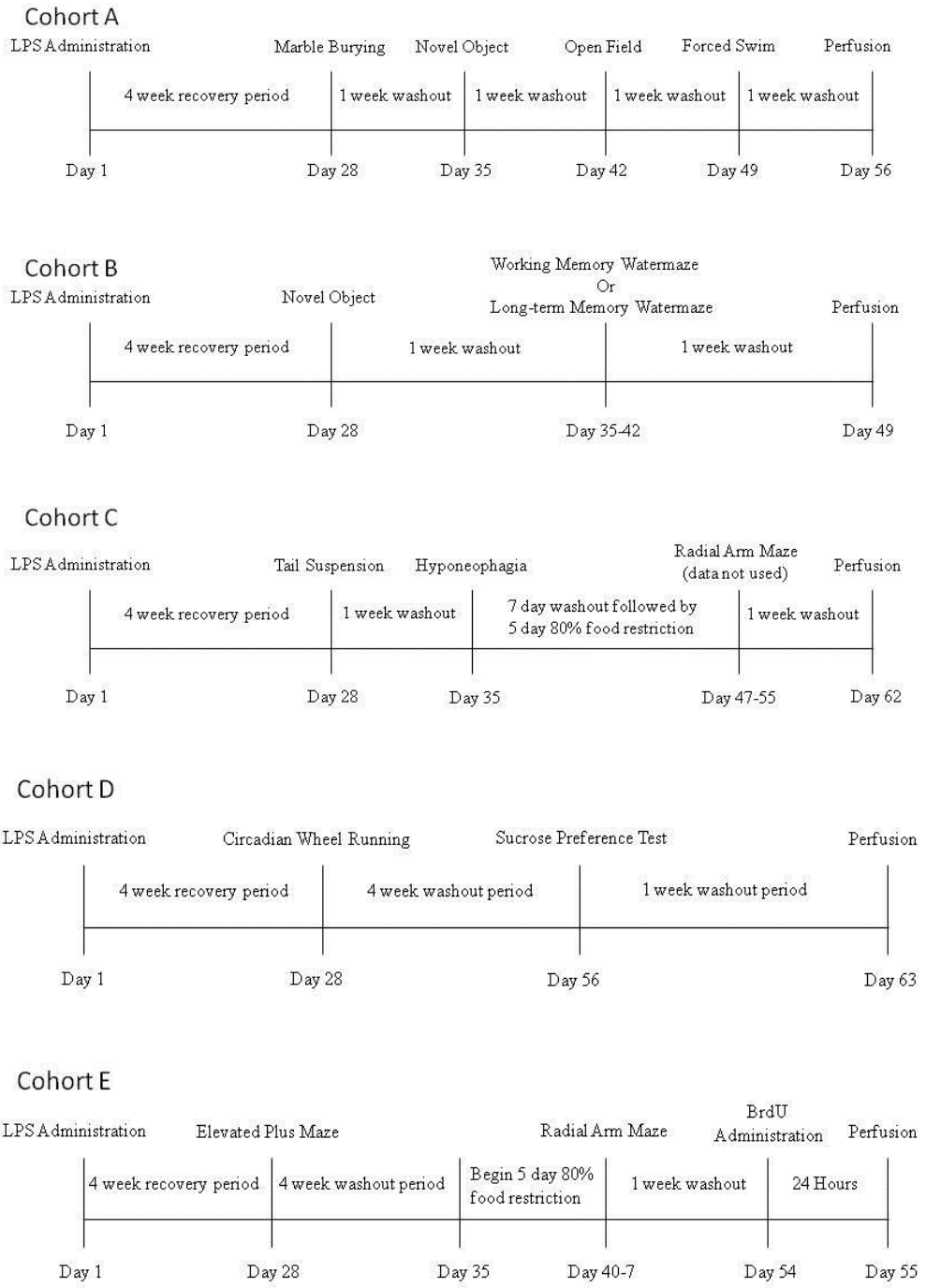



**Figure 2.1 Cohorts and timelines for behavioural experiments**

## 2.3 Results

### *2.3.1 Anxiety-like behaviour*

#### *2.3.1.1 Marble Burying Test*

A two-way ANOVA was used to determine whether or not there was an effect of housing or treatment on the number of marbles buried during the task. It was found that there was no main effect of housing, with the single housed animals (n=16) burying on average 9±0.51 marbles, and the group housed animals (n=16) burying 9.06±0.72 marbles ($F_{1,28}$=0.032, p=0.859). There was also no effect of treatment, with saline-treated animals (n=17) burying 9.06 ± 0.55 marbles, and LPS-treated animals (n=15) burying 9±.71 marbles ($F_{1,28}$=0.007, p=0.933). There was no significant interaction effect between treatment and housing ($F_{1,28}$=2.711, p=0.11). These results are represented in and Figure 2.2.

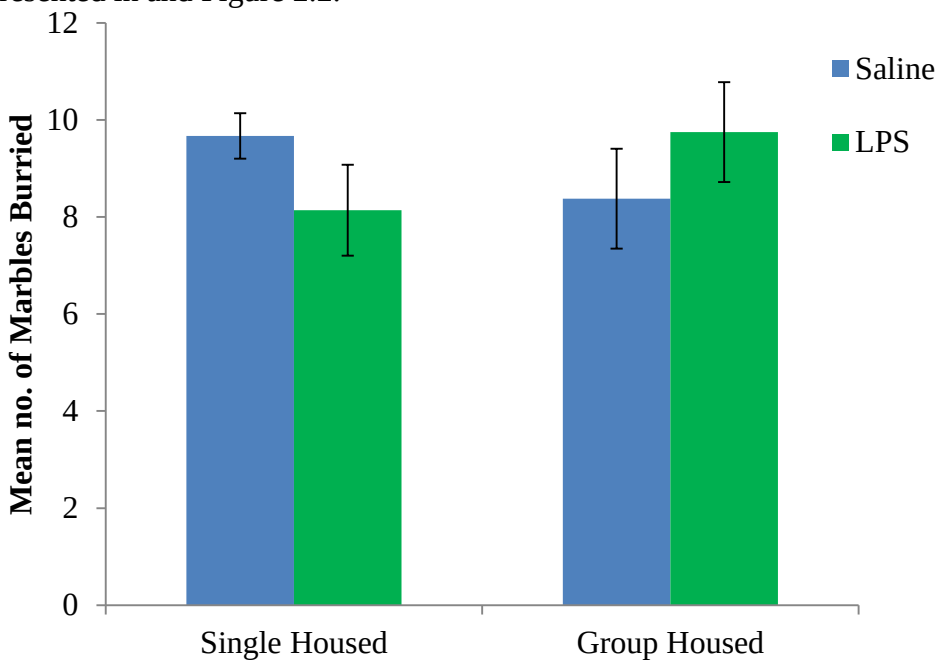



***Figure 2.2 Post-septic mice do not show altered marble burying behaviour*** *Bar graph illustrating the effect of treatment and housing on the number of marbles buried during a 30 minute trial. Error bars represent ± 1 SEM. N=8 group-housed controls, n=9 single-housed controls, n=8 group-housed LPS-treated mice, n=7 single-housed LPS-treated mice.*

*2.3.1.2 Open Field Test*

As shown in Table 1 there was no effect of treatment on any parameter examined in the small arena. In the small arena saline-treated (n=8) animals travelled an average of 889.73±37.35cm while LPS-treated mice (n=7) travelled 804.78±48.79cm ($t_{13}$=1.402, p=0.184). Saline mice travelled at a velocity of 8.0263±.36cm per second while LPS mice travelled at 8.33±.56cm per second ($t_{13}$=-0.455, p=0.657). Saline animals spent 112.6±1.74 seconds moving while LPS animals moved for 113.37±2.4 seconds ($t_{13}$=-0.265, p=0.795). Saline animals spent 30±4.58 seconds in the centre of the arena while LPS animals spent 27.66±4.98 seconds there ($t_{13}$=0.347, p=0.734).

In the large arena there (Table 2) was also no effect of treatment with saline-treated animals moving 909±28.51cm while the LPS animals moved 827.71±56.22cm ($t_{13}$=1.35, p=0.2). Saline animals (n=8) moved at a velocity of 7.99±0.29cm per second while LPS animals (n=7) moved at 8.28±0.28cm per second ($t_{13}$=-0.728, p=0.479). Saline treated mice moved for 113.5±1.75 seconds while LPS-treated animals moved for 113.43±1.34 seconds ($t_{13}$=0.032, p=0.975). Saline-treated animals spent 19.15±3.87 seconds in the centre of the arena while LPS animals spent 20.029±7.74 seconds there ($t_{13}$=-0.159, p=0.876.

*Table 1 Open field exploration in a small arena (50cm$^2$)*

| Treatment | Distance | Velocity | Time Moving | Time in Centre |
|---|---|---|---|---|
| **Saline** | 889.73±37.35cm | 8.0263±.36cm/s | 112.6±1.74s | 30±4.58s |
| **LPS** | 804.78±48.79cm | 8.33±.56cm/s | 113.37±2.4s | 27.66±4.98s |

*Table 2 Open field exploration in a large arena*

| Treatment | Distance | Velocity | Time Moving | Time in Centre |
|---|---|---|---|---|
| **Saline** | 909±28.51cm | 7.99±0.29cm/s | 113.5±1.75s | 19.15±3.87s |
| **LPS** | 827.71±56.22cm | 8.28±0.28cm/s | 113.43±1.34s | 20.029±7.74s |

*2.3.1.3 Hyponeophagia*

An independent t-test was carried out to investigate whether or not there was a main effect of treatment on the latency to eat. There was no effect of treatment observed, with saline animals (n=8) displaying a mean latency to eat of 513±67.64 seconds, and LPS-treated animals (n=8) eating after 415.25±92.67 seconds ($t_{14}$=0.856, p=0.406). These results are illustrated in Figure 2.3.

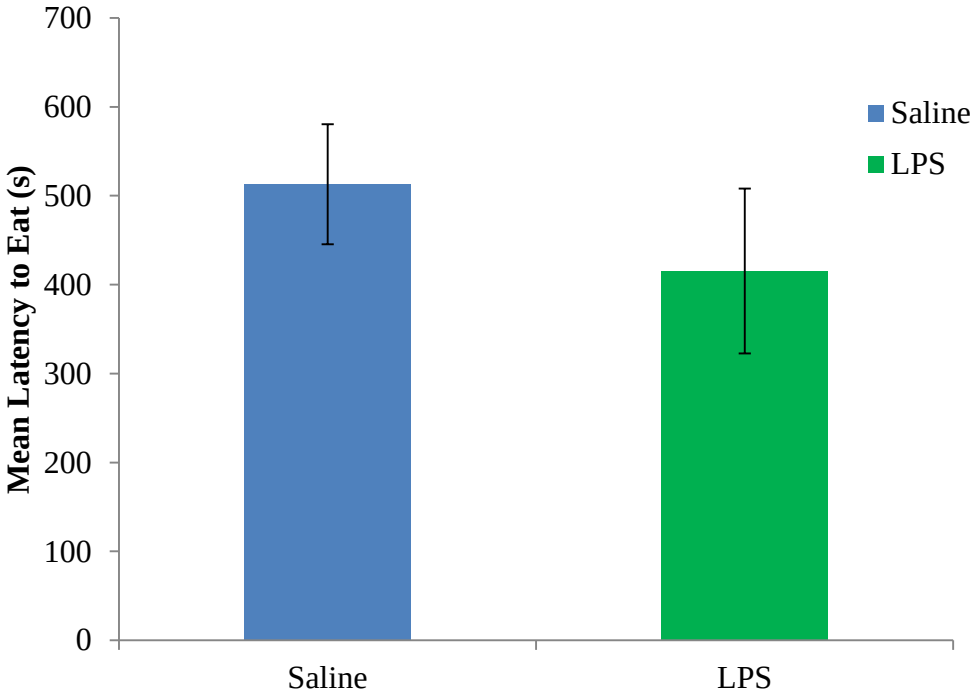



***Figure 2.3 Post-septic mice do not exhibit hyponeophagia.*** *Error bars represent ± 1 SEM.N=8 per group.*

*2.3.1.4 Elevated Plus Maze*

In order to test for the effects of LPS induced sepsis on anxiety-like behaviour in the elevated plus maze the number of open arm entries, closed arm entries and total visits were investigated using independent t-tests. Similarly, time spent in open arms, closed arms and the centre of the maze were also compared using independent t-tests. As seen in Figure 2.4, animals who had received LPS (n=12) made significantly fewer entries to the open arms of the maze than controls (n=12) (5.55±0.87 vs. 9.08±0.9) $t_{21.94}$=2.82, p=0.01. They also made significantly fewer total visits ($t_{22}$=2.81, p=0.009), while there was no significant difference in the number of closed arm entries between groups ($t_{22}$=0.89, p=0.38).

There was no difference between groups in the amount of time spent in the various areas of the maze. LPS-treated animals spent 81.36±16.36 seconds in the open arms, while saline-treated animals spent 100.08±11.07 ($t_{22}$=0.97, p=0.34). LPS-treated animals spent 119.09±14.46 seconds in the closed arms, while saline-treated animals spent 110.31±9.46 seconds in these arms ($t_{22}$=0.52, p=0.61). LPS-treated animals spent 99.54±8.46 seconds in the centre of the maze, while saline-treated animals spent 89.62±6.29 seconds in the centre ($t_{22}$=0.96, p=0.35).

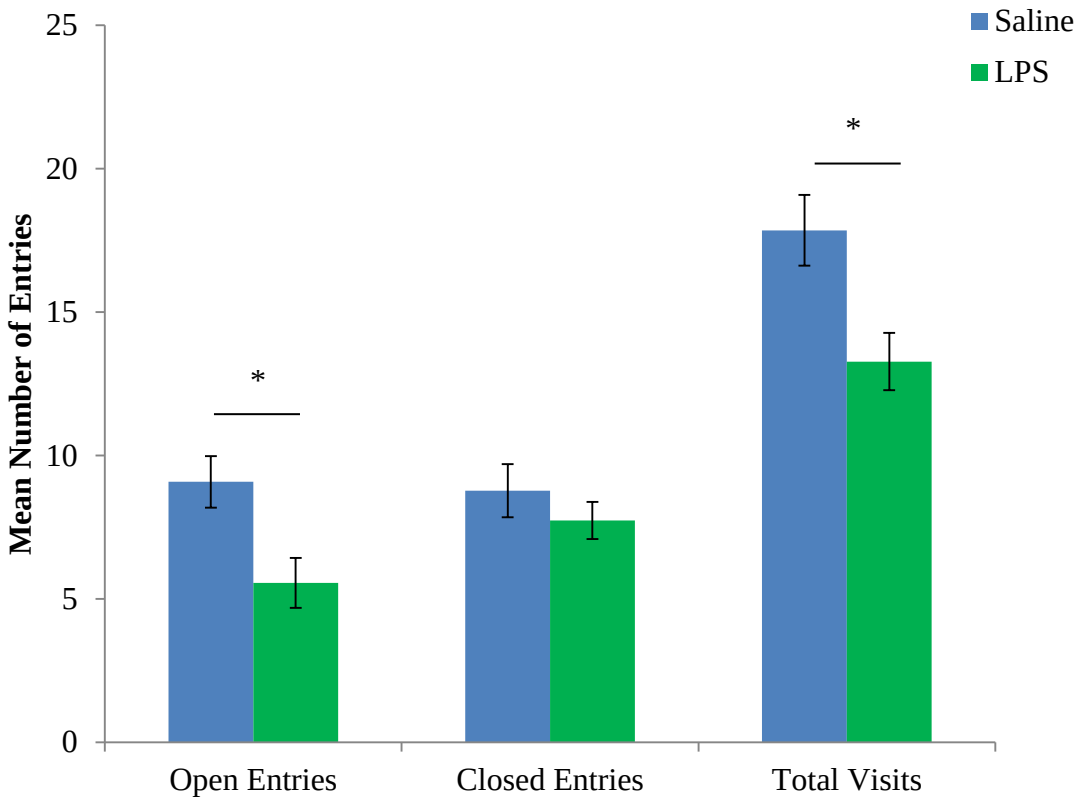



**Figure 2.4 Post-septic mice show reduced elevated plus maze exploration** Bar graph illustrating a significant reduction in the number of open arm entries among post-septic mice in the elevated plus maze. Error bars represent ±1 standard error of the mean, * represents $p<0.05$ in independent t-tests. N=12 per group.

*2.3.2 Depressive-like behaviour*

*2.3.2.1 Forced Swim Test*

A two-way ANOVA was run in order to determine whether or not there was a main effect of either housing or treatment, or an interaction effect between the two variables. There was no main effect of housing, with the single housed animals (n=16) spending an average of 135.06±7.67 seconds immobile, and the group-housed animals (n=16) spending an average of 143.93±4.35 seconds immobile ($F_{1,32}$=0.89, p =0.35). There was also no main effect of treatment with saline-treated animals (n=17) spending an average of 136.23±6.88 seconds immobile, and the LPS-treated animals (n=15) spending an average of 142.6±5.94 seconds immobile ($F_{1,32}$=0.458, p=0.5). There was no interaction effect between the two variables ($F_{1,32}$=0.004, p=0.95). These results are illustrated in Figure 2.5.

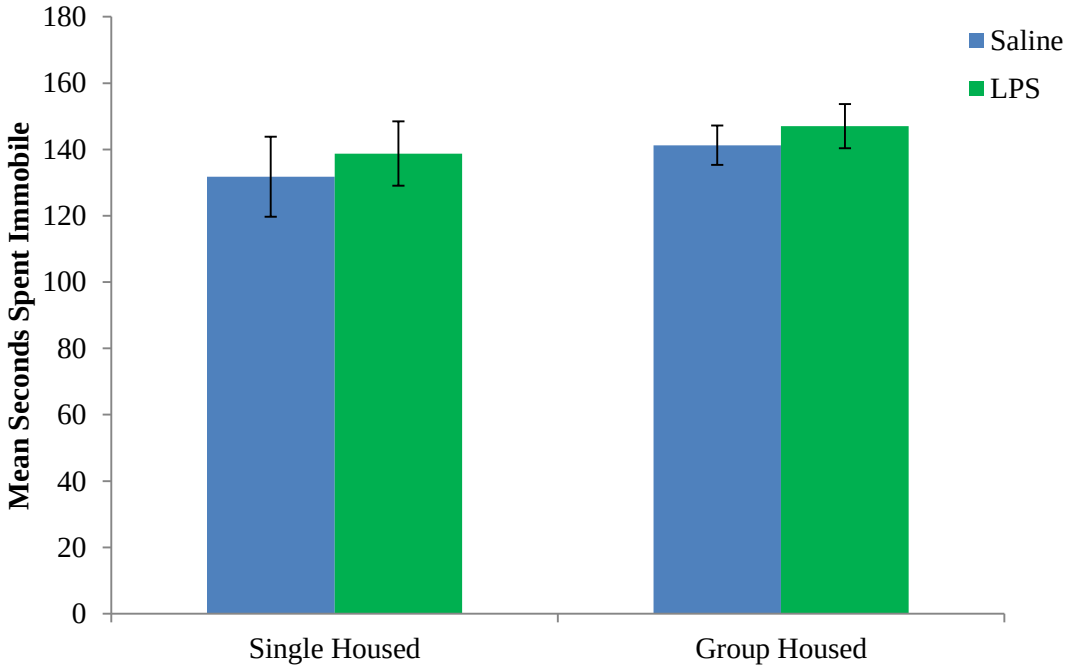



***Figure 2.5 Post-septic mice show no changes in the forced swim test*** *Bar graph illustrating the effect of treatment and housing on time spent immobile in the forced swim test. Error bars represent ±1 standard error of the mean. N=8 group-housed controls, n=9 single-housed controls, n=8 group-housed LPS-treated mice, n=7 single-housed LPS-treated mice.*

*2.3.2.2 Sucrose Preference Test*

As shown in Figure 2.6 an independent t-test revealed a significantly higher sucrose preference among the saline-treated animals (n=10) at 77.9 ±1.6% than the LPS group (n=11) at 68.9±3.5% ($t_{18}$=2.34, p=0.031). In addition, a chi-squared test revealed that a significantly higher portion of the LPS-treated animals could be classes as "anhedonic" according to a 65% cut off for sucrose preference suggested by Strekalova et al (2004) ($x^2_1$=5, p=0.025).

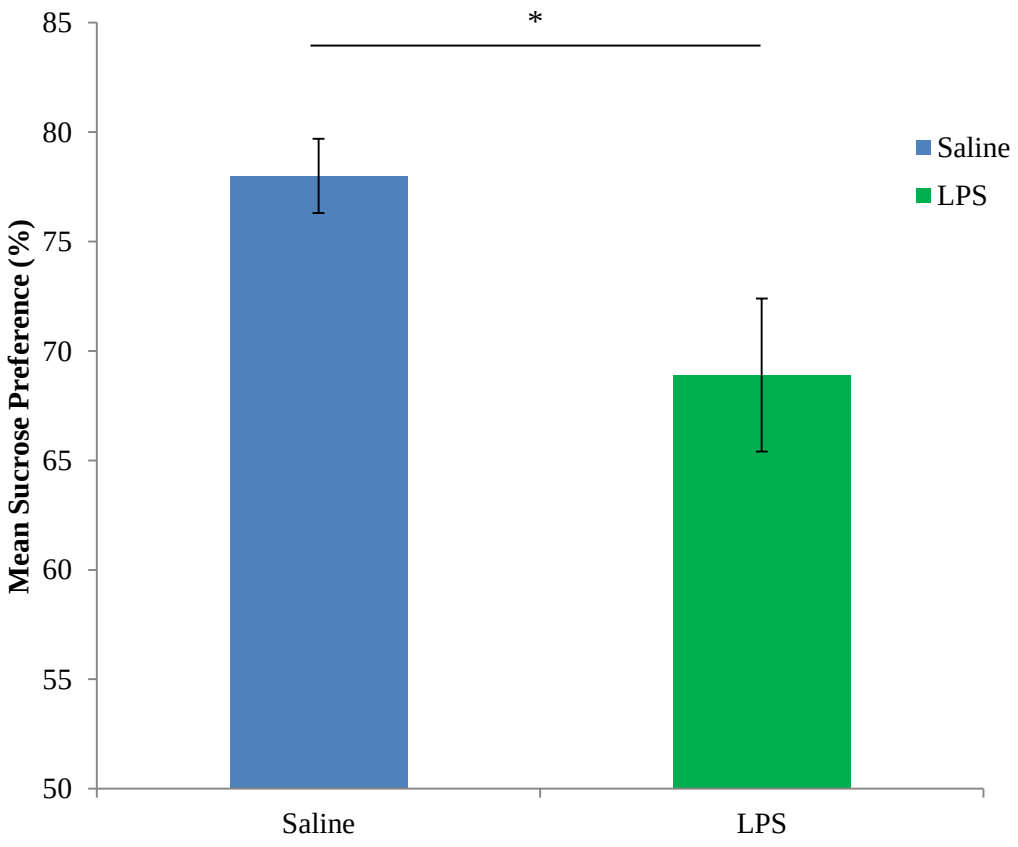



***Figure 2.6 Post septic mice show anhedonia in the sucrose preference test*** *Bar graph illustrating a significant difference between post-septic and control mice in sucrose preference. Error bars represent ±1 standard error of the mean, * represents p<0.05 in an independent t-test. N=10 for saline-treated mice, n=11 for LPS treated mice.*

*2.3.2.3 Tail Suspension Test*

An independent t-test was conducted in order to examine whether or not there was a main effect of treatment on time spent immobile. LPS-treated animals (n=15) spent a significantly longer amount of time immobile than saline-treated (n=16) animals ($t_{29}$=-3.948, p<0.0001). Saline animals spent an average of 158.69 ± 9.76 seconds immobile, while LPS animals spent 210.8 ± 8.8 seconds immobile. This is illustrated in Figure 2.7.

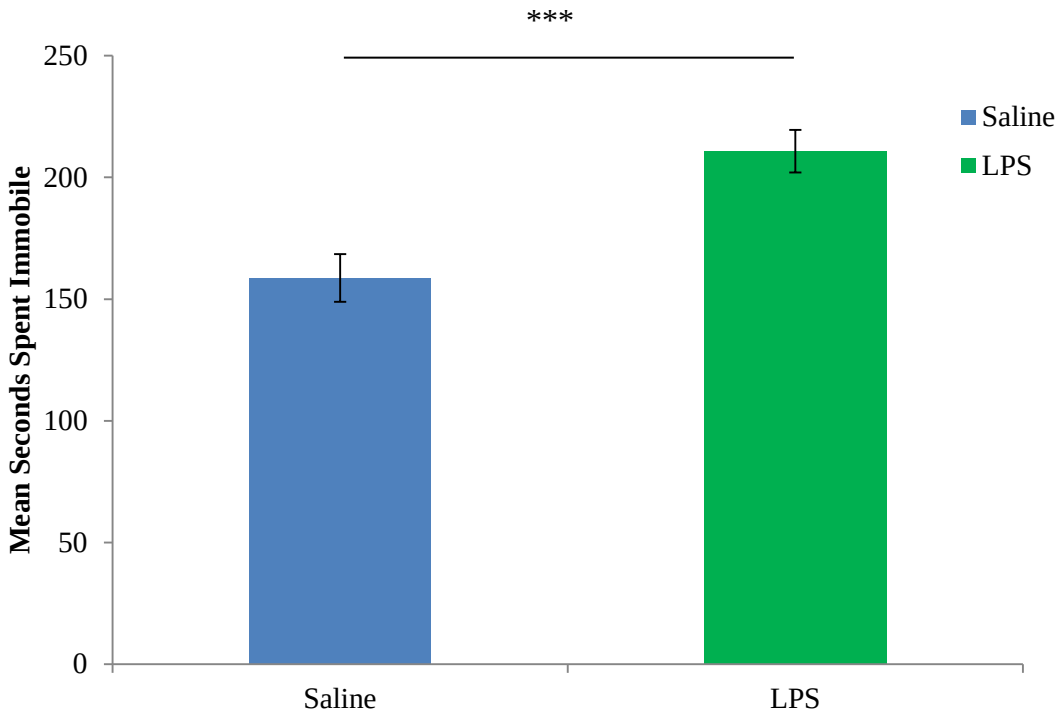



**Figure 2.7 Post-septic mice show behavioural despair in the tail suspension test** Error bars represent ±1 standard error of the mean, *** represents p<0.001 in an independent t-test. N=16 for control mice, n=15 for LPS-treated mice.

*2.3.2.4 Tail Suspension Test with Fluoxetine*

Repeated measures t-tests were conducted in order to examine whether or not the fluoxetine treatment had an effect on time spent immobile in either group. There was no effect of fluoxetine treatment in the saline group (n=10, $t_9$=0.546, p=0.598) with them spending and average of 140.1±16.34 seconds immobile after treatment with saline, and 151±13.64 seconds immobile after a treatment with fluoxetine. In the LPS-treated group, fluoxetine did significantly reduce the time spent immobile however (n=10, $t_8$=2.476, p=0.038). Treatment with fluoxetine reduced the time spent immobile from 167.78±14.29 seconds to 132.89±13.03 seconds. Independent t-tests revealed no significant difference between the groups following the administration of saline ($t_{17}$=-1.262, p=0.222) or fluoxetine ($t_{18}$=1.278, p=0.218). This effect is illustrated in Figure 2.8.

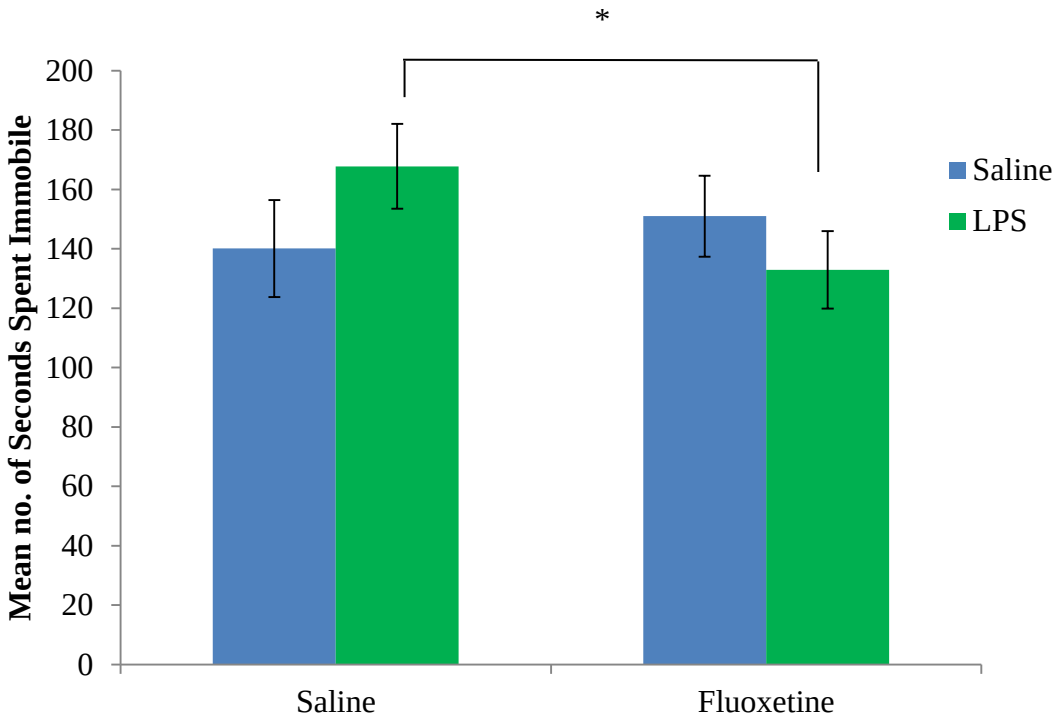



***Figure 2.8 Fluoxetine ameliorates behavioural despair in the tail suspension test*** *Error bars represent ±1 standard error of the mean, * represents p<0.05 in dependent t-test. N=10 per group.*

*2.3.3 Learning and Memory*

*2.3.3.1 Novel Object Recognition*

Significant differences were found on several parameters between the saline (n=20) and LPS-treated (n=18) mice. There were no differences in the amount of time spent exploring, or number of nose touches on either object between the groups in any of the four habituation trials, nor in the total time spent exploring ($t_{36}$=1.097, p=0.28), or total nose touches over all five trials ($t_{36}$=1.914, p=0.064). Exploration in each trial is illustrated in Figure 2.9.

In the fifth trial, where the novel object was introduced, it was found that saline animals spent a significantly longer amount of time exploring the novel object, and touched it more than LPS-treated animals. Saline animals spent 2.43±0.47 seconds exploring the object while the LPS-treated animals spent 1.08±0.22 seconds exploring it ($t_{27.1}$=9.189, p=0.014). Saline animals touched the novel object 3.35±0.43 times, while LPS-treated animals touched it 2.17±0.35 times ($t_{36}$=1.791, p=0.042). Saline animals also explored more overall in trial 5 in terms of both time spent exploring ($t_{30.984}$=5.372, p=0.019), and number of nose touches ($t_{36}$=0.748, p=0.027).

Both the saline-treated animals and the LPS-treated animals did explore the novel object more than the old object in trial five however, illustrated in Figure 2.10. Saline animals spent 1.96 ± 0.48 seconds longer with the novel object ($t_{20.84}$=4.09, p=0.001), and touched it 2.05±0.5 times more ($t_{30.66}$= 4.11, p<0.0005). LPS-treated animals spent 0.81 ± 0.26 seconds longer exploring the novel object ($t_{34}$=3.05, p=0.004), and touched it 1.56±4.15 times more than the familiar object ($t_{34}$=3.75, p=0.001). Important results are laid out in Table 3.

***Table 3 Differences in object exploration in post-septic mice***

| **Measure** | **Saline** | **LPS** | **p-value** |
|---|---|---|---|
| Time Exploring Old object in Trial 5 | 0.48 ± 0.1s | 0.27 ± 0.6s | 0.248 |
| Time Exploring Novel Object in Trial 5 | 2.43 ± 0.47s | 1.08 ± 0.22s | 0.014 |
| Trial 5 Exploration Time | 2.91 ± 0.54 | 1.35 ± 0.33 | 0.019 |
| Trial 5 Nose Touches | 4.65 ± 0.61 | 2.78 ± 0.52 | 0.027 |
| Difference between old object in time touching trial 4 and new object in trial 5 | 1.73 ± 0.41s | 0.28 ± 0.18 | 0.003 |

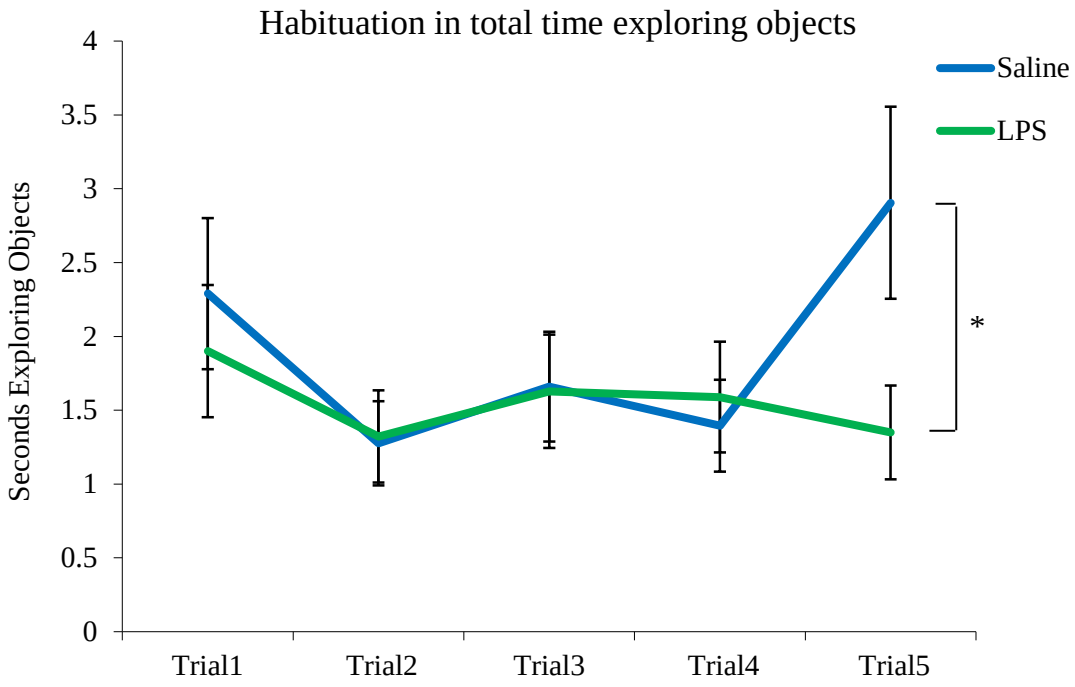



***Figure 2.9 Habituation of mice to objects across 5 trials*** *Line graph illustrating differences in the amount of time spent exploring objects throughout all five trials. Error bars represent ±1 SEM. * represents p<0.05 in independent t-tests. N=20 saline-treated mice, n=18 LPS-treated mice.*

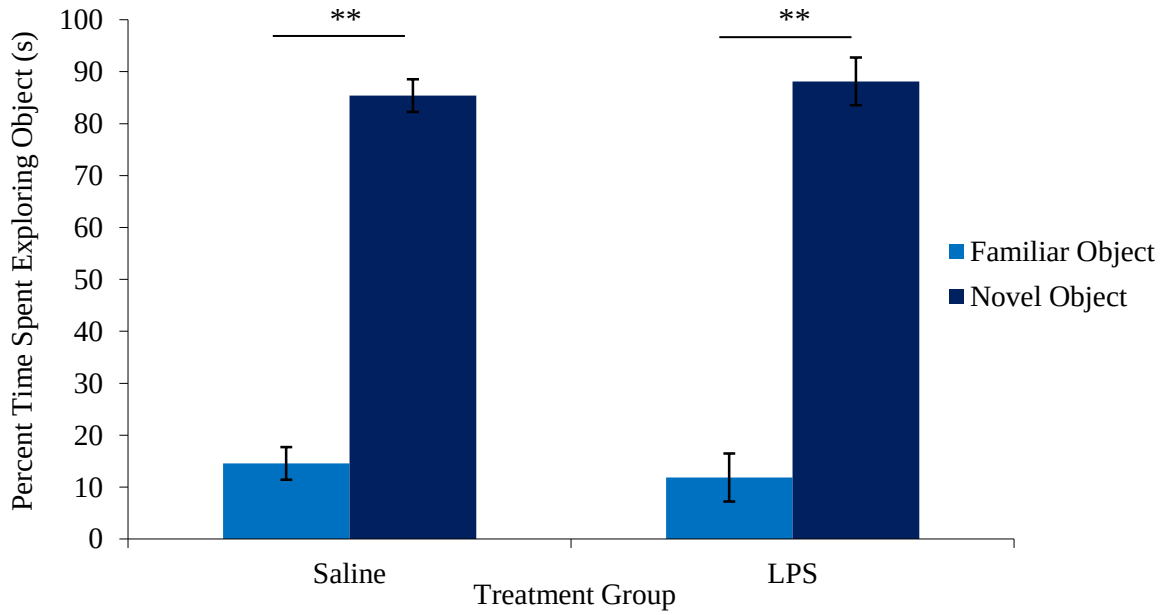



***Figure 2.10 No deficit in object recognition in post-septic mice*** *Bar graph illustrating the difference in the amount of time spent exploring a familiar object in trial 4 versus a novel object in trial 5.Error bars represent ±1 SEM. ** represents p<0.01 in independent t-tests. N=20 saline-treated mice, n=18 LPS-treated mice.*

*2.3.3.2 Radial Arm Maze*

When analysing the latency to complete the maze there was no interaction effect between treatment and time (n=8 per group, $F_{13,286}$=0.46, p=0.943). There was a main effect of time ($F_{13,286}$=11.05, p<0.0001). There was no main effect of treatment ($F_{1,15}$=0.408, p=0.53). These results are illustrated in Figure 2.11. The number of trials before an animal made its first error (returning to an arm where the bait had already been consumed) was also analysed. Here, as illustrated in Figure 2.12, there was a main effect of time ($F_{13,20}$ =5.561, p<0.0001). There was no significant interaction between treatment and time on the first error committed ($F_{13,208}$=0.758, p=0.7). There was also no significant difference between the groups ($F_{1,16}$=0.526, p=0.48).

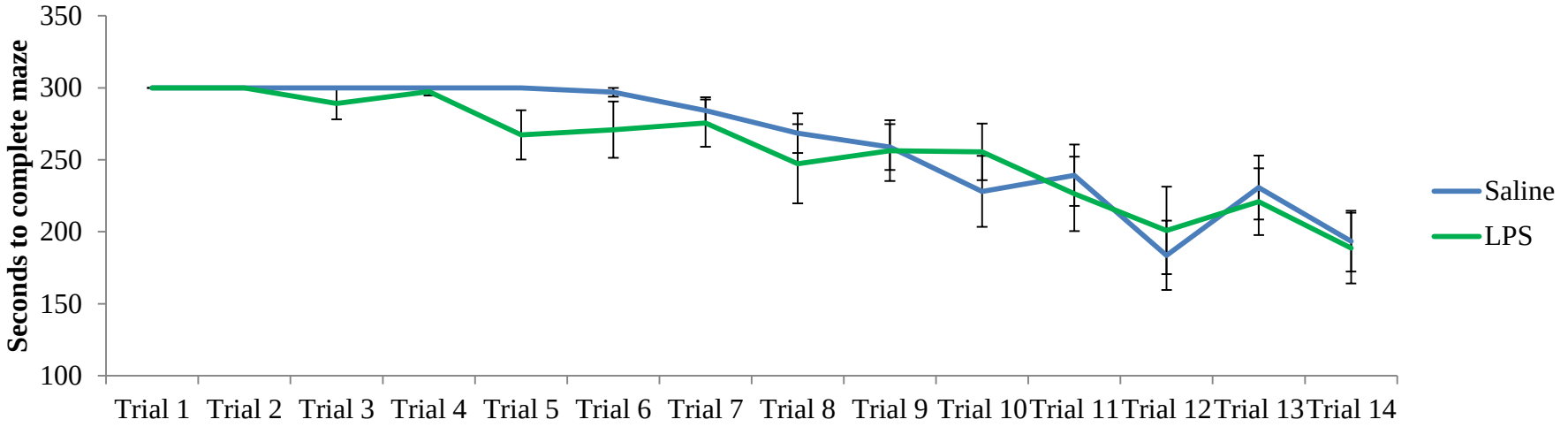



***Figure 2.11 No change in time taken to complete radial arm maze in post-septic mice.*** *Error bars represent ±1 SEM. N=8per group*

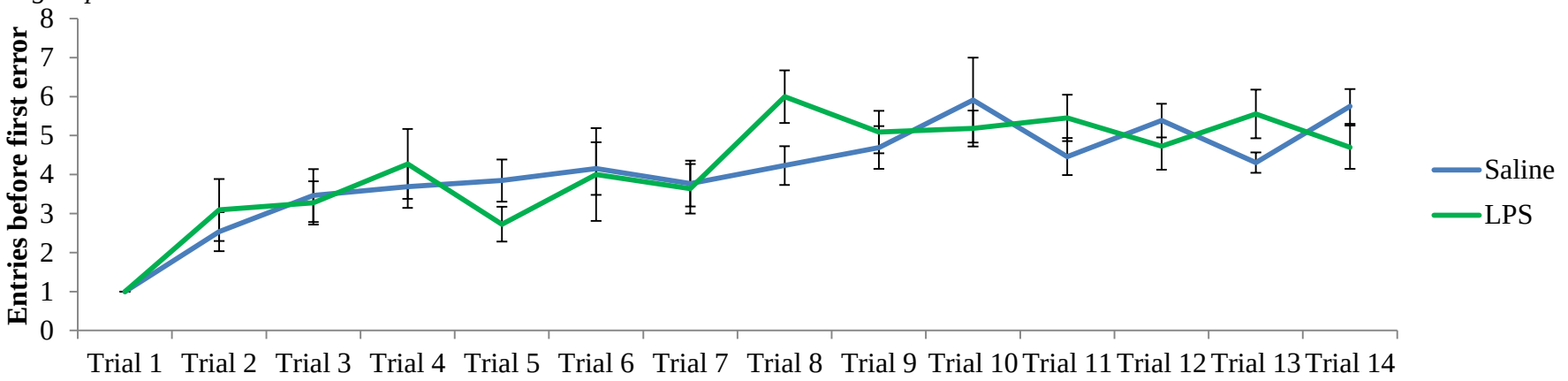



***Figure 2.12 No increase in errors made by post-septic mice*** *Error bars represent ±1 SEM.N=8 per group*

*2.3.3.3 Working Memory Watermaze*

As each trial is a unique test of working memory due to the platform being moved between trials, an independent t-test was done to examine whether there was a difference between the average escape latencies across all trials for saline and LPS animals. There was no significant difference between the groups, with saline-treated animals (n=8) displaying a mean escape latency of 14.78±1.85, while LPS animals (n=7) displayed a mean escape latency of 14.54±2.20 ($t_{88}$=0.092, p=0.927). Independent t-tests also revealed no significant differences between groups within any session (4 trials) – all p>0.05, see Figure 2.13.

In order to examine whether or not there might be a difference in the escape latencies following a 4 hour, or 24 hour delay since the previous trial, a two-way between groups ANOVA was carried out examining the effects of treatment and delay. This revealed no significant main effect of treatment ($F_{1,61}$=0.913, p=0.343), or delay ($F_{1,61}$ = 0.257, p =0.614), and no interaction effect was found ($F_{1,61}$=1.774, p=0.188).

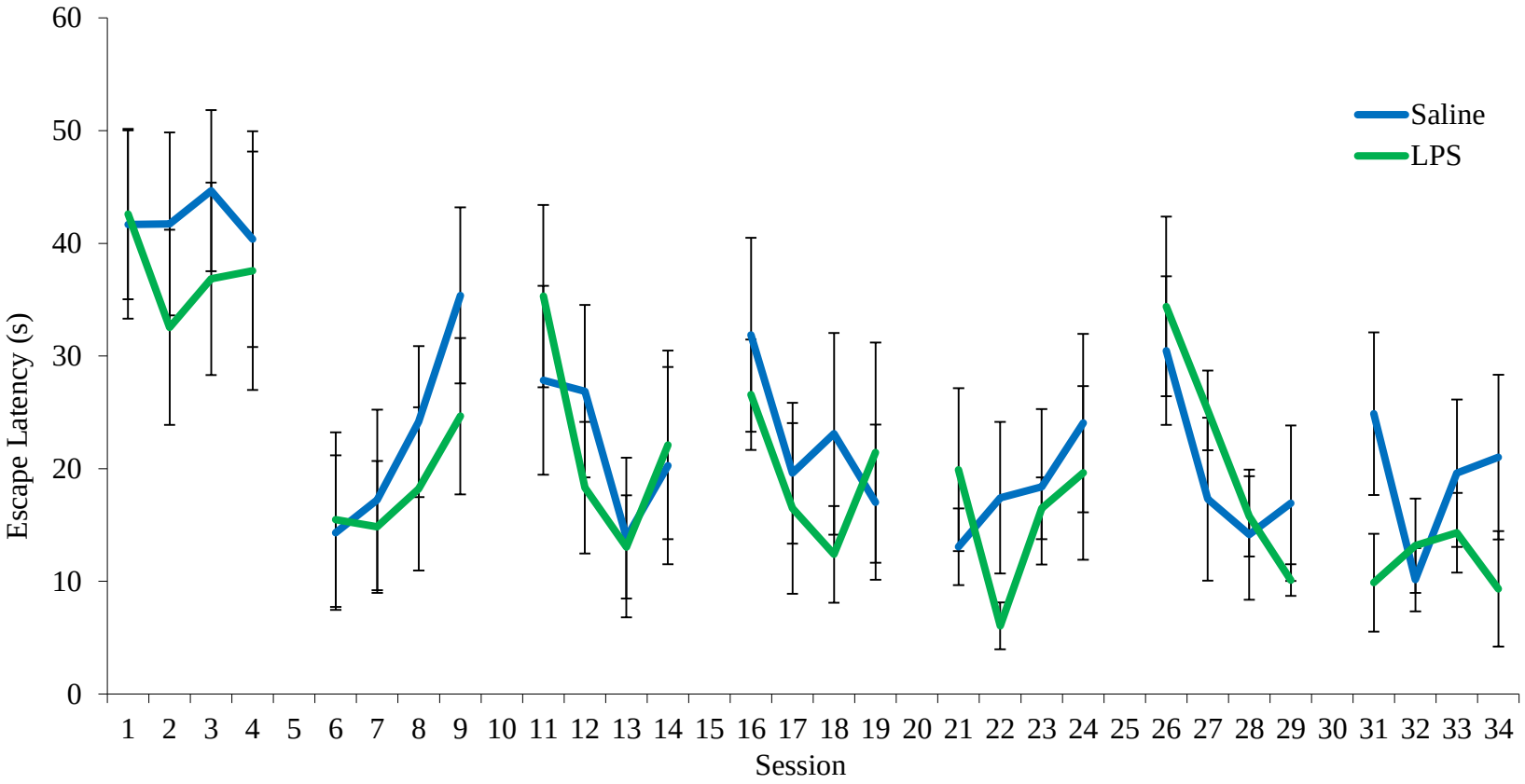



***Figure 2.13 Post-septic mice show no working memory deficits in the watermaze*** *Error bars represent ±1 SEM. N=8 saline-treated mice, n=7 LPS-treated mice.*

*2.3.3.4 Reference Memory Watermaze*

In order to assess whether or not learning took place throughout the trials, both groups (n=8 for saline-treated controls, n=7 for LPS-treated mice) were compared using 3 two way mixed between-within ANOVAs comparing trial number with escape latency, distance swam, and velocity. There was a significant main effect of trial on escape latency ($F_{5,65}$=27.416, p<0.0001), although there was no main effect of treatment ($F_{1,13}$=0.493, p=0.495), or an interaction between the two ($F_{5,65}$=1.013, p=0.417). This is illustrated in Figure 2.14. There was also a main effect of session on the distance swam during trials ($F_{5,65}$=24.916, p<0.0001), but no main effect of treatment ($F_{1,13}$=0.399, p=0.538) or interaction between the variables ($F_{5,65}$=1.124, p=0.356). There was also a main effect of session on velocity across the sessions ($F_{2.85,65}$=3.972, p=0.016), but no effect of treatment ($F_{1,13}$=0.059, p=0.812) or interaction between the variables ($F_{2.85,65}$=1.971, p=0.138).

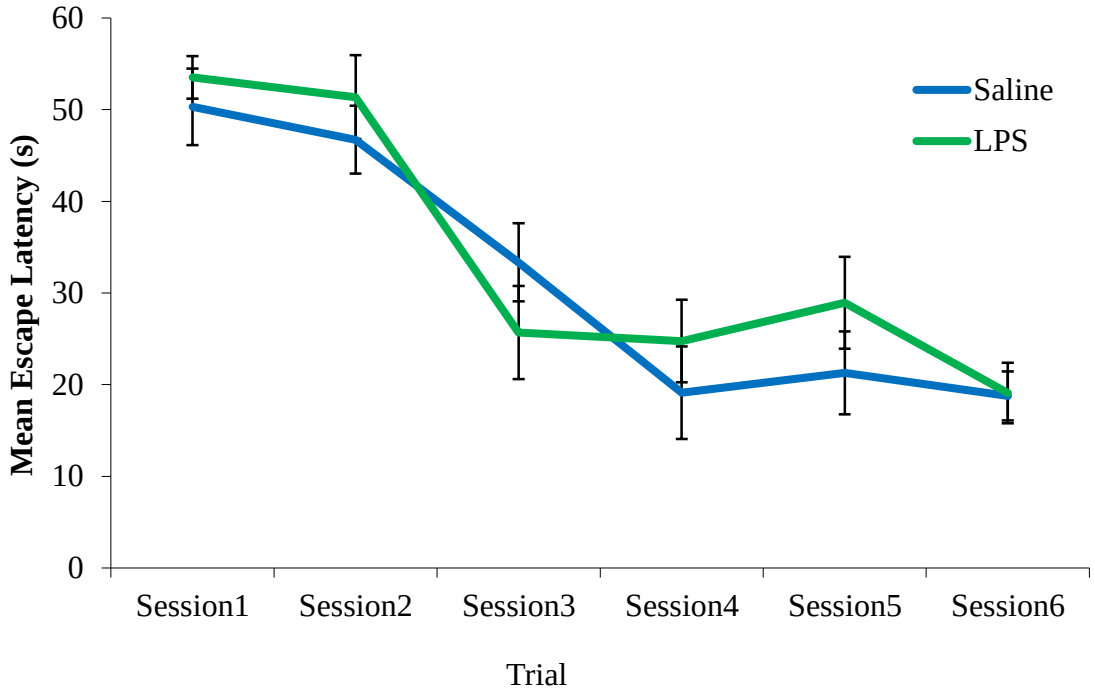



***Figure 2.14 Post-septic mice show no reference memory deficits in the watermaze*** *Error bars represent ±1 SEM. N=8 for saline-treated mice, n=7 for LPS-treated mice.*

Independent t-tests were also conducted to examine whether or not there was a difference between groups in time spent in the North-Eastern quadrant/area where the platform had previously been located, and whether or not there was any difference between groups in terms of the time spent in the outer versus the inner quadrant. There was no difference between groups in the amount of time spent in the North-Eastern quadrant ($t_{13}$ = 0.131, p = 0.898) or area ($t_{13}$ = 1.175, p = 0.261). These differences are illustrated in Figure 2.15. There was also no between-groups difference in the amount of time spent in the inner section of the pool ($t_{13}$ = 1.504, p = 0.157).

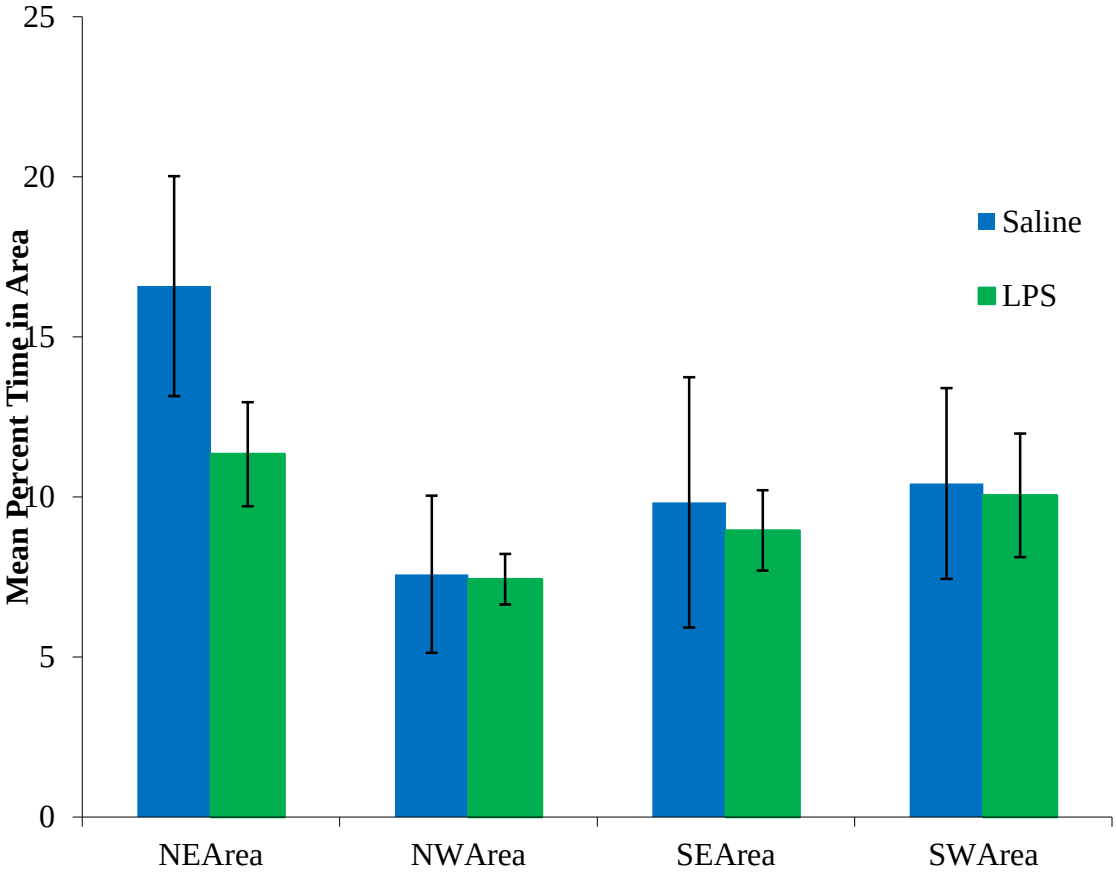



***Figure 2.15 Post-septic mice show no differences in spatial retention*** *Bar graph illustrating no significant between groups differences in the amount of time spent in each area during the retention trial. Error bars represent ±1 SEM. N=8 for saline-treated mice, n=7 for LPS-treated mice.*

## *2.4 Discussion*

### *2.4.1 Anxiety-like Behaviour in Post-septic Mice*

Post-septic mice did not show any discernible differences in anxiety-like behaviour in either the marble burying test or the open field test relative to treatment with saline. Saline and LPS-treated animals buried near-identical numbers of marbles in the first task, which is thought to reflect rodents' anxious or compulsive reaction to novel objects. It has also been argued however that this burying, while a possible predictor of anxiolytics' efficacy (Li, Morrow, & Witkin, 2005), may be simply a measure of digging behaviour which results in the unintentional burying of marbles (Deacon, 2006; Londei, Valentini, & Leone, 1998). Given that treatment with LPS did not induce significantly more thigmotaxic behaviour in the open-field test, it would seem that there was no effect of treatment on the anxious state of the animals in this test.

Interestingly, there was also no effect of housing condition on anxiety-like behaviour in the marble burying task. Some authors have argued that group housing of males may lead to social stress for non-dominant males as they are exposed to territoreal hostility (Arndt, et al., 2009; Karolewicz & Paul, 2001). Others however argue that single housing represents a kind of social deprivation, and group-housed mice have been shown to display significantly less anxiety-like behaviours in the dark-light box test and open field test (Chourbaji, Zacher, Sanchis-Segura, Spanagel, & Gass, 2005). Our results do not support either hypothesis, with no effect of housing on any measure, and no interaction between housing and treatment.

In the hyponeophagia test, which examines an animals' latency to eat a novel food in a novel environment, there was no difference between groups. Two preliminary

trials were run (data not shown) using different environments and food rewards, as well as shorter trials and inter-trial times and it was found that a high number of both the control and sepsis-survivor animals did not eat within the allotted time. Therefore it is possible that, at least using our paradigm, a ceiling effect exists whereby the latency to eat among control animals is already so high, that any effect of LPS treatment on this measure is difficult to distinguish without further methodological alterations.

As previously discussed, the literature on chronic neuroinflammation and post-septic encephalopathy anxiety-like behaviour is sparse and at times contradictory. These results, however, are in agreement with Weberpals et al (2009) who found no effect of a 5mg/kg dose of LPS on open field behaviour in mice 2 months after treatment. Our measures of time moving, distance and velocity also reveal no adverse locomotor effects of LPS treatment one month after recovery in the open field test. This provides evidence that all of our sepsis-survivor mice had fully recovered at this time, and any behavioural alterations are in fact due to cognitive and/or affective changes present in post-sepsis mice, rather than altered locomotion.

Post-septic animals did however exhibit heightened anxiety in the elevated plus maze. Animals surviving CLP-induced sepsis show similarly increased anxiety-like behaviours in the elevated plus maze at 10 days (Calsavara, et al., 2013), and somatic anxious (and depressive) symptoms have been shown to be associated with elevated levels of inflammatory factors among healthy adults (Pitsavos, et al., 2006), clinical populations (Hoge, et al., 2009). This is in contrast to the lack of anxious symptoms present in the hyponeophagia test or marble burying task. Although behavioural tests used to assess affective changes in animals are typically noted as targetting broad categories of behaviour (e.g. anxiety, depression) it is clear that within these categories functional and mechanistic differences are present between the type of test used

(Bourin, Petit-Demoulière, Dhonnchadha, & Hascöet, 2007; Renard, Dailly, David, Hascoet, & Bourin, 2003). Post-septic mice may therefore only be affected in one subset of anxiety-like behaviours.

### *2.4.2 Depressive-like Behaviour in Post-Septic Mice*

Previous research in both humans who have recovered from sepsis and sepsis-survivor rodents has pointed towards and altered emotional processing and increased depressive-like behaviour following recovery from sepsis (Cassol-Jr, et al., 2010; Comim, et al., 2010; Lazosky, Young, Zirul, & Philips, 2010). In accordance with these studies, an increase in the time spent immobile in the tail suspension test was also found, a commonly used model of behavioural despair, following recovery from LPS treatment as compared with saline-treated animals and an decrease in sucrose preference indicative of anhedonia. These increases in anhedonia and behavioural despair represent two key features of major depressive disorder (American Psychiatric Association, 2013).

Importantly, this effect was reversed by treatment with the selective serotonin reuptake inhibitor fluoxetine in the LPS-treated mice. This contributes to the predictive validity of our model. Although other studies have shown that acute neuroinflammation may block the anti-depressant effects of fluoxetine (Wang, et al., 2011), these results provide evidence that fluoxetine can, at least where an acute treatment is concerned, reverse behavioural despair in post-septic mice. It is necessary however to examine the effects of chronic fluoxetine treatment after LPS administration, as in human populations there is a significant latency between beginning treatment with SSRIs such as fluoxetine and a reduction in depressive symptoms (Önder & Tural, 2003).

Confounding these results is the fact that, at baseline, 90 minutes following the administration of saline there was no significant difference in immobility between the saline-treated and sepsis-survivor mice. This suggests that the tail suspension test may be particularly susceptible to acute stressors as has been suggested before (Dunn & Swiergiel, 2008). This cohort of mice had also undergone rather different behavioural testing than those which completed the tail suspension test with no acute treatment – including a food restriction protocol,which may have contributed to high baseline immobility among the saline group.

The forced swim test revealed no effect of LPS treatment on behavioural despair, in addition to no effect of housing condition. Although both the forced swim test and the tail suspension test claim to measure behavioural despair, it is likely that the underlying biology involved in animals' responses to both tests are quite different, with nicotine for example exerting opposite results in the two tests in female C57BL/6J mice (Andreasen & Redrobe, 2009), while many SSRIs which reduce immobility in the tail suspension test fail to do so reliably in the forced swim test (Bai, Li, Clay, Lindstrom, & Skolnick, 2001; Duman & Monteggia, 2006). Furthermore, the forced swim test has been shown to produce alterations in the levels of dopamine, its metabolite DOPAC, and serotonin in male Swiss mice where the tail suspension test does not (Renard, Dailly, David, Hascoet, & Bourin, 2003). Therefore the septic encephalopathy which follows recovery from sepsis in our mice may interfere with the pathways responsible for behavioural despair in the tail suspension test, but not those which control the response in the forced swim test. Our results, when taken together with the absence of locomotor reduction in the LPS-treated mice, suggest that the tail suspension test is not more affected by locomotor responses than the forced swim test as some authors have claimed (Hascoët, Bourin, & Bradwej, 1991).

*2.4.3 Learning and Memory in Post-Septic mice*

Our 5-trial novel object recognition task yielded a number of interesting results. Important to note is that there was no difference between groups in any of the first four habituation trials, or in the amount of time spent exploring either of the two objects, indicating that baseline exploration was similar in both groups of mice, and that there was no object preference among either group. There was a key difference however in that saline-treated animals explored the novel object significantly more than their LPS-treated counterparts. They also explored the item significantly more than they did a familiar object in trial 4, where the LPS animals did not. They had significantly higher levels of exploratory behaviour across the trial as a whole (where no such difference existed in the previous four trials), and had a significantly greater preference for the novel object in terms of the amount of time they spent exploring it than the LPS-treated animals.

All of these results would typically point towards impaired working memory function in the sepsis-survivor mice, as has been previously shown following recovery from sepsis induced via CLP (Cassol-Jr, et al., 2011). The LPS mice did in fact examine the novel object more than the familiar object during the trial however, indicating that the effect is probably not due to a working memory deficit. This result may agree with findings that chronic LPS-infusion into the $4^{th}$ ventricle does not induce short- or long-term working memory deficits in rats (Hauss-Wegrzyniak, Vannucchi, & Wenk, 2000). It is therefore probable that deficits in another process led to the behaviour of LPS-treated mice observed during the novel object recognition test. Acute LPS treatment has been shown to reduce exploratory behaviour towards a novel object hours after locomotor decreases have subsided (Haba, et al., 2012), an effect the authors contribute to a possible decrease in motivation in their mice. Given the reduced exploration in

general in the fifth trial, it is possible that reduced motivation to explore the novel object, although it was correctly recognised as novel. As there was no such effect during the 4 habituation trials, it would seem that this effect is context-dependent.

Supporting the idea that this alteration in the novel object recognition task is not an effect on working memory *per se*, is the fact that no deficits were found in the LPS-treated mice relative to controls in either the radial arm maze, or the working memory watermaze. Although these tests examine spatial working memory, where the novel object recognition task is non-spatial, LPS-treated mice performed just as well as their saline-treated counterparts in all aspects of both tests. There was no increase in errors, time to complete the trials, or any effect on the latency to commit a first error in the radial arm maze, and no effect on escape latencies in the watermaze, while velocity remained the same indicating equivalent locomotor abilities.

It is possible that the working memory deficits observed by Cassol-Jr et al (2011) were unique to the CLP model of septic encephalopathy, or perhaps there is a time dependent effect on working memory. Given that their experiments were carried out 10 days post-sepsis, and other deficits resulting from sepsis have shown to be present after 10 days, but not 30 or 60, that our experiments which were carried out over thirty days later may have missed a possible effect. Although Semmler et al. (2007) and Weberpals et al. (2009) both found working memory deficits in rats and mice respectively in the radial arm maze roughly 2 months after treatment suggesting that methodological factors may play an important role. For example, Weberepals and colleagues (2009) examined radial arm maze behaviour during animals' active phase (subjective night) and used ten minute trials with only 4 out of eight arms baited per trial. Semmler et al. (2007) conducted their experiment with rats, three months post-sepsis, and again used ten minute trials.

In the reference version of the watermaze both groups learned at almost identical rates, indicating that spatial nevigation and memory is conserved in the presence of post-septic encephalopathy. In addition, there was no difference during the retention trial in the length of time spent in the correct quadrant between the groups. This is surprising given the body of research which shows that both acute and chronic neuroinflammation typically lead to deficits in watermaze performance in rodents. In the CLP model of post-septic encephalopathy mice show significant watermaze impairment 15 days after sepsis induction (Hernandes, et al., 2014), although these mice show severe oxidative damage in the hippocampus. It is probable that the chronic neuroinflammation which results from the i.p. dose of LPS is functionally different to that induced via chronic infusion to the 4$^{th}$ ventricle which has previously been shown to disrupt watermaze performance (Cui, et al., 2008, Rosi, et al., 2006). Relevant hippocampal areas may have recovered sufficiently one month post-LPS administration in order to carry out successful spatial learning and memory consolidation in our model, whereas the more aggressive chronic infusion of LPS may lead to a more sustained inflammatory response which interupts spatial memory.

*2.4.4 Conclusion*

In conclusion, the i.p. administration of LPS does not appear to lead to gross alterations in locomotion or memory deficits one month after recovery. There are significant changes in affective behaviour, some of which is reversible with the acute administration of fluoxetine. Also present are alterations in novel object exploration which may be indicative of altered motivation. Our results are somewhat at odds with the existing literature on sepsis-survivor rodents and chronic neuroinflammation, however a number of key differences may explain this. Many of the tests used have been shown to be strain and sex dependent, and were previously carried out using

different doses or different models. Some of the effects seen in other papers may have been specific to certain models, doses or strains, or even particular methodological variations. In addition to this it seems likely that the time since recovery may play an important role in the deficits seen following sepsis.

# Chapter 3. Immunohistochemical Analysis of the Post-Septic Hippocampus

*Abstract*

This chapter sought to assess the extent to which peripheral administration of a septic dose of LPS could produce long-lasting changes within the hippocampus and amygdala. Animals which had previously undergone LPS administration and behavioural testing were sampled and hippocampal and amygdala sections were stained for a protein expression using immunohistochemistry. It is shown that post-septic mice show an upregulation of microglial markers within the hippocampus at both 24 hours and 2 months after LPS treatment. There are also changes to immediate early gene expression at both acute and long-term timepoints. IEG expression is also dampened in the post-septic amygdala. There was no change in astroglial markers and little change in cytokine expression profiles in the hippocampus. To examine hippocampal adult cell proliferation BrdU was administered 24 hours before sampling to one cohort – with findings suggesting a reduced number of BrdU-positive cells in the post-septic subgranular zone. By fourteen months post-sepsis expression levels of microglial markers CD11b and F4/80 match those of controls. It is concluded that peripheral LPS administration has the potential to stimulate long-lasting inflammation within the hippocampus, affect NPC proliferation and alter immediate early gene expression patterns.

*3.1 Introduction*

As outlined in chapter 1, the hippocampus is a brain region known to play a key role in both memory and emotional processing (MacQueen, et al., 2003), both of which may be impaired in patients suffering from post-septic encephalopathy (Lazosky, et al, 2010; Semmler, et al., 2013; Winters, et al., 2010; Ziaja, 2013). Sepsis survivors show hippocampal atrophy (Semmler, et al., 2013) and hippocampal neurons are particularly susceptible to the effects of chronic neuroinflammation (Rosi, Ramirez-Amaya, Hauss-Wegrzyniak, & Wenk, 2004). Given its importance in the behaviours previously described in post-septic mice the aim was to assess the extent to which our systemic administration of LPS affected the hippocampus of sepsis-survivor animals. As LPS is used to induce potent immune responses in a variety of other models, and there are relatively few studies detailing animal models of sepsis, it is also pertinent to consider its effects across this body of literature.

*3.1.1 Acute Immune Responses Triggered by LPS Administration*

LPS can elicit a variety of effects depending on the dose, method and timing of application and investigation, as well as the types of cells or specific area of the brain targeted. Peripheral administration of high-dose LPS leads to a potent inflammatory response, via the NF-κB pathway, mimicking some of the clinical features of sepsis and resulting in depressed EEG activity, low blood pressure, oxidative stress, multiple organ failure and a significant mortality rate (Chang, Tsai, Sheu, Hsieh, & Chiang, 2013; Lin, Chen, Lee, Chen, & Yang, 2010; Okazaki, Tachibana, Koga-Ogawa, & Takeshita, 2013). The hippocampus in particular is highly sensitive to the effects of LPS-induced sepsis in rodents (Semmler, Okulla, Sastre, Dumitrescu-Ozimek, & Heneka, 2005). Two hours following 10mg/kg i.p. LPS administration serum TNFα, IL-6 and IL-10 are significantly upregulated (Zheng, et al., 2012), similarly, 6 hours following 10mg/kg i.p.

LPS administration to C57BL/6J mice TNF-α and IL-1β are greatly upregulated in serum (Chang et al, 2013). In Sprague-Dawley (SD) rats a 10mg/kg dose of LPS has also been shown to significantly upregulate hippocampal, cortical and hypothalamic TNF-α, IL-1 and IL6 as well as $H_20_2$ while decreasing anti-oxidant markers (Chen, et al., 2014). 5mg/kg i.p. administration led to a significant increase in hippocampal IL-1β, but not TNFα mRNA levels in C57BL/6 mice 24 hours post-treatment (Diz-Chavez, Astiz, Bellini, & Garcia-Segura, 2013). In the same study, immunohistochemical analysis of microglial marker Iba-1 also showed that while the absolute number of microglia did not change, a significantly higher percentage of microglia demonstrated a reactive morphology in the DG and CA1, with no increase in GFAP immunoreactivity. 8mg/kg LPS administered intravenously to SD rats resulted in a large increase in cerebral iNOS levels at 1hour, with this increase diminishing at 2 and 3 hours and subsequently returning to high levels at 6 hours post-treatment (Pei, et al., 2013).

LPS can also increase mRNA for cytokine receptors in the brain with a 30μg doses of LPS triggering an increase in hippocampal IL-1RI, IL-6R and TNFαR six hours post treatment (Utsuyama & Hirokawa, 2002). 10mg/kg LPS i.p. has been shown to have peak cytokine (TNFα, IL-6) and chemokine (KC, MIP-2) at 1 – 4 hours post-treatment, with a decline or plateau occurring by 8 hours post-treatment (Remick, Newcomb, Bolgos, & Call, 2000). A 10mg/kg i.p. dose of LPS given to male Wistar rats has also been shown to reduce cerebral glucose uptake in an area-dependent fashion, accompanied by increased TNF-α, IL-1β and iNOS transcription and reduced hippocampal NeuN expression 24 hours post-treatment (Semmler, et al., 2008). Rats treated with 8mg/kg doses of LPS showed significantly higher levels of plasma IL-1 β, IL-6 or TNFα 24 hours or six days post-treatment, with significantly increased IL-1β but no change in IL-6, TNFα or apoptotic cells in cerebral cortex tissue at six days post-

treatment (Plaschke, Bent, Wagner, Zorn, & Kopitz, 2014). LPS (2.25µg/brain) injected intracerebroventricularly results in upregulated TNFα, IL-6 and MCP-1 in the hippocampus at 1 and 4 hours, with levels dropping by 10 hours and close to baseline by 16 hours, while F4/80 expression, indicative of microglial activation, peaked at 2 days post-treatment when cytokine production had ceased to be detectable (Lund, et al., 2006). Overall it appears that systemic administration of LPS leads to rapid upregulation of pro-inflammatory factors and microglial activation within the CNS – although there is evidence for differential susceptibility among brain regions.

*3.1.2 Chronic Immune Responses to LPS Administration*

Fewer studies have examined the long lasting effects of LPS administration – either through repeated low-dose LPS administration or at a later timepoint following a septic administration of LPS. There is considerable variation between the results of these studies, and indeed between the brain regions studied in each experiment. An early study on the effect of a systemic 5mg/kg dose of LPS found that TNF-α protein remained upregulated in the whole brain of mice up to 10 months following treatment – accompanied by persistent microglial activation (Qin, et al., 2007). A repeated i.p. administration of 750-1250µg/kg LPS to female C57BL/6 mice over four months induced anhedonic behaviour accompanied by a reduction in IL-10 and IFN γ and an increase in superoxide and corticosterone – with these effects ameliorated by chronic treatment with fluoxetine (Kubera, et al., 2013). Male Wistar rats treated with 5mg/kg i.p. exhibited significantly higher levels of hippocampal and frontal cortex TNF-α at 7 days post-treatment, with elevated TNF-α persisting at 10 months post-treatment accompanied by an elevated expression of IL-18 (Bossù, et al., 2012). Increased microglial activation and morphological alterations have been seen in the whole brains of C57BL/6 mice 28 days after a dose of only 0.5mg/kg, although this was not

accompanied by significant changes in cytokine expression (Konda, Kohsaka, & Okabe, 2011). Interestingly, an upregulation of CD11b, TNFα and IL-1β expression was seen in the frontal cortex, but not hippocampus, of C57BL/6 mice two months following LPS administration (Weberpals, et al., 2009). Although increased microglial activation was present, there were no changes in GFAP or NeuN levels indicating that there was no long-term neuronal cell loss or change in astrocyte function or number. Fan et al (2012) report sustained microglial activation in the abscence of astrocytic activation or heightened expression of cytokines TNF-α or IL-1β in the hippocampus one week following LPS-induced sepsis in C57 mice using a 5mg/kg dose. Together these findings demonstrate the potential for chronic overactivation of microglia in response to peripheral doses of LPS – although the cytokines which peak rapidly following treatment may not always remain upregulated at later time-points.

### *3.1.3 The Inflammatory Response to Sepsis*

Similar to animal models of LPS-induced sepsis, patients suffering from sepsis also show a hyperinflammatory state with a huge upregulation of proinflammatory cytokines – although this may be slower than that seen in animal models. They then however also show a secondary state of immunosupression characterised by increased apoptosis of immune cells, lower levels of human leukocyte antigen-DR molecule expression on monocytes and macrophages, anti-inflammatory cytokine production, reduced monocyte phagotocytosis and a reduction in the number of pro-inflammatory cytokines released from immune cells (Hutchins, Unsinger, Hotchkiss, & Ayala, 2014; Schefold, Hasper, Volk, & Reinke, 2008; Venet, Lukaszewicz, Payen, Hotchkiss, & Monneret, 2013). It is often during this later immunosuppressed phase of sepsis that patients die – due to second or third infections occurring in conjunction with an impaired immune system.

*3.1.4 Immediate Early Genes in the CNS*

Immediate early genes are rapidly produced in the CNS in response to a wide variety of stimuli and are thought to be key factors in regulating cellular activities necessary for neuronal plasticity (Pérez-Cadahía, Drobic, & Davie, 2011). Three of the most characterised IEGs include activity-regulated cytoskeleton-associated protein (*Arc*), early growth response protein-1 (*egr-1*), and *c-fos*. *Arc* encodes an effector protein which directly influences cell function, *egr1* and *c-fos* on the other hand are classified as regulatory transcription factors, with both genes influencing cells in a more indirect fashion via effects on the transcription of specific downstream genes (Guzowski, Setlow, Wagner, & McGaugh, 2001). *Egr-1* and *arc* are both of central importance to long-term plasticity and memory (Bozon, Davis, & Laroche, 2003; Soulé, et al., 2008), with *egr-1* also implicated in the induction of late-phase *arc* transcription (Penke, Chagneau, & Laroche, 2011). *Arc* has been shown to be both up-regulated, and down-regulated in response to neuroinflammation, with differences emerging between models of inflammation, age of the animals, whether basal *arc* expression or learning-stimulated *arc* is measured and the time at which *arc* measurement takes place (Rosi, 2011).

Though less research exists on the interplay between *egr-1* expression and neuroinflammatory pathways, *egr-1* has been shown to play a role in LPS induced neuroinflammation in addition to its involvement in memory consolidation/formation. Egr-1 has been shown to bind to the promoter region of TNF-α in human monocytic cells following LPS administration (Yao, Mackman, Edgington, & Fan, 1997). In models of cerebral ischemia EGR-1 expression is increased while *egr-1* knockout mice exhibit improved neurological function and reduced inflammatory gene expression (Yi, Park, Kapadia, & Vemuganti, 2007). Little work to date has been done on *egr-1*

expression in the hippocampus following the administration of inflammatory stimuli. It has however been found that a 5µg intracerebroventricular injection of LPS does not increase expression of e*gr-1* or a*rc* expression in the hippocampus, although both were significantly lower in the cortex (Bonow, Aïd, Zhang, Becker, & Bosetti, 2009).

*c-fos* is a transcription factor known to be induced by a number of noxious stimuli (Pérez-Cadahía, Drobic, & Davie, 2011), and has been shown to be acutely upregulated across multiple brain regions upon LPS administration (Wanner, et al., 2013) with levels falling by 24 hours (Frenois, et al., 2007). Owing to its rapid induction in neurons following stimulation *c-fos* has been widely used in neuroscience research as a marker of neuronal activation (Avi, 1997; Cruz, et al., 2013; Kovács, 1998).

### *3.1.5 Aims*

The aim of this chapter was therefore to examine neuroimmune parameters highly relevant to both human sepsis and the behavioural alterations which were previously shown in post-septic mice. Based on previous studies indicating the ability of septic doses of LPS to induce long-lasting neuroinflammatory changes within the hippocampus, it was hypothesised that the peripheral administration of LPS would lead to a chronic activation of microglia and the upregulation of pro-inflammatory factors within our model. It was also hypothesised that these inflammatory changes would affect factors essential to normal hippocampal functioning such as immediate early gene expression and adult cell proliferation. As the amygdala is also a key area in processing emotion c-Fos levels were also examined here to assess neuronal activation in the region.

Immunohistochemical analysis was carried out to examine both the acute and long-term effects of LPS treatment in the hippocampus. Microglial and astrocytic

activation were investigated using the microglial markers cluster of differentiation molecule 11B (CD11b), F4/80 and ionised calcium-binding adaptor (IBA1) in addition to the astrocytic marker glial fibrillary acidic protein (GFAP). The cytokines interleukin(IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) were also examined as well as the enzyme inducible nitric oxide synthase (NOS2/iNOS). Given the importance of the NF-κB pathway in driving LPS-induced inflammation, components of NF-κB signalling phosphorylated (p)-IKK, and p-IκB were then assessed. In addition to these immune factors IEG protein product expression levels, namely ARC, EGR-1 and c-Fos were assessed. C-Fos was also measured in the amygdala at this timepoint. Finally, apoptotic marker cleaved caspase-3 was examined 24 hours post-LPS administration and cell proliferation was assessed using bromodeoxyuridine (BrdU).

*3.2 Methods & Materials*

*3.2.1 Animals*

Male C57BL/6 mice between 8 and 12 weeks old were used in all experiments. Animals were housed two to four per group in plastic cages (12cm x 9cm x 9 cm) with wire lids containing wood-chip bedding and shredded paper for environmental enrichment. All animals had *ad libitum* access to water at all times, and *ad libitum* access to food. Animals were kept in a 12:12 light:dark cycle at 200lux and 21±1°C and 50±5% relative humidity. Where an animal was moribund and deemed unlikely to recover, as occurred in approximately 10% of cases, he was removed from the sample and euthanised.

*3.2.2 Drug Treatments*

All saline and LPS injections were made up fresh on the treatment day, and given intraperitoneally in a final injection volume of 0.125ml. 0.9% sterile saline was made up fresh for control injections, while lipopolysaccharide (serotype0111.B4, Sigma Ireland; Qin et al, 2007) was made up to a 5mg/kg dose in sterile saline. Injections were given intraperitoneally between zeitgeber time (ZT) 06 and 08, where ZT0 is defined as lights on. For the examination of neural precursor cell proliferation BrdU (50mg/kg, Sigma Aldrich) was administered i.p. to animals 24 hours before euthanasia in a volume of 0.3ml.

*3.2.3 Perfusion and Tissue Preparation*

Mice were anaesthetised with 0.1ml sodium pentobarbital (Euthatal, Merial Animal Health, UK), following which they were perfused transcardially with 0.9% saline, and then 4% paraformaldehyde (PFA, Sigma) in 0.1M phosphate buffer (PB), pH 7.4 at 4˚C. Brains were subsequently removed and placed overnight in the same fixative at 4˚C, followed by immersion in 0.1 M PB containing 30% sucrose at 4˚C until

sunk. Following cryoprotection, the brains were then taken from sucrose, cerebellums were removed, and the brains were frozen and sectioned coronally at 30µm in series of 4 using a microtome (Leica, UK), with sections stored in 0.1M PB with 0.1% sodium azide.

*3.2.4 Immunohistochemistry*

Immunohistochemistry followed a standard Avidin-Biotin Complex/Nickel DAB colourmetric protocol (O'Callaghan, Anderson, Moynagh, & Coogan, 2012). Following two ten minute washes in 0.1 M PB and one ten minute wash in 0.1 M PB with 0.3% Triton X (PBX), sections were washed for 20 minutes in 0.1 M PB with 1.5% hydrogen peroxide to inactivate endogenous peroxidases. After a further three ten minute washes (2 x PB, 1 x PBX) sections were blocked with 5% normal horse serum (NHS) or normal goat serum (NGS) for 60 minutes at room temperature. Sections were then incubated with primary antibody in 0.1M PBX containing 2% normal horse serum overnight at 4˚C (primary antibody information given in table 4). The following day sections were once again washed (2 x PB, 1 x PBX) before development with the appropriate biotinylated secondary antibody (1:400) in 0.1M PBX containing 2% NGS or NHS for 70 minutes. During secondary antibody incubation and all subsequent steps sections and solutions were protected from light. Following 3 subsequent ten minute washes (2 x PB, 1 x PBX) sections were developed in ABC for 90 minutes (0.4%; Vector Laboratories, Peterborough, Cambridgeshire, UK). After two more PB washes, and one ten minute wash in 0.1 M Sodium Acetate (Ph 6, Sigma) sections were reacted with 1ml of nickel-enhanced diaminobenzidine, (3, 3'-diaminobenzidene with ammonium nickel chloride, NiDAB, PH6) and 60µl glucose oxidase (5mg/ml) as a catalyst to visualise the protein products of interest. To control for inter-run variability all sections for individual anti-bodies were reacted in the same run, for an equal amount

of time. Once sufficient staining had developed, sections were washed in 0.1M sodium acetate (Ph6) followed by two washes in 0.1M PB (Ph7.4). Sections were subsequently mounted on gelatine coated slides (1% gelatine, 0.05% chromium potassium sulphate) and allowed to dry for 24-48 hours. Slides were then dehydrated via three 3 minute washes in 70%, 90% and 100% ethanol and delipified with three 3 minute washes in 2% Histoclear (National Diagnostics, UK). Slides were then coverslipped with Eukitt (Fluka Analytical).

**Table 4.** *Primary antibodies used in immunohistochemical analysis*

| **Antibody** | **Dilution** | **Raised in** | **Supplier** | **Product Code** |
|---|---|---|---|---|
| Arc (H-300) | 1:500 | Rabbit | Santa Cruz Biotechnology | sc-15325 |
| Egr-1 (C-19) | 1:3000 | Rabbit | Santa Cruz Biotechnology | sc-189 |
| CD11b | 1:1000 | Rat | AbD Serotec, Oxford, UK | MCA74GA |
| F4/80 | 1:100 | Rat | AbD Serotec, Oxford, UK | MCA497GA |
| IBA-1 | 1:3000 | Rabbit | Wako, Denmark | 019-19741 |
| GFAP | 1:1000 | Rabbit | Sigma, St. Louis, MO, U.S.A | G4546 |
| TNF- α | 1:75 | Rat | AbD Serotec, Oxford, UK | MCA1488 |
| IL-1β | 1:50 | Rabbit | Peprotech, U.S.A. | 500-P51 |
| IL-6 (M-19) | 1:200 | Goat | Santa Cruz Biotechnology | sc-1265 |
| NOS2 (M-19) | 1:100 | Rabbit | Santa Cruz Biotechnology | sc-650 |
| p-IKK α/β (Ser 180/ Ser 181)-R | 1:200 | Rabbit | Santa Cruz Biotechnology | sc-23470-R |
| p-IkB- α (Ser32/36) | 1:200 | Rabbit | Santa Cruz Biotechnology | sc-101713 |
| BrdU | 1:200 | Rat | AbD Serotec, Oxford, UK | MCA2060 |

| | | | | |
|---|---|---|---|---|
| Cleaved Caspase-3 (Asp175) | 1:200 | Rabbit | Cell signalling technology, Danvers, MA, USA | #9661 |

Photomicrographs of sections were taken using a digital camera connected to an Olympus BX-51 light microscope at 40x or 100x magnification under constant light intensity. Morphology and number of active microglia stained with IBA1 were assessed by eye under 400x magnification, while for nuclear staining (Cleaved Caspase-3, ARC, and c-FOS) the number of clearly stained immunoreactive cells per area per section were counted via microscope at 100x magnification, and for all other antibodies (except IBA1) image analysis software (ImageJ 1.43, NIH, USA) was used to calculate integrated optical density for photomicrographs of sections of interest. Microphotographs are presented in text with scale bars representing 100µm unless otherwise noted. For each method between 4 and 6 sections of the mid dorso-ventral level of the hippocampus were analysed. Hippocampal sections were assessed across five areas of interest – the dentate gyrus granule cell layer, the dentate gyrus polymorphic cell layer, CA1, CA2 and CA3, with the entire region of interest analysed for each section (Franklin & Paxinos, 1997). Amygdala sections were analysed in an identical manner in the lateral and basolateral nuclei (Franklin & Paxinos, 1997).

*3.2.5 Assessment of the Acute Effects of LPS-induced Sepsis*

10 week old behaviourally naïve mice were treated with either 5mg/kg LPS or 0.9% sterile saline (n=5 per group) at ZT6. 24 hours later these animals were then administered 0.1ml sodium pentobarbital and transcardially perfused as previously described. Sections from these animals were then processed for microglial activation (CD11b, F4/80), astroglial activation (GFAP), as well as IL-6, cleaved caspase-3 and Arc expression.

*3.2.6 Assessment of the Long-lasting Effects of LPS-induced Sepsis*

For all experiments animals were treated with either 5mg/kg LPS or 0.9% sterile saline and allowed to recover for one month. Animals then underwent up to three behavioural tasks and were terminally anaesthetised and perfused no sooner than one week following the cessation of the final task. For the analysis of the effects of LPS induced sepsis at 12 months animals were treated at age 8-12 weeks and subsequently allowed to recover. They then underwent a tail suspension test 12 months later and one week subsequent to this they were terminally anaesthetised and transcardially perfused. All perfusions were carried out at ZT6, and brains were then analysed via immunohistochemistry for microglial (CD11b, F4/80, IBA1) and astroglial (GFAP) activation, IEG expression (Egr-1, Arc, c-Fos), cytokine levels (TNF-α, IL-1β, IL-6), as well as iNOS levels, IKK and IκB expression. Table 5 details which behavioral cohort (as described in chapter 2) mice had been part of prior to perfusion for immunohistochemical analysis.

**Table 5.** *Behavioural cohorts subsequently involved in immunohistochemical analysis.*

| **Cohort** | **Antibodies examined** |
|---|---|
| Cohort A | CD11b |
| Cohort B | F4/80. TNFα, GFAP |
| Cohort C | c-Fos, EGR-1, ARC, iNOS |
| Cohort D | IL-6, IL-1β, p-IKK, p-IκB |
| Cohort E | BrdU |

*3.2.7 Statistical Analysis*

Data for each experiment was collated and analysed with IBM SPSS (version 20). For all experiments independent t-tests were conducted with p values of $< 0.05$

accepted as significant. All data are presented as means ± standard error of the mean (SEM). * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$ in independent t-tests. Scale bars on microphotographs represent 100µm for photos at 40x and 100x magnification and 50µm for photos at 400x magnification. Where Levene's assumption of homogeneity of variance was violated p values and degrees of freedom were corrected. Red arrows show staining considered positive for each antibody.

*3.3 Results*

*3.3.1 Acute effects of LPS-induced sepsis in mice*

*3.3.1.1 CD11b*

CD11b is expressed on the surface of microglia. Shown in Figure 3.1, there was a main effect of LPS treatment on CD11b IOD scores between saline (n=3) and LPS (n=3) treated mice in the DgGr (0.32±0.12 vs. 1.56±.0.29), $t_4$=3.938, p=0.017. A significant difference was also present in the DgPo (0.80±0.19 vs. 3.63±0.24), $t_4$=9.189, p=0.001. In the CA1 region there was again a significant difference (0.29±0.04 vs. 2.17±0.26), $t_{2.08}$=6.738, p=0.019. There was also a significant difference in the CA2 region (0.36±0.07 vs. 3.24±0.29), $t_4$=9.81, p=0.001. Lastly there was a significant difference between saline and LPS-treated animals in the CA3 region (0.58±0.14 vs. 5.1±0.167), $t_4$=20.931, p<0.0001.

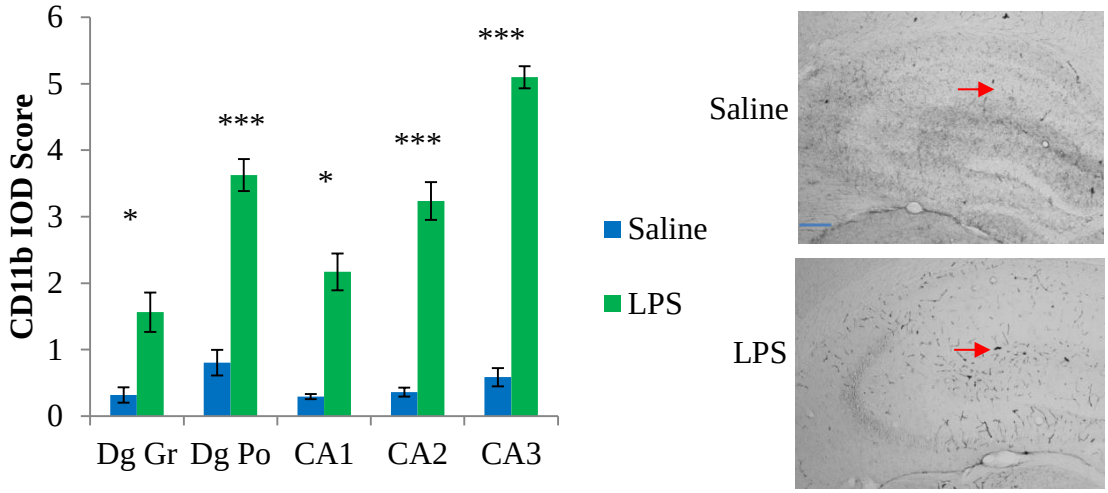



***Figure 3.1 Upregulated CD11b expression 24 hours post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=3) and LPS (n=3) treated mice. */**/*** represents p<0.05/0.01/0.001 respectively in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating CD11b expression in the hippocampus at 40x magnification.*

*3.3.1.2 F4/80*

There was an upregulation of F4/80 – another microglial and macrogphage marker, across all areas of the hippocampus in response to LPS (n=4) compared to saline (n=4) at 24 hours as shown in Figure 3.2. Microglia notably assumed an amoeboid morphology (red arrows) in response to this high dose of LPS. In the dentate gyrus saline-treated animals had a mean IOD score of 1.35±0.27 while LPS-treated animals had a mean score of 11.67±1.95, $t_{3.12}$ = 5.24, p = 0.012. In the DgPo saline-treated animals had a mean score of 2.12±0.32 while LPS-treated animals had a mean score of 23.99±2.4, $t_{3.1}$=9.03, p=0.003. In the CA1 region saline-treated animals had a mean IOD score of 0.94±0.26 while LPS animals had a mean score of 9.93±1.58, $t_{3.16}$=5.61, p=0.012. In the CA2 region saline-treated animals had a mean score of 1.52±0.33 while LPS-treated animals had a mean score of 10.81±1.79, $t_6$=5.11, p=0.002. In the CA3 region saline animals had a mean score of 1.4±0.33 vs. 15.66±0.88 for LPS-treated mice ($t_6$=15.24, p<0.0005).

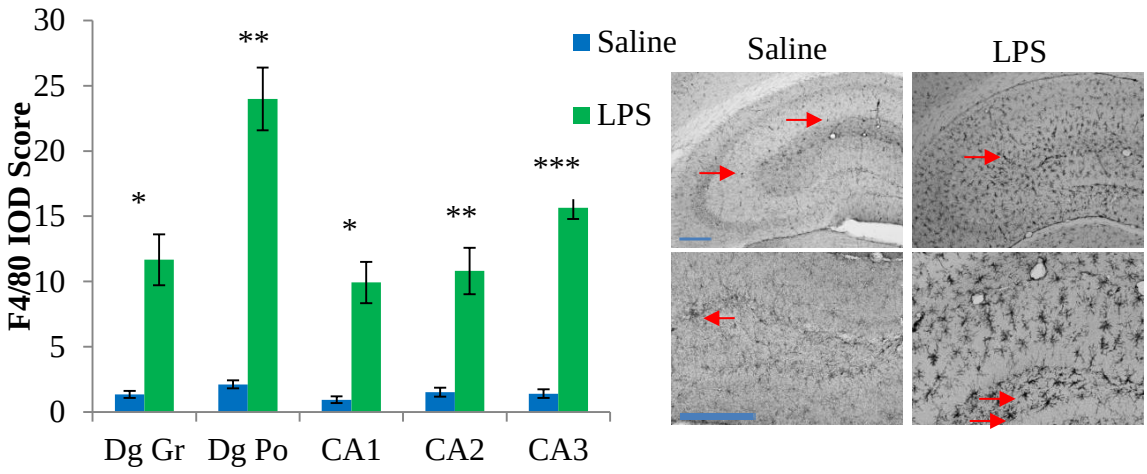



***Figure 3.2 Upregulated F4/80 expression 24 hours post-sepsis*** *Bar graph illustrating mean IOD values for each area of the hippocampus in saline (n=4) and LPS (n=4) treated animals with */**/*** representing p<0.05/0.01/0.001 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating F4/80 expression in the hippocampus at 40x and 100x magnification.*

*3.3.1.3 GFAP*

There was no changes to astrocytes as measured by GFAP IOD in any area of the hippocampus of LPS-treated mice (n=4) in comparison to saline-treated mice (n=4) as shown in Figure 3.3. In the DgGr saline-treated animals had a mean IOD score of 3.51±0.37 while LPS-treated animals had a mean score of 2.81±0.91, $t_6$=0.71, p=0.507. In the DgPo there was a mean score of 3.15±0.28 for saline-treated animals and 5.07±0.99 for LSP treated mice, $t_6$=1.87, p=0.111. In the CA1 region saline-treated animals had a score of 2.13±0.11 while LPS-treated mice had a mean score of 1.73±0.52, $t_{3.268}$=0.76, p=0.477. In the CA2 region saline-treated mice had an IOD score of 5.34±0.51 while LPS-treated mice had a mean score of 5.05±0.91, $t_6$=0.28, p=0.792. In the CA3 region saline-treated mice had a mean score of 5.11±0.84 while LPS-treated mice had a mean score of 7.19±1.45, $t_6$=1.38, p=0.218.

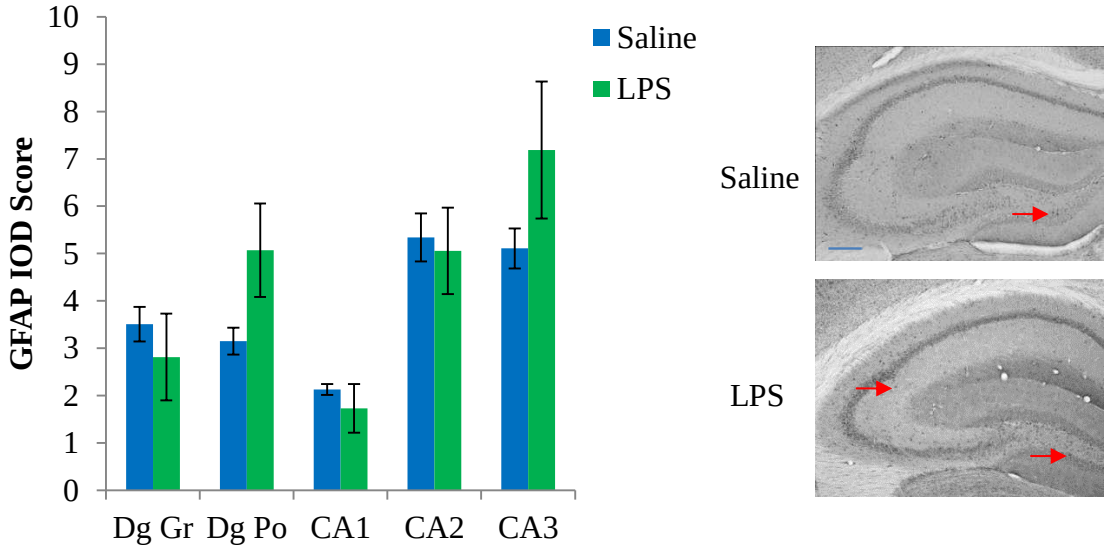



***Figure 3.3 Unaltered GFAP expression 24 hours post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for saline (n=4) and LPS (n=3) treated animals. * represents p<0.05, error bars represent ± 1 SEM. Representative photomicrographs illustrating GFAP expression in the hippocampus at 40x magnification.*

*3.3.1.4 IL6*

When expression of the pro-inflammatory cytokine IL-6 was measured 24 hours post-treatment a significant difference was seen only in the DgGr. As seen in Figure 3.4, in the DgGr saline-treated animals (n=5) had a mean IOD score of 0.45±0.13 while LPS-treated animals (n=5) had a mean score of 1.04±0.09, $t_8$=3.727, p=0.006. In the DgPo saline-treated animals had a mean IOD score of 0.80±0.25 while LPS-treated animals had a mean score of 1.3±0.22, $t_8$=1.52, p=0.167. In the CA1 region saline animals scored 1.5±0.35 while LPS-treated animals scored 1.27±22, $t_8$=0.55, p=0.596. In the CA2 region saline-treated animals scored 1.86±0.58 while LPS-treated mice scored 1.22±0.18, $t_{4.8}$=1.07, p=0.335. Lastly, in the CA3 region saline-treated mice scored 2.71±0.72 while LPS-treated mice scored 1.63±0.11, $t_{4.19}$=1.47, p=0.212.

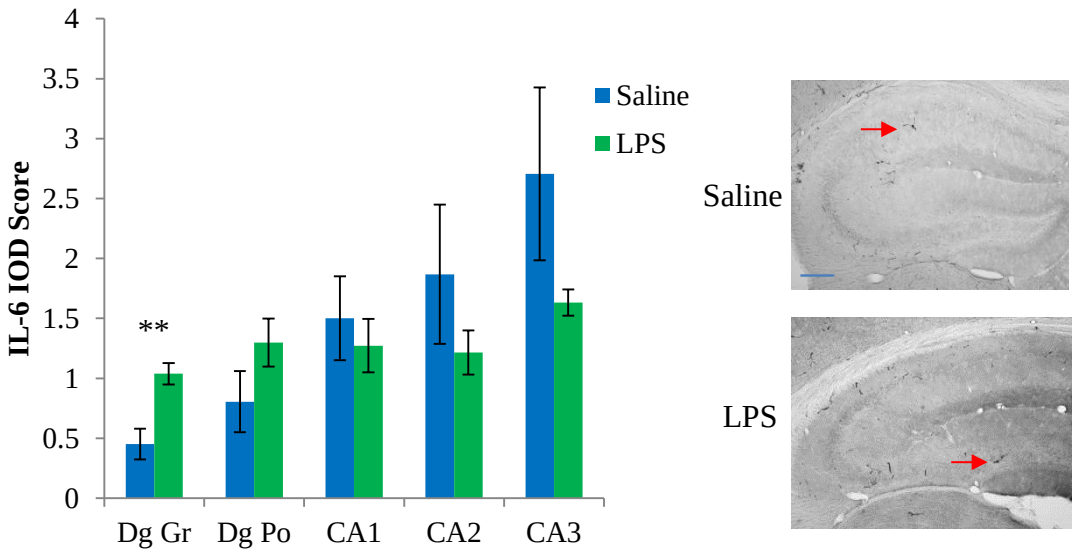



***Figure 3.4 Unaltered IL-6 expression 24 hours post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=5) and LPS(n=5) treated mice with ** representing p<0.01 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating IL6 expression in the hippocampus at 40x magnification.*

*3.3.1.5 Cleaved Caspase-3*

There was a significant upregulation of apoptotic marker cleaved caspase-3 expression in the subgranular zone of the hippocampus 24 hours following LPS administration as can be seen in Figure 3.5. While there was negligible expression of cleaved caspase-3 positive cells in the hippocampus following saline treatment (0.125±0.07, n=5), there was a robust expression of cells positive for cleaved caspase-3 in the LPS-treated animals (10.75±2.33, n=5), $t_{4.007}$=4.561, p=0.01.

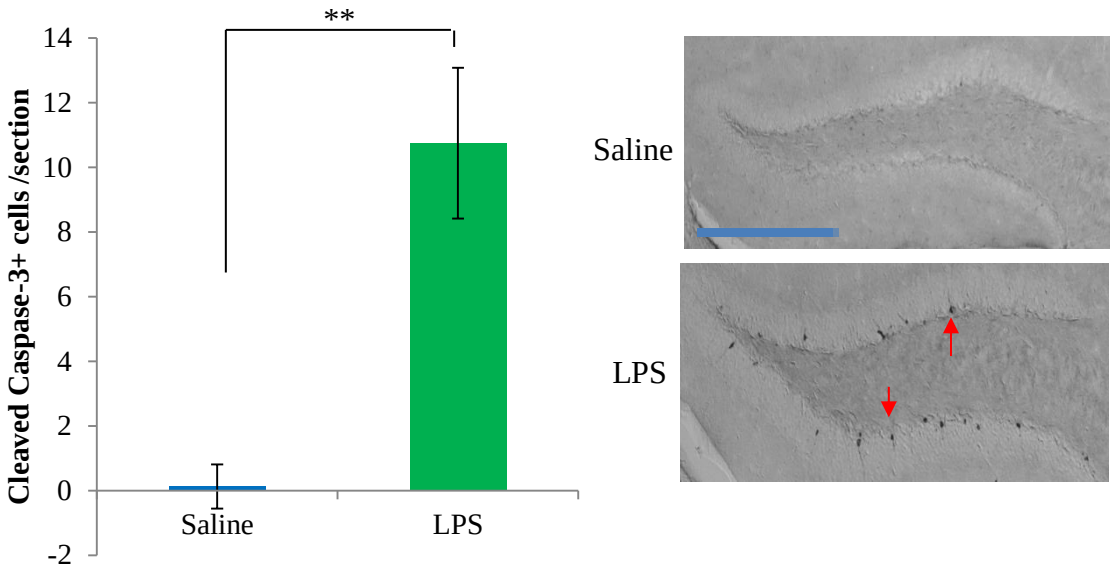



***Figure 3.5 Upregulated cleaved caspase-3 expression 24 hours post-sepsis*** *Cleaved caspase-3 expression in the subgranular zone of the hippocampus at 24 hours post treatment. Bar graph illustrating average number of Cleaved Caspase-3$^+$ cells in the SGZ per hippocampal section in saline (n=5) and LPS (n=5) treated mice with ** representing p<0.01 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating Cleaved Caspase-3 expression in the hippocampus at 100x magnification.*

*3.3.1.6 ARC*

As shown in Figure 3.6, there was a significant reduction in neurons positive for ARC – a key IEG for neuronal plasticity in the dentate gyrus of the hippocampus 24 hours following LPS administration. Saline-treated animals (n=5) had an average of 54.25±3.75 Arc positive cells while LPS-treated animals (n=5) had an average of 23.55±3.054 ARC positive cells in the dentate gyrus $t_8$=6.34, p<0.0005. Arc expression was negligible in hippocampal areas CA1, CA2 and CA3 and is therefore not illustrated.

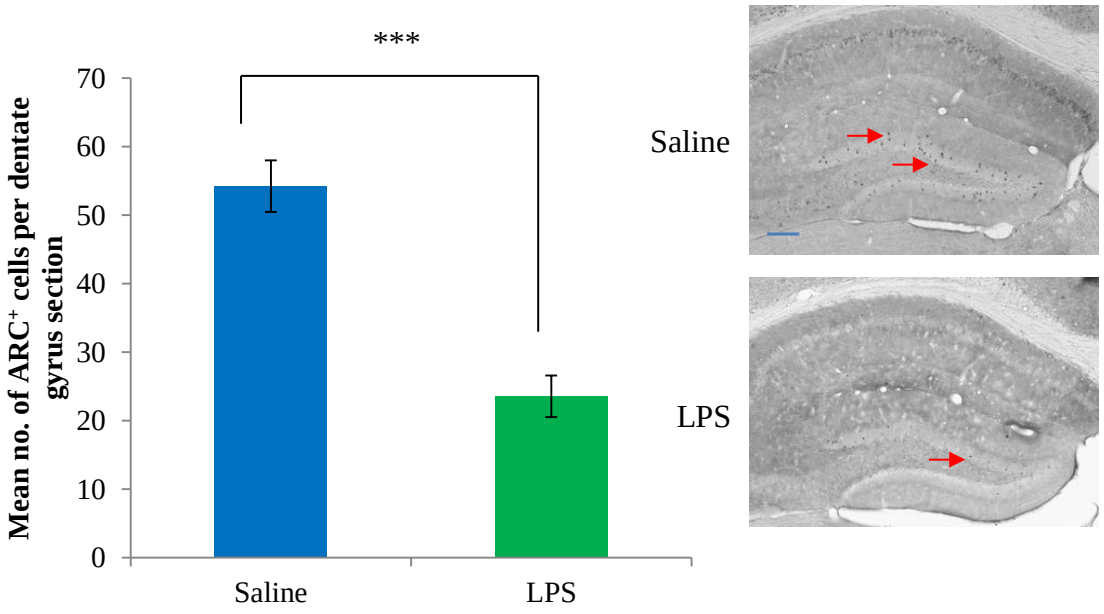



***Figure 3.6 Altered ARC expression 24 hours post-sepsis*** *Bar graph illustrating average ARCc+ cells per dentate gyrus section in both saline (n=5) and LPS (n=5) treated mice. *** represents p<0.001 in an independent t-test, error bars represent ± 1 SEM. Representative photomicrographs illustrating Arc expression in the hippocampus at 40x magnification.*

### *3.3.2 Long-lasting effects of LPS induced sepsis in mice*

#### *3.3.2.1 CD11b*

Microglial activation measured by CD11b indicated an upregulated expression in the DgGr, DgPo, and CA1 of the hippocampus, shown in Figure 3.7. In the DgGr there was a mean IOD score of 1.16±0.23 for saline-treated animals (n=8) and 2.78±0.45 for LPS-treated animals (n=7), $t_{13}$=3.343, p=0.005. In the DgPo saline-treated animals had a mean score of 3.68±0.8 while LPS-treated animals had a mean score of 6.98±0.63, $t_{13}$=3.156, p=0.008. In the CA1 region saline-treated animals had a mean score of 1.56±0.38 while LPS-treated animals had a mean score of 3.46±0.7, $t_{13}$=2.467, p=0.028. There was no difference between saline-treated and LPS-treated animals in the CA2 region (1.76±0.38 vs. 2.37±0.39, $t_{13}$=1.12, p=0.283) or the CA3 (2.67±0.51 vs. 2.47±0.35, $t_{13}$=0.314, p=0.759).

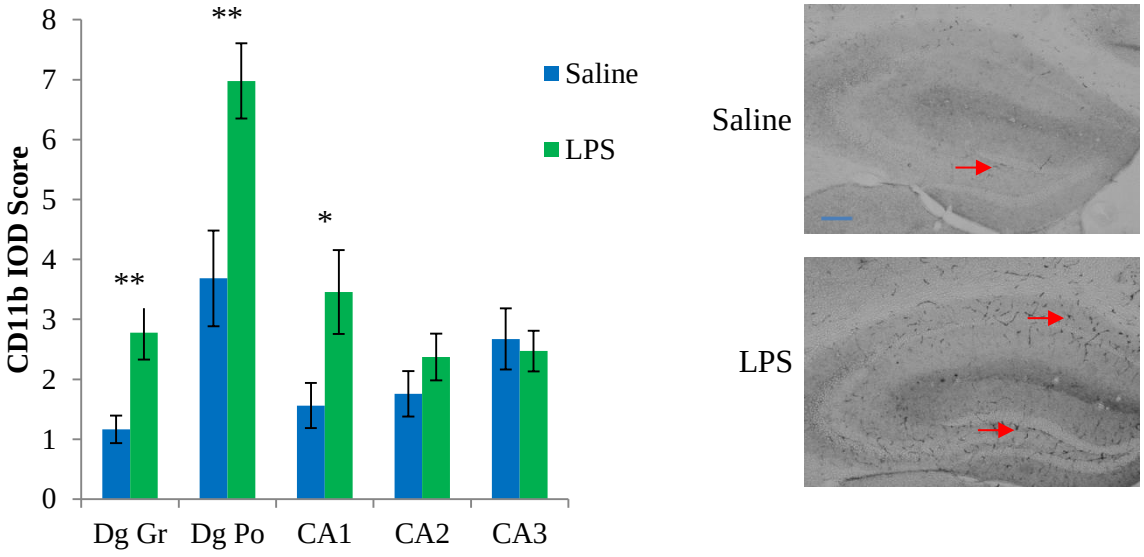



***Figure 3.7 Upregulated CD11b expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=8) and LPS (n=7) treated mice with */** representing p<0.05/0.01 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating CD11b expression in the hippocampus at 40x magnification.*

*3.3.2.2 F4/80*

F4/80 was upregulated throughout the hippocampus 2 months following LPS administration, as can beseen in Figure 3.8. In the DgGr saline-treated animals (n=8) had a mean IOD score of 1.7±0.21 while LPS-treated animals (n=7) had a mean score of 3.82±0.56, $t_{7.76}$=3.55, p=0.008. In the DgPo saline-treated animals had a mean score of 2.57±0.29 while LPS-treated animals had a mean score of 6.51±1.08, $t_{6.89}$=3.52, p=0.002. In the CA1 region saline-treated mice had a mean score of 1.22±0.17 while LPS-treated animals had a mean score of 3.41±0.49, $t_{7.48}$=7.48, p=0.003. In the CA2 region saline-treated animals had a mean score of 2.23±0.25 while LPS-treated animals had a mean score of 5.87±0.75, $t_{7.39}$=7.39, p=0.002. Lastly, in the CA3 region saline-treated mice had a mean score of 1.91±0.19 while LPS-treated animals had a mean score of 4.37±0.72, $t_{6.82}$=3.28, p=0.014.

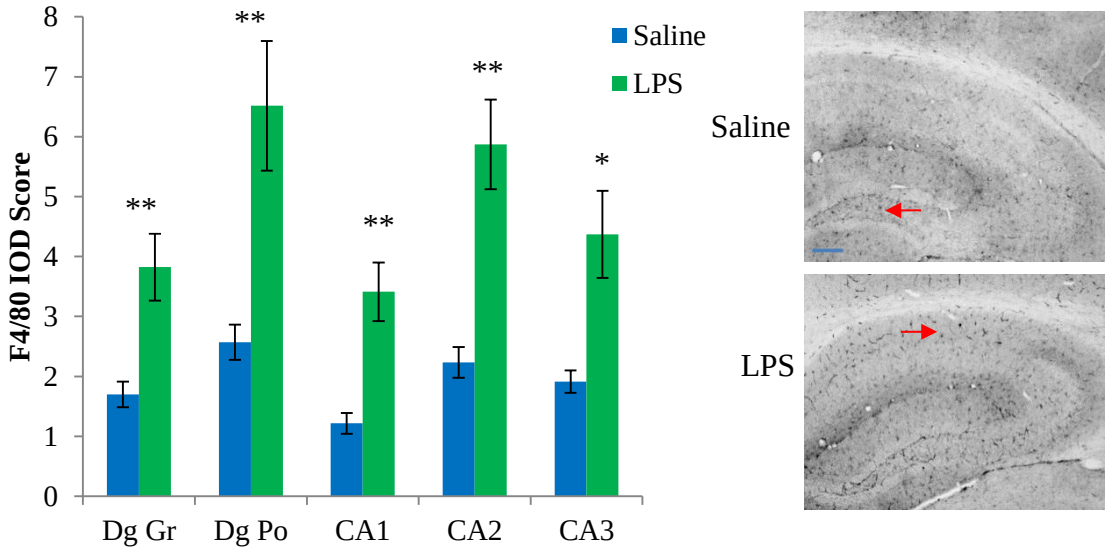



***Figure 3.8 Upregulated F4/80 expression 2 months post-sepsis.*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=7) and LPS (n=8) treated mice. */** represent p<0.05/0.01 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating F4/80 expression in the hippocampus at 40x magnification.*

*3.3.2.3 IBA1*

When microglia in the hippocampus were morphologically examined using IBA1, which is expressed on the surface of all microglia, an increased number of activated microglia (characterised by withdrawn processes and/or enlarged cell bodies, as shown in Figure 3.9) were found in sepsis-survivor animals (n=4) than controls (n=4). In the DgGr saline-treated animals had an average of 6.3±0.52 activated microglia while LPS-treated animals had an average of 10.01±0.64, $t_6$=4.49, p=0.004. In the DgPo saline-treated animals had an average of 5.01±0.64 activated microglia while LPS-treated animals had an average of 7.74±0.69, $t_6$=2.9, p = 0.027. In the CA1 region LPS-treated animals also had a larger number of activated microglia (10.92±1.54 vs. 20.26±2.94), $t_6$=2.81, p=0.031. In the CA2 region saline-treated mice had an average of 0.85±0.05 activated microglia while LPS-treated animals had an average of 1.48±0.29, $t_6$=2.1, p=0.081. In the CA3 region saline-treated animals had an average of 8.26±0.95 activated microglia while LPS-treated animals had 12.75±1.3, $t_6$=2.8, p=0.031.

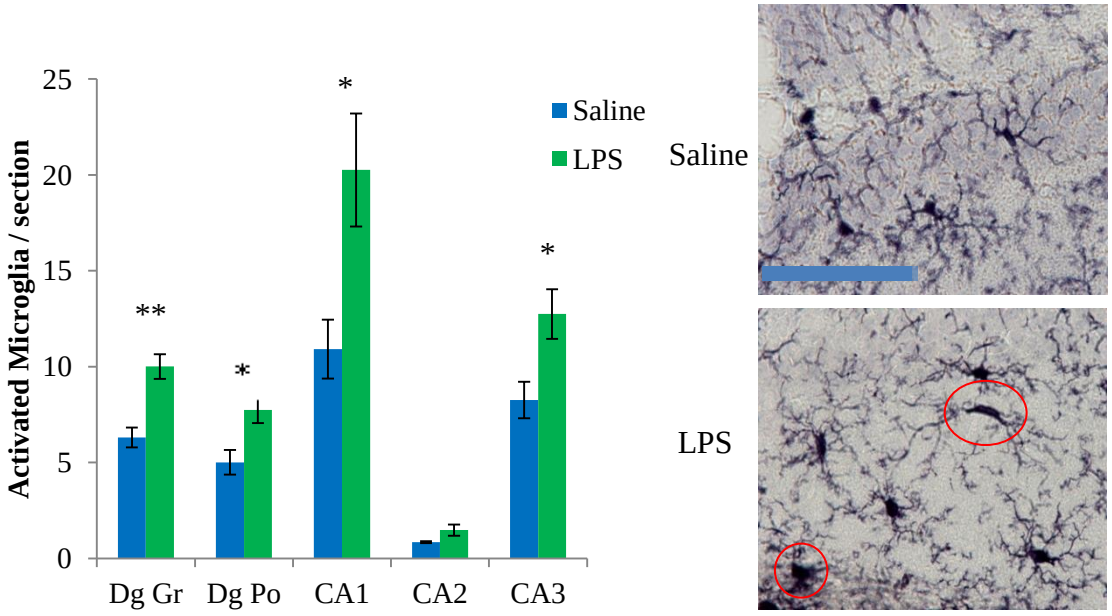



***Figure 3.9 Upregulation of activated microglia 2 months post-sepsis*** *Bar graph illustrating cell counts values for each area of the hippocampus of saline (n=4) and LPS (n=4) treated mice. */** represent p<0.05/0.01 in independent t-tests, error bars show ± 1 SEM. Photomicrographs illustrating IBA1 expression in the hippocampus at 400x magnification, scale bar represents 50µm.Red circles denote activated microglia.*

*3.3.2.4 GFAP*

As illustrated by Figure 3.10, there was no significant difference in GFAP levels – expressed on the surface of astrocytes, between saline (n=4) and LPS (n=4) treated animals after two months. In the DgGr saline-treated animals had mean IOD scores of 5.32±1.69 while LPS-treated animals had scores of 4.53±0.58, $t_{3.71}$=0.44, p=0.67. In the DgPo saline-treated animals had a mean score of 2.17±0.39 while LPS-treated animals had a mean score of 2.92±0.23, $t_6$=1.66, p = 0.148. In the CA1 region saline-treated animals had a mean score of 1.5±0.37 while LPS-treated animals had a mean score of 2.08±0.55, $t_6$=0.88, p=0.411. In the CA2 region saline-treated animals had a mean score of 2.83±0.65 while LPS-treated animals had a mean score of 4.56±0.43, $t_6$=2.2 p=0.07. In the CA3 region saline-treated animals had a mean score of 7±1.69 while LPS-treated animals had a mean score of 9.30±0.77, $t_6$=1.24, p=0.263.

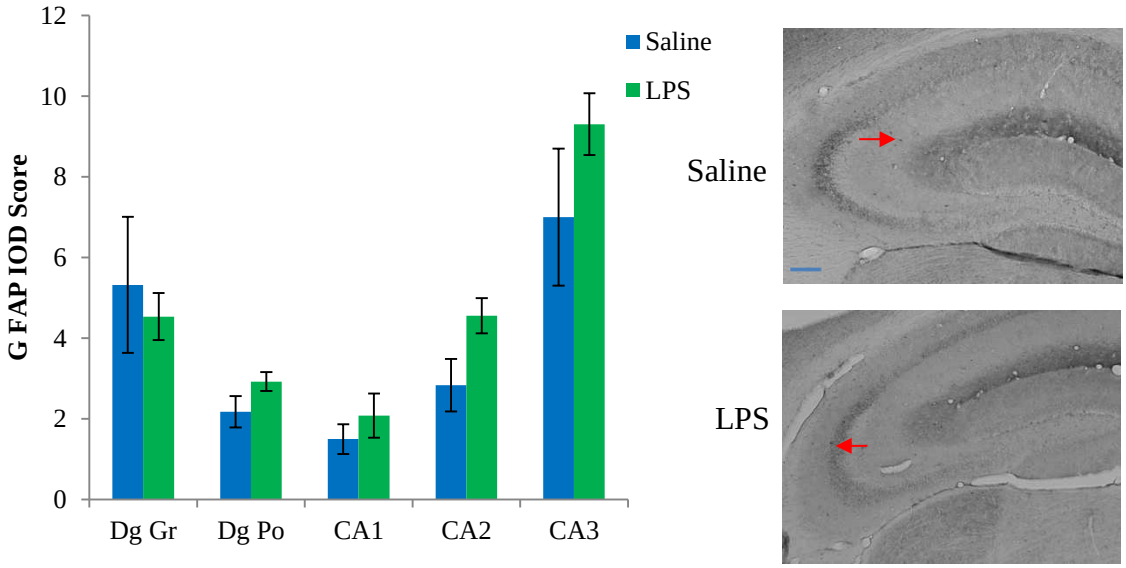



***Figure 3.10 Unaltered GFAP expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=4) and LPS (n=4) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating GFAP expression in the hippocampus at 40x magnification.*

*3.3.2.5 TNFα*

As Figure 3.11 shows, there were no significant differences between groups in the expression of proinflammatory cytokine TNFα at two months post-treatment. In the DgGr saline-treated animals (n=4) had a mean IOD score of 0.57±0.13 while LPS-treated animals (n=4) had a mean score of 1±0.16, $t_6$=2.12, p=0.078. In the DgPo saline-treated animals had a mean score of 1.82±0.41 while LPS-treated animals had a mean score of 2.91±0.19, $t_6$=2.41, p=0.052. In the CA1 region saline-treated animals had a mean score of 0.98±0.23 while LPS-treated animals had a mean score of 1.46±0.43, $t_6$=0.98, p=0.36. In the CA2 region saline-treated animals had a mean score of 0.72±0.25 while LPS-treated animals had a mean score of 0.55±0.14, $t_6$=0.6, p=0.573. In the CA3 region saline-treated animals had a mean score of 0.68±0.25 while LPS-treated animals had a mean score of 0.66±0.28, $t_6$=0.28, p=0.943.

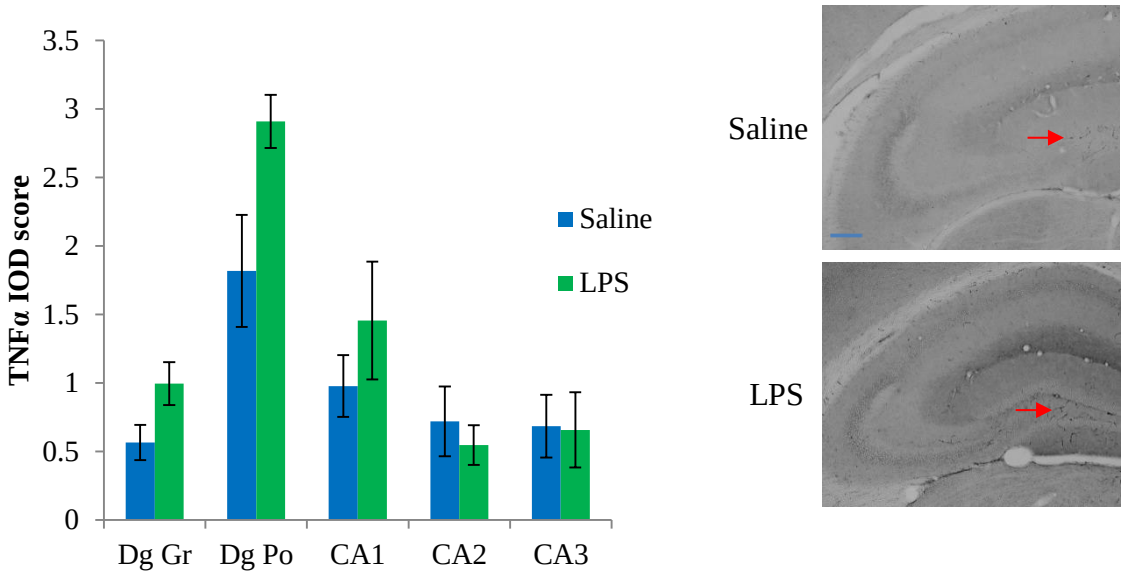



***Figure 3.11 Unaltered TNFα expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for saline (n=4) and LPS (n=4) treated mice with * representing p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating TNFα expression in the hippocampus at 40x magnification.*

*3.3.2.6 IL-1β*

Levels of the pro-inflammatory cytokine IL-1β were measured in the hippocampus 2 months post treatment with a significant difference between saline (n=4) and LPS (n=4) treated animals present only in the CA3 region area, illustrate in Figure 3.12. In the DgGr saline-treated animals had a mean IOD score of 69±10.66 while LPS-treated animals had a mean score of 88.81±6.84, $t_6$=1.52, p=0.18. In the DgPo there was no significant difference between saline and LPS-treated animals (13.29±2.22 vs. 17.97±1.59), $t_6$=1.71, p=0.132. In the CA1 region saline-treated mice had a mean score of 17.62±2.43 while LPS-treated mice had a mean score of 19.42±1.52, $t_6$=0.63, p=0.55. In the CA2 region saline-treated animals had a mean score of 18.59±1.63 while LPS-treated animals had a mean score of 23.89±1.74, $t_6$=2.23, p=0.068. In the CA3 region saline-treated animals had a mean score of 21.52±1.76 while LPS-treated animals had a mean score of 27.58±1.35, $t_6$=2.73, p=0.034.

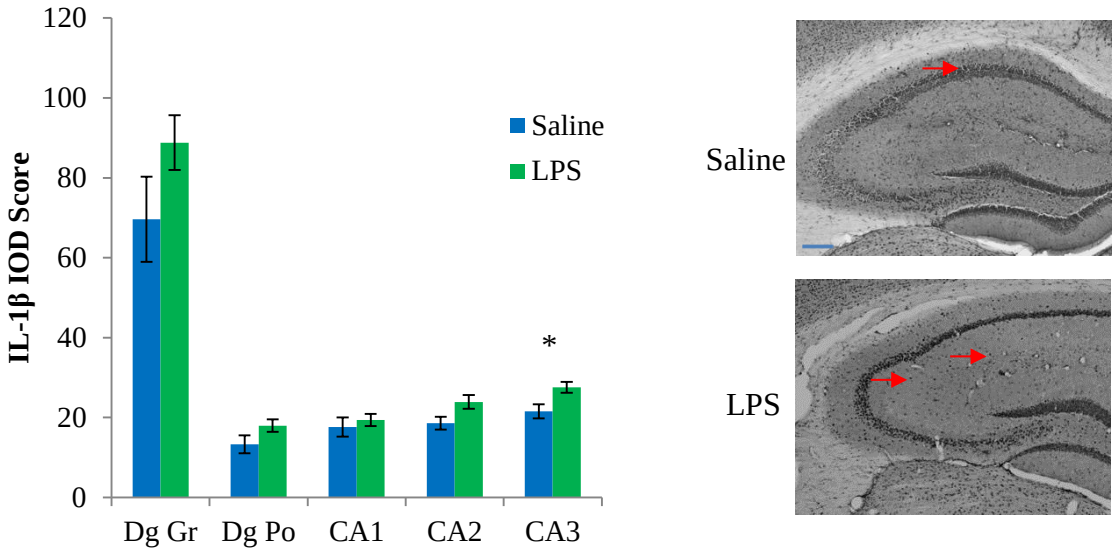



***Figure 3.12 IL-1β expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for saline (n=4) and LPS (n=4) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating IL-1β expression in the hippocampus at 40x magnification.*

*3.3.2.7 IL-6*

When expression of pro-inflammatory cytokine IL-6 was measured via immunohistochemistry there were no significant differences between saline (n=4) and LPS (n=4) treated animals in any area of the hippocampus, illustrated in Figure 3.13. In the DgGr saline-treated animals had a mean score of 1.15±0.17 while LPS-treated animals had a mean score of 1.09±0.277, $t_6$=0.2, p=0.849. In the DgPo saline-treated animals had a mean score of 0.8±0.13 while LPS-treated animals had a mean score of 0.70±0.26, $t_6$=0.35, p=0.736. In the CA1 region saline-treated animals had a mean score of 1.47±0.31 while LPS-treated animals had a mean score of 1.19±0.17, $t_6$=0.8, p=0.455. In the CA2 region saline-treated mice had a mean score of 1.55±0.34 while LPS-treated mice had a mean score of 1.69±0.28, $t_6$=0.31, p=0.769. In the CA3 region saline-treated mice had a mean score of 1.71±0.28 while LPS-treated mice had a mean score of 1.32±0.31, $t_6$=0.91, p=0.396.

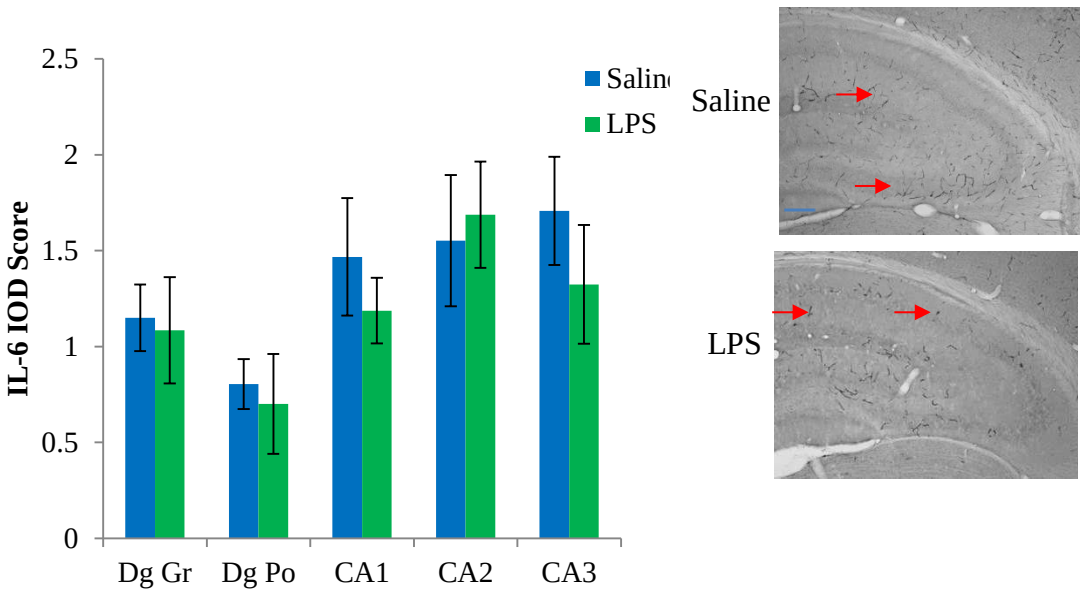



***Figure 3.13 Unaltered IL-6 expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=4) and LPS (n=4) treated mice. * represents p<0.05, error bars represent ± 1 SEM. Photomicrographs illustrating IL-6 expression in the hippocampus at 40x magnification.*

*3.3.2.8 p-IKK*

Analysis of p-IKK levels, an upstream component of the NF-κB pathway, revealed no differences between saline (n=4) and LPS (n=3) treated animals in any area of the hippocampus as can be seen in Figure 3.14. In the DgGr saline-treated animals had a mean score of 2.97±0.22 while LPS-treated animals had a mean score of 1.99±0.42, $t_5$=2.24, p=0.076. In the DgPo saline-treated animals had a mean score of 4.8±1.26 while LPS-treated animals had a mean score of 2.79±01.23, $t_5$=1.34, p=0.237. In the CA1 region saline-treated animals had a mean score of 1.89±0.23 while LPS-treated animals had a mean score of 1.78±0.81, $t_{2.34}$=0.12, p=0.911. In the CA2 region saline-treated animals had a mean score of 5.3±1.17 while LPS-treated animals had a mean score of 4.5±1.05, $t_5$=0.48, p=0.649. In the CA3 region saline-treated animals had a mean score of 7.7±1.38 while LPS-treated animals had a mean score of 6.39±1.31, $t_{4.47}$=0.834, p=0.447.

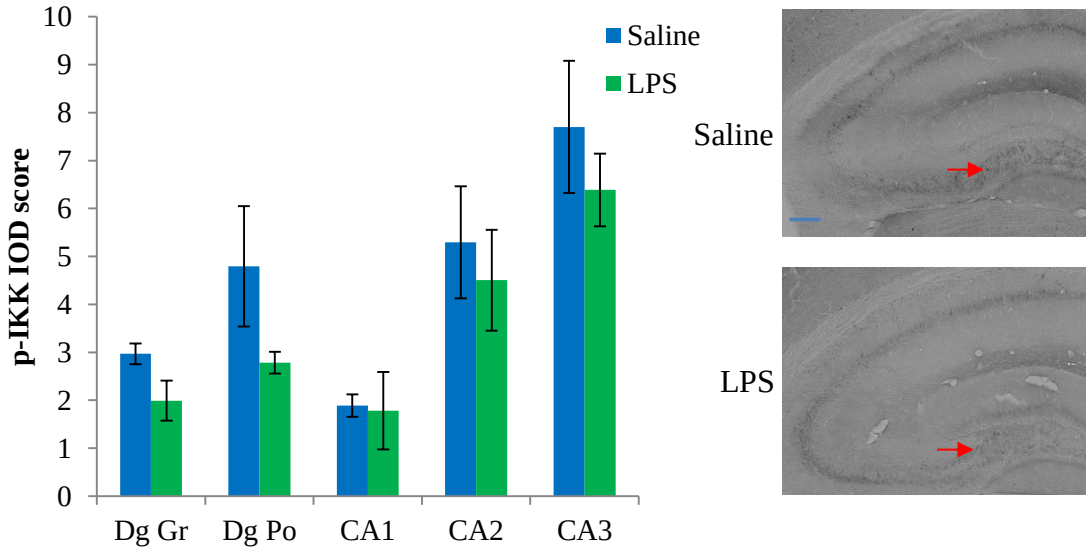



***Figure 3.14 Unaltered p-IKK expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for salinie (n=4) and LPS (n=3) treated animals. * represents p< 0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating p-IKK expression in the hippocampus at 40x magnification.*

*3.3.2.9 p-IκB*

There were no differences in p-IκB levels, which are elevated during NF-κB activation, between saline (n=4) and LPS (n=4) treated animals in the hippocampus at 2 months post-treatment, illustrated in Figure 3.15. In the DgGr saline-treated animals had a mean IOD score of 15.6±1.26 while LPS-treated animals 15.42±1.16, $t_6$=0.1, p=0.921. In the DgPo saline-treated animals had a mean score of 6.37±1.32 while LPS-treated animals had a mean score of 7.07±0.63, $t_{4.283}$=0.48, p=0.655. In the CA1 region region saline-treated animals had a mean score of 10.21±1.79 while LPS-treated animals had a mean score of 11.87±1.6, $t_6$=0.78, p=0.46. In the CA2 region region saline-treated animals had a mean score of 13.86±1 while LPS-treated animals had a mean score of 15.67±2.08, $t_{4.32}$=047, p=0.474. In the CA3 region saline-treated animals had a mean score of 17.73±0.89 while LPS-treated animals had a mean score of 19.75±1.2, $t_6$=1.35, p=0.226.

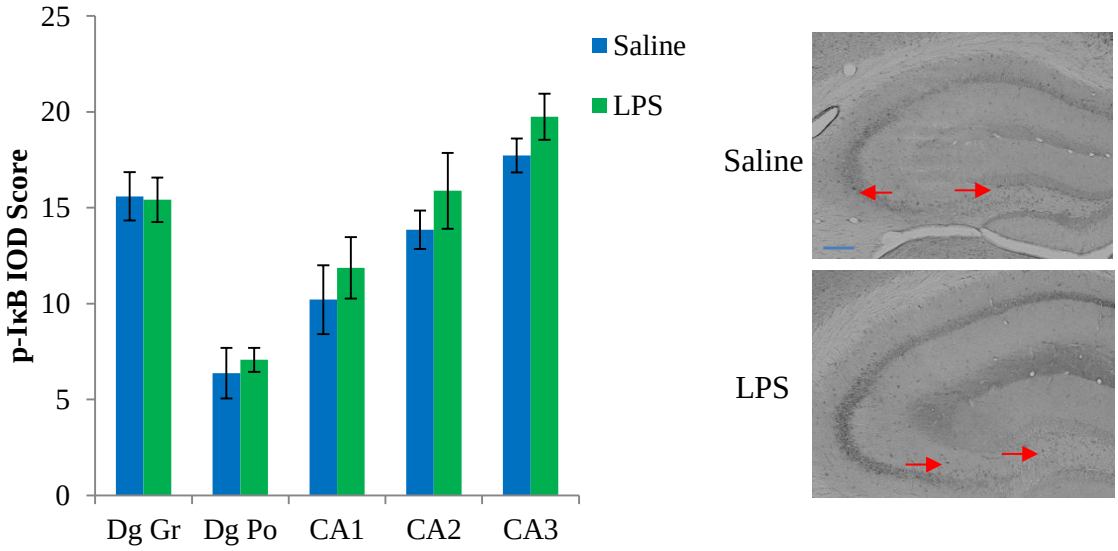



***Figure 3.15 Unaltered p-IκB expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for saline (n=4) and LPS (n=4) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating p-IκB expression in the hippocampus at 40x magnification.*

*3.3.2.10 iNOS*

Immunohistochemical analysis revealed no significant differences between saline (n=4) and LPS (n=3) treated animals in inducible nitric oxide synthase levels in any area of the hippocampus, as shown in Figure 3.16. In the DgGr saline-treated animals had a mean IOD score of 0.15±0.07 while LPS-treated animals had a mean IOD score of 0.11±0.03, $t_5$=0.49, p=0.646. In the DgPo saline-treated animals had a mean score of 0.29±0.04 while LPS-treated animals had a mean score of 0.38±0.1, $t_6$=0.95, p=0.38. In the CA1 region saline-treated animals had a mean IOD score of 0.76±0.1 while LPS-treated animals had a mean score of 0.83±0.21, $t_5$=0.29, p=0.78. In the CA2 region saline-treated animals had a mean score of 3.62±0.67 while LPS-treated animals had a mean score of 5.27±1.54, $t_5$=1.09, p=0.325. In the CA3 region saline-treated animals had a mean score of 0.93±0.23 while LPS-treated animals had a mean score of 1.58±0.17, $t_5$=2.15, p=0.084.

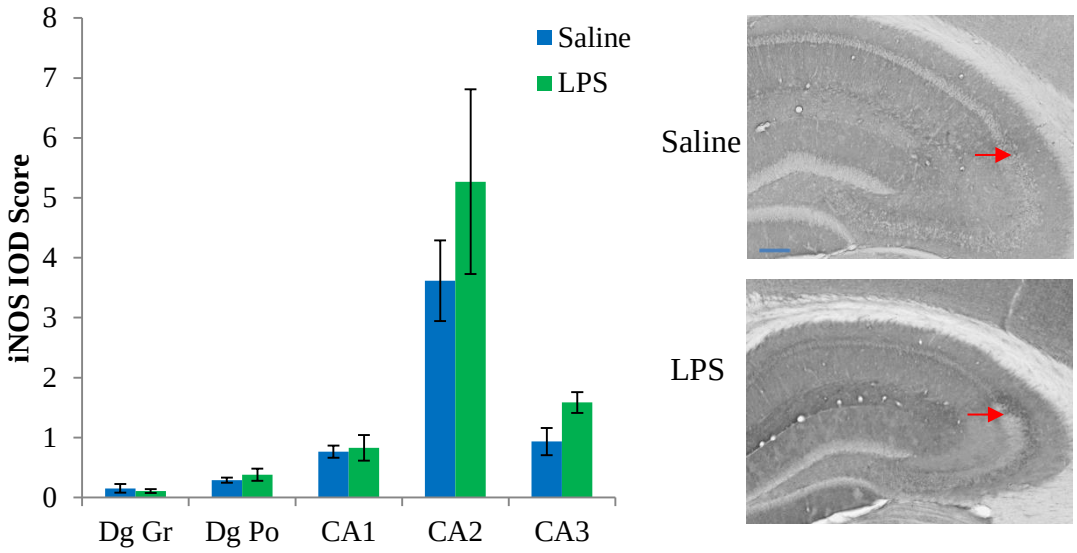



***Figure 3.16 Unaltered iNOS expression 2 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus for saline (n=4) and LPS (n=3) treated mice. * represents p< 0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating iNOS expression in the hippocampus at 40x magnification.*

*3.3.2.11 BrdU*

In order to examine whether or not any alterations in cell proliferation were present in post-septic animals BrdU positive cells were counted in the subgranular zone of the hippocampus 2 months post-treatment. As can be seen in Figure 3.17 cell proliferation appeared reduced in post-septic mice. In saline-treated animals (n=5) there were an average of 13.24±0.79 $BrdU^+$ cells while in the LPS-treated animals (n=6) there was an average of 7.99±1.15 $BrdU^+$ cells per hippocampal section, $t_9$=3.61, p=0.006.

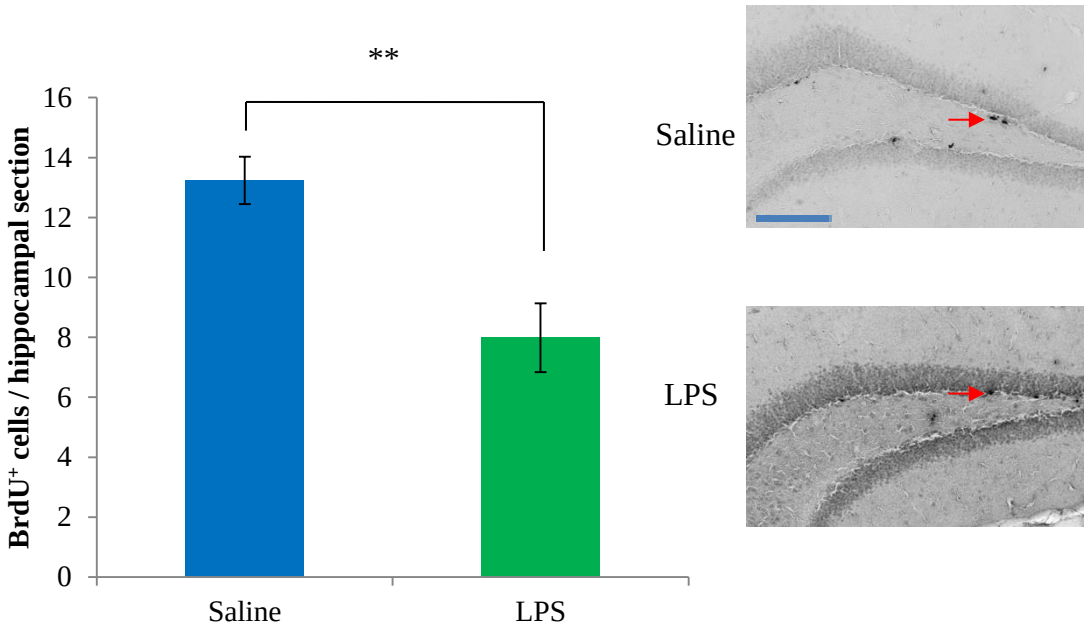



***Figure 3.17 Reduced BrdU incorporation 2 months post-sepsis*** *Bar graph illustrating average number of $BrdU^+$ cells per hippocampal in both saline (n=5) and LPS (n=6) treated animals. ** represents p<0.01 in an independent t-test, error bars represent ± 1 SEM. Representative photomicrographs illustrating BrdU expression in the hippocampus at 100x magnification.*

*3.3.2.12 c-Fos*

Immunohistochemical analysis of neurons positive for activation marker c-Fos in the hippocampus revealed no significant differences between saline (n=8) and LPS-treated animals (n=8) 2 months after treatment, illustrated in Figure 3.18. In the DgGr saline-treated animals had an average of 36.32±3.29 positive cells while LPS-treated animals had an average of 30.79±4.9, $t_{14}$=0.936, p=0.365. In the DgPo saline-treated animals had an average of 1.46±0.45 c-Fos positive cells while LPS-treated animals had an average of 0.87±0.4, $t_{14}$=0.97, p=0.348. In the CA1 region saline-treated animals had an average of 8.44±2.35 c-Fos positive cells while LPS-treated animals had an average of 6.92±1.72, $t_{14}$=0.52, p=0.61. In the CA2 region region saline-treated animals had an average of 1.59±0.47 c-Fos positive cells while LPS-treated animals had an average of 0.94±0.26, $t_{14}$=1.216, p=0.244. In the CA3 region saline-treated animals had an average of 11.54±3.88 c-Fos positive cells while LPS-treated animals had an average of 11.45±3.06, $t_{14}$=0.02, p=0.984.

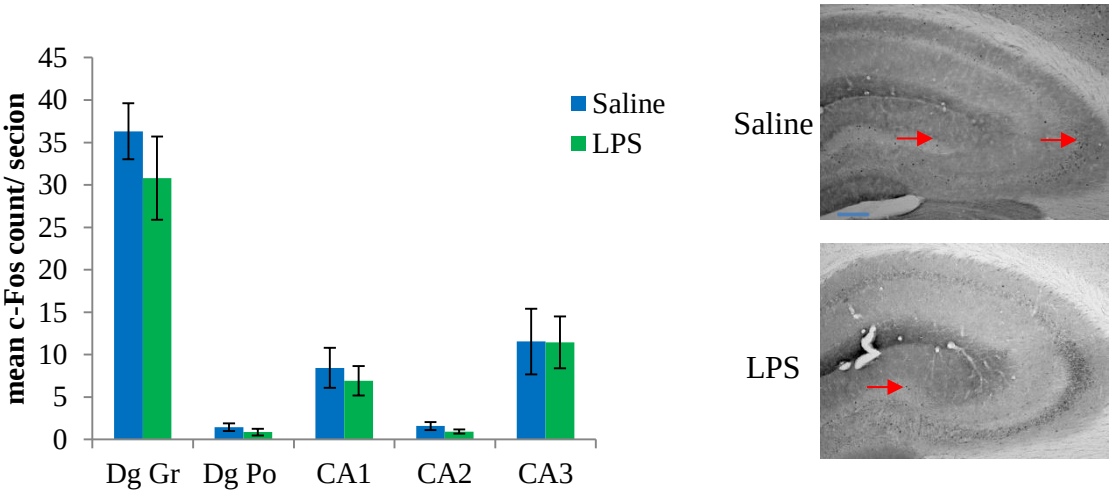



***Figure 3.18 Unaltered c-Fos expression 2 months post-sepsis*** *Bar graph illustrating average number of c-Fos$^+$ cells per hippocampal section for each area of the hippocampus in saline (n=8) and LPS (n=8) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating c-Fos expression in the hippocampus at 40x magnification.*

*3.3.2.13 EGR-1*

Analysis of the immediate early gene EGR-1 expression in the hippocampal neurons of sepsis-survivor mice revealed reduced expression in a number of areas 2 months post treatment, shown in Figure 3.19. In the DgGr saline-treated animals (n=4) had a mean IOD score of 3.92±0.53 while LPS-treated animals (n=4) had a mean score of 1.61 ±0.8, $t_6$=4.29, p=0.005. In the DgPo saline-treated animals had a mean IOD score of 0.94±0.21 while LPS-treated animals had a mean score of 0.55±0.04, $t_{3.21}$=1.822, p=0.16. In the CA1 region saline-treated animals had a mean score of 113.43±12.93 while LPS-treated animals had a mean score of 71.47±7.37, $t_6$=2.82, p=0.03. In the CA2 region saline-treated animals had a mean score of 32.97±4.73 while LPS-treated animals had a mean score of 18.28±3.56, $t_6$=2.48, p=0.048. In the CA3 region saline-treated animals had a mean score of 11.26±2.47 while LPS-treated animals had a mean score of 6.56±0.77, $t_6$=1.81, p=0.12.

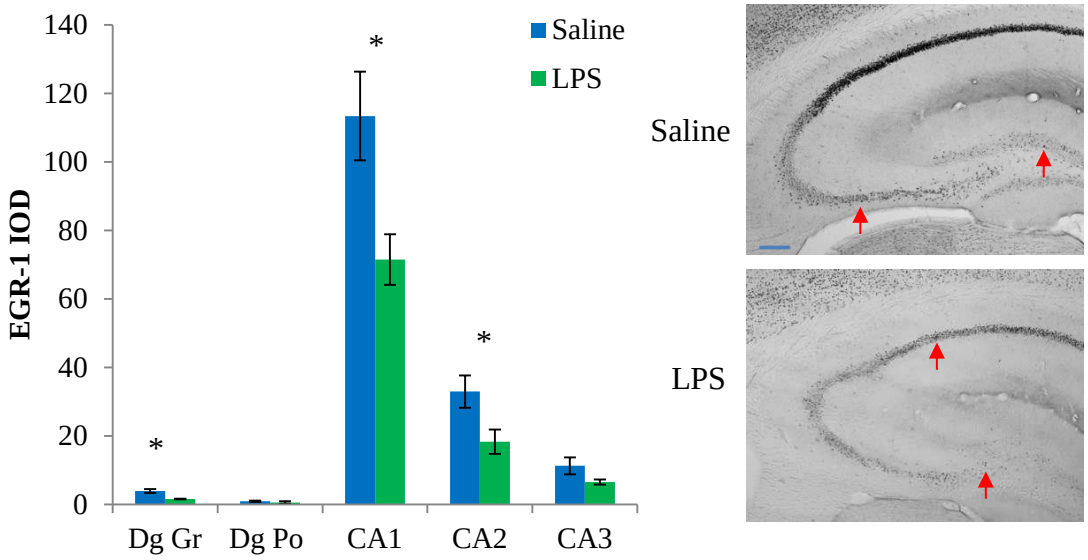



***Figure 3.19 Reduced EGR-1 expression 2 months post-sepsis*** *Bar graph illustrating average number of EGR-1$^+$ cells per hippocampal section for each area of the hippocampus in saline (n=4) and LPS (n=4) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating EGR-1 expression in the hippocampus at 40x magnification.*

*3.3.2.14 ARC*

ARC expression was measured in the dentate gyrus of post septic (n=3) and saline-treated animals (n=4). It was found that there were significantly more $Arc^+$ cells in the dentate gyrus of control animals (25.14±4.47) than sepsis-survivor mice (9.6±1.7), $t_5$=2.84, p=0.036. This is illustrated in Figure 3.20.

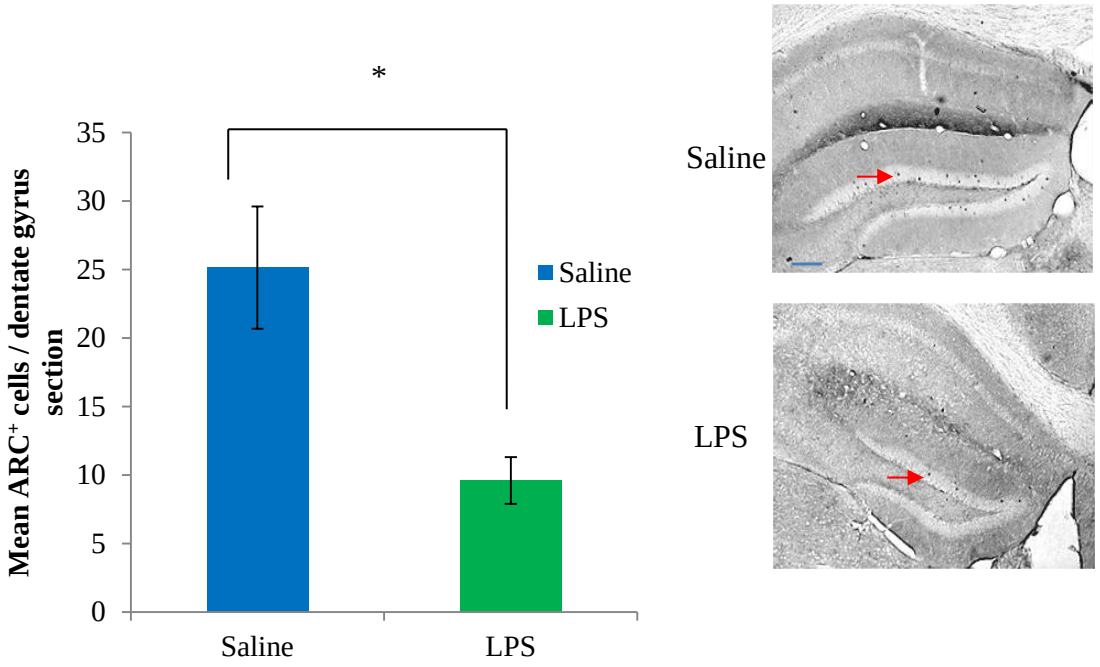



***Figure 3.20 Reduced ARC expression 2 months post-sepsis*** *Bar graph illustrating average number of $ARC^+$ cells per dentate gyrus section in saline (n=4) and LPS (n=3) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating Arc expression in the hippocampus at 40x magnification.*

*3.3.2.15 Amygdala c-Fos*

Analysis of c-Fos in neurons of the lateral and basolateral amygdala was also conducted. Here it was found that post-septic animals (n=8) had significantly lower levels of c-Fos protein product than controls (n=7) in both the lateral ($t_{13}$=2.275, p=0.041) and basolateral regions of ($t_{13}$=2.624, p=0.021). These changes are shown in Figure 3.21.

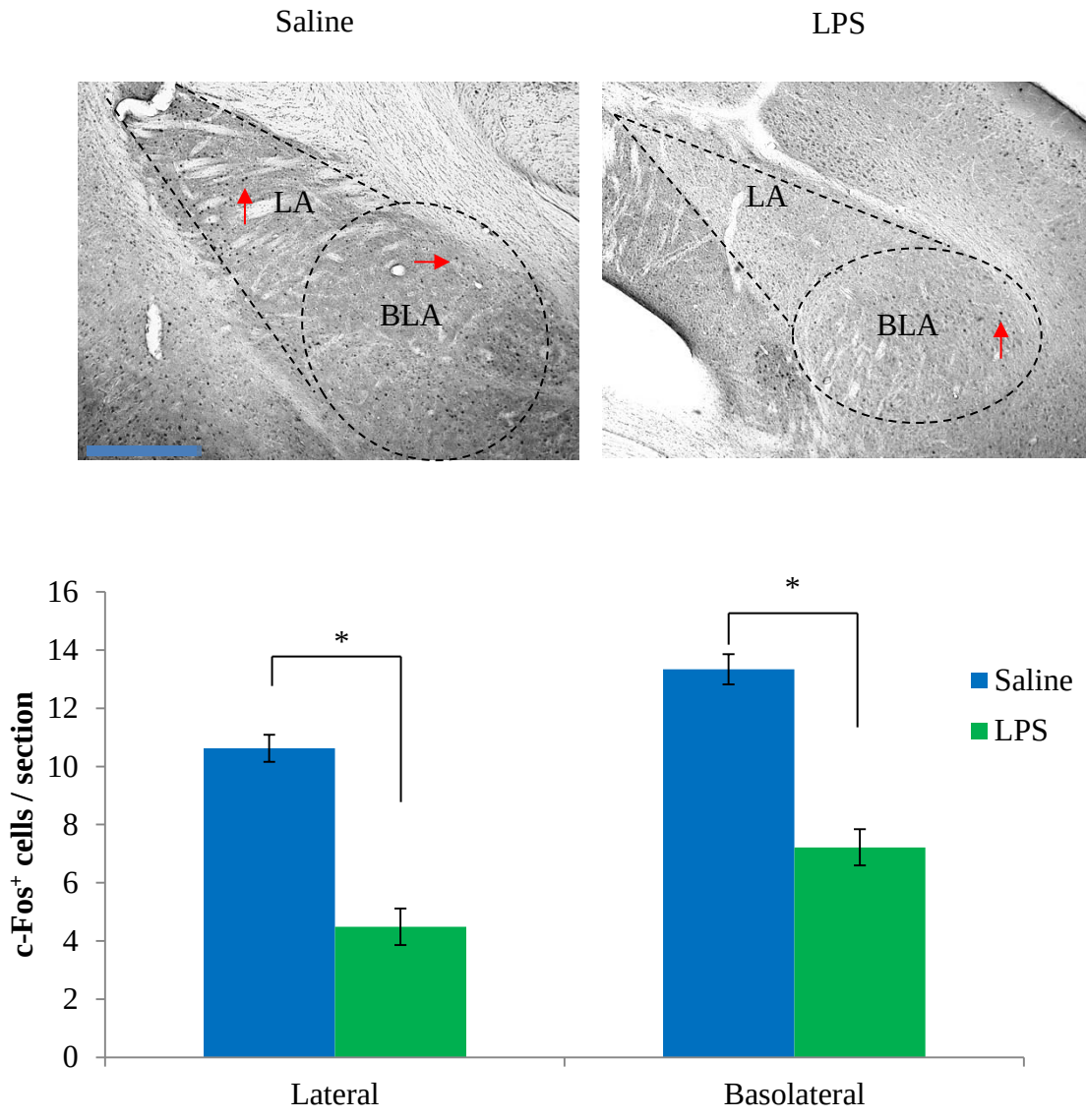



***Figure 3.21 Decreased Amygdala c-Fos expression 2 months post-sepsis*** *Bar graph illustrating average number of c-Fos$^+$ cells per amygdala section in saline (n=8) and LPS (n=7) treated mice with * representing p<0.05 in independent t-tests, error bars represent ± 1 SEM. Representative photomicrographs illustrating c-Fos expression in the amygdala at 100x magnification.*

### *3.3.3 Microglial activation 12 months post-LPS administration*

#### *3.3.3.1 CD11b*

No differences existed between post-septic (n=7) and control (n=7) animals in any area of the hippocampus at 12 months post-treatment, as can be seen in Figure 3.22. In the DgGr saline-treated animals had a mean IOD score of 3.45±0.53 vs. 4.03±0.75 for LPS-treated mice, $t_{12}$=0.625, p=0.544. In the DgPo saline-treated animals had a mean score of 10.05±1.3 while LPS-treated animals had a mean score of 8.3±1.2, $t_{12}$=0.99, p=0.343. In the CA1 region saline-treated animals had a mean score of 5.49±1.22 while LPS-treated animals had a mean score of 4.52±0.64, $t_{12}$=0.71, p=0.494. In the CA2 region saline-treated animals had a mean score of 9.47±2.71 while LPS-treated animals had a mean score of 6.6±0.92, $t_{12}$=1, p=0.336. In the CA3 region saline-treated animals had a mean score of 10.57±2.04 while LPS-treated animals had a mean score of 9.11±1.34, $t_{12}$=0.603, p=0.558.

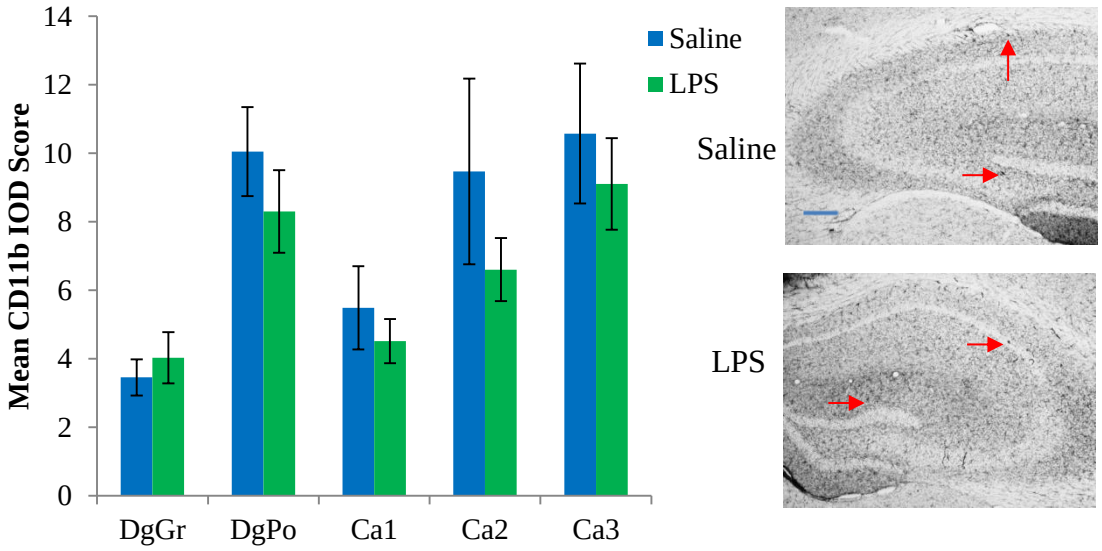



***Figure 3.22 Unaltered CD11b expression 12 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=7) and LPS (n=7) treated mice. * represents p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating CD11b expression in the hippocampus at 40x magnification.*

*3.3.3.2 F4/80*

There was no significant difference in F4/80 levels, illustrated in Figure 3.23, in any region of the hippocampus at 12 months post LPS administration. In the DgGr saline-treated (n=7) animals had a mean score of 7.46±0.91 while LPS-treated animals (n=7) had a mean score of 7.88±0.88, $t_{12}$=0.333, p=0.745. In the DgPo saline-treated animals had a mean score of 13.56±1.47 while LPS-treated animals had a mean score of 12.69±1.19, $t_{12}$=0.456, p=0.657. In the CA1 region saline-treated animals had a mean score of 6.44±1.1 while LPS-treated animals had a mean score of 10.65±3.24, $t_{12}$=1.232, p=0.242. In the CA2 region saline-treated animals had a mean score of 7.33±1.19 while LPS-treated animals had a mean score of 9.25±1.26, $t_{12}$=1.11, p=0.287. In the CA3 region saline-treated animals had a mean score of 2.92±1.11 while LPS-treated animals had a mean score of 7.56±1.16, $t_{12}$=1.17, p=0.265.

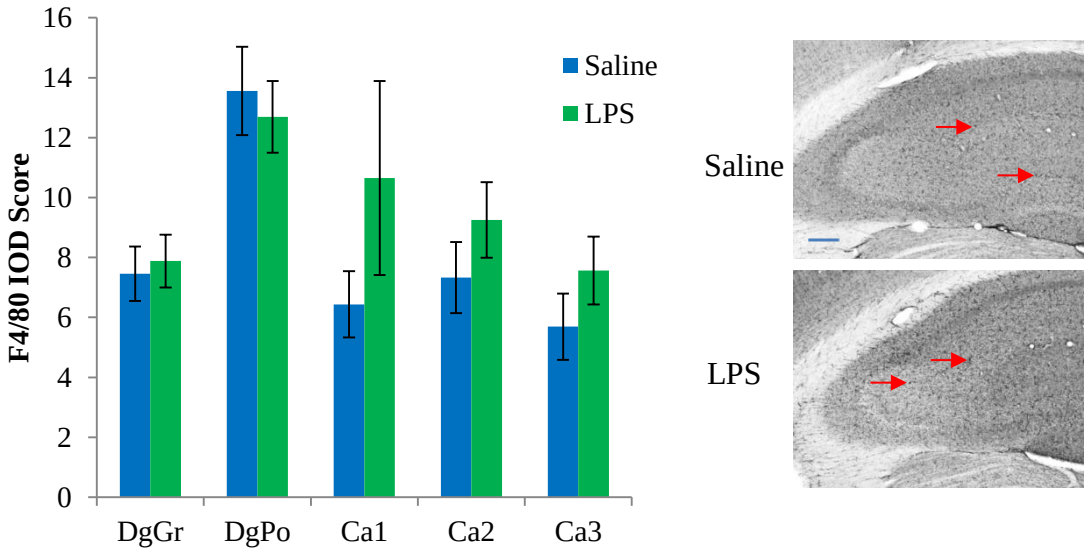



***Figure 3.23 Unaltered F4/80 expression 12 months post-sepsis*** *Bar graph illustrating IOD values for each area of the hippocampus in saline (n=7) and LPS (n=7) treated mice. * represent p<0.05 in independent t-tests, error bars represent ± 1 SEM. Photomicrographs illustrating F4/80 expression in the hippocampus at 40x magnification.*

*Table 6 Protein expression across experimental timepoints*

| **Antibody** | **24 Hours** | **Long-term** | **14 months** |
|---|---|---|---|
| Arc | Down-regulated in LPS (DgGr) | Down-regulated in LPS (DgGr) | Not examined |
| Egr-1 | Not examined | Down-regulated in LPS (DgGr, CA1, CA2) | Not examined |
| c-Fos | Not examined | No changes in hippocampus, down-regulated in amygdala. | Not examined |
| CD11b | Up-regulated in LPS (all areas) | Up-regulated in LPS (DgGr, DgPo, CA1) | No changes |
| F4/80 | Up-regulated in LPS (all areas) | Up-regulated in LPS (all areas) | No changes |
| IBA-1 | Not examined | Up-regulated in LPS (all areas except CA2) | Not examined |
| GFAP | No changes | No changes | Not examined |
| TNF- α | No changes | No changes | Not examined |
| IL-1β | | Up-regulated in LPS (CA3) | Not examined |
| IL-6 | Upregulated iin LPS (DgGr) | No changes | Not examined |
| NOS2 | Not examined | No changes | Not examined |
| p-IKK α/β | Not examined | No changes | Not examined |
| p-IkB- α | Not examined | No changes | Not examined |
| BrdU | Not examined | Down-regulated in LPS (DgGr) | Not examined |
| Cleaved Caspase-3 | Up-regulated in LPS (SGZ) | Not examined | Not examined |

*3.4 Discussion*

These results indicate that a 5mg/kg dose of LPS is sufficient to produce a potent inflammatory response accompanied by possible apoptosis in the hippocampus at 24 hours post-treatment, leading to sustained microglial activation, altered IEG expression, and a reduction in neural precursor cell proliferation up to two months later – with microglial activation appearing to return to baseline levels by 14 months post-treatment. Hippocampal cytokine levels at both 24 hours, and two months post-treatment appear to be close to those of control animals.

*3.4.1 The Acute Response to LPS-induced Sepsis*

At 24 hours post-treatment there is a significant upregulation of activated microglia (F4/80, CD11b) in the hippocampus, accompanied by increased cleaved caspase-3 expression and a reduction in the production of Arc. These microglia assume an amoeboid morphology typical of acutely activated microglia. There was no upregulation in astrocytes (GFAP) and IL-6 production was upregulated only within the granule cell layer. Since the typical profile of activated microglia assumes the production of certain pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 (Huang, He, Chen, Zhang, & Chen, 2013; Kao, et al., 2011; Lund, et al., 2006; Smith, Das, Ray, & Banik, 2012), it was surprising that increased IL-6 was present only within the granule cell layer and not other areas of the hippocampus at 24 hours post-treatment, despite the large upregulation of activated microglia throughout the hippocampus. Several studies show however that *in vivo* administration of LPS leads to peak cytokine levels at much earlier timepoints than 24 hours. For example i.cv. administration of LPS leads to peak cerebral IL-6 and TNFα levels between 1-4 hours (Lund, et al., 2006; Zujovic, Schussler, Jourdain, Duverger, & Taupin, 2001), while i.p. administration of a 100µg/kg dose to SD rats induced upregulated hippocampal cytokine production at 2

hours with levels generally dropping by 4 hours (Roche, Diamond, Kelly, & Finn, 2007) and a 2.5mg/kg i.p. administration leads to increased cytokine levels at 2 and 6 hours post-LPS but not 24 hours later in whole-brain samples from male NMRI mice (Buiesmans, et al., 2013). Although our dose of LPS is higher than these studies it may still elicit a cytokine response which peaks earlier than 24 hours.

In addition to microglial activation an acute reduction in basal Arc expression is also seen within the hippocampus of post-septic animals. Aged rats treated with *E. coli* show a reduction in resting levels of hippocampal Arc (but not c-Fos) 24 hours post-treatment, an effect which was blocked by anti-inflammatory IL-1RA administration (Frank, et al., 2010). Mice chronically over-expressing IL-1β have also been shown to have significantly reduced basal, and conditioning induced, levels of Arc in the hippocampus (Hein, et al., 2010). Newly born activated microglia have been shown to negatively correlate with behaviourally induced Arc levels following whole-body radiation (Nelson, et al., 2014). Thus it appears that in the acute state of LPS-induced sepsis, activated microglia contribute to a reduction in resting levels of Arc in the dentate gyrus.

There was a large upregulation of cleaved caspase-3 within the granule cell layer of the hippocampus of post-septic animals while levels are almost undetectable in the control group. Cleaved caspase-3 is typically associated with apoptotic cell death (Elmore, 2007: Kim, et al., 2014), indicating that apoptosis may be taking place within the hippocampus 24 hours following sepsis induction. TUNEL analysis has also shown apoptosis in rats 24 hours after a 10mg/kg dose of LPS (Semmler et al, 2005), although cognitive deficits have also been observed in rodents following sepsis induction without neuronal cell loss (Weberpals, et al., 2009). Caspase-3 may also play other, non-

apoptotic, roles in the adaptive immune system however (Yi & Yuan, 2009) which could also explain its significant upregulation following LPS administration.

*3.4.2 Long-lasting Changes in Immune Factors Post-Sepsis*

A large number of immune factors were examined in order to elucidate the long-lasting effects of LPS-induced sepsis on hippocampal processes. Microglia appear to remain upregulated at 2-months post-LPS, showing a deramified morphology compared to the ramified microglia present in the control animals and the amoeboid microglia present acutely following LPS administration. Similar to the results at 24-hours post-treatment, no upregulation of astrocytic activity was seen using GFAP staining. Apart from increased IL-1β expression in the CA3 region, there were also no changes in cytokine levels, NF-κB precursor proteins (p-IKK, p-IκB), or iNOS levels in any area of the hippocampus at this timepoint. By 12 months F4/80 and CD11b levels in post-septic animals were no different to those of controls. As increased inflammation and microglial activation is known to be a factor in normal aging (Liu, Wu, Hayashi, & Nakanishi, 2012) it is possible that either LPS-induced inflammation was resolved at this timepoint, or that microglial activation in control animals had reached similar levels by this age.

As other researchers have demonstrated upregulation of TNF-α production at 10 months after similar doses (Bossù, et al., 2012; Qin, et al., 2007), and Fan, et al. (2012) and Konda, Kohsaka, & Okabe (2011) show increases in activated microglia without cytokine over-expression at 7 days and 4 weeks post-treatment it is unclear which factors lead to prolonged increases in cytokine production following a single administration of LPS. It is known that regional variation exists in the expression of inflammatory parameters in the brain following systemic LPS administration (Weberpals, et al., 2009; Bossù, et al., 2012; Fan, et al., 2012), which may explain why

analysis of the whole brain may yield different results to our examination of only the hippocampus, see Qin, et al. (2007. Also of note is that while other studies examined cytokine levels using ELISA (Bossù, et al., 2012; Konda, et al., 2011; Qin, et al., 2007), immunohistochemistry is used in the present work toexamine these factors. It has previously been illustrated that, even in near identical samples, large discrepancies can exist between immunohistochemistry and ELISA measurements (Ferrier, et al., 1999).

Given that there is no upregulation of p-IKK or p-IκB, two upstream components of the NF-κB signalling pathway it appears that NF-κB production does not remain upregulated at 2 months following LPS administration. This coincides with unaltered levels of TNF-α expression, a key driver of the NF-κB pathway (Schütze, Wiegmann, Machleidt, & Krönke, 1995) and mirrors results found using the same LPS administration model but examining the suprachiasmatic nucleus (O'Callaghan et al., 2012). There also appears to be no upregulation of iNOS as detected by immunohistochemistry in any area of the hippocampus. This is again in contrast to Weberpals, et al. (2009) who showed increased iNOS levels at 2 months in whole brain samples of animals treated with 5mg/kg dose of LPS, although Fan et al. (2012) showed that the same dose elicited increased acute iNOS production which returned to baseline by 7 days. Differences may also exist between our study and other experiments due to differences in the strain of *E. coli* from which the LPS was isolated.

### *3.4.3 Immediate Early Genes*

In addition to alterations in inflammatory parameters LPS also induced alterations in key IEGs involved in neuronal activity and synaptic plasticity. Similar to the acute timepoint, at 2 months post-LPS administration there was a reduction in the expression of Arc within the dentate gyrus. There was also a reduction in EGR-1 within the DgGr, CA1 and CA2 with no changes present in c-Fos expression in any region.

Both Arc and Egr-1 have also been used as markers of neuronal activity and their transcription has previously been shown to be altered by various forms of LPS administration with differential expression occurring based on animals' age, activity prior to measurement, time of measurement and other factors (Bonow et al, 2009; Rosi, 2011; Rosi, et al., 2005). Neurons express c-Fos in response to many stimuli and it has previously been shown to be upregulated in response to acute treatment with LPS (Haba, et al., 2014; Ma, Matsuwaki, Yamanouchi, & Nishihara, 2014). It is likely that c-Fos expression may only be altered at timpoints closely following LPS administration. c-Fos does not appear to be expressed in the hippocampus at 6 or 24 hours after LPS administration, although c-Fos expression in several other areas such as the amygdala was at the same timepoints (Frenois, et al., 2007). In our examination of the amygdala it was found that LPS actually reduced c-Fos levels, possibly mirroring the underactivation of the amygdala seen in general anxiety disorder (Yassa, Hazlett, Stark, & Hoehn-Saric, 2012). It is difficult to know if this may explain any of the anxiety behaviours present in post-septic mice however as rodent anxiety behaviour is more typically associated with an overactivation of the amygdala (Duncan, Knapp, & Breese, 1996). Although it is unclear how LPS affects the expression of these IEGs in this model, they illustrate subtle changes in hippocampal function among post-septic mice.

Staining with BrdU revealed that post-septic mice show a long-lasting reduction in cell proliferation within the hippocampus. Acute and chronic treatments with LPS have previously been shown to reduce hippocampal neurogenesis in the rodent hippocampus by reducing both cell proliferation (Fukioka & Akema, 2010; Wu, et al., 2013) and the survival of newborn cells (Bastos, Moriya, Inui, Katura, & Nakahata, 2008; Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003). Indeed, an inverse correlation has been observed between the number of microglia present within the

hippocampus and cell proliferation (Gebara, Sultan, Kocher-Braissant, & Toni, 2013). Neurogenesis is strongly implicated in the pathology of affective disorders, although the experimental interruption of neurogenesis in the rodent hippocampus is not, by itself, always sufficient to produce increased anxiety-like and depressive-like symptoms, with stress appearing to play an important role (Petrik, Lagace, & Eisch, 2012). As BrdU expression was examined after only a single 24 hour pulse it is unknown whether LPS-induced sepsis affected the survival and differentiation of NPCs or their integration into existing hippocampal networks.

### *3.4.4 Interactions between glia and synapses – implications for post-septic encephalopathy*

Though these experiments describe acute and long-lasting changes in the hippocampus of post-septic animals, the mechanisms through which these changes occur remain unclear. The high prevalence of activated microglia in the post-septic hippocampus is likely to have a deleterious effect on normal brain functioning in these animals. Intact neuron-astrocyte-microglial signalling is essential for normal neuronal circuit structure and homeostasis (Cerbai, et al., 2012; Wake, Moorhouse, Miyamoto, & Nabekura, 2013) which is likely to be compromised in this model given the elevated levels of activated microglia. Although neither cytokine levels nor NF-κB pathway activation appeared elevated in the hippocampus of post-septic animals at 2 months post-treatment, microglia may also be detrimental to neuronal function via mechanisms not measured here such as the production of reactive oxygen species (Hernandes, et al., 2014), increased indoleamine 2,3-dioxygenase production (Corona, et al., 2013) altered chemokine expression (Rivest, 2009) or changes to mitochondrial function (Noh, Jeon, & Seo, 2014). LPS treatment is also known to affect blood-brain barrier permeability (Jangula & Murphy, 2013) and several aspects of cerebrovascular functioning (Gavins,

et al., 2012) which may continue to disrupt normal brain processes long after the acute effects of LPS-induced sepsis have passed.

In other studies a 5mg/kg dose of LPS does not appear to induce long-lasting neuronal loss, although pre- and post-synaptic protein levels are altered in the hippocampus (Fan, et al., 2012; Weberpals, et al., 2009) and it is shown here that post-septic mice exhibit reduced levels of IEG proteins essential to synaptic plasticity. Although the present experiments show increased cleaved caspase-3 expression in the post-septic brain, this is not definitively indicative of apoptosis, with TUNEL analysis in the same model showed no signs of apoptosis in the suprachiasmatic nucleus (O'Callaghan, Anderson, Moynagh, & Coogan, 2012). The reduction of adult cell proliferation in post-septic animals in this study may also contribute to this pathology. As adult-born cells are continually produced and incorporated into synaptic networks within the hippocampus a disruption, or reduction, of this process may disrupt hippocamal functioning.

*3.4.5 Relevance to Human Sepsis*

It is known that the administration of high doses of LPS induces a more rapid, and intense, hyper-inflammatory response than that seen in human sepsis (Buras, Holzmann, & Sitkovsky, 2005). Another critical component of sepsis in humans is the hypo-inflammatory state which follows the initial hyperinflammatory response, involving immune factors such as IL-10 and transforming growth factor (TGF)-β. Although IL-6 is a key factor in clinical sepsis, and its levels have been positively correlated with severity and mortality in septic patients (Hack, et al., 1989), these results show elevated hippocampal IL-6 levels 24 hours after LPS administration in the granule cell layer only, possibly due to their return to basal levels prior to our sampling time in other areas. Neuronal apoptosis as marked by cleaved caspase-3 immunocytochemistry,

iNOS expression and microglial activation are present in patients who died from septic shock at higher levels than other ICU fatalities (Lamar, Hurley, Hayman, & Taber, 2011; Sharshar, et al., 2004) which is reflected in some of the changes in our post-septic mice. One caveat may also be that while many studies examining neuroimmune changes in the brains of septic patients rely on post-mortem tissue, in our experiments those animals which survive sepsis-induction are assessed. Although substantial research has been done into the immediate neurological alterations present during septic encephalopathy, the long-lasting pathophysiology of post-septic encephalopathy in humans remains unclear, making direct comparisons difficult.

### *3.4.6 Conclusion*

In conclusion it is shown that a peripheral administration of a septic dose of LPS induces a rapid (24 hour) microglial activation in the hippocampus as well as cleaved caspase-3 activation and increased IL-6 expression in the granule cell layer. There is also a reduction in the immediate early gene Arc, but no changes to astrocytic marker GFAP. 2 months after recovery from these acute effects microglia maintain an activated, deramified morphology throughout the hippocampus. Save for increased IL-1β expression in the CA3 region, there is no over-expression of pro-inflammatory cytokines. IEGs key to synaptic plasticity (Arc, Egr-1) are downregulated within the hippocampus of post-septic animals. There was also a reduction in adult cell proliferation in the subgranular zone of the hippocampus which may be contributing to disrupted hippocampal functioning. These changes mimic some of the changes seen in human cases of sepsis, although it does not mirror the more long-lasting upregulation of pro-inflammatory cytokines and inducible nitric oxide expression.

# Chapter 4. Can Pharmacological Disruption of the NF-κB Pathway Reduce Post-Septic Symptoms?

*Abstract*

In chapters two and three it was demonstrated that LPS administration could lead to long-lasting inflammation in the hippocampus, accompanied by reduced cell proliferation and IEG expression as well as changes to affective behaviour. The NF-κB pathway is known to be highly important in the inflammatory response to LPS. It was therefore hypothesised that if this pathway was disrupted prior to sepsis induction the subsequent post-septic symptoms may be dampened. PDTC was chosen as our pharmacological inhibitor of NF-κB. Animals were treated either with saline or PDTC prior to sepsis induction or control treatment and their health was then monitored. After one month animals underwent behavioural testing. They then were given a BrDU treatment 24 hours to being sampled and processed for immunohistochemistry. LPS treatment again induced significant changes to affective behaviour, while PDTC pre-treatment was shown to protect against post-septic deficits in the novel object and tail suspension tests as well as the elevated plus maze. Post-septic mice pre-treated with PDTC also show less activated microglia, more BrdU incorporating cells and immediate early gene expression than post-septic mice which had not been administered PDTC. It is concluded that inhibition of the NF-κB pathway can protect against some of the effects of LPS-induced sepsis highlighting the importance of NF-κB signalling in this disorder.

## 4.1 Introduction

In previous chapters it has been shown that post-septic animals exhibit depressive-like and anxious-like behaviours in addition to elevated levels of microglial activation, reduced IEG levels and impaired adult cell proliferation in the hippocampus. As previously discussed, LPS is recognised following peripheral administration by TLR-4 receptors, leading to a potent inflammatory response in the CNS driven primarily by NF-κB. It was therefore hypothesised that pre-treatment with pyrrolidine dithiocarbamate (PDTC) could potentially ameliorate some of the deficits seen in post-septic mice. PDTC is a potent inhibitor of the NF-κB pathway, thought to exert its effects by inhibiting IκBα degradation (Liu, Ye, & Malik, 1999), thereby preventing the translocation of NF-κB dimers to the cell nucleus and resulting pro-inflammatory gene transcription.

PDTC itself is a metal chelating compound which can act, dose-dependently, as a potent anti-oxidant (Moellering, McAndrew, Jo, & Darley-Usmar, 1999) and anti-inflammatory (Shao, Lee, Huang, Liao, & Lin, 2004) agent. At high levels however it can exert pro-oxidative effects as well as induce apoptosis (Erl, Weber, & Hansson, 2000; Moellering, McAndrew, Jo, & Darley-Usmar, 1999). Preclinical toxicity assessment indicates that, although well tolerated at low doses, it has an $LD_{50,\ intravenous}$ of 282.4mg/kg in mice – exerting toxic effects on both the central and peripheral nervous system (Chabicovsky, et al., 2010). Its derivative diethyldithiocarbamate has been shown to be clinically tolerated in humans (Reisinger, et al., 1990). It therefore appears to have high utility as a modulator of the inflammatory response, although only at specific doses.

Though most researchers attribute its neuroprotective effects to NF-κB inhibition, some authors have suggested that PDTC may exert beneficial effects independent of, or despite failing to, inhibit NF-κB signalling (Huang, et al., 2008; Malm, et al., 2007) such as by activating the metal-activated heat shock factor 1 (HSF1) and inhibiting IL-6 activity (Song, et al., 2011). Importantly however in Malm et al. (2007) NF-κB was not upregulated in their transgenic Alzheimer's disease model relative to controls and Huang et al. (2007) also hypothesised that NF-κB does not play a role in the pathophysiology of their model. If PDTC inhibits NF-κB activation primarily in models which involve significant NF-κB overactivation then it is less surprising that PDTC did not supress NF-κB activation in the above experiments, but exerted its effects through other mechanisms. Song et al. (2011) for example show that, apart from it's effects on NF-κB, PDTC also promotes the activation of a large number of cytoprotective genes. Interestingly, in Huang's (2007) study discrepancies did exist at certain timepoints between heightened NF-κB levels despite restored IκBα levels, and attenuated NF-κB levels despite reduced IκBα expression suggesting that in some casse NF-κB activation may occur independently of IκBα expression.

*4.1.1 The effect of PDTC on microglia and neurons in culture*

Many experiments have reported PDTC's anti-inflammatory effects on microglial and neuronal cell lines. In human endothelial cells PDTC has been shown to reduce IL-6 and IL-8 transcription in response to LPS (Muñoz, et al., 1996). In murine BV-2 microglia cells LPS treatment leads to an increase of NF-κB protein in the cell nucleus versus cytosol, accompanied by increased TNF-α, IL-6 and NO production, an effect which is blocked when cells are pre-incubated with PDTC (Yuan, Ren, Liu, Wang, & Liu, 2014). This effect is mirrored in rat hippocampal neurons where PDTC has been shown to block the translocation of NF-κB to the cell nucleus upon LPS

administration – which also attenuated the transcription of IL-1β and TNF-α (Zhao, Zhou, Xu, & Zhang, 2014). In murine BV2 microglial cells PDTC administration has been also shown to significantly reduce microglial expression of activation marker CD11b (Roy, et al., 2008) as well as production of iNOS following LPS treatment (Kumar, et al., 2014). In rat microglial cells PDTC was shown to inhibit LPS induced production of COX-2 and Prostaglandin $E_2$ (Bauer, et al., 1997).

PDTC is clearly therefore a potent inhibitor of NF-κB driven pro-inflammatory responses in microglia *in vitro*. Importantly however, LPS can still induce inflammation in rodent microglia via NF-κB independent pathways such as c-Jun N-terminal Kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) dependent pathways (Uesugi, Nakajima, Tohyama, Kohsaka, & Kurihara, 2006). Particularly, TNF-α production seems to occur despite NF-κB inhibition with PDTC (Nakajima, Matsushita, Tohyama, & Kurihara, 2006).

### *4.1.2 The effects of PDTC in system level models of sepsis and other inflammatory disorders*

Though PDTC has not been studied in human sepsis, it has been shown to be effective in several studies involving the administration of septic doses of LPS. Specifically, PDTC has been shown to improve survival rate, protect against multiple organ dysfunction and attenuate pro-inflammatory cytokine and iNOS levels following septic doses of LPS (Lauzurica, et al., 1999; Meisner, Schmidt, Schywalsky, & Tschaikowsky, 2000; Németh, Haskó, & Viz, 1998; Yao, Xu, Yao, Yu, & Sheng, 2006). PDTC's ability to exert powerful anti-inflammatory and antioxidant effects in models of LPS-induced sepsis suggest that it may protect against long-lasting behavioural and neuroimmune deficits seen in post-septic mice.

PDTC has also been examined in animal models of CLP-induced sepsis. A 100mg/kg dose of PDTC 2 hours prior to CLP inhibited NF-κB activation leading to a reduction in renal cytokine and NOS2 production and ultimately reduced hypotension and renal failure (Höcherl, Schmidt, Birgül, & Bucher, 2010). PDTC (100mg/kg) has also been shown to inhibit NF-κB production and therefore plasma levels of TNF-α, IL-1β, IL-6, and IFN-γ (Raspé, Höcherl, Rath, Sauvant, & Bucher, 2013). An early study however showed that the same dose of PDTC failed to improve survival rates or macrophage function following CLP, although C3H/HeN mice were used here rather than the C57/BL6 strain used in later studies (Joshi, et al., 2002). These studies provide support for PDTC's utility as an anti-inflammatory not just in vitro but also in whole system models.

*4.1.3 The effects of PDTC on behaviour in inflammatory disease models*

Although PDTC has not previously been shown to protect against LPS-induced cognitive or affective impairment, it has been shown to protect against cognitive impairment and anxiety-like behaviours in other models involving inflammatory components such as acute restraint stress (Sharma, Gilhotra, Dhingra, & Gilhotra, 2011), diabetes (Zhao, Huang, Wang, & Zhong, 2013), neurosurgery (Zhang, Jiang, & Zuo, 2014), maternal immune stimulation (Song, et al., 2011) and neonatal hypoxia-ischemia (Wang, Zhao, Peng, & Zuo, 2013). In the APP/PS1 transgenic model of Alzheimer's disease a 7-month oral administration of PDTC was found to rescue spatial learning (Malm, et al., 2007). Elevated gliosis in APP/PS1 mice was not affected by PDTC administration however. In a scopolamine-induced model of dementia PDTC has been shown to reduce IL-1β expression, rescue decreased cortical dopamine levels and ameliorate cognitive performance in the Y-maze (Abd-El-Fattah, Abdelakader, & Zaki,

2014). PDTC therefore appears to be a promising candidate for effecting behavioural change in post-septic animals.

### 4.1.4 Aims

Given PDTC's ability to improve many of the acute effects of LPS-induced sepsis it was hypothesised that it may ameliorate some of the long-lasting effects present in our post-septic mice. Although PDTC may have limited clinical utility given the necessity of pre-administration to protect against sepsis, this approach may shed light on the contribution of NF-κB to the persistent deficits in sepsis-survivors. Therefore, control and post-septic animals which either had received saline or PDTC were tested on a number of behavioural measures which had previously shown to be affected by LPS administration before examining hippocampal levels of microglial activation, EGR-1 expression and adult cell proliferation. We also assessed EGR-1 expression in the amygdala of these mice, given this area's importance in anxiety-like behaviours and the previous results showing reduced c-Fos expression here.

*4.2 Methods*

*4.2.1 Animals*

For the purpose of all experiments male C57BL/6 mice (Charles River, Kent, UK) aged between 8 and 16 weeks were used. Animals were group housed in a 12:12 light:dark cycle for 2 weeks prior to LPS administration. Food and water were available *ad libitum*, temperature was 21 ±1°C and humidity was 50 ±10%. Animals remained housed in groups of 2-4 in polypropylene cages (33cm long x 15 cm wide x 13cm high) with wood chip bedding and environmental enrichment (shredded paper and cardboard tubes). All procedures were approved by the Research Ethics Committee, National University of Ireland Maynooth, and were licensed by the Department of Health and Children, Ireland under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU.

*4.2.2 Drug treatments*

All injections were prepared fresh on the treatment day, and given intraperitoneally in a final injection volume of 0.1 – 0.15ml. 0.9% sterile saline was made up fresh for control injections, while lipopolysaccharide (serotype 0111.B4, Sigma Ireland; Qin et al, 2007) was made up to a 5mg/kg dose in sterile saline. PDTC (Sigma Ireland) was administered in a 200mg/kg dose (Liu, et al, 1999) 10 minutes before LPS treatment. Animals were once again returned to their homecage and their health was scored at 1 hour, 4 hours, 24 hours, 48 hours and 1 week post treatment. Animals were scored based on weight reduction, activity in the cage (response to stimulus, spontaneous movement), general appearance (e.g. staring coat, hunching, shivering), behaviour (motility, alertness), and clinical signs (breathing etc.). To assess neural precursor cell proliferation in the hippocampus mice which had one month

previously undergone sepsis-induction were injected i.p. with 50mg/kg BrdU (Sigma Ireland) and transcardially perfused 24 hours later.

*4.2.3 Behavioural testing*

One month after the administration of LPS animals were tested the novel object recognition task followed by the elevated plus maze and then the tail suspension test with one week between tests. They were then singly housed and two days later underwent the sucrose preference test. All behavioural tests were carried out as previously described in Chapter 2.

*4.2.4 Immunohistochemical analysis*

One week after the sucrose preference test mice were anaesthetised with 0.1ml sodium pentobarbital (Euthatal, Merial Animal Health, UK), following which they were perfused transcardially with 0.9% saline, and then 4% paraformaldehyde (Sigma Ireland) in 0.1M phosphate buffer (PB), pH 7.4 at 4˚C. Brains were then cryoprotected in 0.1M PB containing 30% sucrose, frozen and sectioned coronally at 30µm. Immunohistochemistry for IBA-1, Egr-1 and BrdU was then carried out following a standard Avidin-Biotin Complex/Nickel DAB colourmetric protocol as previously described in Chapter 3.

*4.2.5 Statistical Analysis*

Data were analysed in SPSS version 20 (IBM) with the type of test used indicated for each data set. All data are presented as means ± standard error of the mean (SEM). * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$. Where statistical assumptions were violated adjusted values are presented. Scale bars on microphotographs represent 100µm for photos at 40x and 100x magnification and 50µm for photos at 400x magnification.

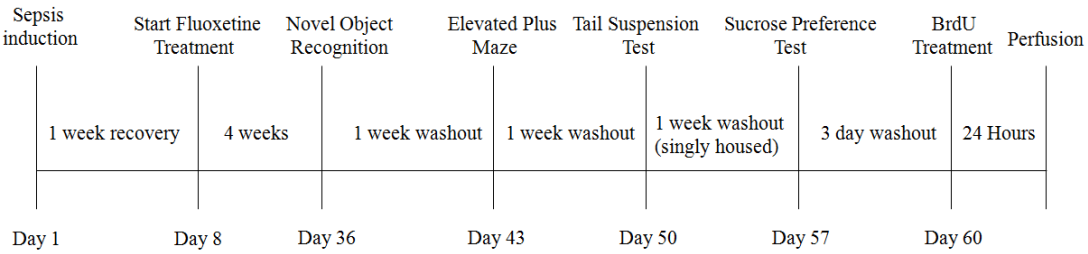



**Figure 4.1 Timeline for PDTC experiments.**

*4.3 Results*

*4.3.1 Health Ratings After Sepsis Induction*

A three-way time x PDTC x sepsis ANOVA revealed a significant main effect of time ($F_{4,140}$=64.082, p<0.0005), sepsis induction ($F_{1,35}$=157.174, p=0.0005) and PDTC treatment ($F_{1,35}$=33.97, p<0.0005). Significant interaction effects were present between all three variables (p<0.0005 in all cases).

Subsequent analysis with two-way sepsis x PDTC ANOVAs at each timepoint indicated there was a main effect of LPS treatment at 4 hours ($F_{1,35}$=37.088, p<0.0005), 24 hours ($F_{1,35}$ = 357.854, p <0.0005), 48 Hours ($F_{1,35}$=91.182, p<0.0005) and 1 week ($F_{1,35}$=9.096, p<0.005) but not at one hour post-treatment ($F_{1,35}$=0.042, p=0.838). There was a main effect of PDTC treatment one hour ($F_{1,35}$=186.136, p<0.0005) and 4 hours ($F_{1,35}$=24.827, p<0.0005) with a trend towards significance at 48 hours post-treatment ($F_{1,35}$=3.991, p=0.054). There was no main effect of PDTC treatment at 24 hours post-treatment ($F_{1,35}$=3.187, p=0.085) or one week post-treatment (F=0.364, p=0.55). There was an interaction effect between the two treatments at 1 hour ($F_{1,35}$=15.087, p<0.0005), 4 hours ($F_{1,35}$=6.812, p=0.013), 24 hours ($F_{1,35}$=4.118, p=0.05) but not at 48 hours ($F_{1,35}$=2.782, p=0.104) or one week ($F_{1,35}$=0.364, p=0.55).

Animals who received PDTC exhibited a sharp reduction in mobility and irresponsiveness to stimulus lending to a high score at one hour post-treatment (9±0.91 for animals subsequently treated with saline and 7.11±0.42 for those subsequently treated with LPS). By four hours those treated with PDTC followed by saline were returning to baseline (4.8±1.04) while those treated with PDTC followed by LPS remained elevated at 7±0.44. While both animals receiving PDTC and saline and saline followed by saline were essentially back to normal at 24 hours with scores of 0.5±1.67

and 0 respectively, animals who received saline followed by LPS peaked at a score of 8.6±0.62 while those who received PDTC followed by LPS scored 7.11±0.42. At 48 hours PDTC+LPS animals scored 4.78±0.83 while Saline+LPS animals scored 7±0.91. These numbers are illustrated in Figure 4.2.

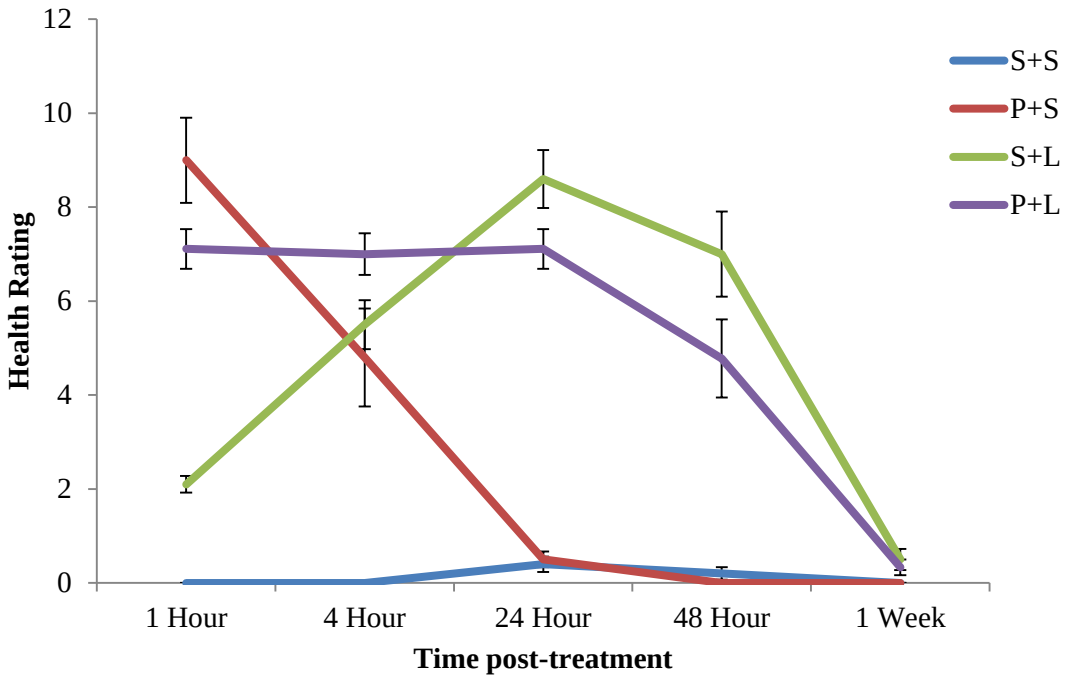



***Figure 2.1 Health ratings following sepsis induction*** *Mean health rating at each timepoint following drug administration. Error bars represent ±1 SEM.(S+S= saline & saline, P+S = PDTC + Saline, S+L = Saline + LPS, P+L = PDTC + LPS). N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.2 Tail Suspension Test*

As shown in Figure 4.3 a two-way ANOVA showed no main effect of sepsis induction on time spent immobile ($F_{1,34}$=1.988, p=0.168) and no main effect of PDTC treatment ($F_{1,34}$=1.824, p=0.186). There was however an interaction effect between the two treatments ($F_{1,34}$=12.616, p=0.001). Animals who received a saline treatment followed by a second saline treatment spent a mean time of 93.3±9.395 seconds immobile, those who received PDTC followed by saline spent 115.2±8.799 seconds immobile, animals treated with saline and subsequently LPS were immobile for 142.67±13.25 seconds while those who received PDTC before LPS were immobile for 93.89±7.75 seconds. Post-hoc analysis with Tukey's test revealed a significant difference between post-septic and control mice (p=0.029) while post-septic mice and their counterparts who were pre-treated with PDTC had a p value of 0.051.

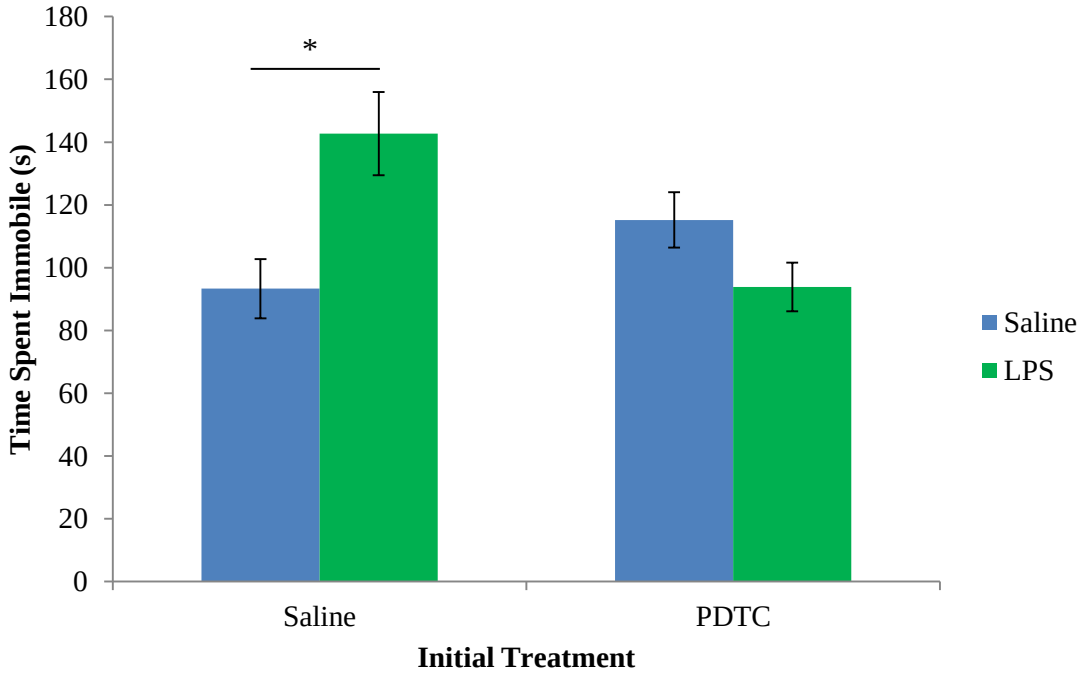



***Figure 4.3 PDTC protects against increased behavioural despair in post-septic mice***

*Mean time spent immobile (seconds) in the tail suspension test. * represents p<0.05 in Tukey analysis. Error bars represent ±1SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.3 Sucrose Preference Test*

As illustrated in Figure 4.4 there was no main effect of PDTC treatment on the percentage of sucrose consumed ($F_{1,33}$=3.317, p=0.078). There was a trend towards a main effect of LPS treatment on sucrose consumed ($F_{1,33}$=4, p=0.054). There was no interaction effect between these two variables ($F_{1,33}$=0.014, p=0.907). Animals who received two saline treatments had a mean sucrose preference percentage of 76.59±3.06% while those who received PDTC followed by saline had a similar percentage of 81.71±1.84%. Post-septic animals who received saline prior to LPS had a mean sucrose percentage of 70.22±2, while those who received PDTC prior to LPS had a preference of 76.05±4.34%.

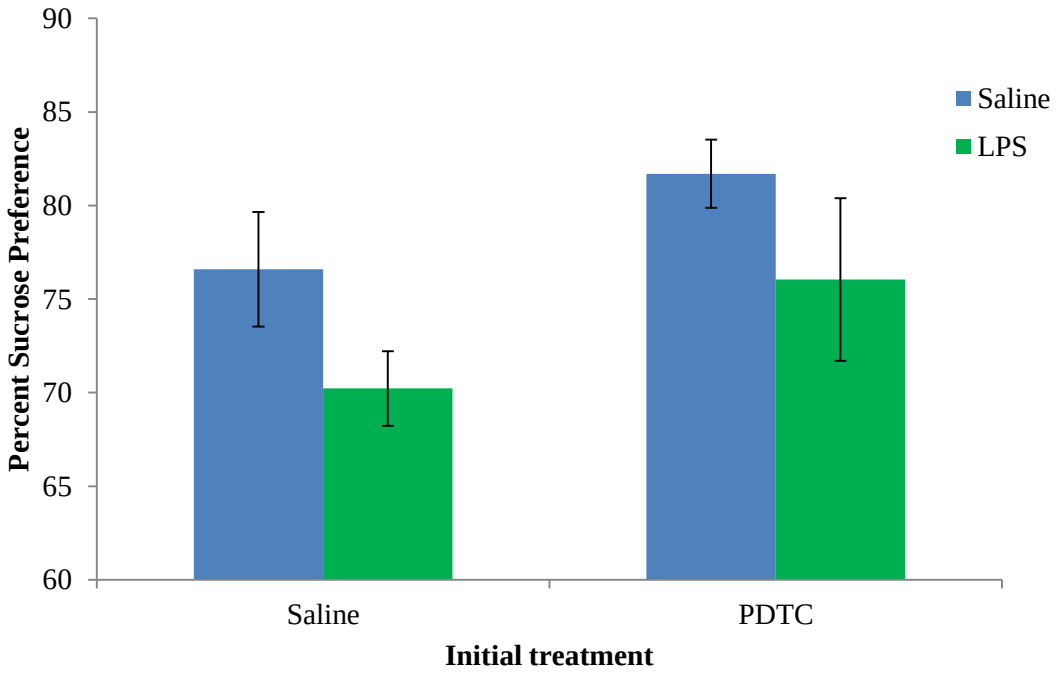



***Figure 4.4 Anhedonia is unchanged following LPS or PDTC administration***. *Bar graph displaying mean percentage sucrose preference for all groups. Error bars represent ±1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=8), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.4 Elevated Plus Maze*

As shown in Figure 4.5, in the elevated plus maze there was no main effect of PDTC treatment on any of the four measures. There was a main effect of LPS treatment on time spent in the close arm of the maze ($F_{1,35}$=4.154, p=0.049), but not on time spent in the open arm ($F_{1,35}$=2.083, p=0.158). There was an interaction effect present between PDTC and LPS treatment on both time spent in the open arms ($F_{1,35}$=6.699, p=0.014) and time spent in the closed arms ($F_{1,35}$=9.178, p=0.005). Post-hoc analysis with Tukey's test for time spent in the open arm showed differences only between post-septic mice and saline treated controls (p=0.032). For time spent in the closed arm Tukey's post-hoc test indicated a significant difference again only between post-septic mice and saline-treated controls (p=0.005).

Animals that were treated with two saline treatments (n=10) had an average of 9.7±0.94 open arm entries and 12.5±1.18 closed arm entries, spending 98.2±13.38 seconds in the open arms and 115.3±5 seconds in the closed arms. Animals who received a PDTC treatment followed by a saline treatment (n=10) had 8±0.99 open arm entries and 12.7±0.99 closed arm entries on average, with 72.6±11.6 seconds spent in the open arms and 147.2±13.27 spent in the closed arms. Post-septic animals who received saline followed by LPS treatments (n=10) had 7.3±0.42 open arm entries and 12.7±0.76 closed arm entries, spending 56.5±5.38 seconds in the open arms and 166.3±6.61 seconds in the closed arm. Those who received a PDTC treatment followed by an LPS treatment (n=9) had 8.56±0.84 open arm entries and 11.67±1.08 closed arm entries, spending 84.44±8.83 seconds in the open arms and 137.22±13.16 seconds in the closed arms.

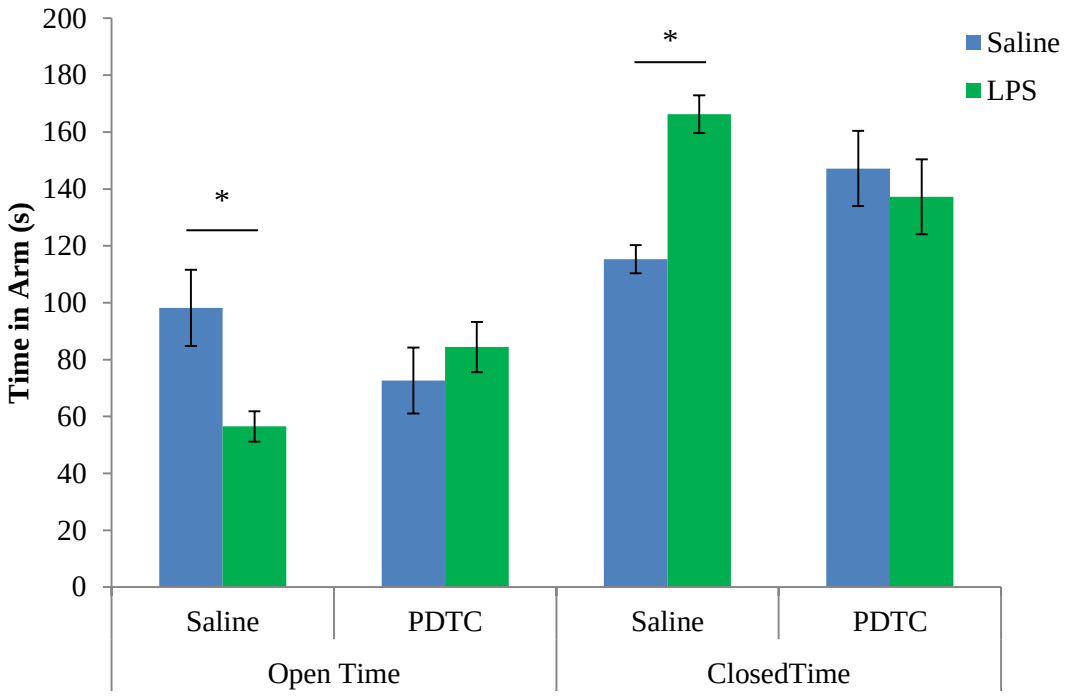



***Figure 4.5 PDTC protects against increased anxiety in the elevated plus maze in post-septic mice.*** *Bar graph illustrating time spent in each arm of the Elevated Plus Maze by each group. * represents p<0.05 post-hoc analysis, error bars represent ± 1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.5 Novel Object Recognition*

A mixed between within ANOVA with PDTC and LPS treatments as between groups factors and trial number as a within groups variable indicated that from trial 1 to trial 4 there was a main effect of trial number on seconds spent exploring both objects ($F_{3,105}$=13.021, p<0.0005), but not of PDTC treatment ($F_{1,35}$=0.894, p=0.351) or LPS treatment ($F_{1,35}$=0.611, p=0.44). There were no interaction effects present indicating that all groups habituated to the objects at similar levels.

When analysing the percent time spent exploring the novel object relative to the familiar object in the fifth trial it was observed that there was a main effect of PDTC treatment ($F_{1,35}$= 5.094, p=0.03), but not LPS treatment or any interaction between the two treatments. Animals treated with saline followed by saline had a 76.92±9.26% preference for the novel objects, those treated with PDTC followed by saline showed 90.03±1.66% preference, animals who received saline followed by LPS showed 71.95±5.42% percent preference, and those treated with PDTC followed by LPS had a 84.38±2.33% preference.

As illustrated in Figure 4.6, in terms of total exploration time in the fifth trial there was a main effect of LPS treatment ($F_{1,35}$=11.153, p=0.002) but not PDTC treatment ($F_{1,35}$= 2.295, p=0.139) or any interaction effect ($F_{1,35}$=0.27, p=0.607). Animals receiving two saline treatments had a total exploration time of 8.01±1.31 seconds, those receiving PDTC followed by saline 9.07±1.25, those receiving saline followed by LPS 3.9±5.07 and those receiving PDTC followed by LPS 6.07±0.97. Tueky post-hoc tests revealed significant differences between post-septic and control mice (p=0.043 and post-septic mice and mice receiving PDTC followed by saline (p=0.007).

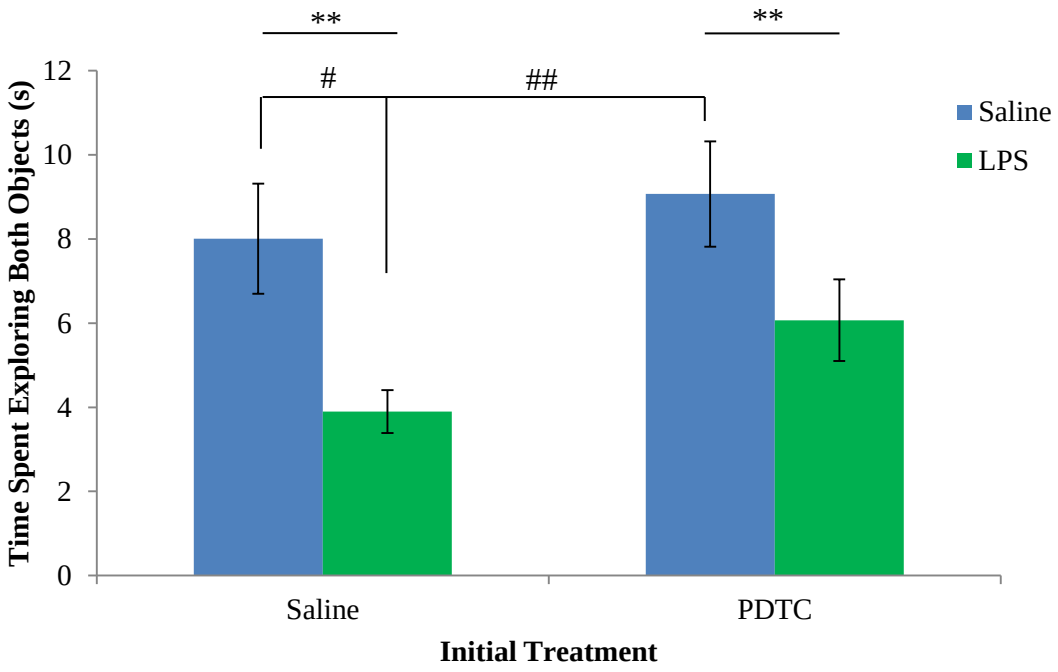



***Figure 4.6 PDTC protects against reduced exploration in the novel object recognition test in post-septic mice.*** *Bar graph illustrating the effects of both treatments on time spent exploring both objects in the fifth trial. ** represents a main effect of LPS (p<0.01), #/## represent post-hoc differences (p0.05/0.01), error bars represent ± 1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.6 IBA*

There was a main effect of PDTC treatment on IBA1 expression in the granule cell layer of the dentate gyrus ($F_{1,35}$=45.157, p<0.0005) the CA1 ($F_{1,35}$=22.616, p<0.0005) and the CA3 ($F_{1,35}$=6.145, p=0.016) but not the polymorphic cell layer ($F_{1,35}$=3.504, p=0.07) or the CA2 ($F_{1,35}$=1.928, p=0.174). There was also a main effect of LPS treatment in the granule cell layer of the dentate gyrus ($F_{1,35}$=27.275, p<0.0005), the CA1 ($F_{1,35}$=24.447, p< 0.0005) and the CA3 ($F_{1,35}$=6.145, p=0.018) but again not within the polymorphic cell layer ($F_{1,35}$=0.399, p=0.532) or the CA2 ($F_{1,35}$=2.678, p=0.111). As illustrated in Figure 4.7 there were significantly higher levels of microglia showing altered cell body shapes and deramification in the post-septic hippocampus. Finally there was an interaction effect between both treatments in the granule cell layer ($F_{1,35}$=12.241, p=0.001) the CA1 ($F_{1,35}$=22.098, p<0.0005) and the CA3 ($F_{1,35}$=9.998, p=0.003) but not the polymorphic cell layer ($F_{1,35}$=0.173, p=0.68) or CA2 ($F_{1,35}$=0.02, p=0.889).

Post-hoc analysis with Tukey's test revealed that in the DG and CA1 post-septic mice had higher levels of activated microglia than all other groups (p<0.0005 for all cases). In the CA3 region post-septic mice had significantly more activated microglia than controls (p=0.002), controls pre-treated with PDTC (p=0.002) and mice treated with PDTC and then LPS (p=0.005). There were no other significant differences.

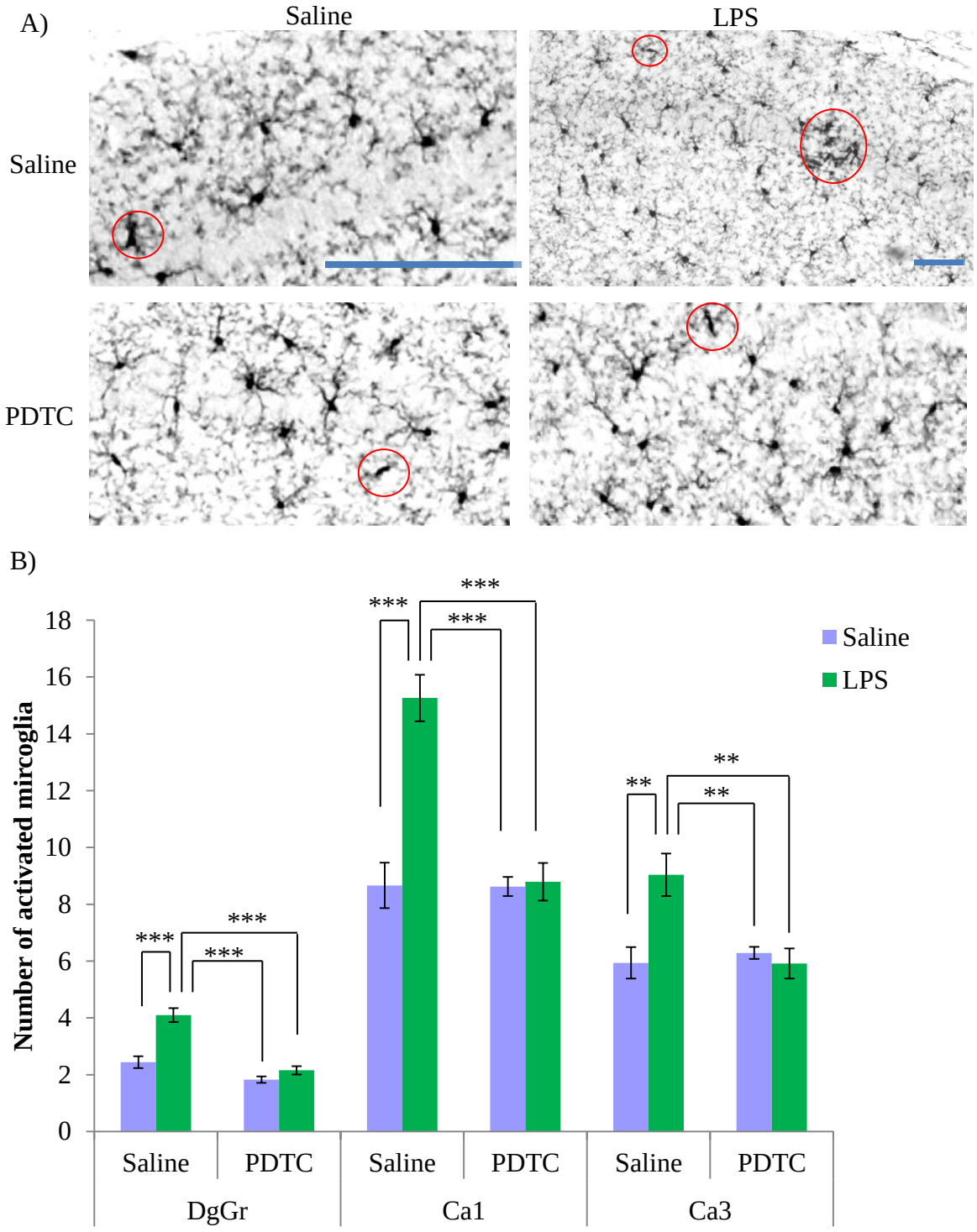



***Figure 4.7 PDTC ameliorates microglial activation in post-septic mice*** *A) Representative microphotographs (x400 magnification) illustrating microglia exhibiting altered cell body morphology (red circles) in the hippocampus. B) Bar graph illustrating the number of activated microglia in the hippocampus of each group. **/*** represent significant post-hoc differences with p<0.01/0.001. Error bars represent ±1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.7 EGR-1*

A main effect of PDTC treatment was present within the granule cell layer of the dentate gyrus ($F_{1,35}$=5.258, p=0.028) and CA3 ($F_{1,35}$=6.291, p=0.017), but not the polymorphic cell layer ($F_{1,35}$=0.265, p=0.61) the CA1 ($F_{1,35}$=1.824, p=0.186) or the CA2 ($F_{1,35}$=0.442, p=0.511). There was also a main effect of LPS treatment in the CA1 region ($F_{1,35}$=26.272, p<0.0005) the CA2 ($F_{1,35}$=09.814, p=0.003) and the CA3 ($F_{1,35}$=20.554, p<0.0005) but the granule cell layer did not reach significance ($F_{1,35}$=3.719, p=0.062) and there was no effect in the polymorphic cell layer ($F_{1,35}$=1.065, p=0.309). There was a significant interaction effect between the two treatments in the granule cell layer ($F_{1,35}$=4.699, p=0.037), the polymorphic cell layer ($F_{1,35}$=4.506, p=0.41), the CA1 ($F_{1,35}$=6.486, p=0.015), the CA2 ($F_{1,35}$=7.003, p=0.012) and the CA3 ($F_{1,35}$=14.516, p=0.001). These results are illustrated in Figure 4.8.

Post-hoc analysis with Tukey's test revealed significant differences in the DG between post-septic mice (n=10) and controls (n=10, p=0.028), post-septic mice and controls pre-treated with PDTC (n=10, p=0.023) and post-septic mice and mice given PDTC followed by LPS (n=9, p=0.018). In CA1 post-septic mice differed from controls (p<0.0005), PDTC treated controls (p<0.0005) and PDTC pre-treated post-septic mice (p=0.048). In CA2 post-septic mice differed from controls (p=0.01) and PDTC pre-treated controls (p=0.047). In CA3 post-septic mice differed from controls (p<0.0005), PDTC pre-treated controls (p<0.0005) and PDTC pre-treated post-septic mice (p=0.001).

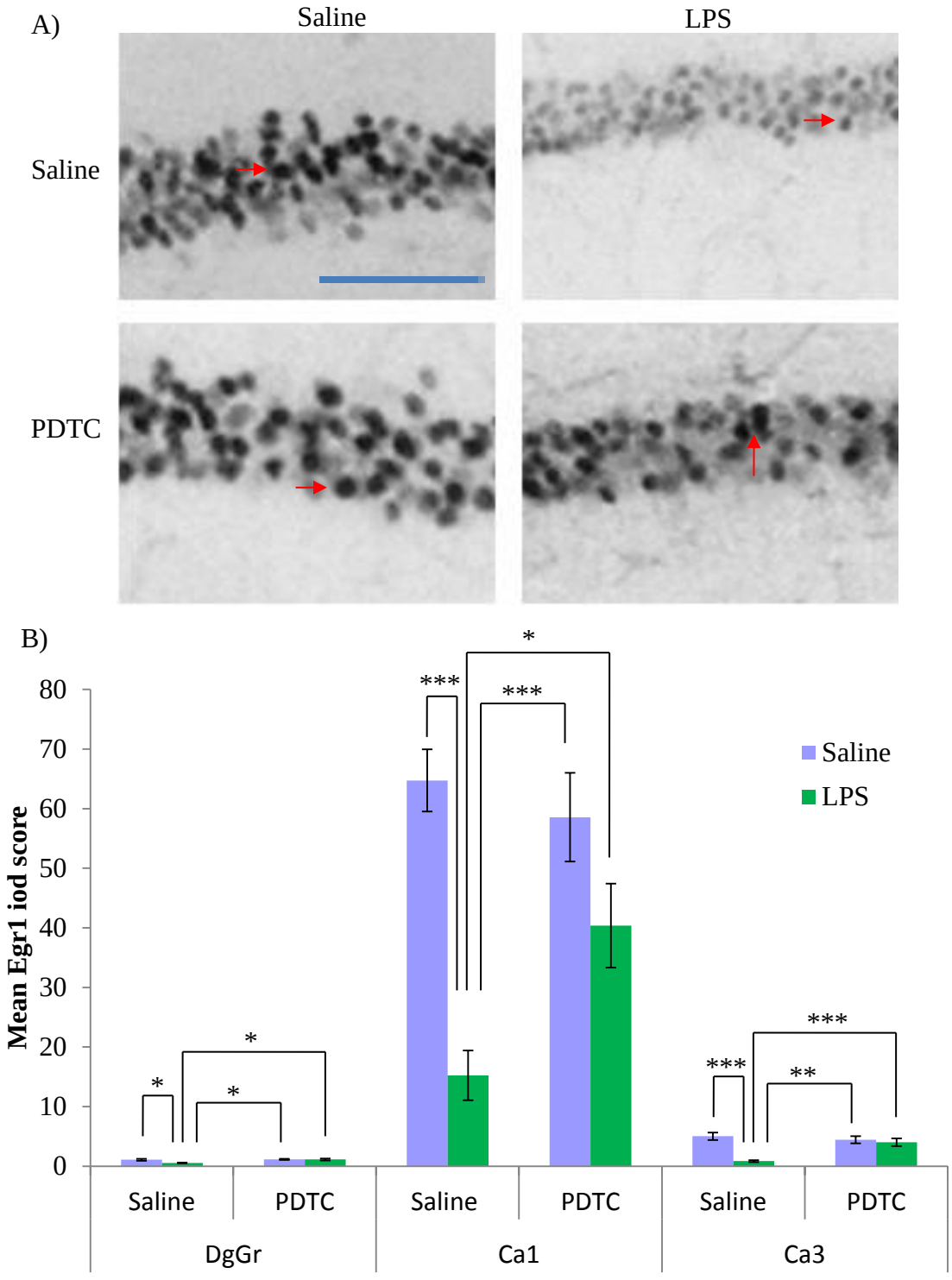



***Figure 4.8 PDTC ameliorates reduced EGR-1 expression in post-septic mice*** *A) Representative micrographs (x400 magnification) illustrating EGR-1 expression in the CA1 region. 4) Bar graph illustrating EGR-1 expression in the hippocampus of each experimental group. */**/*** represents post-hoc differences with p<0.05/0.01/0.001, error bars represent ±1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.8 Amygdala EGR-1 Expression*

Amygdala EGR-1 expression was analysed in both the lateral and basolateral regions. As illustrated in Figure 4.9, a two way ANOVA indicated a main effect of PDTC in the lateral ($F_{1,35}$=18.245, p<0.0005) but not basolateral ($F_{1,35}$=0.887, p=0.353) regions. There was a main effect of sepsis induction in both the lateral ($F_{1,35}$=10.546, p=0.003) and basolateral ($F_{1,35}$=44.395, p<0.0005) regions. A significant interaction effect was not present in the lateral amygdala ($F_{1,35}$=2.266, p=0.141) although it was present in the basolateral amygdala ($F_{1,35}$= 5.068, p=0.141).

Post-hoc analysis with Tukey's test revealed a significant difference in the lateral amygdala between post-septic mice and controls (p=0.009), post-septic mice and PDTC treated controls (p<0.0005) and post-septic mice and those treated with PDTC before LPS (p=0.002). In the basolateral amygdala there were differences between post-septic mice and controls (p<0.0005) and PDTC pre-treated controls (p<0.0005) as well as between mice treated with PDTC before LPS and PDTC pre-treated controls (p=0.02).

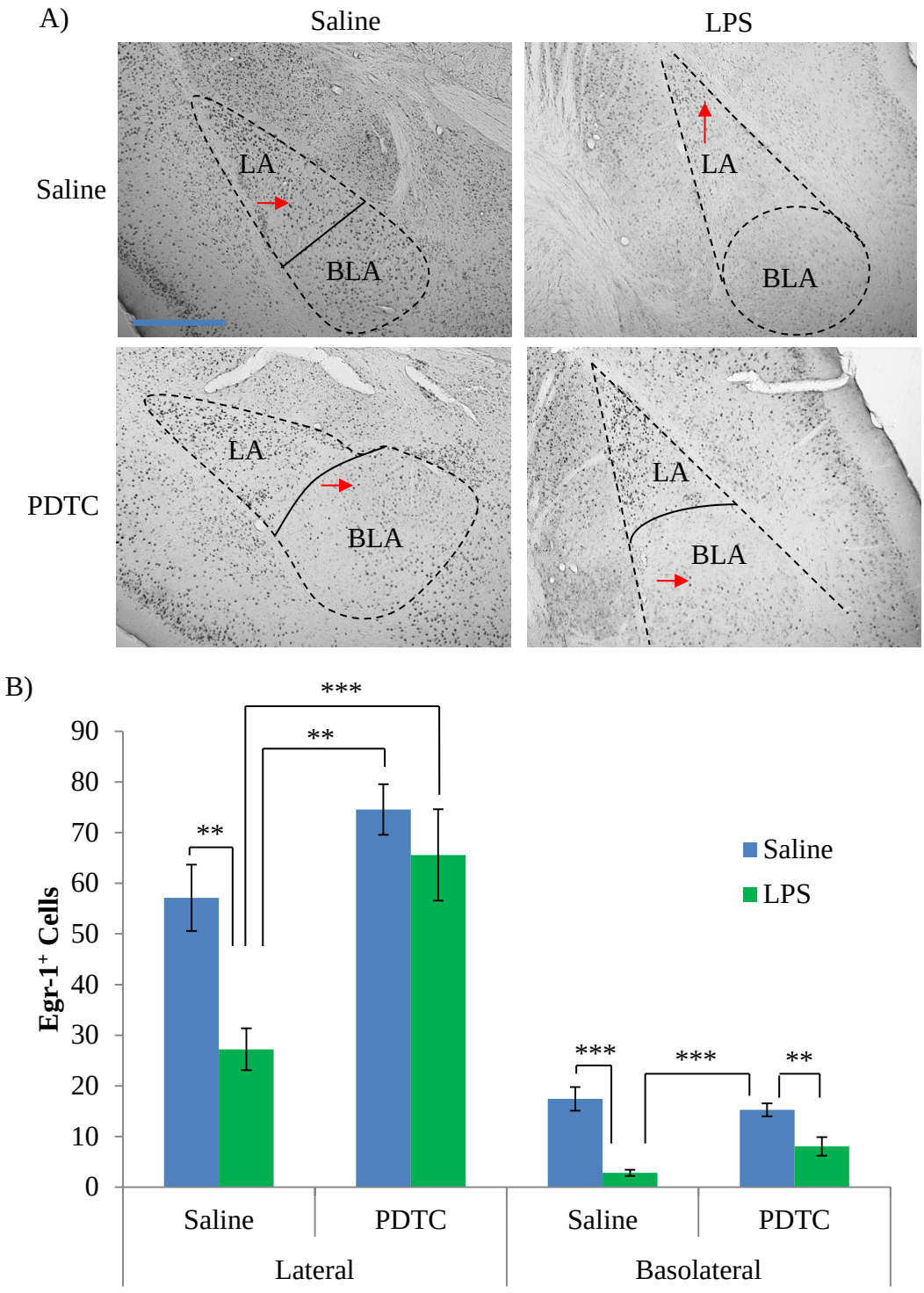



***Figure 4.9 PDTC alters amygdala EGR-1 expression in post-septic mice*** *A) Representative micrographs illustrating Egr-1 expression in the amygdala B) Bar graph illustrating Egr1 expression in the amygdala of each experimental group. */**/*** represent signficicant differences in Tukey post-hoc tests with p<0.05/0.01/0.001 Error bars represent ±1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.3.9 BrdU*

There was no main effect of PDTC treatment on BrdU expression in the hippocampus ($F_{1,35}$=3.273, p=0.079), there was a main effect of LPS treatment ($F_{1,35}$= 7.195, p=0.011) but no interaction effect between the two variables ($F_{1,35}$=0.438, p=0.512). Tukey post-hoc analysis revealed a significant difference only between post septic animals and those treated with PDTC followed by saline (p=0.014). Animals treated with two saline treatments (n=10) had an average of 22.91±2.17 BrdU positive cells, those treated with PDTC followed by saline (n=10) had an average of 25.43±2.92 BrdU positive cells. Post septic animals who received saline followed by LPS (n=10) had an average of 15.59±1.63 BrdU positive cells while those who received PDTC prior to LPS (n=9) had an average of 21±1.7 BrdU positive cells. These results are illustrated in Figure 4.10.

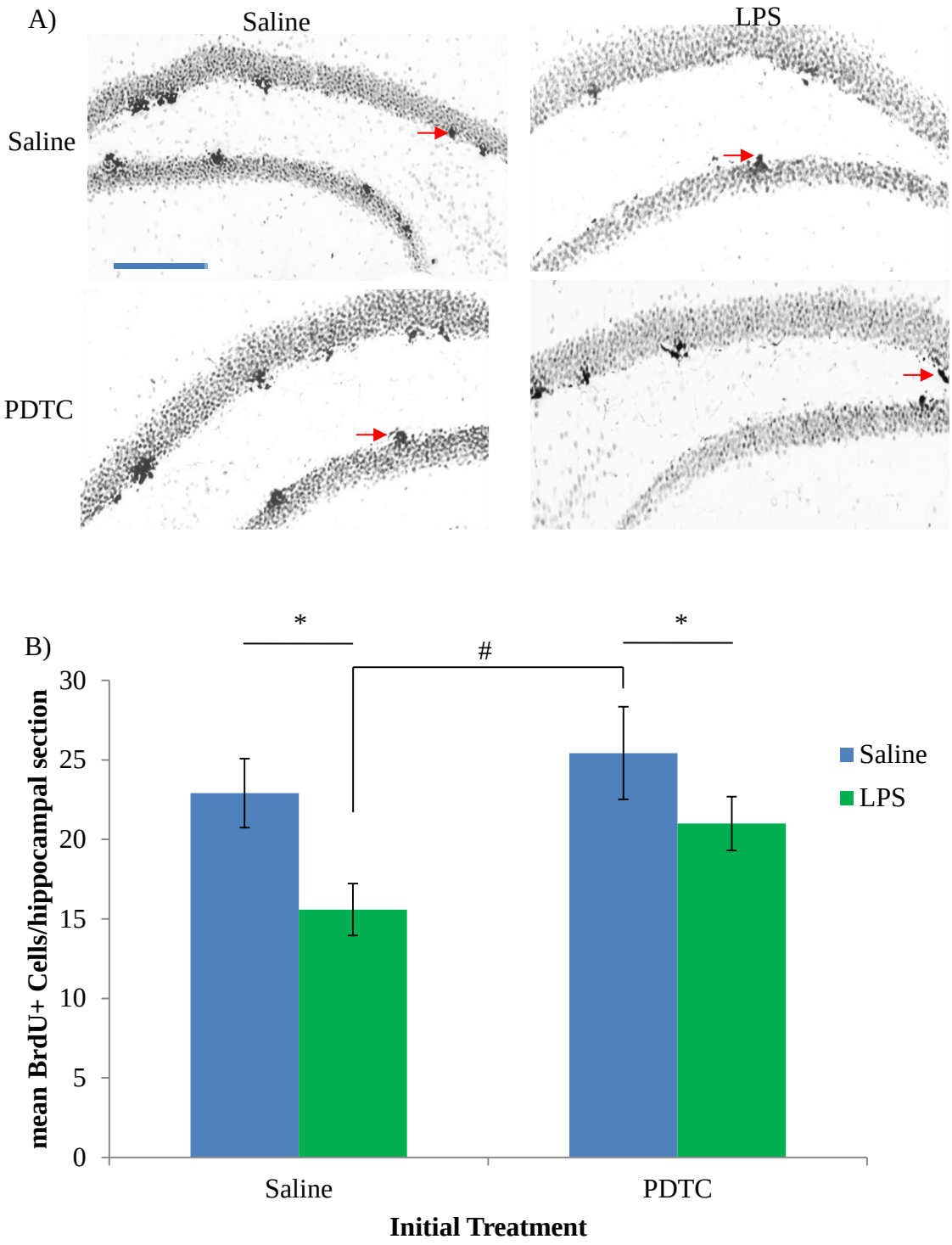



***Figure 4.10 PDTC may not alter BrdU incorporation in post-septic mice*** *A) Representative microphotographs (100x magnification) illustrating BrdU$^+$ cells in the dentate gyrus. B) Bar graph illustrating number of BrdU positive cells in the SGZ of each treatment group. * represents main effect of LPS treatment ($p<0.05$), # represents Tukey post-hoc difference ($p<0.05$), error bars show ±1 SEM. N numbers for each group are as follows – saline/saline (n=10), saline/LPS (n=10), PDTC/saline (n=10), PDTC/LPS (n=9).*

*4.4 Discussion*

These experiments illustrate the effects of PDTC on long lasting behavioural and neuroimmune deficits following LPS-induced sepsis. Although PDTC administration acutely affected mice one to four hours post-treatment it appeared to lessen the severity of sepsis experienced by those also administered LPS by 24 hours and onwards. Importantly, PDTC did not completely block the acute effects of LPS administration, with all animals still clearly exhibiting symptoms at 48 hours post-treatment. The inhibitory effect of PDTC on NF-κB is dose dependent (Liu, Ye, & Malik, 1999; Satoh, et al., 1999) – and as it is neurotoxic at high doses it may be impossible to administer a dose sufficient to completely block the NF-κB signalling present following large doses of LPS. As previously discussed, while LPS elicits potent and rapid inflammatory responses via the MAL-MyD88 pathway NF-κB signalling may also be driven by the later phase TRAM-TRIF pathway. It is therefore also possible that LPS elicited NF-κB signalling via the TRAM-TRIF pathway after PDTC became inactive within the CNS. The activation of other transcription factors apart from NF-κB, such as JNK and p38MAPK, may also play a role here in driving an inflammatory response.

*4.4.1 Behavioural effects of PDTC pre-treatment during LPS-induced sepsis*

As in previous experiments, LPS administration led to long-lasting affective changes in post-septic mice. Compared with mice treated only with saline, post-septic mice showed significantly higher immobility in the tail suspension test, a significant reduction in time spent in the open arms of the elevated plus maze and reduced exploration during the novel object recognition task with an effect on sucrose preference just under significance. Again post-septic mice did not exhibit significantly lower preference for the novel object but rather showed lower overall exploration in the fifth

trial. This constellation of affective changes is again typical of some of the core components of depressive episodes as discussed in previous chapters.

Though PDTC alone induced acute behavioural changes, possibly symptoms of some neurotoxicity, it did not evoke any long-lasting behavioural changes in mice save for possibly increasing novel object preference in the novel object recognition task. It is unclear exactly why pre-treatment with PDTC could have increased this index above control levels. As no other cognitive tests were conducted the effects of PDTC on cognitive behaviour in the present model cannot be assessed. Other results are consistent with the findings of other behavioural studies using control groups treated only with PDTC similarly report it having no effect on behaviour in isolation compared with vehicle treated controls (Sharma, et al., 2011; Song, et al., 2011; Zhang, et al., 2014), even following 7 months of PDTC administration (Malm, et al., 2007)– although some do not include control groups treated only with PDTC (Abd-El-Fattah, et al., 2014; Zhao,et al., 2013; Wang, et al. 2013). It therefore appears that PDTC has no long-lasting affect, adverse or otherwise, in healthy control animals.

In the novel object recognition task, the sucrose preference task and the elevated plus maze post-septic animals who had received pre-treatment with PDTC appeared to show behaviour which was somewhere in between that of post-septic mice and saline-treated controls, while in the tail suspension test immobility levels were on par with non-septic controls. Although PDTC did not completely return behaviour to baseline levels, significant differences between post-septic and non-septic animals in the control group were greatly diminished in the group pre-treated with PDTC. If, as hypothesised, elevated microglial activation plays a role in effecting lost-lasting behavioural changes in post-septic mice then the amelioration of this with PDTC may have led to improvements in affective behaviours. Also of possible relevance is the fact that PDTC

did not fully restore cell proliferation in the subgranular zone of the hippocampus. Neurogenesis is hypothesised to play an important, although not necessarily causative, role in the pathophysiology of affective states (Petrik, Lagace, & Eisch, 2012).

### *4.4.2 Using anti-inflammatories to elicit changes in affective behaviour*

Though it may initially seem surprising that anti-inflammatory agents could elicit a anti-depressive and anxiolytic effects – in fact many currently established treatments for affective disorders have been shown to also have anti-inflammatory components. For example fluoxetine (Liu, et al., 2011), as well as several other SSRIs (Tynan, et al., 2012), attenuate the release of inflammatory molecules in microglia stimulated with LPS *in vitro*. In fact, the commonly used SSRI fluoxetine can increase survival rates in mice pre-treated with the drug before a lethal dose (50mg/kg) of LPS (Roumestan, et al., 2007). Although there is evidence that fluoxetine exerts anti-inflammatory effects by inhibiting NF-κB signalling (Zhang, et al., 2012), others have demonstrated increased NF-κB and inflammatory activity when microglial cells are treated with fluoxetine (Ha, et al., 2006). These findings provide context for the use of potent anti-inflammatory treatments in eliciting anti-depressant effects.

### *4.4.3 The effects of sepsis and PDTC on neuroimmune parameters in the post-septic hippocampus*

Post-septic animals displayed elevated levels of microglial activation relative other groups within the dentate gyrus, CA1 and CA3 areas. These animals displayed a greater number of microglial cells featuring deramified processes and/or highly dense or elongated cell bodies typical of activated microglia (Walker, et al., 2014). This appeared to be ameliorated by the administration of PDTC prior to sepsis induction. PDTC administration alone did not lead to any significant changes relative to saline-treated controls.

Given the multiple pathways through which peripherally administered LPS may affect the CNS, it is possible that PDTC exerted its effects either by inhibiting inflammatory signalling within the periphery leading to less severe CNS effects, by affecting the CNS directly or both. PDTC is known to cross the blood-brain barrier (Chen, Liu, Liang, Lin, & Lin-Shiau, 2000), offering protection against ischemia following intraperitoneal administration (Nurmi, et al., 2004a) and reducing microglial activation even when administered up to six hours after onset of ischemia (Nurmi, et al., 2004b). It is therefore likely that in the present model PDTC is affecting the brain directly during the acute stages of LPS-induced septic encephalopathy. As PDTC is anti-inflammatory, anti-oxidative and in certain conditions neuroprotective independently of either of these properties (Song et al, 2011) it is not possible to delineate from the data collected here its exact mechanism of action in the present model.

Microglia are maintained in an active state by their own release of factors such as reactive oxide species, nitric oxide and cytokines (Lijia, Zhao, Wang, Wu, & Yang, 2012). This state can become harmful if unresolved, as may be happening in the post-septic brain. Given PDTC's anti-oxidant and anti-inflammatory following endotoxin challenge (Németh, Haskó, & Viz, 1998), the reduction in these signalling factors may have led to a return to resting state following the acute effects of sepsis rather than the prolonged activation seen in post-septic animals who did not receive PDTC. Interestingly, in another model PDTC promotes the production of anti-inflammatory cytokine IL-10 within the hippocampus (Abd-El-Fattah, et al., 2014). If present here, increased IL-10 production could drive anti-inflammatory responses and a quiescence of microglial activation (Hu, et al., 1999). The relatively acute timeframe in which PDTC is active within the body (Chabicovsky, et al., 2010) indicates that it most likely

attenuated the rapid upregulation of inflammatory factors involved in LPS-induced sepsis. A timecourse analysis of both the effects of PDTC and LPS on these factors would be particularly interesting here.

In line with previous results, LPS administration led to a significant reduction in EGR-1 expression in the hippocampus, particularly within the Cornus Ammonis region. In the granule cell layer this difference just failed to reach significance, despite the presence of significantly higher levels of activated microglia as indicated by IBA-1 staining. PDTC treatment reverted the expression close to normal levels in post-septic animals while not exerting an effect in non-septic mice. As Egr-1 is a transcription factor with a wide variety of biological functions it is hard to delineate exactly what changes in levels of this IEG may represent. For example, although it is often used as a marker of neuronal activation and plasticity within the brain (Guzowski, Setlow, Wagner, & McGaugh, 2001; James, Conway, & Morris, 2006), in other contexts it functions as a transcription factor involved in cell growth and differentiation, apoptosis and TNF-α activation (Calogero, et al., 2001). PDTC has been shown to increase *egr-1* RNA in HL-60 (acute myloid lukemia) cells (Della Ragione, et al., 2002), but it has not been examined in terms of its effects on neuronal EGR-1 expression. Here, EGR-1 is measured at resting levels rather than in response to any stimuli or learning paradigm. Given EGR-1's rapid induction and degradation (Mokin & Keifer, 2005), and PDTC's terminal elimination half-life of around 20 hours (Chabicovsky, et al., 2010) any effect of PDTC on EGR-1 expression either in post-septic or control animals is likely due to lasting changes within the hippocampus itself.

EGR-1 expression was also decreased within the basolateral and lateral amygdala of post-septic mice – areas highly implicated in anxiety like behaviour (Duncan, Knapp, & Breese, 1996). Previous studies have shown that EGR-1 expression

is induced in the lateral amygdala following footshock training (Malkani & Rosen, 2000) but not due to predator stress (Rosen, Adamec & Thompson, 2005) indicating that Egr-1 in this case may be involved in the learning of an anxiety-inducing task. There is a paucity of data however regarding EGR-1 expression in anxious strains of mice rather than following anxiety-inducing experiences, and to our knowledge there are no other experiments examining the effects of LPS on EGR-1 expression in the amygdala. It is therefore difficult to assess the implications of a reduction in amygdala EGR-1 expression in the present experiment – although interestingly PDTC pre-treatment did protect against the reduction in lateral Egr-1 expression.

As previously outlined, IEGs such as *egr-1* are sometimes suppressed in situations involving prolonged neuroinflammation factors (Bonow et al, 2009; Rosi, 2011; Rosi, et al., 2005). As PDTC ameliorated microglial activation within the hippocampus in the current experiments, increased EGR-1 expression in post-septic animals given PDTC is likely a downstream consequence of this. Normal microglial functioning is essential for proper neuronal activity/plasticity, and in turn behaviour and cognition (Morris, Clark, Zinn, & Vissel, 2013). The upregulation of active microglia in the hippocampus of post-septic animals appears to affect neuronal activity as measured by EGR-1 – an effect which is ameliorated by the suppression of microglial activation via PDTC. This may then be reflected in the return to normal behaviour in post-septic animals who were pre-treated with PDTC. The precise mechanisms underlying these changes are not clear from the present data however.

Post-septic animals in the present study showed a significant reduction in the expression of BrdU within the subgranular zone of the hippocampus – a commonly used marker of neuronal progenitor cells (Lehner, et al., 2011). In the current experiment, utilising a 24 hour pulse with only a single treatment of BrdU, this reduction represents

a decrease in the production of adult-born cells but does not inform us about their eventual differentiation or integration into existing neural networks. Although PDTC attenuated microglial activation in post-septic animals it did not protect against the subsequent decrease in cell proliferation. As previously discussed, neurogenesis is reduced in the presence of activated microglia – and this reduction is implicated in the pathophysiology of mood disorders. One possibility may be that the LPS-induced sepsis was not sufficiently attenuated to affect cell proliferation. Therefore it is somewhat surprising that affective behaviour was ameliorated in the absence of repaired cell proliferation.

Though impaired neurogenesis is strongly implicated in affective disorders – interruption of neurogenesis is not always sufficient to produce depressive-like or anxious-like behaviour in rodents, with stress playing an important role (Petrik, Lagace, & Eisch, 2012). As the affective changes in the present study are not the effect of stress, it is possible that decreases in cell proliferation are simply co-occurring with – but not causative of, these behavioural changes. Although NF-κB signalling itself is a regulator of neurogenesis (Bortolotto, Cuccurazzu, Cononico, & Grilli, 2014; Denis-Donini, et al., 2008), PDTC treatment alone in the current experiment did not affect cell proliferation 2 months post-treatment. It therefore appears that the disruption of NF-κB signalling did not have any long-term negative impact on cell proliferation.

### *4.4.4 Conclusion*

In this experiment it is shown that pre-treatment with NF-κB inhibitor PDTC protects against some of the long last neuroimmune and affective changes present in the post-septic mouse. PDTC pre-treatment reduced the severity of sepsis experienced by animals, attenuated microglial activation and prevented decreases in Egr-1 expression following LPS administration – although it failed to protect against reductions in adult

cell proliferation. Increased depressive-like and anxious-like behaviour in post-septic animals was also significantly improved in animals pre-treated with PDTC despite cell proliferation not returning to baseline levels. PDTC itself did not elicit any long lasting changes in control mice. These results point towards the involvement of the NF-κB pathway in effecting the changes present in post-septic mice and highlight it as a potential therapeutic target.

Though the present experiment both re-affirms the potential of LPS induced sepsis to produce long-lasting behavioural and neuroimmune effects and elucidates a potential role for NF-κB in that pathophysiology of these effects, it also is not without certain limitations. In particular, as no examination of NF-κB levels either at sepsis induction or when animals were sampled was conducted, it cannot definitively said that inhibition of this pathway is what led to these effects. In addition to this, as post-septic encephalopathy is likely a whole brain condition and the present experiment looks only at a specific region, differences present within other areas may be contributing significantly to the behavioural effects seen here. Elucidating the precise role of microglial activation, EGR-1 underexpression and decreased cell proliferation in affecting behaviour is also not possible here meaning the results are descriptive in nature rather than offering a mechanistic explanation.

Given the limitations of the current study it would be useful in the future to look for example at the timecourse involved in LPS-induced septic encephalopathy and how this is affected by PDTC treatment. Also of interest is whether elevated inflammation in the hippocampus may influence NPC cells to differentiate at a higher rate to astrocytes, and whether the survivability of these cells is compromised in the presence of microglial overactivation. Future work should also seek to assess the efficacy of potential therapeutics which can be administered after the onset of sepsis.

# Chapter 5. Investigating the Potential of Fluoxetine in Improving Outcomes in Post-Septic Mice

*Abstract*

Previous chapters demonstrated that LPS-induced sepsis reliably produces long-lasting changes within the affective domain. These behavioural changes are accompanied by significant microglial activation and reduced adult cell proliferation in the hippocampus of post-septic mice. Fluoxetine is a common anti-depressant which also has potent anti-inflammatory effects. It was hypothesised that chronic treatment with fluoxetine could ameliorate the effects of LPS-induced sepsis in mice. 28 days of fluoxetine treatment reduced microglial activation and increased levels of BrdU expressing cells in post-septic mice. It also increase immediate early gene expression in the hippocampus of post-septic and control mice. Fluoxetine improved affective behaviour in the tail suspension test, novel object recognition test and elevated plus maze among post-septic but not control mice. In the sucrose preference test there was no effect of LPS induction and fluoxetine paradoxically lowered preference levels. It is concluded that fluoxetine can reduce the inflammation present in the post-septic hippocampus as well as improve related depressive-like and anxiety-like behaviours.

## 5.1 Introduction

Previous chapters have described the long lasting effects of LPS-induced sepsis on a variety of neuroimmune parameters as well as a constellation of affective behaviours. Excessive microglial activation within the post-septic hippocampus appears to play an important role in these changes. It was shown in the preceding chapter that inhibition of the NF-κB pathway with PDTC could ameliorate most of the long-lasting changes within the post-septic mouse, reducing levels of microglial activation and returning behaviour close to control levels. Unfortunately however, owing to the necessity to administer PDTC before the onset of sepsis and the potential for neurotoxic side effects, it has limited clinical utility. An alternative potential therapeutic was therefore sought which could be administered after the onset of sepsis and which is clinically tolerated. Given that post-septic mice appear to show changes primarily in affective behaviour, as well as excessive microglial activation and inhibited cell proliferation, the selective serotonin uptake inhibitor (SSRI) fluoxetine was chosen as a potential treatment due to its ability to effect change in all three of these parameters.

### *5.1.1 Anti-inflammatory effects of fluoxetine*

Fluoxetine, first approved for use in the 1980s, is now the most commonly prescribed anti-depressant medication (Perez-Caballero, Torres-Sanchez, Bravo, Mico, & Berrocoso, 2014). Early studies showed fluoxetine's efficacy as a selective serotonin re-uptake inhibitor (Wong, Horng, Bymaster, Hauser, & Molloy, 1974), binding to the serotonin transporter and thereby inhibiting the reuptake of serotonin into the presynaptic cell which then leads to elevated levels of extracellular serotonin. This was in contrast to other monoamine uptake inhibitors available at the time which also affected norepinephrine and dopamine levels. The inhibition of serotonin reuptake was initially thought to be fluoxetine's primary mechanism of action. More recently

however it has been shown that there is a significant discrepancy between the time fluoxetine takes to alter brain serotonin levels - with peak inhibition occurring around 4 hours (Perez-Caballero, et al., 2014), and the ~4 weeks it takes to produce an anti-depressive effect in patients (Thompson, 2002). This has led to the investigation of alternative neural mechanisms through which chronic fluoxetine treatment may effect anti-depressant changes.

Of primary relevance to the affective changes in post-septic encephalopathy is fluoxetine's ability to module inflammatory responses. *In vitro* fluoxetine has been shown to attenuate microglial activation and the subsequent production of proinflammatory cytokines and nitric oxide as well as p38 MAPK phosphorylation and NF-κB expression following stimulation of BV2 microglial cells with LPS (Liu, et al., 2011). Similarly, in cultured rat glial cells fluoxetine protects against LPS induced NF-κB activation, iNOS expression as well as elevated TNF-α, COX-2 and IL-1β levels (Lim, et al., 2009). In primary mixed glial cultures from rat pups fluoxetine also dose-dependently inhibited the production of TNF-α and IL-1β while inhibiting the translocation of NF-κB p65 subunit to cell nuclei (Obuchowicz, Bielecka, Paul-Samojedny, Pudełko, & Kowalski, 2014). Interestingly, fluoxetine here also prevented morphological changes associated with LPS administration in glial cultures. In rat neuronglia cultures fluoxetine protects against microglial activation following LPS administration and subsequent neurotoxicity (Zhang, et al., 2012). In BV-2 microglial cells which are unstimulated by LPS however fluoxetine may actually increase NF-κB DNA binding and subsequent TNF-α and IL-6 expression (Ha, et al., 2006).

Several *in vivo* studies have also demonstrated an anti-inflammatory and neuroprotective role for fluoxetine. In one particularly relevant study researchers administered various doses of fluoxetine either preventatively or curatively to BALB/c

mice who then received a lethal (50mg/kg) dose of LPS. Although fluoxetine administered preventatively significantly increased mortality this effect did not occur when the drug was administered after sepsis was induced (Roumestan, et al., 2007). Interestingly, the authors also showed that fluoxetine could also inhibit TNF-α production in human monocytes – the primary sources of TNF-α production during septic shock. In a model of Parkonsinosian symptoms, rats administered intranigral LPS injections following 18 doses of fluoxetine (5 or 10mg/kg) show significantly greater numbers of dopaminergic neurons and inhibited microglial activation compared with controls receiving LPS but not fluoxetine (Chung, et al., 2010). In another model of Parkinson's disease fluoxetine administration attenuated dopamine neuron death, microglial activation and ensuing pro-inflammatory cytokine and reactive oxygen species production following MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) administration (Chung, et al., 2011). In BALB/c mice acute fluoxetine dose dependently increased serum IL-10 and decreased serum TNF-α (Ohgi, Futamura, Kikuchi, & Hashimoto, 2013).

While fewer studies have been done regarding the anti-inflammatory and neuroprotective effects of chronic fluoxetine treatments, 28 days of fluoxetine administration (5mg/kg, i.p.) led to an anti-inflammatory profile with reduced IFN-γ IL-6, CXCL1, CX3CL1 expression in the hypothalamus accompanied by an increase in the anti-inflammatory cytokine IL-4, but not IL-10 (Alboni, Benatti, Montanari, Tascedda, & Brunello, 2013). Although these studies shed light on the anti-inflammatory effects of fluoxetine in various cell lines as well as animal models of inflammatory disorders – the effects of chronic treatments with fluoxetine on inflammatory parameters within the hippocampus is still relatively unclear.

*5.1.2 Anti-depressive effects of anti-inflammatories – problems when they combine?*

While it could be suspected that anti-inflammatory and anti-depressant medications might work synergistically, however the results of investigations into this have been mixed. Apart from increasing the risk of gastrointestinal problems (Mort, Aparasu, & Baer, 2012), serotonin syndrome (Hersh, Pinto, & Moore, 2007) when used in conjunction certain NSAID and anti-depressant combinations have been shown to lead to poorer remission rates and anti-depressant effects. One particularly important study demonstrated that the administration of ibuprofen significantly inhibited the antidepressant effect of fluoxetine, in addition to tricyclic and MAOI antidepressants, in a number of behavioural models as well as attenuating the effectiveness of SSRI citalopram in humans (Warner-Schmidt, Vanover, Chen, Marshall, & Greengard, 2011). There is also some evidence that fluoxetine may impair the anti-inflammatory abilities of NSAIDs indomethacin and celecoxib in a rat paw oedema model (Abdel-Salam, Baiuomy, & Arbid, 2004).

The COX-2 selective NSAID celoxib has also been shown to work synergistically with the atypical anti-depressant bupropion in an inflammatory model of depression (Maciel, Silva, Morrone, Calixto, & Campos, 2013), as well as enhance serotenergic output when used in conjunction with fluoxetine (Johansson, Falk, Marcus, & Svensson, 2012) and reboxetine with celecoxib actually showed significantly more efficacy in ameliorating depressive symptoms in patients with major depression than reboxetine alone (Müller, et al., 2006). Interestingly, despite inhibiting the effects SSRIs, ibuprofen did not affect the antidepressant potency of bupropion in a number of behavioural models (Warner-Schmidt, Vanover, Chen, Marshall, & Greengard, 2011). While NSAIDs typically exert their effects acting within the periphery and spinal cord – rofecoxib, another COX-2 specific drug, may act through the central serotenergic

system. This drug does not inhibit the anti-inflammatory effect of fluoxetine to the same level as other NSAIDs (Abdel-Salam, et al., 2004).

Another possible explanation put forward is that it is not the NSAIDs *per se* that impair response to anti-depressants, but rather the disease which the NSAIDs are treating (Gallagher, et al., 2012; Shelton, 2012). As comorbidites are associated with lower remission rates in depression (Otte, 2008), it may be that those patients required to take an NSAID concomitantly with anti-depressants are at a greater risk in general for poor treatment outcomes. This does not however explain the differing effects of various anti-depressant and NSAID combinations in animal models of depressive-like behaviour, although each of these models are different from each other, and different from the human condition of affective disorders.

### *5.1.3 Inflammation in Affective Disorders*

Several studies have looked at levels of inflammatory cytokines in depressed patients. For example, before treatment with fluoxetine a sample of 20 depressed patients showed higher serum levels of the inflammatory cytokines IL-1β, IL-6, IFNγ and TNFα as well as serotonin transporter 5-HTT than healthy-matched controls. Following three months of fluoxetine treatment both IFNγ and 5-HTT levels were significantly reduced – although only 8 participants returned for the second analysis (Tsao, Lin, Chen, Bai, & Wu, 2006). Other studies have also found elevated levels of plasma IL-6 (Maes, et al., 1995), IL-1β (Diniz, Teixeira, Talib, Gattaz, & Forlenza, 2010) and TNF-α (Lanquillon, Krieg, Bening-Abu-Shach, & Vedder, 2000). Interestingly some studies have shown that certain cytokines may be predictive of or associated with particular symptoms of affective discords. For example there is an association between TNF-α and dissociative symptoms in major depression patients while IL-1β can correlate with the severity of depression and increased suicidality

(Bizik, et al., 2014; Lichtblau, Schmidt, Schumann, Kirkby, & Himmerich, 2013; Thomas, et al., 2005). Aside from plasma cytokine levels, post-mortem studies in suicide victims have shown elevated levels of pro-inflammatory cytokines such as IL-6, TNFα and IL-1β in prefrontal cortex (Pandey, et al., 2012) compared with matched controls.

### *5.1.4 Neurogenesis and BDNF in Sepsis/Inflammatory Disorders*

Though neurogenesis and BDNF levels are both well studied in affective disorders, research examining these two factors in models of sepsis and post-septic encephalopathy is scarce. One study showed that CLP actually led to an increase in neurogenesis within the SVZ, but no change in the SGZ, 24 hours post sepsis-induction (Bakirci, Kafa, Uysal, & Kurt, 2011). A sub-septic, although still large, peripheral 1mg/kg dose of LPS has been shown to lead to a decrease in newborn neurons 7 days later, accompanied by significant microglial activation (Monje, Toda, & Palmer, 2003). The administration of *E. Coli* to early postnatal rats (P4) led to an acute decrease in BrdU positive cells as well as a sustained reduction in BrdU+NeuN positive cells throughout the hippocampus suggesting that this systemic infection both reduced NPC proliferation and the survivability of newborn neurons. A 5mg/kg dose of LPS administered to female C57BL/6 mice led to significant reductions in the levels of newbord neurons (DCX + NeuN positve), but not NPC proliferation, at 7 days following LPS treatment as well as significant reductions at 28 days accompanied by a net reduction in neurogenesis (Omerod, et al., 2013).

BDNF levels have been shown to significantly decrease in the hippocampus of rats submitted to CLP at 48 hours but not 12 or 24 hours (Comim, et al., 2014). In two further studies by the same group BDNF levels are not significantly lower in CLP rats than sham operated rats at 10 days post-operation, despite persistent memory deficits

(Cassol-Jr, et al., 2011) but were significantly lower at 17 days post-treatment, although this effect was reversed by subchronic (14 day) imipramine treatment (Comim, et al., 2010). Other studies have shown that reduced BDNF expression is present in the hippocmapus of CLP survivors up to one month after CLP occurs (Biff, et al., 2013; Wu, et al., 2013). Interestingly, in patients admitted to hospital with sepsis, lower plasma BDNF is actually associated with mortality (Ritter, et al., 2012).

### *5.1.5 Aims*

The aim of this experiment was therefore to examine whether the long-lasting effective changes in post-septic animals could be ameliorated by chronic treatment with the SSRI fluoxetine. It was hypothesised that fluoxetine treatment could reduce microglial activation in the hippocampus following post-septic encephalopathy similarly to its anti-inflammatory effects seen in other models. As neurogenesis is thought to be important for the behavioural effects of fluoxetine it was examined whether the drug could upregulate cell proliferation in this model.

## *5.2 Materials and Methods*

### *5.2.1 Animals*

For the purpose of all experiments male C57BL/6 mice (Charles River, Kent, UK) aged between 8 and 16 weeks were used. Animals were group housed in a 12:12 light:dark cycle for 2 weeks prior to LPS administration. Food and water were available *ad libitum* and temperature was 21 ±1°C and humidity was 50 ±10%. Animals remained housed in groups of 2-4 in polypropylene cages (33cm long x 15 cm wide x 13cm high) with wood chip bedding and environmental enrichment (shredded paper and cardboard tubes) except during the sucrose preference test which necessitates single housing. All procedures were approved by the Research Ethics Committee, National University of Ireland Maynooth, and were licensed by the Department of Health and Children, Ireland under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU.

### *5.2.2 Drug treatments*

All injections were prepared fresh on the treatment day, and given intraperitoneally in a final injection volume of 0.1 – 0.15ml. 0.9% sterile saline was made up fresh for control injections, while lipopolysaccharide (serotype 0111.B4, Sigma Ireland; Qin et al, 2007) was made up to a 5mg/kg dose in sterile saline. Fluoxetine (10mg/kg, Tocris Bioscience, UK) was administered orally in standard drinking water beginning one week after LPS administration. Fresh fluoxetine was given every second day and animals and water were weighed to ensure the correct dosage was maintained. Animals were given fluoxetine for 28 days before testing began and throughout the duration of behavioural testing. To assess adult cell proliferation in the hippocampus mice which had one month previously undergone sepsis-induction

were injected i.p. with 50mg/kg BrdU (Sigma Ireland) and transcardially perfused 24 hours later.

*5.2.3 Behavioural testing*

Five weeks after the administration of LPS animals were tested the novel object recognition task followed by the elevated plus maze and then the tail suspension test. They were then singly housed and two days later underwent the sucrose preference test. All behavioural tests were carried out as previously described in Chapter 2.

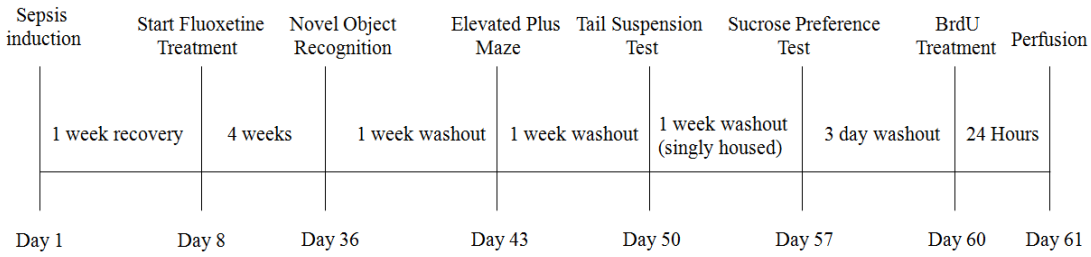



**Figure 5.1 Timeline of fluoxetine experiments.**

*5.2.4 Immunohistochemical analysis*

One week after the sucrose preference test mice were anaesthetised with 0.1ml sodium pentobarbital (Euthatal, Merial Animal Health, UK), following which they were perfused transcardially with 0.9% saline, half the brain was then placed in 4% paraformaldehyde (Sigma Ireland) in 0.1M phosphate buffer (PB), pH 7.4 at 4˚C. Brains were then cryoprotected in 0.1M PB containing 30% sucrose, frozen and sectioned coronally at 30µm. Immunohistochemistry for IBA-1, Egr-1 and BrdU was then carried out following a standard Avidin-Biotin Complex/Nickel DAB colourmetric protocol as previously described in Chapter 3.

*5.2.5 Statistical Analysis*

All data were analysed in SPSS version 20 (IBM) with the type of test used indicated for each data set. All data are presented as means ± standard error of the mean (SEM). * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$.Where data violated statistical assumptions correcte values are presented. Scale bars on microphotographs represent 100µm for photos at 40x and 100x magnification and 50µm for photos at 400x magnification.

*5.3 Results*

*5.3.1 Tail Suspension Test*

Illustrated in Figure 5.2, a two-way between groups ANOVA with sepsis and fluoxetine as independent variables revealed a main effect of sepsis induction in the tail suspension test $F_{1,37}$=31.848, p<0.0005. A main effect of fluoxetine administration was also present $F_{1,37}$=13.624, p=0.001 as well as an interaction effect between the two variables $F_{1,37}$=36.61, p<0.0005. Post-hoc analysis via Tukey test revealed a significant difference between untreated post-septic animals and the three other groups (p<0.0005) but no other significant differences.

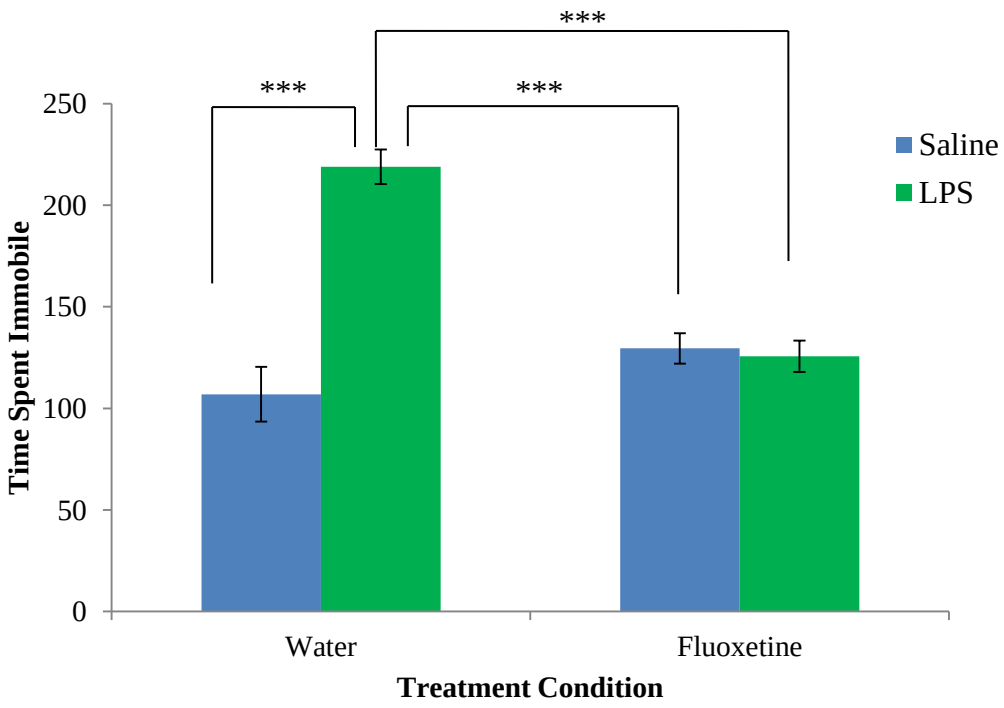



***Figure 5.2 Fluoxetine treatment attenuates behavioural despair in post-septic mice.*** *Bar graph illustrating mean time spent immobile in the tail suspension test for each group. Error bars represent 1x SEM, *** represent significant post-hoc differences at p<0.001. SEM. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.2 Sucrose Preference Test*

A two-way ANOVA with sepsis induction and fluoxetine treatment as between groups variables revealed no main effect of sepsis induction on sucrose preference $F_{1,37}$=1.738, p=0.196, illustrated in Figure 5.3. There was a main effect of fluoxetine administration $F_{1,37}$=4.603, p=0.039 with no interaction effect between the two variables $F_{1,37}$=.811, p=0.374. Post-hoc tests showed no differences between any groups.

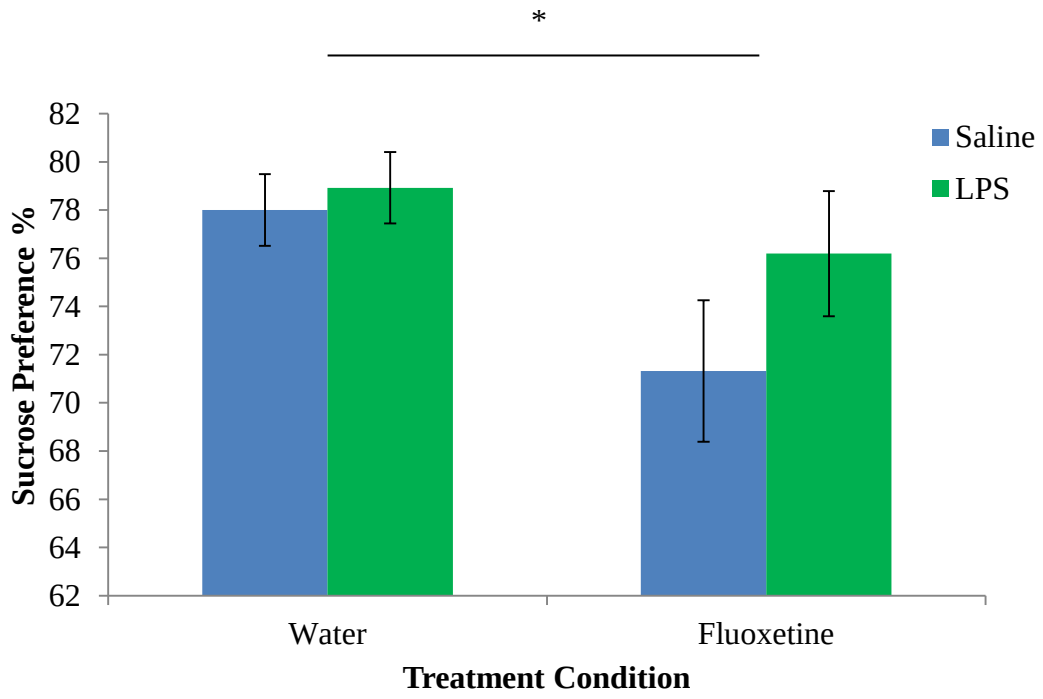



***Figure 5.3 Reduced sucrose preference in fluoxetine administered mice*** *Bar graph illustrating sucrose preference % in each group. Error bars represent 1x SEM and * represents a main effect of fluoxetine administration (p<0.05). Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.3 Elevated Plus Maze*

As shown in Figure 5.4, a two-way between groups ANOVAs for sepsis induction and fluoxetine treatment were carried out to investigate results in the elevated plus maze. There was a main effect of sepsis induction on time spent in the open arms ($F_{1,37}$=7.303, p=0.01) with a trend towards a main effect on number of entries to open arms ($F_{1,37}$=3.829, p=0.058) but not closed entries ($F_{1,37}$=3.315, p=0.077) or time in closed arms ($F_{1,37}$=0.716, p=0.321). There was a main effect of fluoxetine on closed arm entries ($F_{1,37}$=5.103, p=0.03) but not open entries ($F_{1,37}$=1.861, p=0.181), time in open arms ($F_{1,37}$=0.163, p=0.689) or closed arms ($F_{1,37}$=1.01, p=0.953). An interaction effect was not present for either open arm ($F_{1,37}$=1.486, p=0.231), closed arm entries ($F_{1,37}$=0.008, p=0.929), time in open arms ($F_{1,37}$=1.883, p=0.178) or closed arms ($F_{1,37}$=0.003, p=0.953).

Post-hoc analysis revealed that there was a significant difference in time spent in the open arms between untreated post-septic mice (n=11) and untreated controls (p=0.029) while the other groups were not significantly different (n=10 per group).

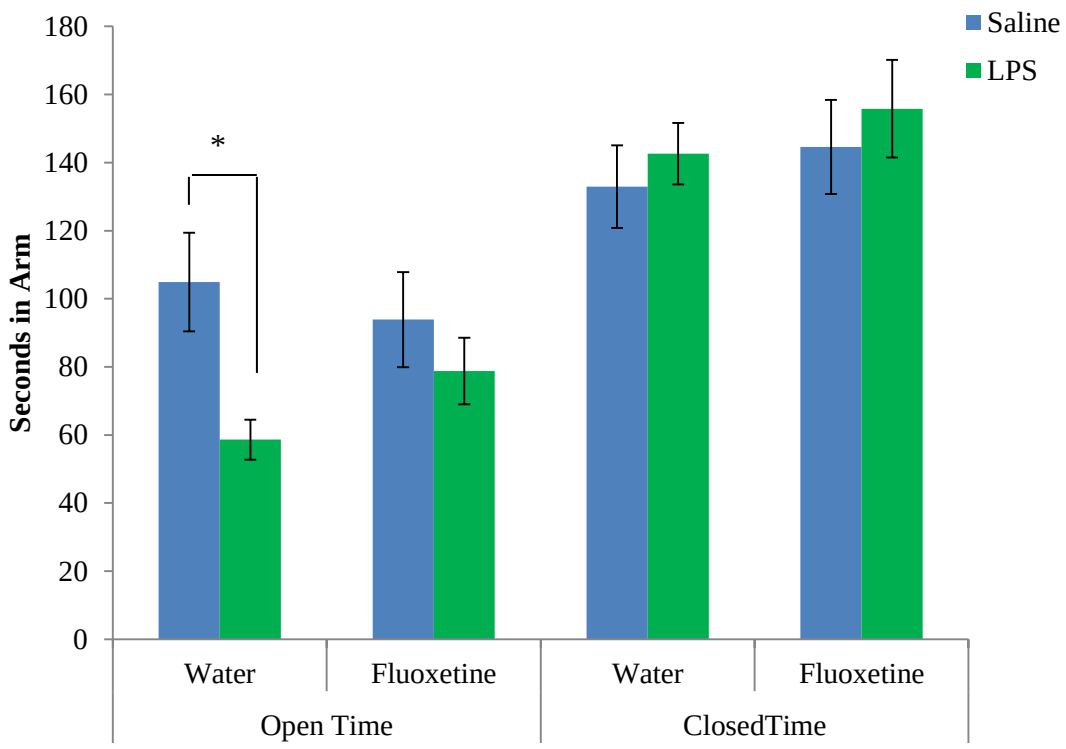



***Figure 5.4 Fluoxetine treatment increases elevated plus maze exploration in post-septic mice.*** *Bar graph illustrating time spent in each arm during the elevated plus maze. Error bars represent 1x SEM and * represents significant post-hoc difference $p<0.05$. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.4 Novel Object Recognition*

A three way mixed between within ANOVA with sepsis induction and fluoxetine administration as between groups variables and trial number as a within groups variable revealed a significant effect of trial number ($F_{3,111}$=42.72, p<0.05), but not sepsis induction ($F_{1,37}$=0.511, p=0.479) although there appeared to be a main effect of fluoxetine administration ($F_{1,37}$=6.485, p=0.015). There were no significant interaction effects present (p>0.05 in all cases). These effects are illustrated in Figure 5.5.

There was no main effect of sepsis induction ($F_{1,37}$=2.601, p=0.115), fluoxetine treatment ($F_{1,37}$=, p=0.278) or interaction effect ($F_{1,37}$=3.681, p=0.063) for percentage preference on time exploring the novel object. For total time exploring the novel object there was a significant sepsis x fluoxetine interaction ($F_{1,37}$=11.985, p=0.001), but not main effect of sepsis ($F_{1,37}$=2.627, p=0.114) or fluoxetine ($F_{1,37}$=0.266, p=0.609).

As illustrated in Figure 5.6 post-hoc analysis with Tukey tests showed that untreated post-septic mice (n=11) had significantly lower time spent exploring in the fifth trial than both untreated controls (n=10, p=0.003) and fluoxetine treated post-septic mice (n=10, p=0.04).

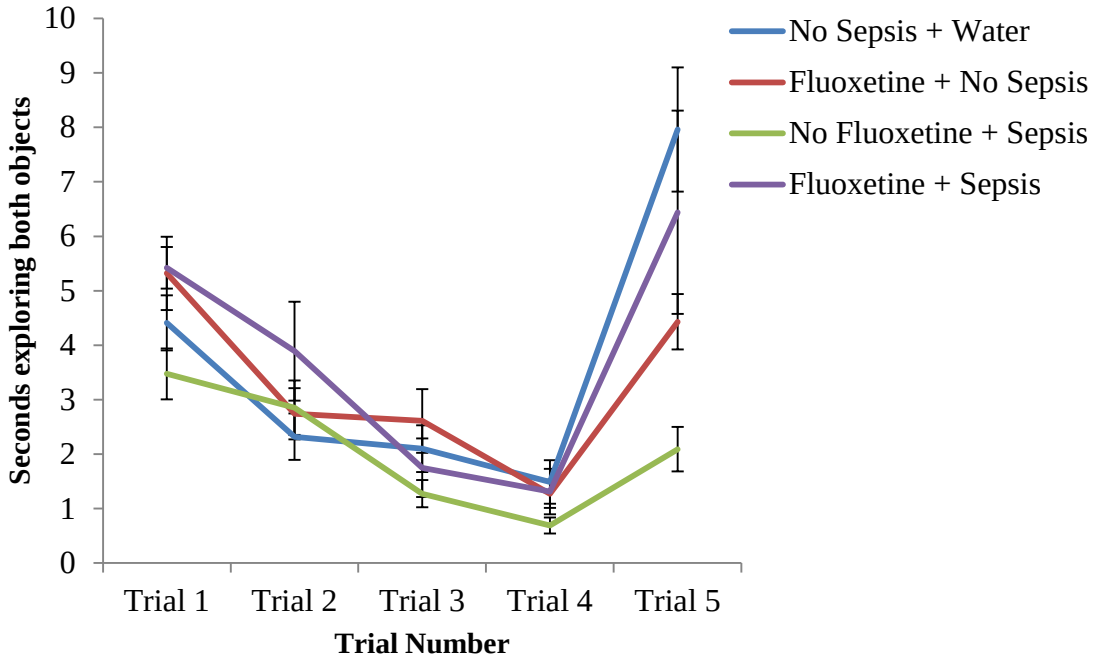



***Figure 5.5 Object explorations across all five trials.*** *Line graph displaying habituation to both objects over the five trials of the novel object recognition task.*

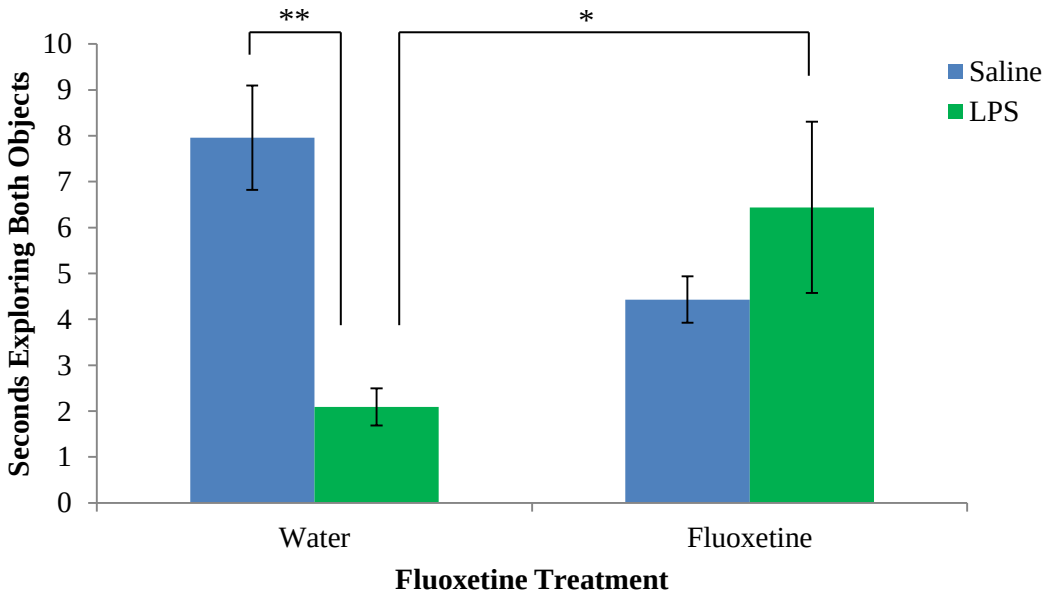



***Figure 5.6 Fluoxetine treatment promotes object exploration in post-septic mice****. Time spent exploring both objects in the fifth trial. Error bars represent 1xSEM, */** denotes p<0.05/0.01 in post-hoc analysis. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.5 EGR-1*

Two-way mixed between within ANOVA were conducted in order to analyse the effects of sepsis and fluoxetine administration on EGR-1 expression throughout the hippocampus. Illustrated in Figure 5.7, there was no main effect of sepsis induction in the dentate gyrus granule cell layer ($F_{1,37}$=0.215, p=0.645), polymorphic cell layer (($F_{1,37}$=1.549, p=0.221), CA1 ($F_{1,37}$=3.33, p=0.076), CA2 ($F_{1,37}$=0.065, p=0.801) or CA3 ($F_{1,37}$=1.824, p=0.185). There was a main effect of fluoxetine in all areas of the hippocampus – granule cell layer ($F_{1,37}$=30.2, p<0.0005), polymorphic cell layer ($F_{1,37}$=24.061, p<0.0005), CA1 ($F_{1,37}$=22.018, p=0.0005), CA2 ($F_{1,37}$=24.475, p<0.0005) and CA3 ($F_{1,37}$=19.344, p<0.0005). A sepsis x fluoxetine interaction was present within the CA1 region ($F_{1,37}$=6.301, p=0.017), the CA2 ($F_{1,37}$=4.405, p=0.043) and the CA3 ($F_{1,37}$=6.128, p=0.018).

Post-hoc analysis with Tukey tests revealed that in the granular layer untreated post-septic mice were significantly different to post-septic mice treated with fluoxetine (p<0.0005) and controls treated with fluoxetine (p<0.001), while controls treated with fluoxetine were also significantly different to untreated controls (p<0.016). Similarly in the polymorphic cell layer untreated post-septic mice were significantly different to post-septic mice treated with fluoxetine (p<0.001) and controls treated with fluoxetine (p<0.0005), while controls treated with fluoxetine were also significantly different to untreated controls (p<0.043). In CA1 untreated post-septic mice were significantly different to controls (p=0.018), post-septic mice treated with fluoxetine (p<0.0005) and controls treated with fluoxetine (p<0.0005). In CA2 untreated post-septic mice were siginificantly difference to fluoxetine treated post-septic mice (p<0.0005) and fluoxetine treated controls (p=0.004). In CA3 untreated post-septic mice were significantly

different to controls ($p=0.045$), post-septic mice given fluoxetine ($p<0.0005$) and fluoxetine treated controls ($p=0.001$).

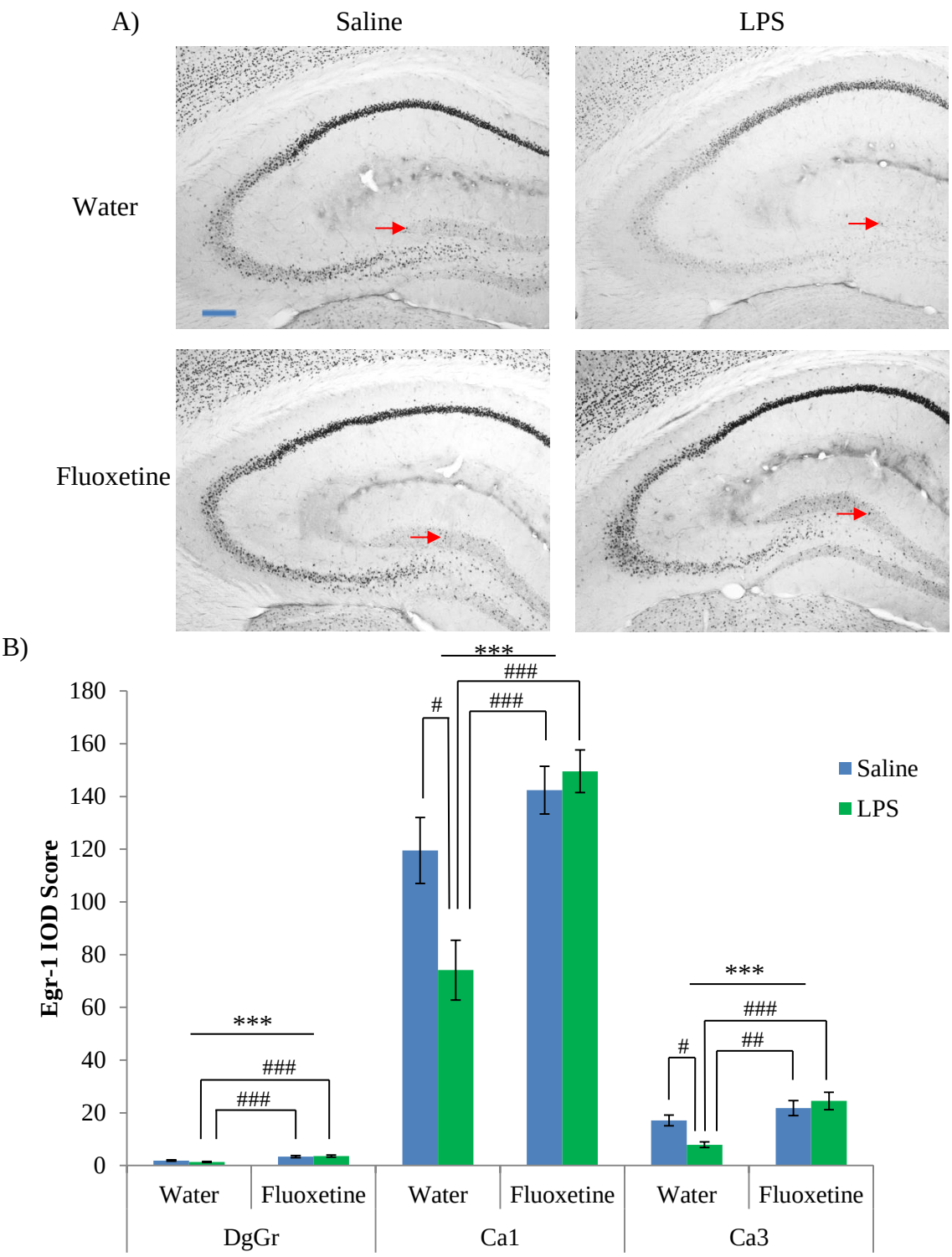



***Figure 5.7 Fluoxetine treatment increases EGR-1 expression in post-septic and control mice*** *A) Representative micrographs (40x magnification) illustrating EGR-1 expression. B) Bar graph illustrating EGR-1 expression in the hippocampus of each experimental group. */**/*** represents main effects, #/##/### represent post-hoc differences with p<0.05/0.01/0.001. Error bars represent ±1 SEM. SEM. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.6 IBA-1*

A two-way between groups ANOVA was conducted to analyse the effects of sepsis induction and fluoxetine treatment on IBA-1 expression in the hippocampus. There was a main effect of sepsis in the granule cell layer ($F_{1,37}$=9.766, p=0.003), the polymorphic cell layer ($F_{1,37}$=9.701, p=0.004) the CA1 ($F_{1,37}$=79.237, p<0.0005), CA2 ($F_{1,37}$=5.802, p=0.021) and CA3 ($F_{1,37}$=54.479, p<0.0005). There was also a main effect of fluoxetine in the granule cell layer ($F_{1,37}$=62.289, p<0.0005), the polymorphic cell layer ($F_{1,37}$=29.636, p<0.0005), the CA1 ($F_{1,37}$=83.439, p<0.0005), the CA2 ($F_{1,37}$=17.538, p<0.0005) and the CA3 ($F_{1,37}$=39.518, p<0.0005). There was an interaction effect between the two variables in the granule cell layer ($F_{1,37}$=10.128, p=0.003), the polymorphic cell layer ($F_{1,37}$=22.116, p<0.0005), the CA1 ($F_{1,37}$=58.318, p<0.0005) and the CA3 ($F_{1,37}$=25.015, p<0.0005) but not the CA2 ($F_{1,37}$=2.602, p=0.115).

Post-hoc analysis with Tukey tests revealed that in the granular layer untreated post-septic animals (n=11) were significantly different from the three other groups (n=10 per group, p<0.0005). The same differences were present in the polymorphic cell layer and the CA1 (p<0.0005). In CA2 untreated post-septic mice had significantly more microglia than untreated controls (p=0.032), fluoxetine treated controls (p<0.0005) and fluoxetine treated post-septic mice (p<0.0005). In CA3 untreated post-septic mice were significantly different to the three other groups again (p<0.0005). These differences are illustrated in Figure 5.8.

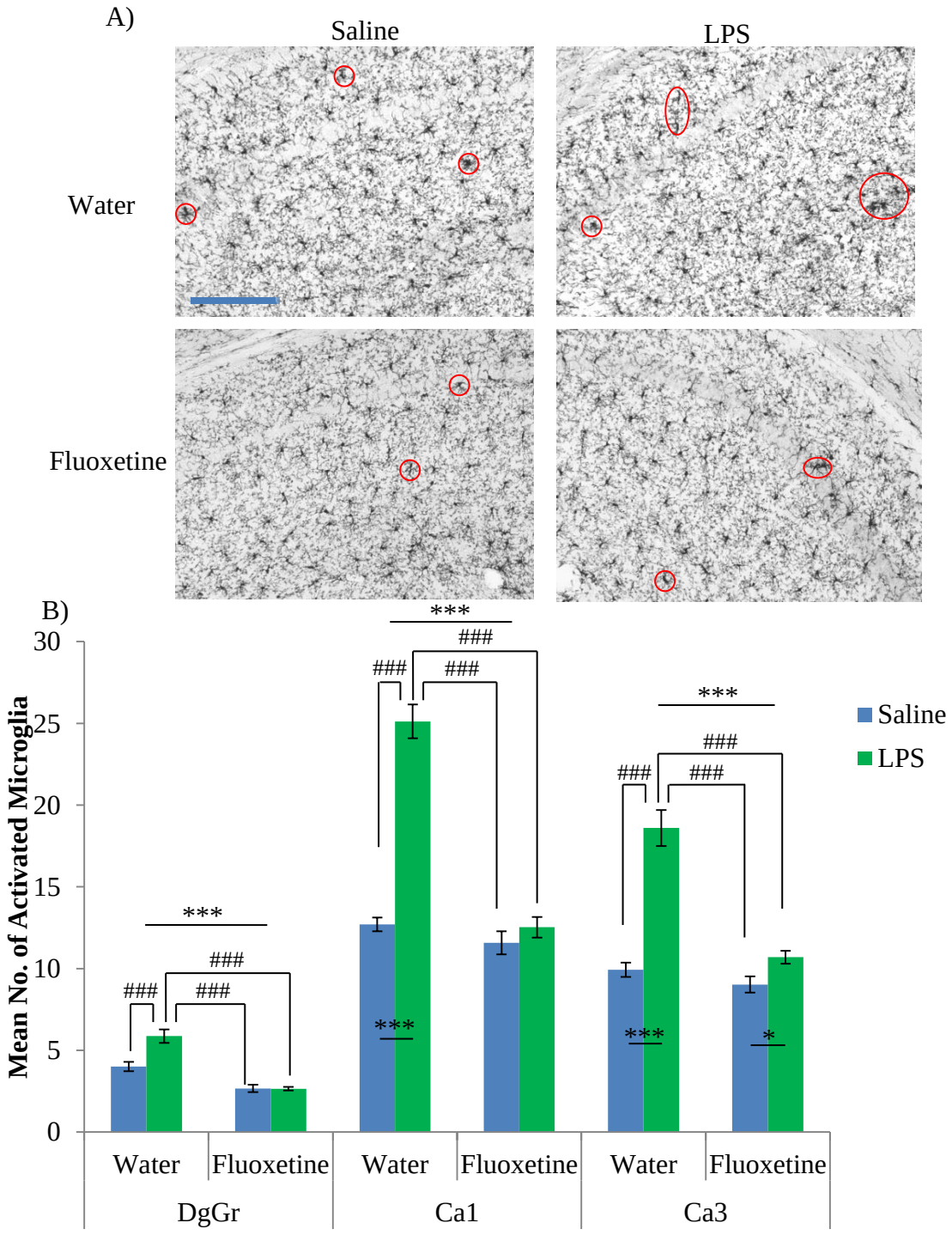



***Figure 5.8 Fluoxetine treatment reduces microglial activation in post-septic mice*** *A) Representative micrographs (100x magnification) illustrating IBA1 expression in the CA1 region. Red circles highlight microglia with activated morphology B) Bar graph illustrating hippocampal IBA1 expression. */**/*** represents main effects, #/##/### show post-hoc differences at p<0.05/0.01/0.001. Error bars represent ±1 SEM. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.3.7 BrdU*

A two way between groups ANOVA was conducted in order to assess the effects of sepsis induction and fluoxetine treatment on BrdU levels within the subgranular zone of the dentate gyrus. Illustrated in Figure 5.9, there was a main effect of sepsis induction ($F_{1,37}$=29.774, p<0.0005), fluoxetine treatment ($F_{1,37}$=6.89, p=0.013) and an interaction between the two variables ($F_{1,37}$=15.288, p<0.0005). Post-hoc analysis with a Tukey test revealed that there was a difference in BrdU expression between untreated post-septic animals and each of the other three groups (p<0.0005) but no other significant differences.

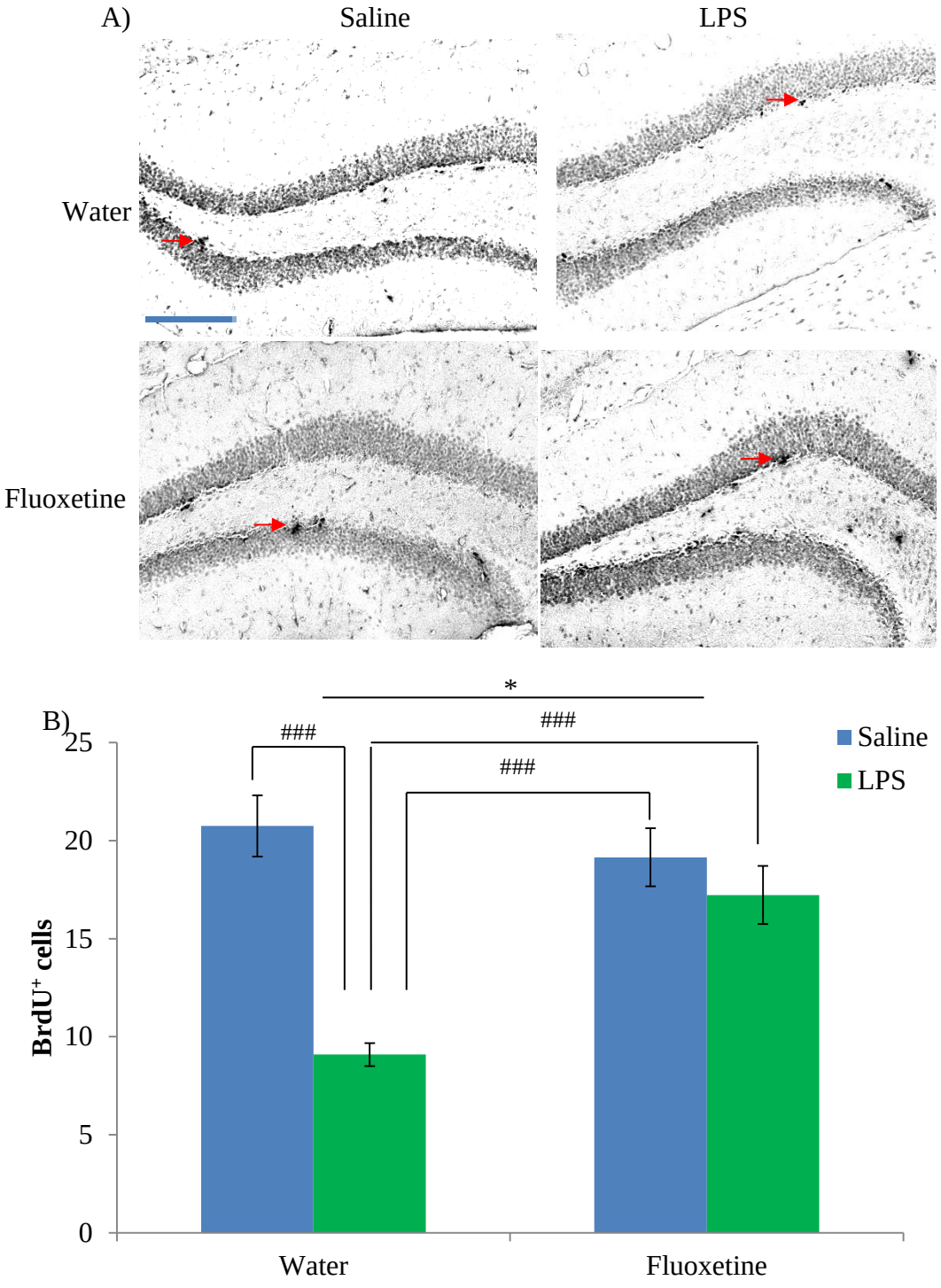



***Figure 5.9 Fluoxetine promotes cell proliferation in post-septic mice.*** *A) Representative micrographs (100x magnification) illustrating BrdU expression in the SGZ. B) Bar graph illustrating BrdU$^+$ cells in the SGZ each experimental group. * represents main effect, ### represents post-hoc differences $p<0.05/0.001$. Error bars represent ±1 SEM. Saline/Water n=10, Sepsis/Water n=11, Saline/FLX n=10, Sepsis/FLX n=10.*

*5.4 Discussion*

In previous chapters it has been shown that LPS induces long lasting affective changes which can be ameliorated with the pre-administration of anti-inflammatory agents. These results demonstrate that these behavioural deficits and the long-lasting neuroimmune changes which co-occur with them can be ameliorated by chronic treatment with the SSRI fluoxetine. Affective changes were evidenced in post-septic animals' behaviour in the tail suspension test, elevated plus maze and novel object recognition test as had been seen in previous cohorts of animals. These behavioural changes occurred in conjunction with excessive microglial activation, altered IEG levels and cell proliferation as previously seen. Although fluoxetine has previously been used in other models of inflammatory depressive states, this is the first time that it has been used to treat the long-lasting behavioural deficits associated with LPS-induced sepsis.

*5.4.1 Fluoxetine Treatment Rescues Affective Behaviour in Post-Septic Mice*

The tail suspension test is a well validated model of depressive-like behavioural despair. Although not previously used in models of sepsis associated encephalopathy, chronic fluoxetine has previously been shown to successfully reduce immobility times in a number of other inflammatory models of depressive-like symptoms – swiss albino mice submitted to chronic unpredictable mild stress (Dhingra & Bhankher, 2014), neonatal immune activation in NMRI mice (Doosti, et al., 2013) and acute LPS administration to CD-1 mice (Ji, et al., 2014). Fluoxetine in this model did not appear to affect immobility time in control mice. This is in line with some other experiments which show that non-depressed animals do not show reduced immobility time following fluoxetine administration (Doosti, et al., 2013; Wang, et al., 2011). Others however have shown reductions in immobility time even in control animals (Mutlu, et al., 2012; Wang, et al., 2013). Whether or not fluoxetine affects immobility in

control animals is therefore likely to vary according to both strain and dose (Tang, et al., 2014). Using a genetic model of helplessness, mice specifically bred for their high or low scores in the tail suspension test, it was shown that chronic fluoxetine administration elicited anti-depressant effects in the helpless mice while not affecting behaviour in the non-helpless mice (Naudon, Yacoubi, Vaugeois, Leroux-Nicollet, & Costentin, 2002). Therefore in this context it appears that fluoxetine is effective in attenuating immobility time in the post-septic mice, without affecting behaviour in non-septic animals.

Anxiety-like responses were examined in the elevated plus maze. As before, post-septic animals explored open arms of the maze significantly less than non-septic controls, while this difference was not present among animals who had been treated with fluoxetine. Although anxiety disorders are highly comorbid with major depressive disorder (Kessler, et al., 2003), and fluoxetine has demonstrated anxiolytic effects in a variety of populations (Clark, et al., 2005; Faramarzi, et al., 2008; Shu, Huang, Fones Soon Leng, Strijckmans Crutel, & Kim, 2014) – as with the tail suspension test, a variety of effects have been reported in the elevated plus maze. Chronic and subchronic treatments with 20mg/kg fluoxetine has been shown to increase mice's time spent in the open arms of the maze (Nollet, et al., 2012; Kurt, Arik, & Celik, 2000) and produce modest increases in the same measure among chronically stressed rats (Lapmanee, Charoenphandhu, & Charoenphandhu, 2013) while other groups have showed no effect of chronic fluoxetine on behaviour in the EPM (Griebel, Cohen, Perrault, & Sanger, 1999; Robert, Drapier, Bentué-Ferre, Renault, & Reymann, 2011; Silva & Brandão, 2000). Anxiogenic effects of fluoxetine have also been observed in the elevated plus maze following chronic treatments (Silva, Alves, & Santarem, 1999; Duran, et al., 1999).

Given the variety of models used to assess anxiety-like behaviour, and the different biological underpinnings of each of these models, it is not surprising that fluoxetine has shown conflicting effects across the literature. As with the tail suspension test, fluoxetine may differentially effect EPM behaviour depending on whether the animal in question is showing anxiety-like behaviour or not. For example, neonatal LPS administration leads to decreased time spent in the open arms and decreased open arm entries – both of which are dose-dependently ameliorated with fluoxetine (Majidi-Zolbanin, Azarfarin, Samadi, Enayati, & Salari, 2013). While in the same experiment certain doses of fluoxetine were actually anxiogenic in nature in control animals. In the present work, prolonged microglial activation is likely to be responsible, at least in part, for increased anxiety like behaviour in post-septic mice. It therefore follows that attenuating this microglial activation also heLPS to ameliorate the anxiety behaviour that co-occurs with it. If anxiety-like behaviour is the result of another aetiology which is not affected by fluoxetine however responses may differ in that context.

In the novel object task similar results were observed to those in previous chapters among the control animals submitted to sepsis. These animals did show preference for the novel object, but lower overall exploration – an effect which has been examined in the context of decreased motivation. This effect was attenuated in those animals treated with fluoxetine. For the first time, in those not treated with fluoxetine, there was actually a small effect of sepsis induction present on preference for the novel object. As there can be much variability between animals in behavioural testing (Lewejohann, Zipser, & Sachser, 2011), as well as some variation in the severity of sepsis suffered by each animals this cohort may have been more susceptible to a mild cognitive effect than previous cohorts.

As the novel object recognition task is primarily used as a measure of motivation here, it is difficult to compare it with other studies using fluoxetine in the same test. There is some evidence that chronic fluoxetine treatment can actually reduce preference for the novel object in similar NOR tasks (Carlini, et al., 2012; Valluzzi & Chan, 2007) although significantly different methodology was employed. Other studies have shown that fluoxetine has no effect in control animals, and can actually rescue performance in experimental models showing impaired cognitive/affective performance (LeGates, et al., 2012; Tellez, Rocha, Castillo, & Meneses, 2010). Here an effect of fluoxetine on object preference was not seen, but rather an amelioration of the decrease in overall exploration appeared present in post-septic animals. This suggests that fluoxetine can be useful in restoring motivated behaviours and exploration in post-septic animals – in line with its other beneficial effects on various affective constructs.

Surprisingly, in this experiment LPS-induced sepsis did not bring about any long-lasting changes to sucrose preference. In fact, there was actually a small effect of fluoxetine in the opposite direction – suggesting that here fluoxetine administration actually reduced sucrose preference levels. No group however had preference scores below 65% which is the often used percentage point for defining "anhedonia" (Briones, et al., 2012). This is particularly surprising given that fluoxetine has previously been reported to increase sucrose preference in a number of depressive models such as chronic unpredictable mild stress (Dhingra & Bhankher, 2014) and repeated LPS administration (Kubera, et al., 2013), There have been other reports of chronic fluoxetine leading to decreased sucrose preference in some other models however. For example, Sprague Dawley rats given 10mg/kg chronic fluoxetine showed significantly decreased preference compared with controls (Brenes & Fornaguera, 2009) and chronic fluoxetine (7.5mg/kg) also failed to restore 1% sucrose consumption in a model of

interferon-α induced anhedonia (Sammut, Bethus, Goodall, & Muscat, 2002). It remains difficult to understand why LPS failed to alter sucrose preference as in previous chapters. As with the novel object presence it may be simply differences in individual cohorts' responses to LPS-induced sepsis.

*5.4.2 Fluoxetine has Anti-Inflammatory Effects in Post-Septic Mice*

Fluoxetine had a significant effect on microglial activation within the hippocampus. Chronic treatment led to an attenuation of microglial activation throughout the hippocampus – in line with other studies discussing fluoxetine's anti-inflammatory properties. Although fluoxetine failed to protect against mortality when administered after lethal doses of LPS (Roumestan, et al., 2007), these results indicate that it has utility in attenuating the long-lasting glial activation present in post-septic animals. These results are also important because the anti-inflammatory properties of fluoxetine are typically examined in the context of acute treatments, whereas here its potential to modulate inflammation over a chronic administration period is illustrated. This is in line with Alboni et al (2013) who showed that a 5mg/kg dose of fluoxetine reduced a broad range of inflammatory markers and led to an anti-inflammatory cytokine expression profile.

Chronic fluoxetine administration has previously been shown to promote anti-inflammatory mRNA expression, particularly IL-4, while inhibiting pro-inflammatory signalling in rodents (Alboni, et al., 2013). As the IL-4 is upregulated by fluoxetine, and this in turn stimulates CD200 expression – an important deactivating signaller for microglia (Lyons, et al., 2007), this could represent one potential pathway through which fluoxetine inhibits microglial activation. In the present model such an effect may have contributed towards quiessing microglial activation following LPS-induced sepsis. Subchronic fluoxetine has also been shown to reduce microglial production of NADPH,

iNOS and ROS which attenuate oxidative stress associated with microglial activation (Chung, et al., 2010). In vitro studies also suggest that fluoxetine may inhibit microglial activation via mechanisms such as NF-κB, COX-2 and pro-inflammatory cytokine suppression (Lim, et al., 2009; Liu, et al., 2011; Obuchowicz, et al., 2014). Future work is needed however to ascertain whether the effects of fluoxetine when chronically administered may differ from its properties during *in vitro* application.

Post-septic animals showed reduced EGR-1 expression within the CA1 region and CA3 while fluoxetine treatment led to a robust increase in EGR-1 expression in both saline-treated and LPS-treated animals throughout the hippocampus. EGR-1 expression can be modulated by BDNF via its downstream target ERK (Warren, et al., 2011). Acute fluoxetine administration has previously been shown to lead to increased *egr-1* expression in the cingulated and motor cortex (Van Waes, Beverley, Marinelli, & Steiner, 2010) as well as the central amygdale (Slattery, et al., 2005) with no effect in the hippocampus (Humblot, Thiriet, Gobaille, Aunis, & Zwiller, 1998). While hypothalamic *egr-1* transcription appears to be affected by chronic fluoxetine (Conti, et al., 2007), the effects of chronic fluoxetine administration on hippocampal EGR-1 expression have not previously been examined. These results also highlight once again the potential for regional differences to occur within the hippocampus following post-septic encephalopathy with significant change present only within certain areas.

The serotonergic system has been shown to be involved in inducing e*gr-1* expression, with lesions of the serotonin system inhibiting cocaine induced expression (Bhat, Cole, & Baraban, 1992). Fluoxetine also potentiates methylphenidate-induced *egr-1* upregulation (Van Waes, Beverley, Marinelli, & Steiner, 2010), with serotonin possibly promoting increased transcription driven by dopaminergic signalling. As EGR-1 here is measured at baseline levels and not in response to a stimulus it is unclear

whether such a synergistic effect could be taking place here. Another possibility is that elevated EGR-1 expression represents increased neuronal activity in the hippocampus due to increased neuronal proliferation and the incorporation of these cells into hippocampal networks. Although fluoxetine did not lead to increased proliferation in non-septic controls it may have affected survival or differentiation which are not measured here.

### *5.4.3 Fluoxetine Promotes Adult Cell Proliferation in Post-Septic Mice*

In contrast with PDTC treatment, fluoxetine administration restored cell proliferation following LPS-induced sepsis in the present experiment. Fluoxetine by itself did not appear to stimulate increased cell proliferation in control animals. This is in contrast to a number of other studies in rodents which have shown that fluoxetine can increase NPC proliferation even in control animals (Malberg, Eisch, Nestler, & Duman, 2000; Nollet, et al., 2012; Wang, 2011). Others however have reported no change using BrdU as a marker of cell proliferation in non-depressed animals (Huang, Bannerman, & Flint, 2008; Marlatt, Lucassen, & van Praag, 2010; Tyler, 2014). Some of these differences are likely to stem from significant differences in protocols used to assess NPC proliferation with BrdU. In the present experiment a single BrdU injection 24 hours before sampling labels. While this informs us that the rate of generation of cells is decreased in the post-septic hippocampus during a 24 hour period it does not inform us about their differentiation, survival, migration or incorporation into hippocampal networks. Many of the other studies showing increases in proliferation following fluoxetine treatment used either repeated administrations of BrdU and/or much longer time points between administration and sampling. They therefore mark a wider range of newborn cells, and so fluoxetine's other effects – such as promoting survivability, may explain increased numbers of cells incorporating BrdU over longer periods of time. As

with the behavioural effects of fluoxetine there is also considerable strain differences in its neurogenic effects. Miller and colleagues (2008) for example showed that fluoxetine significantly increased proliferation in DBA/2J and 129S1 mice – but not A/J or SWR/J mice.

Though fluoxetine has not been examined in the context of LPS induced sepsis it has been shown to be effective in restoring proliferation in another depressive model involving long lasting inflammation resulting from prenatal LPS exposure (Lin & Wang, 2014). The effect of fluoxetine in the present experiment may be attributable either to its direct promotion of cell proliferation, or to its attenuation of microglial suppression of proliferation. Fluoxetine upregulates levels of extracellular serotonin which in turn leads to increased NPC proliferation (Encinas, Vaahtokari, & Enikolopov, 2006; Ohira & Miyakawa, 2011). Activated microglia on the other hand disrupt hippocampal neurogenesis during inflammatory states (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003; Monje, et al,. 2003). This is in contrast to microglia which have polarised towards the M2 anti-inflammatory phenotype and may actually promote neurogenesis (Ekdahl, Kokaia, & Lindvall, 2009; Vukovic, Colditz, Blackmore, Ruitenberg, & Bartlett, 2012). If fluoxetine either reduces microglial activation, or polarises microglia towards a neuroprotective phenotype – for example by increasing IL-4 production, then this may be one potential mechanism through which it restores proliferation in the post-septic brain.

### *5.4.4 Conclusion*

In conclusion, it is clear that when fluoxetine is administered after sepsis induction it can help to improve many of the effects associated with post-septic encephalopathy. Although the mechanisms by which fluoxetine exerts its anti-depressant effects are not yet well understood – in this instance it successfully

ameliorates ongoing microglial activation in the hippocampus and also promotes cell proliferation. As BDNF appears to be a key factor associated with both affective and inflammatory disorders it would be interesting to examine the levels of this neurotrophin in post-septic animals and its modulation by fluoxetine. These results, in conjunction with those of previous chapters, also illustrate the potential for variation in the symptoms experienced by post-septic animals – particularly in the case of behavioural effects. There is much potential for further investigation to be done regarding the link between fluoxetine, inflammation and affective behaviours in post-septic encephalopathy. For example, little is known about changes key monoamine neurotransmitters such as serotonin in post-septic encephalopathy, and although changes to cell proiferation are present, no information is available regarding the survival and integration of these newborn cells in the hippocampus.

# Chapter 6. Are Microglia Primed in the Post-Septic Hippocampus?

*Abstract*

Microglia are known to survey and adapt to their environment, changing their morphology and function as needed. The results in chapter three revealed that microglia appeared to take on an activated morphology in the hippocampus subsequent to LPS administration and that this persists for up to two months. These microglia did not however appear to be over-producing pro-inflammatory cytokines. As such it was suspected that LPS–induced sepsis may result in microglia assuming a primed phenotype. These microglia would exhibit exaggerated responses to a subsequent inflammatory response – particularly in terms of IL-1β expression. It was also hypothesised that behavioural responses to a low dose of LPS might also differ between post-septic and control mice.

To test this sepsis was induced in mice as in prior experiments. After their recovery these mice were then tested for behavioural responses to a 100µg/kg dose of LPS, and brains were collected to examine markers of microglial activation at 4 hours post-treatment, as well as IL-1β expression and IEG activation at 2 and 9 hours post-treatment. Post-septic and control mice showed no differences in behavioural responses to the acute low dose LPS in the open field and tail suspension tests. Immunohistochemistry revealed that prior sepsis induction did increase mice's responses to a second dose of LPS in terms of microglial activation (F4/80, CD11b) and IEG expression (EGR-1, c-Fos) but not IL-1β expression. This effect was time and region specific. It is suggested that, as IL-1β expression was not elevated, these differences may be due to increased underlying inflammation in post-septic mice rather than the presence of primed microglia.

## *6.1 Introduction*

### *6.1.1 Microglia Phenotypes in the CNS*

As previously described in chapter one, microglia may rapidly change their morphology and phenotype in response to a variety of signals within their microenvironment. Microglia in a resting (or ramified) state remain relatively immobile, but survey their surroundings via a large number of highly motile processes, completing a comprehensive screening of the entire brain parenchyma every couple of hours (Nimmerjahn, Kirchhoff, & Helmchen, 2005). These resting microglia are in direct contact with neurons, astrocytes and blood vessels (Nimmerjahn et al, 2005). A variety of signals can cause resting microglia to undergo morpho-functional change (Walker, et al., 2014). While pro-inflammatory factors influence microglial phenotype, inducing a shift from the ramified state to a deramified active state as previously discussed, several other CNS factors can also induce changes in microglial state. Microglia extend their processes in reaction to adenosine triphosphate (ATP) released from injured cells (Ohsawa, et al., 2010), microglial ramification is also induced by the presence of astrocytes *in vitro* (Wilms, Hartmann, & Sievers, 1997). In contrast, components of the extracellular matrix such as fibronectin and vitronectin can contribute to the deramification of resting microglia (Milner & Campbell, 2003).

Though microglia in the healthy adult brain are typically neuroprotective and support neuronal functioning (Streit, 2002), they have the potential to switch to deleterious, neurotoxic phenotypes in chronic inflammatory states. Apart from shifting between resting and activated phenotypes, microglia may also assume a "primed" phenotype during stress (Munhoz, et al., 2006), aging (Henry, Huang, Wynne, & Godbout, 2009) or during inflammatory CNS diseases (Cunningham, 2012). These primed microglia are thought to exhibit an exaggerated response to subsequent immune

challenges, including an increased expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, increased IκBα expression as well as elevated production of iNOS and neuronal cell death (Cunningham, Wilcockson, Campion, Lunnon, & Perry, 2005; Henry et al, 2009; Weber, Frank, Sobesky, Watkins, & Maier, 2013). Morphologically, primed microglia share similarities with activated microglia (deramification), although they do not express heightened levels of pro-inflammatory cytokines in the absence of immune challenges (Choi & Won, 2011; Perry & Teeling, 2013). Their detection therefore relies on the examination of their response to secondary immune challenges.

*6.1.2 LPS-Induced Microglial Priming*

Though priming can occur following a number of neuroinflammatory stimuli or conditions - LPS has been used in several experimental paradigms to study microglial priming both *in vivo* and *in vitro*. Typically, animals or macrophages which have either been treated with a large dose of LPS, are aged or are undergoing inflammatory diseases are given secondary, low doses of LPS or other immunostimulatory agents which elicit a more severe pro-inflammatory response than that seen in models which have not been primed (Perry & Teeling, 2013; Pestka & Zhou, 2006). Enhanced IL-1β production is the most typical marker of microglial priming, although IL-6 and TNFα production are also heightened (Islam & Pestka, 2006; Wynne, Henry, & Godbout, 2009). Recently it has also been reported that super low dose (0.5μg/kg) LPS priming can lead to significantly elevated mortality and TNFα production in mice later subjected to an extremely high dose (25mg/kg) of LPS (Deng, Maitra, Morris, & Li, 2013)

The exaggerated inflammatory response exhibited by primed animals in response to subsequent immune activation is accompanied by a significantly altered behavioural and cognitive response. Aged mice subjected to a 0.33mg/kg dose of LPS

express significantly higher hippocampal levels of IL-1β transcription, IL-6 and IL-1β mRNA accompanied by an extended decrease in social behaviour and food intake than that seen in adult mice (Godbout, et al., 2005). The same dose has also been shown to increase depressive-like behaviours in the forced swim test and tail suspension test along with increased plasma IL-6, Indoleamine 2,3-dioxygenase (IDO) production and serotonin turnover rate compared with adult mice (Godbout, et al., 2007). Cognition is also impaired in this model with older mice showing deficits in a hippocampus-dependent learning task along with exaggerated Il-1β, IL-6 and TNFα mRNA production in hippocampal neuronal cell layers (Chen, et al., 2008). Microglial priming has also been shown to be involved in producing delirium-like cognitive deficits in mice modelling prion disease (ME7 mice) subsequently administered a 100μg/kg dose of LPS (Murray, et al., 2012).

As of yet there have been few studies into the precise mechanism which switches microglia to a primed phenotype. Excessive activation of the alternative complement system has been suggested as one cause of microglial priming – with mice knocked out for *Crry* (a key regulatory gene for the complement system) exhibiting chronic complement component 3 overactivation along with primed microglia in the CNS which over-express pro-inflammatory cytokines and iNOS in response to LPS challenge (Ramaglia, et al., 2012). It has also been suggested that super-low doses LPS may prime microglia by reducing levels of RelB - an NF-κB component which can suppress pro-inflammatory signalling (Deng, Maitra, Morris, & Li, 2013).

### *6.1.3 Neuroprotective Effects of LPS-Activated Microglia*

Though microglia primed by serious inflammatory insult can assume a primed phenotype, LPS-activated microglia may also play a protective role during subsequent injury or pathology. This may occur when relatively mild harmful stimuli (such as low

doses of LPS) precede a more harmful secondary insult – typically hypoxia/ischaemia or traumatic brain injury, and has been termed "preconditioning" (Shpargel, et al., 2008). For example, in as study by Chen et al (2012) pre-treatment for four days with 1mg/kg LPS i.p. induced microglial activation but protected mice from neuronal cell death following experimental brain injury with microglia ensheathing neurons in the CNS. Pre-treatment with LPS has also been shown to significantly attenuate cytokine production and apoptosis, as well as improve survival rates in mice subsequently subjected to septic peritonitis (Feterowski, Weighardt, Emmanuilidis, Hartung, & Holzmann, 2001). Aside from experimental brain injury models, LPS preconditioning has also been noted to attenuate cerebral ischemic damage in a number of models (Ahmed, et al., 2000; Dawson, Furuya, Gotoh, Nakao, & Hallenbeck, 1999; Tasaki, et al., 1997). This effect appears to be maximally beneficial 3-4 days post-LPS administration (Tasaki, et al., 1997) but is maintained as far as 14 days (Furuya, Zhu, Kawahara, Abe, & Kirino, 2005) and may be a result of microglia attenuation by low dose LPS treatment in the post-ischemic brain (Rosenzweig, et al., 2004).

Evidence from studies of preconditioning with LPS suggests that sustained microglial production of anti-inflammatory factors may be key in eliciting this neuroprotective effect. Yu et al (2010) illustrated that both IL-4 and IL-13 remain upregulated for much longer in the hippocampus of animals preconditioned with LPS before induction of ischemia relative to those who were not pre-treated with LPS. This effect was accompanied by a relative reduction in microglial and astrocytic activation as measured by GFAP and IBA-1 expression (Yu, et al., 2010). This may be due to altered TLR-4 signalling in response to secondary stimuli throughout the preconditioned brain with interferon regulatory factor (IRF)-3 and IRF-7 of particular importance (Stevens, et al., 2011).

### *6.1.4 LPS Tolerance*

Related to the idea of microglial preconditioning is that of endotoxin tolerance. Endotoxin tolerance describes the desensitization of the immune system by prior low-dose LPS exposure to subsequent LPS challenge leading to reduced production of inflammatory mediators and mortality (Ishiyama, et al., 2006). This tolerance involves the suppressed transcription of a broad number of LPS target genes (Mages, Dietrich, & Lang, 2008) as well as both a reduction in pro-inflammatory cytokine production and an accompanying increase in anti-inflammatory cytokine production (Soszynski, 2002). LPS tolerance also affects animals' responses to LPS in terms of depressive-like behaviours, with tolerance to LPS-induced anhedonia exhibited by rats by the 7$^{th}$ consecutive day of endotoxin administration (Barr, et al., 2003). Early research characterised both an early phase tolerance, and a later phase tolerance which appears after several days and may persist for weeks following the initial insults (Greisman & Hornick, 1975). This phenomenon is measurable in septic patients, with leukocytes from these patients showing reduced LPS-reactivity (Cavaillon, Adrie, Fitting, & Adib-Conguy, 2003).

### *6.1.5 Aims*

The aim of this chapter was therefore to investigate whether the administration of a septic dose of LPS would alter the neuroimmune response in the hippocampus to a subsequent low dose of LPS, and if so, whether this would affect the behavioural response to the second LPS treatment. Since a low dose of LPS given onto a background of existing neuroinflammation typically appears to prime an exaggerated inflammatory response it was hypothesised that this may occur within the context of the long-lasting neuroinflammation present following LPS-induced sepsis. Chosen for examination were microglial markers CD11b and F4/80 as well as immediate early gene

protein products c-FOS, EGR-1 and ARC, and finally the cytokine IL-1β given its importance as a marker of microglial priming. Locomotion and anxiety-like behaviour in the open field test as well as depressive-like behaviour in the tail suspension test were also assessed.

*6.2 Methods and Materials*

*6.2.1 Animals*

Male C57/bl6 mice were group housed in a 12:12 light dark cycle at 21±1˚C and 50±10 humidity with food and water available ad libitum for 2 weeks before LPS administration. Animals remained housed in groups of 2–4 in polypropylene cages (33 cm long × 15 cm wide × 13 cm high) with wood chip bedding and environmental enrichment (shredded paper and cardboard tubes).

*6.2.2 Behavioural Priming Experiments*

Animals which had previously received septic doses of LPS (n=7) or vehicle (n=9) 3-4 months prior were treated i.p. in a counterbalanced fashion with either 0.9% sterile saline or 100µg/kg LPS. Two hours later these animals then underwent open field testing. Nine hours after treatment animals were tested in the tail suspension test as previously described. These timepoints were chosen due to the delay in the onset of depressive-like behaviours relative to sickness behaviour following acute LPS treatment (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). All behavioural tests were conducted as previously described in chapter 2. The experimental timelines are shown in Figure 6.1.

*6.2.3 Assessment of microglial markers*

In order to assess whether treatment with a low dose of LPS elicited an exaggerated response in the hippocampus of post-septic animals compared with control animals, animals which had previously undergone treatment with 5mg/kg of LPS (n=7) or vehicle (n=9) were administered a 100µg/kg dose of LPS at ZT1-2 before being perfused 4 hours later at ZT5-6. Brains were then collected and processed immunohistochemically for microglial markers CD11b and F4/80 as previously described in chapter 2.

*6.2.4 Assessment of neuroimmune parameters in the hippocampus*

Animals which had previously been administered either 5mg/kg of LPS (n=7) or vehicle (n=9) were injected i.p. with a 100µg/kg dose of LPS. Mice were then perfused either 2 or 9 hours later in order to coincide with behavioural timepoints. Brains were collected and processed for immunohistochemical analysis of c-Fos, Egr-1 and Arc as well as pro-inflammatory cytokine IL-1β as previously described. Four groups of animals were therefore examined – vehicle + 100µg/kg LPS (n=4) and post-septic + 100µg/kg LPS (n=3) at 2 hours, and vehicle + 100µg/kg LPS (n=5) or post-septic + 100µg/kg LPS (n=4) at 9 hours.

*6.2.5 Immunohistochemistry*

Hippocampal sections were divided in four sections to be stained for three immediate early gene markers (EGR-1, c-FOS and ARC) as well as the cytokine IL-1β. Immunohistochemistry followed a standard Avidin-Biotin Complex/Nickel DAB colourmetric protocol as previously described in chapter 3. Following two ten minute washes in 0.1 M PB and one ten minute wash in 0.1 M PB with 0.3% Triton X (PBX), sections were washed for 20 minutes in 0.1 M PB with 1.5% hydrogen peroxide to inactive peroxidises. After a further three ten minute washes (2 x PB, 1 x PBX) sections were blocked with 5% normal horse serum. Sections were then incubated with primary antibody in 2% normal horse serum overnight (EGR-1 1:3000, c-FOS 1:2000, ARC 1:500, IL1-β 1:50). The following day sections were once again washed (2 x PB, 1 x PBX) before development with anti-rabbit biotinylated secondary antibody (1:400) for 70 minutes. Following 3 subsequent ten minute washes (2 x PB, 1 x PBX) sections were developed in ABC for 90 minutes (0.4%; Vector Laboratories, Peterborough, Cambridgeshire, UK). After two more PB washes, and one ten minute wash in 0.1 M Sodium Acetate (Ph 6) sections were reacted with nickel-DAB to visualise the protein

products of interest. To control for inter-run variability all sections for individual antibodies were reacted in the same run, for an equal amount of time. Photomicrographs of sections were taken using a digital camera connected to an Olympus BX-51 light microscope under constant light intensity. For ARC and c-FOS staining the number of clearly stained immunoreactive cells per area per section were counted via microscope, while for EGR-1 and IL1-β image analysis software (ImageJ 1.43, NIH, USA) was used to calculate integrated optical density for photomicrographs of sections of interest. For each method between 4 and 6 sections of the mid dorso-ventral level of the hippocampus were analysed. Hippocampal sections were assessed across five areas of interest – the dentate gyrus granule cell layer, the dentate gyrus polymorphic cell layer, CA1, CA2 and CA3 (Franklin & Paxinos, 1997).

*6.2.6 Statistical Analyses*

All results were analysed in IBM SPSS Statistics version 20. Two-way ANOVAs were run with timepoint and treatment as between groups variables, and t-tests were run to follow up specific differences of interest. Alpha levels below 0.05 were considered significant for all tests. All data are presented as means ± standard error of the mean (SEM). Where data violated homogeneity of variances corrected p/t values are presented. Scale bars on microphotographs represent 100µm for photos at 40x and 100x magnification and 50µm for photos at 400x magnification.

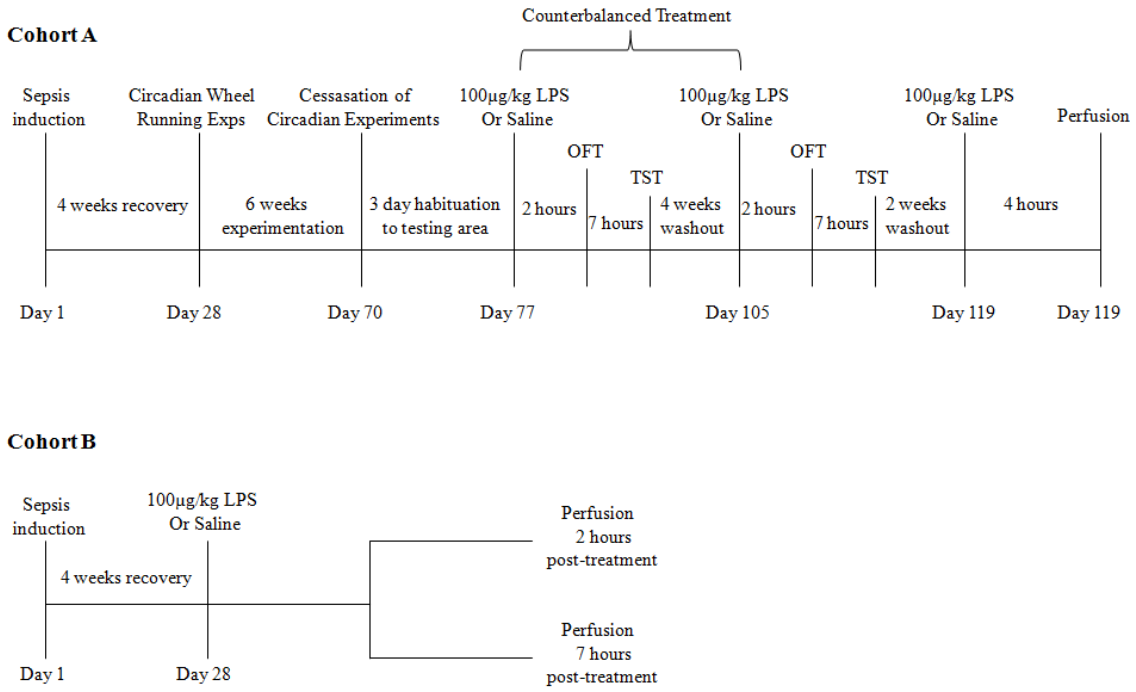



**Figure 6.1 Timeline of priming experiments.**

*6.3 Results*

*6.3.1 EGR-1*

A two way ANOVA revealed that there was no significant interaction between prior sepsis induction and timepoint across any of the five areas examined ($p>0.05$ in all cases). As illustrated in Figure 6.2 there was a significant effect of prior sepsis induction in the dentate gyrus granule cell layer ($F_{1,12}=16.462$, $p=0.016$), the CA1 ($F_{1,12}=4.892$, $p=0.047$), and the CA2 regions ($F_{1,12}=18.435$, $p=0.001$). There was a significant effect of time in all regions (Dentate gyrus granule cell layer ($F_{1,12}=22.786$, $p<0.0001$, dentate gyrus polymorphic layer $F_{1,12}=35.957$, $p<0.0001$, CA1 $F_{1,12}=22.948$, $p<0.0001$, CA2 $F_{1,12}=39.927$, $p<0.0001$, CA3 $F_{1,12}=20.222$, $p=0.001$).

To assess groupwise differences at each timepoint independent t-tests were carried out. These indicate that significant differences existed at 2 hours in the CA1 region ($t_5= 3.509$, $p=0.017$) and the CA2 ($t_5=3.187$, $p=0.024$) with LPS-treated animals (n=3) showing significantly more activation in these areas than saline-treated animals (n=4) (IOD = 151.982 ± 2.139, 71.4724 ± 5.20502 and 124.1504 ± 6.52986 and 54.6577 ± 2.51846 respectively). While at the 9 hour timepoint a significant difference was present in the CA2 region region ($t_7=2.858$, $p=0.024$) with LPS-treated animals (n=4) remaining higher compared to saline-treated (n=5) controls (47.6356±2.50181 versus 34.6755±47.6356).

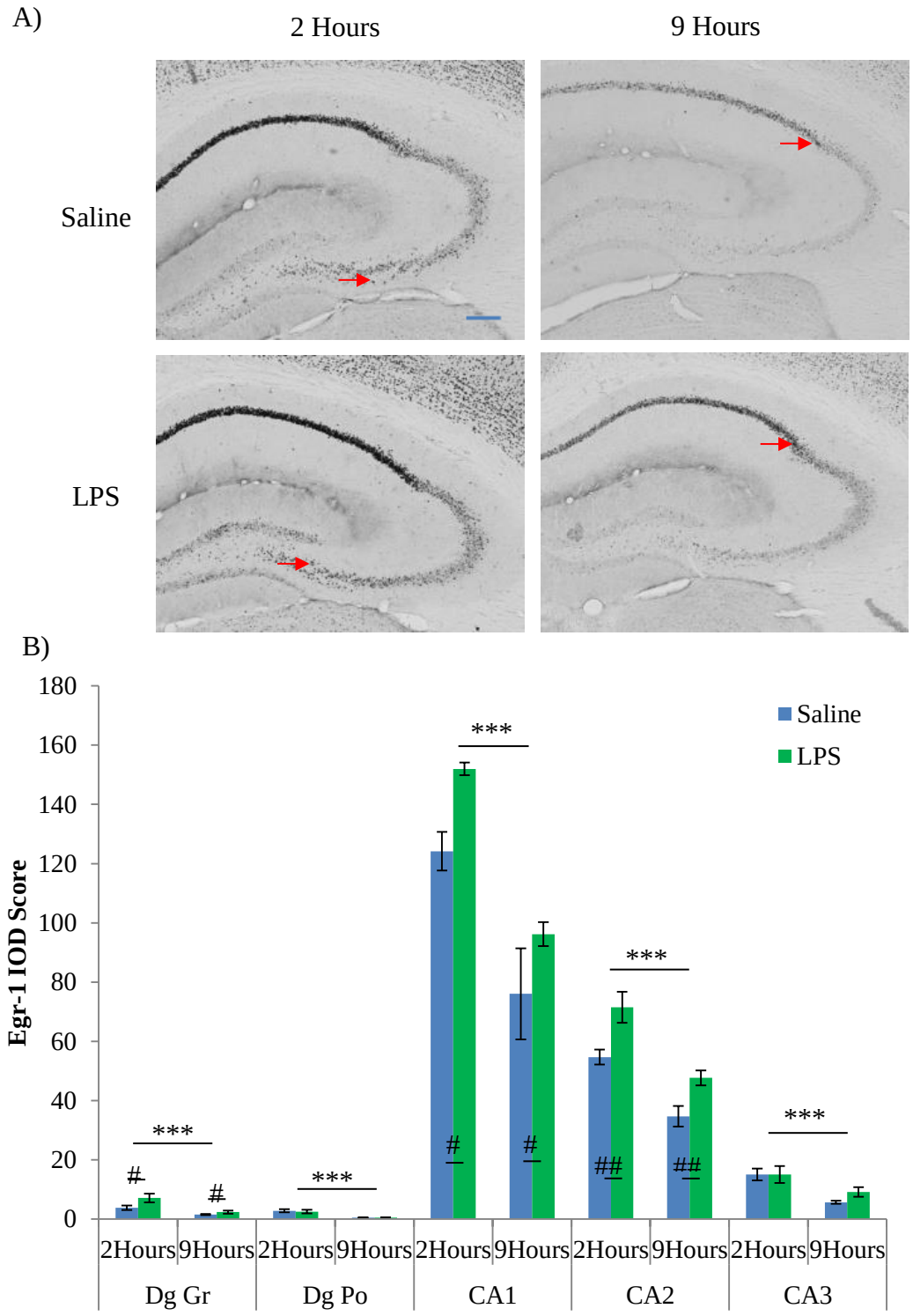



***Figure 6.2 EGR-1 expression is heightened in post-septic mice following a secondary LPS challenge*** *A) Representative photomicrographs of Egr-1 expression in hippocampal sections. B) Bar graph illustrating mean Egr-1 expression (IOD) in each hippocampal subsection at 2 or 9 hours post-treatment. Error bars represent 1x SEM, */**/*** and #/##/### represents p<0.05/0.01/0.001 for main effects of time and LPS respectively. Control/2hours (n=4) and post-septic/2hours (n=3), and control/9hours (n=5) post-septic/9hours (n=4).*

*6.3.2 c-Fos*

A two-way ANOVA revealed no significant interaction effect between prior sepsis induction and time in any area of the hippocampus – DgGr $F_{1,12}$=0.224, p=0.63; DgPo $F_{1,12}$=0.001, p=0.976; CA1 $F_{1,12}$=1.326, p=0.272; CA2 $F_{1,12}$=0.393, p=0.543; CA3 $F_{1,12}$=0.045, p=0.836. As shown in Figure 6.3 a significant effect of prior sepsis induction was present in the dentate gyrus granule cell layer ($F_{1,12}$=6.476, p=0.026) with post-septic mice showing higher c-Fos expression and the Ca1 ($F_{1,12}$=7.311, p=0.019) with saline-treated controls showing higher c-Fos expression, but not the DgPo $F_{1,12}$=0.793, p=0.391, the CA2 $F_{1,12}$=3.242, p=0.097 or the CA3 $F_{1,12}$=0.969, p=0.344. There was a significant effect of time in all areas apart from the DgGr ($F_{1,12}$=1.296, p=0.277) – DgPo ($F_{1,12}$=19.225, p=0.001); CA1 ($F_{1,12}$=12.652, p=0.004); CA2 ($F_{1,12}$=24.082, p<0.0005); CA3 ($F_{1,12}$=22.76, p<0.0005).

Independent T-tests revealed that at the two hour timepoint there was a significant difference in the number of immunoreactive cells in the granule cell layer of the dentate gyrus between saline-treated animals (n=4, 40±4.88) and LPS-treated animals (n=3, 73±3.54) $t_5$=5.083, p=0.004. At the nine hour timepoint there was a significant difference in the number of immunoreactive cells in the CA1 region region of saline animals (n=5, 12.5±1.41) and LPS animals (n=4, 3.08±1.01) $t_7$=5.163, p=0.001.

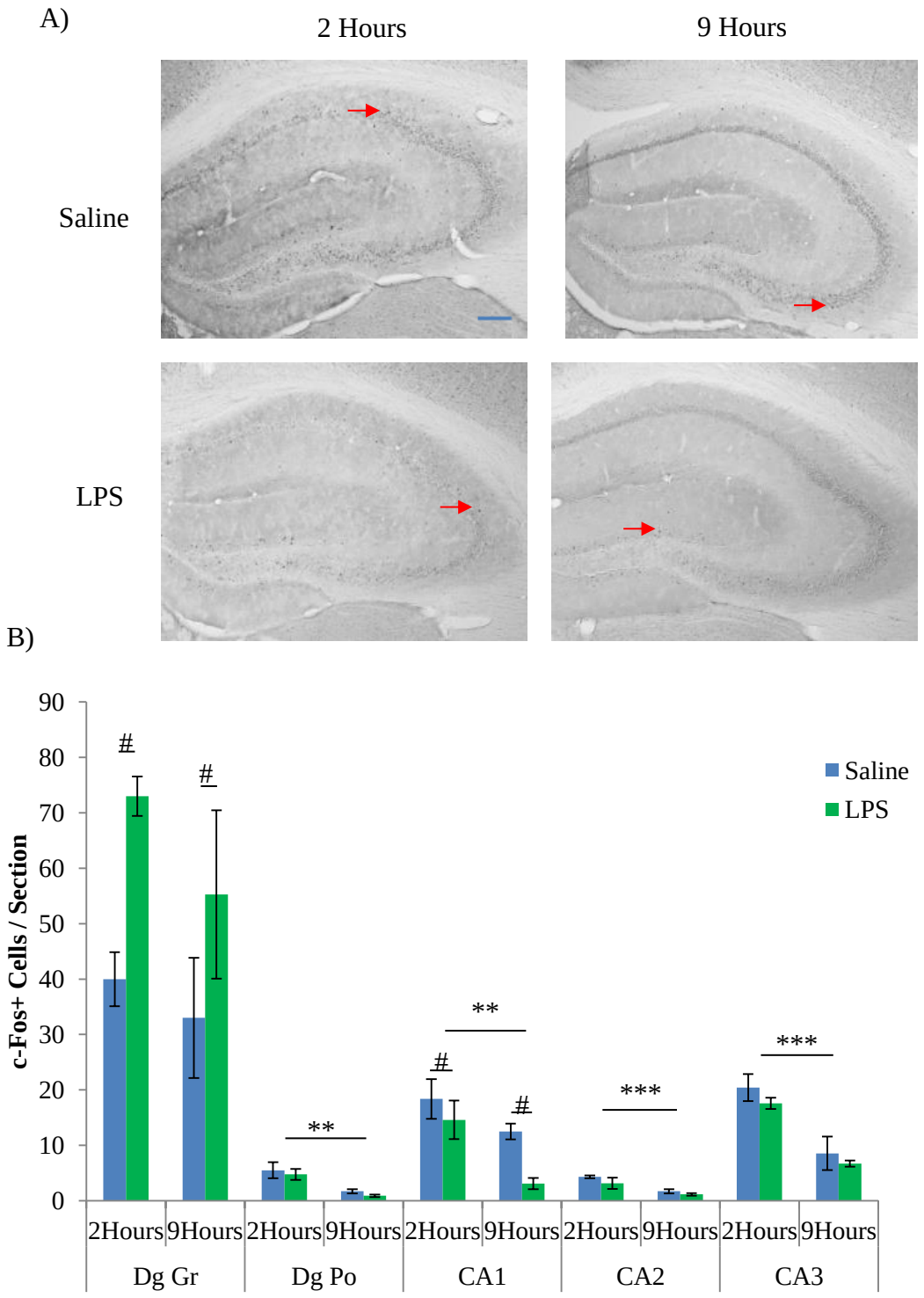



***Figure 6.3 c-Fos expression is heightened in post-septic mice following a secondary LPS challenge*** *A) Representative photomicrographs of c-Fos expression in hippocampal sections. B) Bar graph illustrating mean no. of c-Fos$^+$ cells per hippocampal subsection at 2 or 9 hours post-treatment. Error bars represent 1x SEM, */**/*** and #/##/### represents p<0.05/0.01/0.001 for main effects of time and LPS respectively. Control/2hours (n=4) and post-septic/2hours (n=3), and control/9hours (n=5) post-septic/9hours (n=4).*

*6.3.3 ARC*

As illustrated in Figure 6.4 a two-way ANOVA revealed no significant interaction between timepoint and prior sepsis induction ($F_{1,12}$=1.789, p=0.206). There was no significant effect of prior sepsis induction ($F_{1,12}$=0.541, p=0.476) or timepoint ($F_{1,12}$=0.646, p=0.437).

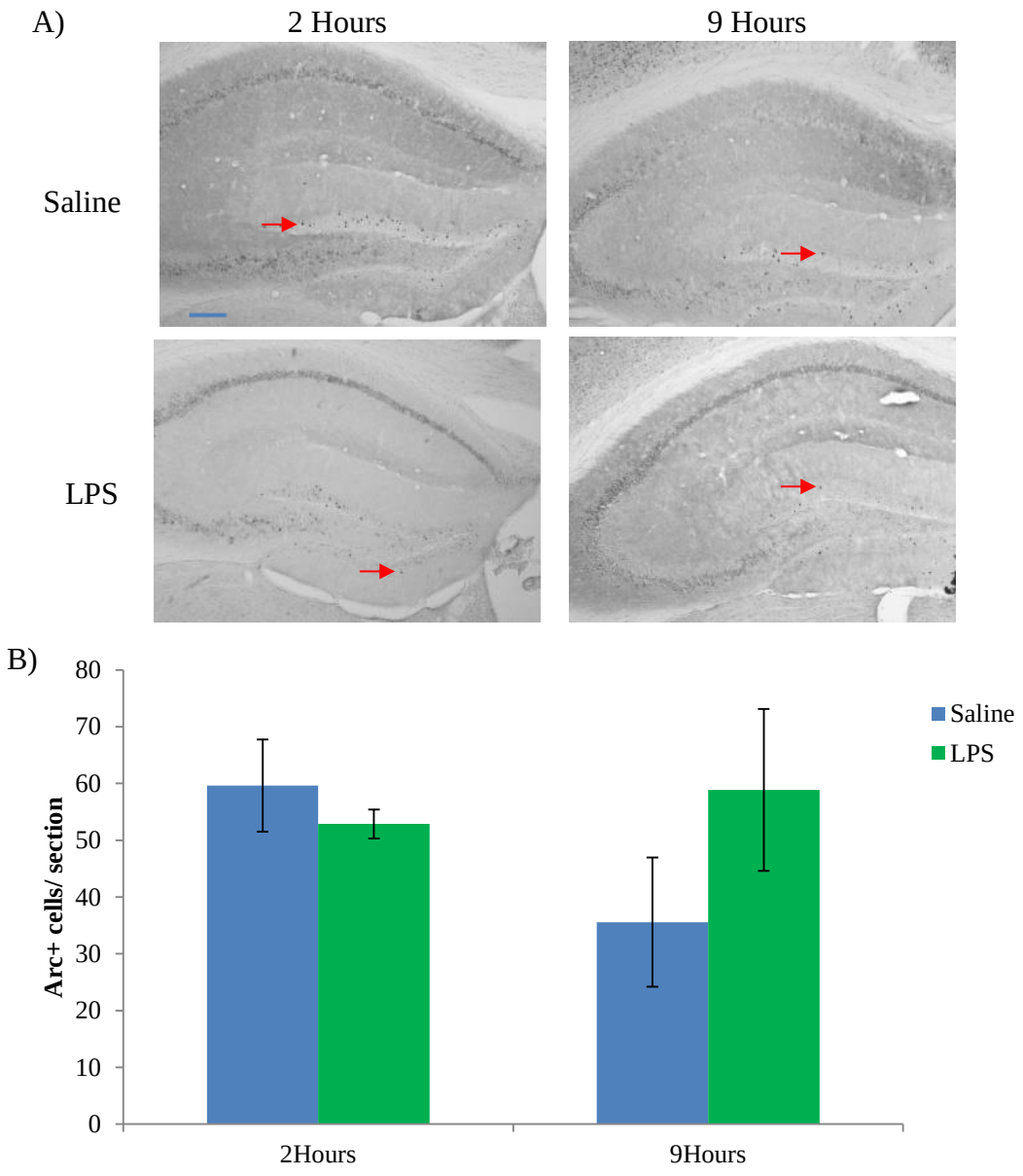



***Figure 6.4 ARC expression is not altered in post-septic mice following a secondary LPS challenge*** *A) Representative photomicrographs of Arc expression in hippocampal sections. B) Bar graph illustrating mean no. of Arc$^+$ cells per Dentate Gyrus at 2 or 9 hours post-treatment. Error bars represent 1x SEM, Control/2hours (n=4) and post-septic/2hours (n=3), and control/9hours (n=5) post-septic/9hours (n=4.*

*6.3.4 IL-1β*

A two-way ANOVA, illustrated in Figure 6.5, revealed no significant interaction between prior sepsis induction and time in any area of the hippocampus – DgGr $F_{1,12}$=0.088, p=0.772; DgPo $F_{1,12}$=2.4, p=0.147; CA1 $F_{1,12}$=2.231, p=0.161; CA2 $F_{1,12}$=3.424, p=0.089; $F_{1,12}$=0.664, CA3 p=0.431. There was also no main effect of treatment in any area of the hippocampus DgGr $F_{1,12}$=0.002, p=0.963; DgPo $F_{1,12}$=0.345, p=0.568; CA1 $F_{1,12}$=0.347, p=0.567; CA2 $F_{1,12}$=0.143, p=0.712; CA3 $F_{1,12}$=0.974, p=0.343. As illustrated in Figure 6.4 there was a main effect of time in the DgGr only $F_{1,12}$=12.644, p=0.004; but not the DgPo $F_{1,12}$=1.938, p=0.621; CA1 $F_{1,12}$=0.374, p=0.552; CA2 $F_{1,12}$=1.208, p=0.293; CA3 $F_{1,12}$=0.014, p=0.908. When groups were split by time there were no significant differences between saline and LPS-treated animals in any area of the hippocampus at either time (Control/ 2hours n=4, and post-septic/2hours n=3, and control/9hours n=5 post-septic/9hours n=4).

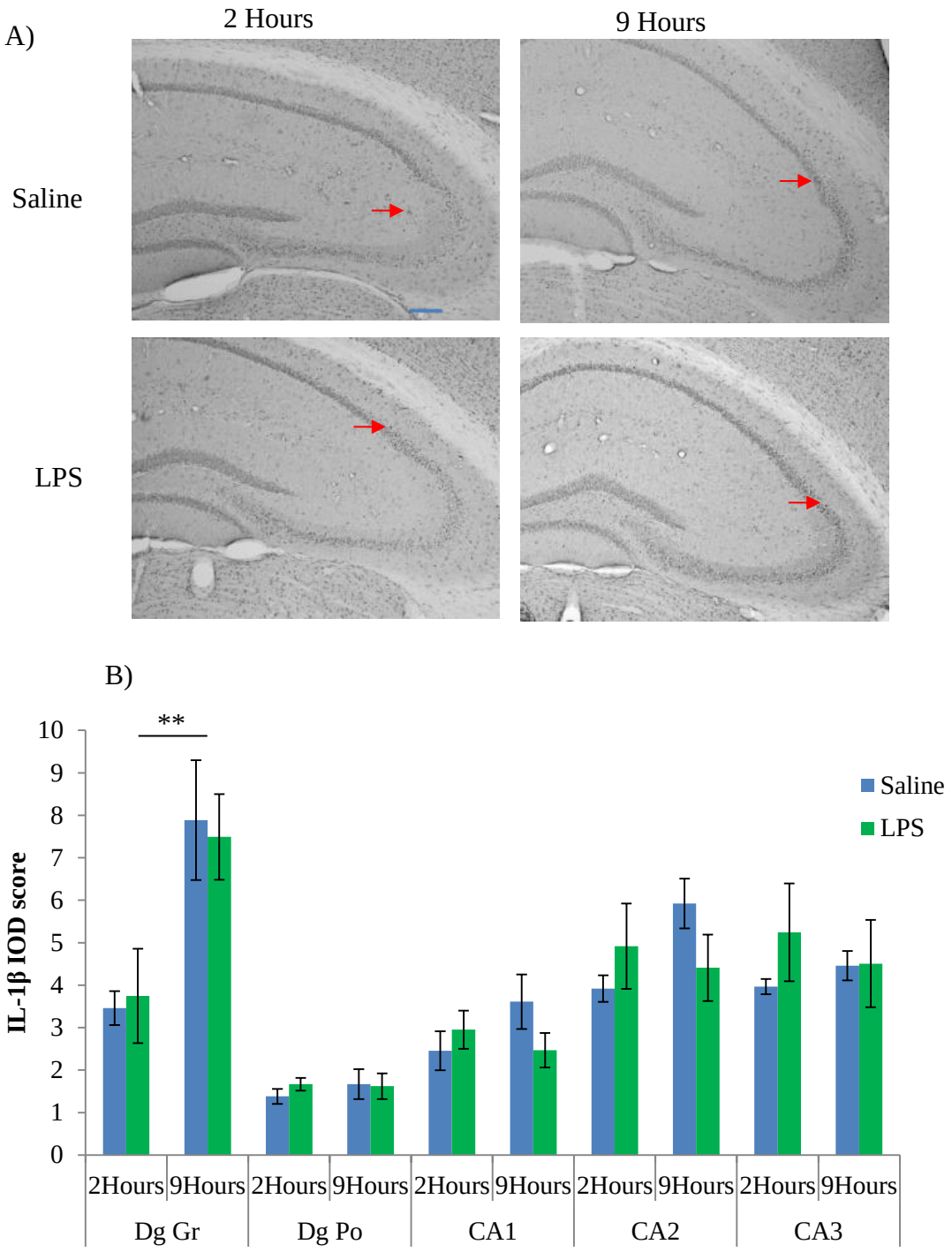



***Figure 6.5 Il-1β expression is heightened in post-septic mice following a secondary LPS challenge*** *A) Representative photomicrographs of Il-1β expression in hippocampal sections. B) Bar graph illustrating mean IL-1β expression (IOD) in each hippocampal subsection at 2 or 9 hours post-treatment. Error bars represent 1x SEM, ** represents main effect of time ($p<0.01$). Control/2hours (n=4) and post-septic/2hours (n=3), and control/9hours (n=5) post-septic/9hours (n=4).*

*6.3.5 F4/80*

As illustrated in Figure 6.6, F4/80 scores were significantly higher in post-septic animals (n=7) than controls (n=8) within the CA1 region ($t_{13}$=2.765, p=0.016), the CA2 $t_{13}$=3.44, p=0.004 and the CA3 ($t_{13}$=2.489, p=0.027). These differences were not statistically significant in the DgGr ($t_{13}$=1.449, p=0.171) or the DgPo ($t_{8.134}$=1.915, p=0.091).

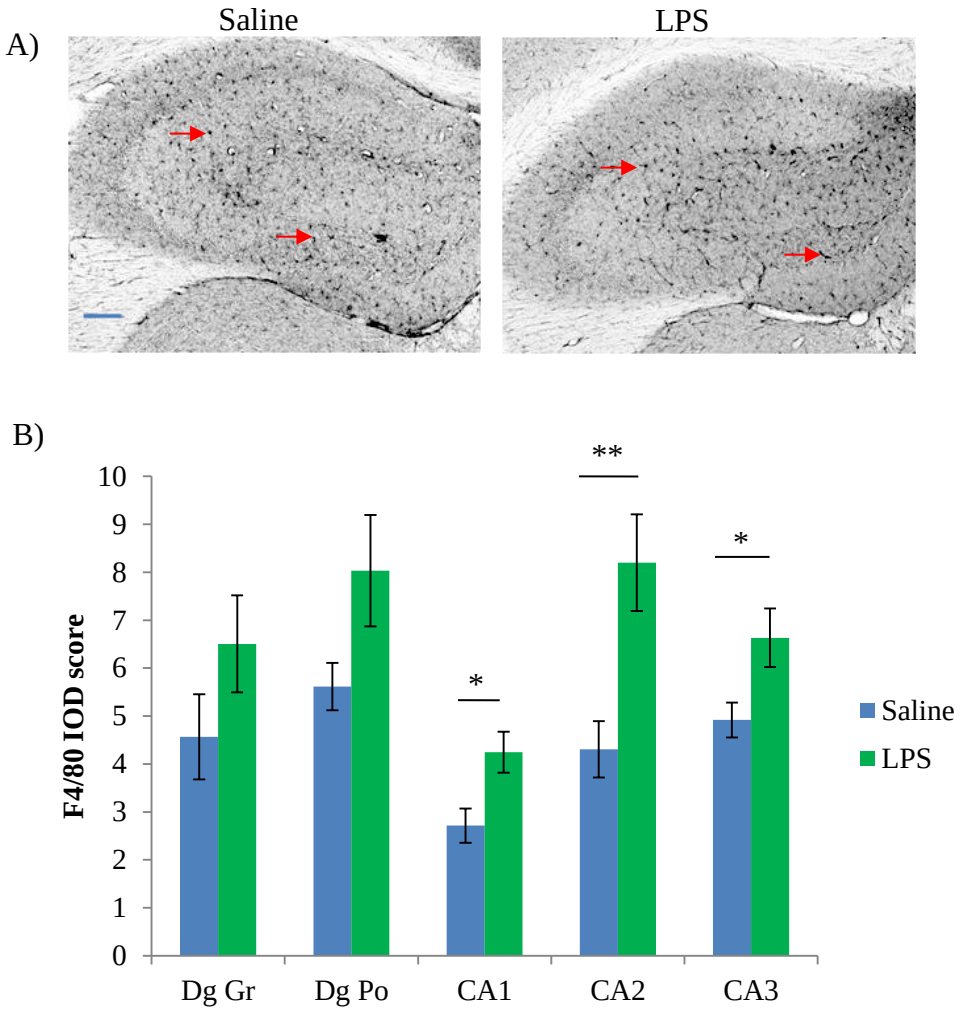



***Figure 6.6 F4/80 expression is heightened in post-septic mice following a secondary LPS challenge*** *A) Photomicrographs illustrating F4/80 expression across the hippocampus following a 100µg/kg dose to animals previously treated with a 5mg/kg dose or vehicle. B) Bar graph illustrating mean IOD scores for F4/80 across each area of the hippocampus. Error bars represent 1x SEM and */** indicates p<0.05/0.01 in independent t-tests. Control animals n=7, post-septic animals n=8.*

*6.3.6 CD11b*

CD11b expression was significantly elevated in post-septic animals (n=7) compared to saline-treated animals (n=9) within the CA1 region ($t_{14}$=2.23, p=0.043), the CA2 ($t_{14}$=5.392, p<0.0005) and the CA3 ($t_{8.31}$=2.342, p=0.046). These differences were not statistically significant within the DgGr ($t_{8.157}$=2.252, p=0.054) or the DgPo ($t_{14}$=1.698, p=0.143). This is illustrated in Figure 6.7.

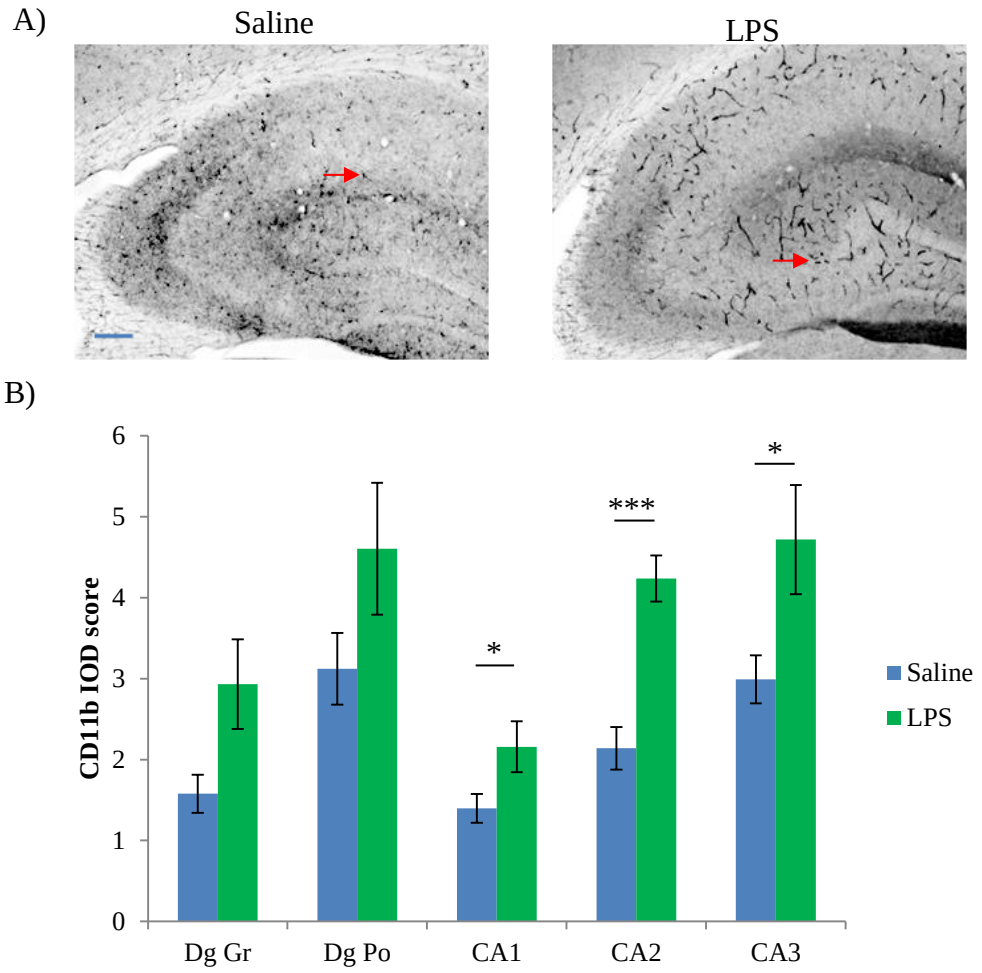



***Figure 6.7 CD11b expression is heightened in post-septic mice following a secondary LPS challenge*** *A) Photomicrographs illustrating CD11b expression across the hippocampus following a 100µg/kg dose to animals previously treated with a 5mg/kg dose or vehicle. B) Bar graph illustrating mean IOD scores for CD11b across each area of the hippocampus. Error bars represent 1x SEM and */*** indicates p<0.05/0.001 in independent t-tests. Control animals n=7, post-septic animals n=9.*

*6.3.7 Open Field Test*

A mixed between-within ANOVA indicated that animals spent significantly less time moving after acute LPS administration ($F_{1,14}$=154.042, p<0.0005), although there was no main effect of prior sepsis induction ($F_{1,14}$=1.012, p=0.332) or interaction effect ($F_{1.14}$=0.003, p=0.955). Both groups (control n=7, post-septic n=9) also spent significantly less time in the inner area of the arena following LPS administration ($F_{1,14}$=7.412, p=0.017) although again there was no main effect of prior sepsis induction ($F_{1,14}$=1.117, p=0.308) or interaction effect between the two variables ($F_{1,14}$=0.797, p=0.387). Velocity was significantly reduced following LPS administration in both groups ($F_{1,14}$=97.072, p<0.0005), with no effect of sepsis ($F_{1,14}$=0.528, p=0.479) or interaction effect ($F_{1,14}$=0.002, p=0.967). Distance moved was also significantly reduced by LPS administration ($F_{1,14}$=97.851, p<0.0005) with no effect of sepsis ($F_{1,14}$14=0.516, p=0.484) or interaction effect ($F_{1,14}$=0.004, p=0.95). These results are illustrated in Figure 6.8.

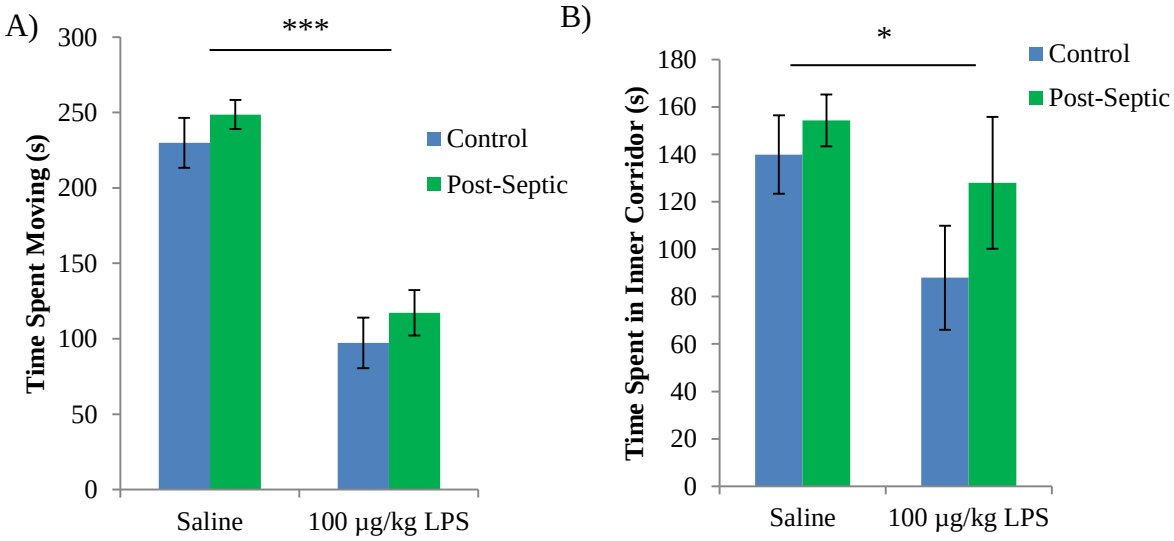



***Figure 6.8 Post-septic mice show identical responses to secondary LPS challenge in the open field test*** *A) Bar graph illustrating number of seconds moving during 300 second trial 2 hours following treatment B) Bar graph illustrating number of seconds spent in the inner corridor of the arena during a 300 second trial hours following treatment. Error bars represent 1xSEM and */*** represent main effect of prior sepsis induction p<0.05/0.001. Control n=7, post-septic n=9 for both saline & 100µg/kg LPS treated groups.*

*6.3.8 Tail Suspension Test*

As illustrated in Figure 6.9, in the tail suspension test there was a significant increase in time spent immobile nine hours following LPS administration in both groups ($F_{1,14}$=16.923, p=0.001). Although there was a main effect of prior sepsis induction ($F_{1,14}$=5.587, p=0.033), there was no interaction effect between the two variables ($F_{1,14}$=0.212, p=0.653). Independent t-tests showed that control animals (n=7) and post-septic animals (n=9) did not differ significantly in their time spent immobile following either saline ($t_{14}$=1.59, p=0.0135), or LPS administration ($t_{14}$=2.02, p=0.063).

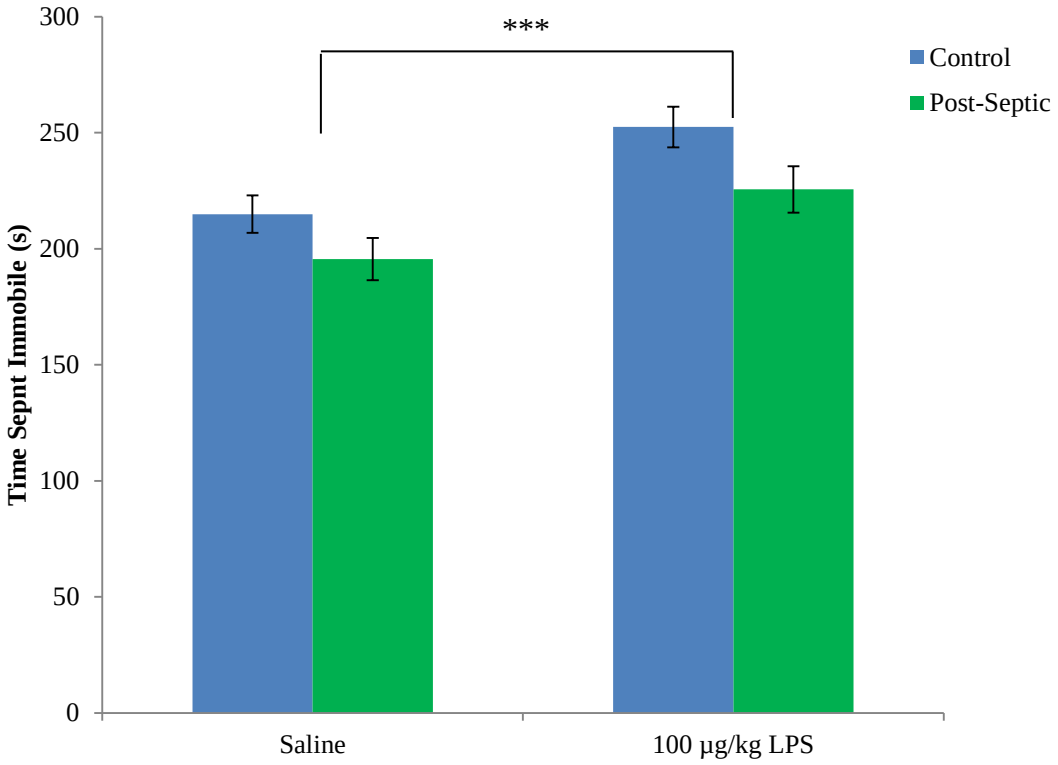



***Figure 6.9 Post-septic mice show identical responses to secondary LPS challenge in the tail suspension test.*** *Bar graph illustrating a significant increase in time spent immobile 9 hours after treatment with 100µg/kg LPS than with vehicle. Error bars represent 1xSEM and *** represent main effect of acute LPS administration p<0.001. Control n=7, post-septic n=9 for both saline & 100µg/kg LPS treated groups.*

*6.4 Discussion*

These experiments investigated whether animals which had previously survived sepsis would exhibit an altered behavioural and/or neuroimmune response to subsequent challenge with low dose LPS. In previous chapters a sustained inflammatory state is described in the post-septic hippocampus, accompanied by significant changes in depressive-like and anxiety-like behaviour. As other studies have shown that similar paradigms where low doses of LPS elicit primed responses in animal models of chronic neuroinflammatory states – such as exaggerated IL-1β expression and increased depressive-like behaviour (Godbout, et al., 2005; Perry & Teeling, 2013; Wynne et al., 2009), it was hypothesised that such an effect may occur in post-septic animals. Therefore, post-septic and control animals' responses to secondary insult with LPS in terms of IEG expression, microglial activation, pro-inflammatory cytokine expression and behavioural responses were examined.

*6.4.1 Are Neuroimmune Responses Primed Following LPS-Induced Sepsis?*

Though there was no change in Arc expression, altered responses to LPS administration in terms of Egr-1 and c-Fos expression in the hippocampus of post-septic animals were noted – two common markers of neuronal activation. Interestingly, there was no change in Arc levels within the dentate gyrus at either timepoint or between treatment groups. These changes appeared to be particularly apparent in the granule cell layer and the CA1 region. In the dentate gyrus regions, post-septic animals exhibited higher levels of IEG expression in response to LPS administration at 2 hours while in the CA1 region Egr-1 expression was increased while c-Fos expression was lower in post-septic mice. By nine hours post-treatment post-septic animals actually show a significantly decreased level of c-Fos expression in comparison to control animals. There were also significant differences in IEG levels throughout the hippocampus when

animals were sampled at either 2 or 9 hours post-treatment. In both groups IEG expression was significantly higher at 2 hours post-LPS administration than at 9 hours throughout the hippocampus. This likely reflects the relatively rapid timeframe in which IEGs are expressed in response to CNS stimulation (Pérez-Cadahía, Drobic, & Davie, 2011).

These differences in the expression patterns of IEGs following secondary LPS administration may show regional differences in the susceptibility of the hippocampus to the effects of inflammatory stimulus. Although related, these IEGs are functionally different, and as in response to behavioural training – may show differential temporal induction in the context of inflammatory stimuli also (Lonergan, Gafford, Jarome, & Helmstetter, 2010).

As previous chapters showed no change in c-Fos levels and decreased levels of Egr-1 and Arc in the post-septic hippocampus, it is interesting that the expression of these protein products is elevated upon subsequent LPS administration. Although c-Fos is known to be expressed throughout the brain in response to acute LPS administration (Wanner, et al., 2013) and Egr-1 is induced in murine macrophages in response to stimulation with LPS (Coleman, Bartiss, Sukhatme, Liu, & Rupprecht, 1992), neither marker has previously been used to examine response to secondary insults in models of chronic neuroinflammation. As with our previous experiments there is substantial variation in the response seen between different areas of the hippocampus. Here the granule cell layer of the dentate gyrus and the CA1, which is primarily composed of pyramidal cells (Mizuseki, Royer, Diba, & Buzsáki, 2012), appear particularly affected. As these proteins are primarily markers of neuronal activation they may represent a potentiated neural response to LPS rather than microglial priming *per se*.

Microglial activation was examined at 4 hours post-LPS administration showing a significant upregulation of CD11b and F4/80 expression in the hippocampus of post-septic versus control animals throughout the Cornus Ammonis region. Although this may reflect over-activation of microglia in post-septic animals a central hallmark of primed microglia – IL-1β over-expression was not present among post-septic animals. Primed microglia have previously been described as morphologically identical to activated microglia, only identifiable by the over-expression of pro-inflammatory cytokines in response to stimulation (Cunningham, Wilcockson, Campion, Lunnon, & Perry, 2005; Islam & Pestka, 2006; Wynne, Henry, & Godbout, 2009). It is therefore possible that elevated levels of CD11b and F4/80 are simply indicative of the chronic neuroinflammation present in post-septic animals regardless of acute LPS-administration. The examination of other pro-inflammatory factors such as TNF-α or IL-6 for example may shed more light on the microglial response to subsequent LPS administration in the post-septic hippocampus.

### *6.4.2 Are Behavioural Responses Primed Following LPS-induced Sepsis*

As priming has previously been shown to lead to altered behavioural responses in terms of decreased social behaviour, food intake, and increased behavioural despair in the tail suspension test and forced swim test (Godbout, et al., 2005; Godbout, et al., 2007) it was decide to examin behaviour in the open field test and tail suspension test at 2 and 9 hours post-LPS respectively. Although there was a clear effect of acute LPS administration on behaviour in both the open field test and the tail suspension test these effects did not differ based on prior sepsis induction. In both groups animals significantly decreased their mobility and time spent in the central area of the open field test, and similarly exhibited increased immobility in the tail suspension test. Unlike in the prior experiments, post-septic mice did not show higher immobility than control

animals in the tail suspension following acute administration of either saline or LPS. As levels of immobility in control animals were relatively high compared with other instances of this experiment it is possible that the acute stress of injections and open field testing itself were a confounding factor. Immobility in the tail suspension test has previously been shown to be very sensitive to acute stressors, even in experimentally naïve animals (Dunn & Swiergiel, 2008) which may explain the heightened levels in this experiment.

Given that animals showed such drastic reductions in movement it may be that this dose (100µg/kg) of LPS elicited reduced motility as part of a febrile response to LPS (Dantzer, 2001; Hart, 1988) rather than any true increase in anxiety-like or depressive-like behaviour which is thought to develop later than sickness behaviour (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). LPS doses as low as 5µg/kg have been shown to significantly reduce mobility in mice (Silvia, et al., 2009), although this effect becomes much more potent at doses above 50µg/kg suggesting that any effect of prior sepsis induction on a behavioural response to LPS may be masked by the administration of a dose which is either too low or too high. It may therefore be useful to study behavioural priming following relatively low doses of inflammatory agents, or to use later timepoints than those examined here.

### *6.4.3 Conclusion*

It therefore appears that prior sepsis induction does not lead to a primed microglial or behavioural response to a 100µg/kg dose of LPS. The responses elicited also do not seem indicative of LPS-tolerance or microglial pre-conditioning. Rather LPS induced high levels of sickness behaviours in both groups accompanied by microglial activation which likely appeared elevated in post-septic animals due to the altered level of activation already present before a secondary dose was administered. It

may also be that while there is no altered response in terms of affective behaviour, post-septic animals might be more susceptible to a primed effect in terms of cognitive behaviours, not measured in this experiment, which are highly affected in post-septic patients (Iwashyna, Ely, Smith, & Langa, 2010).

# Chapter 7. Disruption of Hippocampal Circadian Rhythmicity in Post-Septic Mice

*Abstract*

Circadian rhythms are present in organisms at almost every level from protein expression to behaviour and are essential for proper functioning. These near 24 hour cycles are severely disrupted during sepsis and are also affected in sufferers of mood disorders. Microglia themselves show circadian variation in their responses to inflammatory stimuli. It was hypothesised that post-septic mice may show variation in key circadian proteins which are important to maintaining rhythmicity in the hippocampus. The expression of these proteins (CLOCK, PER1 & PER2), as well as c-Fos, was examined at 6 timepoints across a 24 hour cycle in post-septic and control mice. In order to assess the role of circadian factors in the microglial response to LPS a second experiment was also carried out. Here mice with a heterozygous knockout for circadian protein BMAL1 in macrophages were treated with 100µg/kg LPS before undergoing behavioural testing and then immunohistochemical analysis.

The first experiment revealed that post-septic mice showed dampened rhythms in the hippocampal expression of PER1 and PER2 across a 24 hour cycle – with some regional differences within the hippocampus. c-Fos and CLOCK expression did not oscillate across the 24 hour cycle and there were no differences between post-septic and control mice. The latter experiment revealed that mice with a knockdown for BMAL1 in macrophages showed subtle depressive-like behaviours in some tests but overall did not show large differences in response to LPS treatment. These results shed some light on the effects of LPS-induced sepsis on circadian rhythms in the mouse hippocampus.

*7.1 Introduction*

As demonstrated in previous chapters, LPS-induced sepsis leads to prolonged microglial activation, alterations in hippocampal functioning and accompanying behavioural changes. The mechanisms through which these changes are brought about are not clear. One potential target for investigation is the disruption of circadian rhythmicity following sepsis induction. Circadian clocks are present both within the CNS and the periphery, regulating near 24 hour cycles of physiology and behaviour, with the master circadian pacemaker the suprachiasmatic nucleus (SCN) residing within the hypothalamus (Partch, Green, & Takahashi, 2014). Although the SCN itself does not directly control cognitive or affective behaviours, it synchronises clocks in other brain regions, such as the hippocampus, which are more directly involved in these processes (Cho, 2012).

Within the SCN circadian oscillations are driven by both positive and negative transcriptional and translational feedback loops which control the production of essential clock proteins (Kwon, Choe, Son, & Kim, 2011; Reppert & Weaver, 2002). A positive feedback mechanism occurs when the BMAL1:CLOCK and BMAL1:NPAS2 dimers promotes the transcription of target genes *Per1, Per2* and *Per3* as well as *Cry1* and *Cry2* (Debruyne, Weaver, & Reppert, 2007). PER:CRY protein dimers in turn negatively regulate their own transcription by translocating to the nucleus and interacting with CLOCK:BMAL1 to stop activation (Reppert & Weaver, 2002). Also important in regulating clock rhythmicity are REV-ERBα which represses *Bmal1* transcription and RORa and RORb which activate it (Liu, et al., 2008). Many circadian genes can also serve alternative functions outside of the SCN, for example *Rev-Erbα* can modulate aspects of macrophage function highlighting the interplay between the circadian and immune systems (Sato, et al., 2014).

*7.1.1 Circadian Rhythms and Clock Gene Production in the Hippocampus*

It is thought that cell populations within areas such as the hippocampus may also function as circadian oscillators but that these are maintained in synchrony by the master clock within the SCN (Wang, et al., 2009). Although direct neural input from the SCN to the hippocampus has not yet been identified, hippocampal cells produce the clock gene proteins PER1, PER2, CRY1, CRY2, CLOCK and BMAL1 (Jilg, et al., 2009). Some cell populations within the HPC even show endogenous clock gene rhythmicity. For example, pyramidal cells from the hippocampus display Per2 rhythmicity in isolation from the SCN (Wang, et al., 2009). Evidence from electrophysiological study of CA1 pyramidal neurons in C57 mice and Wistar rats offer further insight into the role of circadian regulation in hippocampal function, showing that these neurons have significant diurnal variations in LTP (Chaudhury, Wang, & Colwell, 2005; Nakatsuka & Natsume, 2014). Circadian rhythms within the hippocampus are now known to control functions essential for memory formation and consolidation such as mitogen-activated protein kinase (MAPK) and cAMP signal transduction signalling (Eckel-Mahan, 2012), as well as dendritic spine formation and stabilisation following periods of learning via control of glucocorticoid levels (Liston, et al., 2013). Further evidencing the role of circadian genes in hippocampal function, mice knocked out for *Per1* show severe deficits in hippocampal-dependent spatial learning (Jilg, et al., 2009).

In humans circadian rhythms have also been shown to be important for regulating both cognitive and affective functioning (Wright, Lowry, & LeBourgeois, 2012), both of which depend on the hippocampus. Aside from their role in normal functioning, circadian disturbances and clock gene abnormalities have been implicated in affective disorders such as major depression (McCarthy & Welsh, 2012) and diseases

involving significant cognitive impairment such as Alzheimer's disease (Cermakian, Lamont, Boudreau, & Boivin, 2011). One hippocampal function important to both memory formation and emotion is the adult proliferation of NPCs within the subgranular zone of the hippocampus. Interestingly, there are molecular clocks present in these NPC cells and proliferation has been shown to be rhythmic, with the clock genes *Per2* and *Bmal1* of particular importance (Bouchard-Cannon, Mendoza-Viveros, Yuen, Kærn, & Cheng, 2013). The clock gene *Bmal1* has also been shown to be essential to regulating the survival of newly proliferated NPCs within the SGZ, with *Bmal1* KO mice actually showing increased survival of newborn cells (Rakai, Chrusch, Spanswick, Dyck, & Antle, 2014). Dysregulation of BMAL1 and PER2 proteins leads to significant alterations in proliferation and is also accompanied by cognitive deficits.

### *7.1.2 Does the hippocampus exhibit independent rhythmicity or is it dependent on SCN input?*

One important question regarding the hippocampal circadian clock is whether its rhythmicity is controlled purely by the SCN or whether it can maintain endogenous rhythms without SCN input. Although peripheral clocks within the brain and other organs are synchronised by neural or humeral signalling from the master timekeeper the SCN, some organs can maintain rhythmic oscillations despite SCN lesion (Tahara, et al., 2012). While these clocks may maintain their own individual rhythms without SCN input, genetic ablation of SCN function leads to desynchronicity between peripheral clocks in the absence of any zeitgeber (Husse, Leliavski, Tsang, Oster, & Eichele, 2014). Clock genes *Per2, Cry1* and *Cry2* (Navigatore-Fonzo, Delgado, Golini, & Anzulovich, 2014) have shown endogenous rhythmicity within the hippocampus of rats kept in constant darkness and Jilg et al. (2009) illustrated that C3H mice express the majority of the central clock genes in the hippocampus also. As already noted, *Per2*

oscillates rhythmically in pyramidal cells cultured in isolation from SCN input (Wang, et al., 2009) but while *Per1* (Abe, et al., 2002) has been shown to be strongly expressed in dentate gyrus tissue isolated from the SCN it was not rhythmic, and CA1, CA2 and CA3 tissue failed to show *Per1* production at all. Although the oscillation of some factors may occur independent of SCN input, the rhythmic expression of MAP kinase and adenylyl cyclase as well as memory consolidation have been shown to depend on a functioning SCN (Phan, Chan, Sindreu, Eckel-Mahan, & Storm, 2011), and rhythmicity of PER2 expression in the DG has been shown to be abolished following SCN lesion (Lamont, Robinson, Stewart, & Amir, 2005). Therefore while some hippocampal cells populations may show rhythmic expression of clock genes in isolation from the SCN, proper hippocampal functioning still depends on SCN input. Despite this, the precise mechanism by which the SCN outputs to the hippocampus has yet to be defined. The hippocampus does show sensitivity to melatonin, a hormone under circadian control (Perreau-Lenz, et al., 2003) which can modulate serotonin signalling (Monnet, 2002), and subiculum synchronization by the SCN may also influence rhythmicity within the hippocampus (Cho, 2012).

One group of factors which may link the circadian system, the inflammatory response and cognitve/affective functioning are glucocorticoids – steroid hormones which are regulated by the circadian system, whose receptors are expressed in almost all tissue (Kadmiel & Cidlowski, 2013) and which are upregulated in response to life stressors (Sauro, Jorgensen, & Pedlow, 2003). Glucocorticoids may play a key role in the disruption to hippocampal rhythmicity during both psychiatric and inflammatory disorders, controlling both *Per1* and *Per2* oscillation in the dentate gyrus (Gilhooley, Pinnok, & Herbert, 2011) and CA1 (Conway-Campbell, et al., 2010) as well as Per2 rhythmicity within the central amygdala (Segall, Milet, Tronche, & Amir, 2009).

Importantly in the context of the present research, glucocorticoids are also able to modulate adult neurogenesis, with over expression suppressing the proliferation of neural progenitor cells (Mirescu & Gould, 2006). Glucocorticoids are implicated in the pathophysiology of major depression (Anacker, Zunszain, Caravalho, & Pariante, 2011), with cortisol for example showing flattened rhythms of expression in depressed women (Jarcho, Slavich, Tylova-Stein, Wolkowitz, & Burke, 2013). Cortisol rhythms are also dampened in chronic inflammatory conditions such as athlerosclerosis – with higher levels of inflammatory cytokines correlating with more disrupted rhythmicity (DeSantis, et al., 2012). Others have suggested that the increase in inflammatatory markers present in depression may drive HPA-axis hyperactivity (Carvalho, et al., 2014) – an effect which could subsequently disrupt the expression of circadian genes in the hippocampus and other areas.

### *7.1.3 Is There a Circadian Aspect to the Inflammatory Response?*

Aside from its role in maintaining proper hippocampal functioning, the circadian system also shows significant bidirectional communication with various aspects of the immune system. As with peripheral macrophages (Keller, et al., 2009), microglia themselves actually contain their own molecular clock, with murine microglia rhythmically expressing *Per1*, *Rev-erbα*, *Per2* and *Bmal1* when isolated from mice (Hayashi, et al., 2013). These microglia also exhibit circadian expression of Cathepsin S which controls the diurnal changes in synaptic activity and spine density in neurons, illustrating yet another mechanism through which circadian oscillations can influence factors controlling cognition. Indeed, the immune response as a whole has been demonstrated to vary depending on the time of day at which a challenge occurs. Marpegan et al. (2009) showed a diurnal variation in mice's susceptibility to LPS-induced mortality and their immune responses to such a challenge. The authors found

that mice had significantly higher mortality following LPS administered at ZT11 rather than ZT19, a timepoint at which they also produced significantly higher levels of the cytokines IL-1β and IL-6 as well as chemokines MCP1 and MIP1α. Spengler et al. (2012) showed that the circadian protein CLOCK actually forms complexes with NF-κB subunit p65 and promotes its transcription independent of BMAL1. As NF-κB drives the innate immune response to LPS this explains in part the large diurnal variations mice exhibit in susceptibility to LPS challenge. This circadian regulation of the immune system is also present humans, with pro-inflammatory factors highest during night-time, and a shift towards an anti-inflammatory profile present during the day (Lange, Dimitrov, & Born, 2010).

As the circadian system appears to gate immune factors in healthy contexts as well as in response to pathogens researchers have also examined immune responses in situations of circadian disruption. This disruption can be achieved in animal models by altering the length of the light or dark periods of a normal 12:12 light:dark cycle, or by phase-shifting animals by advancing or delaying the onset of the light or dark part of their cycle – similar to what occurs during shift work and jet lag in humans. For example, Castanon-Cervantes et al. (2010) showed that four consecutive 6-hour phase advances in mice led to significant reductions in survival following LPS challenge as well as exaggerated inflammatory response in terms of serum cytokines IL-1β, IL-12 and IL-13 with reduced IL-10 production. Chronic phase shifting has also been shown to alter circadian clock gene expression in natural killer (NK) cells , disrupting the expression of cytokine IFN-γ and altering their functional response to tumour challenge (Logan, et al., 2012). Destruction of clock functioning by bilateral SCN lesion leads to excessive immune responses to LPS (Guerrero-Vargas, et al., 2014). These studies

illustrate the necessity of proper circadian functioning for maintaining an appropriate and effective immune response to pathogens.

*7.1.4 How Does Inflammation Impact on Circadian Factors?*

While the circadian system can control immune factors during normal functioning and in response to inflammatory challenge, the immune system can also exert effects on circadian parameters, even leading to desynchronicity in pathological states. A relatively low dose of LPS (50µg/kg) induces phase shifts, along with c-Fos activation in the SCN primarily through a TLR-4 dependent pathway (Paladino, Leone, Plano, & Golombek, 2010). Septic doses of LPS have also been shown to induce long-lasting alterations in circadian parameters such as accelerated re-entrainment to six hour phase advances and exaggerated phase advances following photic stimulation (O'Callaghan, Anderson, Moynagh, & Coogan, 2012). These effects may be driven by the NF-κB pathway with the NF-κB subunit RelB having been shown to suppress circadian expression of BMAL1, and the suppression of light-induced phase advances by NF-κB inhibition with pyrrolidine dithiocarbamate (Bellet, Zocchi, & Sassone-Corsi, 2012; Marpegan, et al., 2004). The cytokine TNFα and its receptor tnfr1, which are known to activate NF-κB also appear to be important in driving the circadian response to LPS stimulation (Paladino, Mul Fedele, Duhart, Marpegan, & Golombek, 2014). Inhibition of the NF-κB pathway with sulfasalazine blocks LPS-induced phase delays (Marpegán, Bekinschtein, Costas, & Golombek, 2005). These studies illustrate the bi-directionality of the immune-circadian signalling and the potential for disruption in each system produce deleterious effects in the other.

*4.1.5 Linking Inflammatory Disorders, Psychiatric Illness and Circadian Parameters*

In humans, circadian dysfunction is implicated in the pathogenesis and symptomatology of several immune diseases such as rheumatoid arthritis (Takagi,

Inada, & Naito, 2013; Yoshida, Hashimoto, Sakai, & Hashiramoto, 2014), and multiple sclerosis (Najafi, et al., 2013). Many symptoms of immune disease are experienced in diurnal patterns, with some more apparent in early morning or late night – corresponding to rhythmic oscillations in immune factors within the body (Scheiermann, Kunisaki, & Frenette, 2013). These findings are important because disrupted circadian rhythmicity and subsequent sleep disturbance contribute towards impaired immune functioning in humans (Faraut, Boudjeltia, Vanhamme, & Kerkhofs, 2012; Irwin, et al., 1996). Circadian disruption and sleep disturbances are also a factor in both the pathogenesis and symptomatology of numerous psychiatric illnesses (Jagannath, Peirson, & Foster, 2013). This includes diseases such as major depression (Souêtre, et al., 1989), anxiety disorders (Monti & Monti, 2000; Papadimitriou & Linkowski, 2005) and schizophrenia (Wulff, Dijk, Middleton, Foster, & Joyce, 2012). One particularly interesting study using a time of death analysis in post-mortem depressive patients illustrated that the rhythmicity of several key clock genes including *Bmal1*, *Per1/2/3*, and *Rev-Erbα* was blunted in cortical and limbic regions of depressive patients relative to controls (Li, et al., 2013). As these conditions also often involve inflammatory components (Najjar & Pearlman, 2014; Rosenblat, Cha, Mansur, & McIntyre, 2014) a link may also be present here between neuroinflammation and disrupted circadian rhythmicity.

This cross talk between the immune and circadian systems is particularly pertinent in the case of sepsis given that severe circadian disruption is present in the acute stages of sepsis, and data from animal models showing both that the severity of sepsis is affected by the circadian time at which sepsis is induced (Silver, Arjona, Walker, & Fikrig, 2012; Walker, Bozzi, & Fikrig, 2011) and that disruption of rhythmicity by *Bmal1* deletion leads to increased mortality ad disease severity

(Cornonata, et al., 2014). Analysing melatonin metabolite 6-sulfatoxymelatonin (6-SMT) in both ICU patients suffering from sepsis and non-septic ICU patients, Mundigler et al. (2002) showed that this circadian disruption was specific to sepsis and not simply an effect of environmental disruption such as noise, light or carer interactions. Interestingly, the authors followed up these authors 1-4 weeks after recovery from sepsis and found that they still failed to show circadian rhythmicity in aMT6s secretion. Another study of 6-SMT levels and lighting variability in septic patients showed again that rhythmicity is disrupted and also that light exposure in patient care rooms failed to entrain circadian rhythms (Verceles, et al., 2012), although the authors also highlight the importance of considering the modulatory effects many medications used during sepsis can have on melatonin secretion. An antioxidant, melatonin administration has actually been shown to improve clinical outcomes in septic newborns (Gitto, et al., 2001) and a strong negative correlation exists between disease severity and nocturnal melatonin levels in severe sepsis (Perras, Kurowski, & Dodt, 2006). An in depth analysis spanning six days measuring heart rate as well as systolic, diastolic and mean blood pressure in a large sample (n=2690) found that while survivors tended to show circadian oscillations in these functions, rhythmicity was lost among non-survivors (Cheng, et al., 2014).

### *7.1.6 Aims*

Given these associations between inflammation, affective behaviours and circadian rhythms these experiments aimed to assess whether hippocampal rhythmicity may be altered in post-septic animals. Levels of PER1, PER2 and CLOCK protein products as well as the marker of neuronal activation c-Fos were examined, as these are all endogenously expressed in the mouse hippocampus (Jilg, et al., 2009), at six timepoints over a 24 hour cycle. As microglia also contain their own intrinsic clock

(Hayashi, et al., 2013) conducted behavioural and neuroimmunological investigation was also conducted with a circadian mutant mouse with a heterozygous knockout for *Bmal1* in myeloid-lineage cells. It was hypothesised that these mice may exhibit an altered response to a low dose of LPS given the importance of correct clock functioning in gating an appropriate immune response.

*7.2 Materials and methods*

*7.2.1 Experiment 1. The effects of sepsis on hippocampal rhythmicity*

*7.2.1.1 Animals*

For the purpose of all experiments male C57BL/6 mice (Charles River, Kent, UK) aged between 8 and 10 weeks were used. After two weeks of group housing following arrival to acclimatise to conditions, animals were singly housed in polypropylene cages (33cm long x 15 cm wide x 13cm high) with steel running wheels (11.5 cm diameter) in a 12:12 light:dark cycle for 2 weeks prior to LPS administration. Food and water were available *ad libitum*, temperature was 21 ±1°C and humidity was 50 ±10%. All procedures were approved by the Research Ethics Committee, National University of Ireland Maynooth, and were licensed by the Department of Health and Children, Ireland under statutory instrument (S.I.) No. 543 of 2012 and the European directive 2010/63/EU.

*7.2.1.2 Drug Treatments*

All saline and LPS injections were made up fresh on the treatment day, and given intraperitoneally in a final injection volume of 0.125ml. 0.9% sterile saline was made up fresh for control injections, while lipopolysaccharide (serotype0111.B4, Sigma Ireland; Qin et al, 2007) was made up to a 5mg/kg dose in sterile saline. Injections were given intraperitoneally between zeitgeber time (ZT) 06 and 08, where ZT0 is defined as lights on. Mortality and significant moribundity requiring euthanasia occurred within approximately 10% of animals following the induction of sepsis.

*7.2.1.3 Assessment of circadian clock gene expression*

One month following either LPS (5mg/kg) or saline treatment animals were placed in DD (constant darkness) for two cycles following which they were sampled

every four hours across the third 24 hour DD cycle (n=3-4 per group). Brains were therefore collected at CT2, CT6, CT10, CT14, CT18 and CT22. Animals were administered sodium pentobarbital (0.1ml / animal) under dim red light (<1 lux) before being transcardially perfused with saline followed by 0.4% PFA as previously described. Brains were then cryoprotected in 30% sucrose and coronally sectioned at 30µm as previously described. Immunohistochemical analysis was conducted as in previous chapters using the following antibodies – PER1 (1:500, goat, Santa Cruz), PER2 (1:1000, rabbit, Alpha Diagnostics), CLOCK (1:500, goat, Santa Cruz) and c-Fos (1:2000, rabbit, Santa Cruz). Between four and six section were analysed per mouse with the number of immunoreactive cells per area of interest counted as previously described.

### *7.2.2 Experiment 2. Effects of myeloid-specific Bmal1 knockdown*

#### *7.2.2.1 Animals*

Male mice (n=11) with heterozygous BMAL1 deletion in the myeloid cell lineage (*Bmal1*$^{+/-}$) and wild-type counterparts (n=10) were received from Trinity College Dublin and were group housed for 2 weeks in a 12:12 light:dark cycle at 21±1˚C with 50±10% humidity and food and water available *ad libitum*. These animals were produced via a cross between *LysMCre* animals and floxed *bmal1* mice, as per (Gibbs, et al., 2012). At age 8 weeks, they were taken to the testing area 3 days before experimental testing begun to allow for habituation and control for stress induced by moving. Experimental timelines for these mice are shown in Figure 7.1.

#### *7.2.2.2 Behavioural phenotyping*

Animals were given either a sterile saline or 100µg/kg dose of LPS (0.125ml) between ZT2.5 and ZT3.5 and were two hours later (ZT4.5-5.5) introduced to a novel

arena (diameter 60cm) and allowed to explore for five minutes during which time distance, velocity and time in the inner and outer corridors were recorded. They were then returned to their homecage and one hour later they were introduced to an elevated plus maze. Animals were tested in the elevated plus maze for 5 minutes – recording their time spent in the open and closed arms, as well as number of entries to the open and closed arms. 24 hours after treatment animals were tested in the tail suspension test.

Five weeks later, when animals were deemed to be ready to be re-tested, the same experiments were carried out, but those who were treated with saline the first time were given LPS, and vice versa. For the elevated plus maze, in order to combat one-trial tolerance (Schneider, Ho, Spanagel, & Pawlak, 2011), when the animals were re-tested the environment was changed significantly by the introduction of novel light sources and extra-maze stimuli, as well as a altered direction of the maze.

The forced swim test was carried out a week after the previous experiments, this time in the animal house itself. There was no treatment prior to testing, rather animals were tested only for a between-groups effect. During the light phase of their light:dark cycle mice were placed singly in a 2 litre clear plexiglass beaker (12.5cm x 23.5cm) filled to a height of 12cm with 1.25l water (27±1°C), such that their tails could not touch the bottom of the beaker. Each test lasted six minutes, with the immobility being marked in the final four minutes only. Immobility was defined as an absence of movement, except slight leg movements to stay afloat. Animals were then lightly dried and placed under a warm lamp before being returned to their home cage.

The sucrose preference test was carried out one week after the forced swim test. Following procedures set out in (Strekalova, Spanagel, Bartsch, Henn, & Gass, 2004) animals housed either in twos or threes were given *ad libitum* access to two water

bottles at 10am, one containing tap water, the other containing a 1% sucrose in tap water solution. Both bottles and bottle tops were washed before being filled, they were then sealed to prevent leakage, tested for liquid availability and weighed before being placed in the cage. Twelve hours later, the placement of the bottles was reversed to control for any side-preference in drinking. After 24 hours had passed, both bottles were then weighed again. Where liquid was found to have leaked in a cage data for that animal was not considered.

*7.2.2.3 Assessment of BMAL-1 expression in microglia*

One week after the sucrose preference test animals were transcardially perfused and brains were processed as previously described. For double staining with BMAL1 and IBA-1 brains were first stained using standard NiDAB protocol for BMAL1(rabbit, 1:500, sc-48790) as previously described. Following three washes with 0.1M PB, sections were then blocked for one hour in 0.1M 0.03% PBX containing 5% NGS before being incubated overnight at 4˚C in 0.1M 0.03% PBX containing 2% NGS and a 1:3000 dilution of IBA-1 (Rabbit, Wako). The following day, sections were washed and incubated with anti-rabbit secondary as previously described before colour development with NovaRed (Vector labs, SK-4800). This staining was carried out using a VECTOR NovaRED peroxidise substrate kit according to manufacturer's instructions. Sections were then washed in 0.1M PB before being mounted, dehydrated, delipified and coverslipped. For analysis, the number of microglia within a single field of view in the dorsal CA1 area was calculated via an Olympus BX-51 light microscope at 40x magnification. This was repeated for 4 sections per animal. The number of microglia which were also stained positive for BMAL1 were then analysed and this was used to calculate the percentage values used for statistical analysis.

*7.2.3 Statistical Analysis*

All statistical analysis was conducted in IBM SPSS version 20. For the assessment of clock gene rhythmicity data were analysed using two-way ANOVAs with time and treatment as between groups factors. To examine the level of BMAL1 in microglia independent t-tests were utilised with genotype as the independent variable. For behavioural tests with LPS two-way ANOVAs were conducted with genotype as a between groups factor and treatment as a within groups factor, while for testing in naïve animals independent t-tests were used to examine the effects of genotype. P values of < 0.05 were accepted as significant for all tests. Where statistical assumptions were violated corrected values are presented. All data are presented as means ± standard error of the mean (SEM). * represents p<0.05, ** represents p<0.01 and *** represents p<0.001. Scale bars on microphotographs represent 100µm for photos at 40x and 100x magnification and 50µm for photos at 400x magnification.

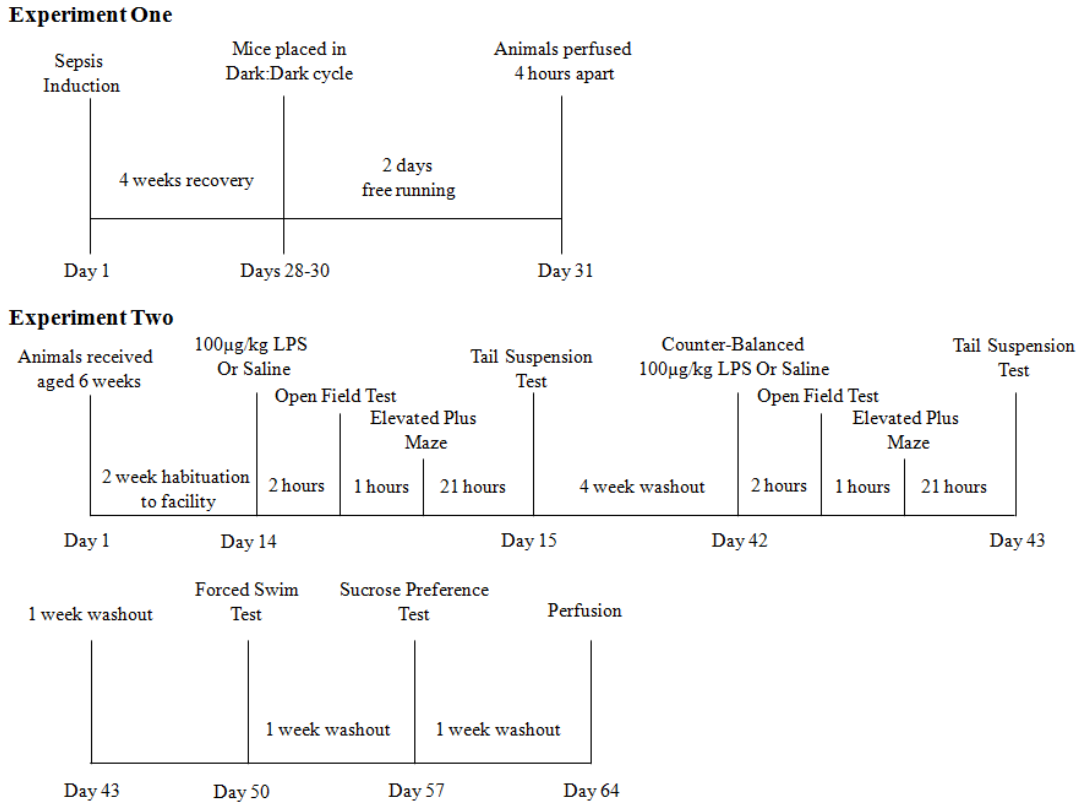



**Figure 7.1 Timeline for circadian experiments.**

*7.3 Results*

*7.3.1 Effect of Sepsis on Hippocampal Clock Gene Expression*

*7.3.1.1 PER1*

Two-way ANOVAs were conducted for each hippocampal subregion with n=3 mice per group per timepoint for each area. As shown in Figure 7.2, there was no main effect of treatment ($F_{1,24}$=0.04, p=0.843), timepoint ($F_{5,24}$=1.46, p=0.239) or interaction effect ($F_{5,24}$=1.331, p=0.285) in the granule cell layer. In the polymorphic cell layer there was no effect of treatment ($F_{1,24}$=1.238, p=0.277), or interaction effect ($F_{5,24}$=0.139), or timepoint ($F_{5,24}$=2.559, p=0.054). In the CA1 region there was a main effect of treatment ($F_{1,24}$=5.288, p=0.03), time ($F_{5,24}$=13.301, p<0.0005) and an interaction effect ($F_{5,24}$=5.312, p=0.002). In the CA2 region there was no main effect of treatment ($F_{1,24}$=1.104, p=0.304), although there was a main effect of time ($F_{5,24}$=2.976, p=0.031) and an interaction effect ($F_{5,24}$=2.846, p=0.037). Lastly, in the CA3 region there was no main effect of treatment ($F_{1,24}$=0.346, p=0.562) although there was a main effect of time ($F_{5,24}$=4.747, p=0.004) and no interaction effect ($F_{5,24}$=1.813, p=0.148). Where analysed as a whole there was no main effect of treatment ($F_{1,24}$=1.091, p=0.307) although there was a main effect of time ($F_{5,24}$=7.16, p<0.0005) and interaction effect present ($F_{5,24}$=3.19, p=0.024).

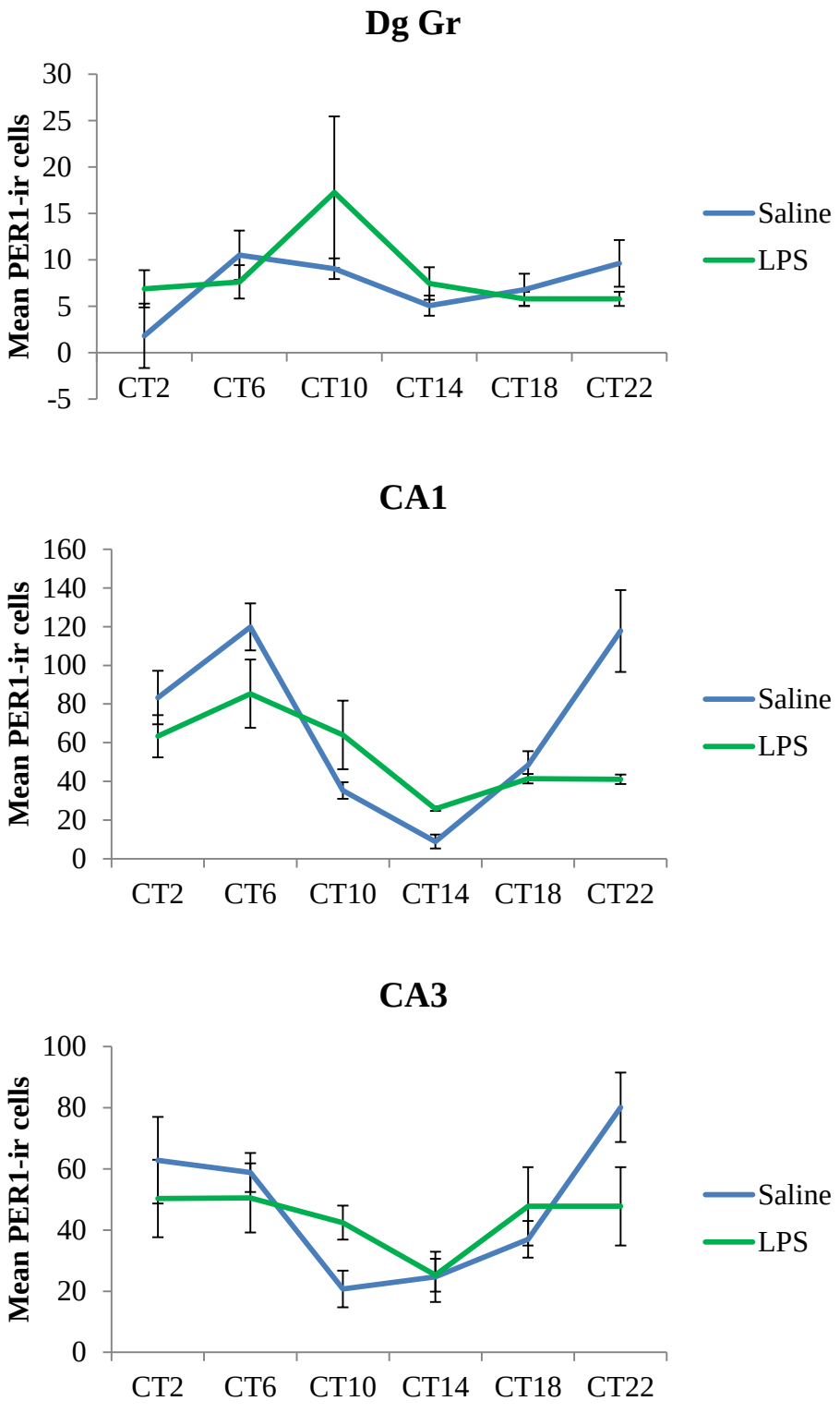



**Figure 7.2 PER1 expression across a 24-hour cycle.** Line graphs illustrating mean PER1 expression in DgGr, CA1 and CA3 at each of the six timepoints. Error bars represent 1xSEM. N=3 per group per timepoint.

*7.3.1.2 PER2*

Two-way ANOVAs were conducted for each hippocampal subregion with n=3 mice per group per timepoint for each area. There was no significant effect of timepoint in the granule cell layer ($F_{5,24}$=0.647, p=0.666) although there was a main effect of treatment ($F_{1,24}$=5.694, p=0.025) but no interaction ($F_{5,24}$=0.768, p=0.582). In the polymorphic cell layer there was a main effect of time ($F_{5,24}$=4.642, p=0.004), but not treatment ($F_{1,24}$=0.784, p=0.385) and no interaction effect ($F_{5,24}$=0.906, p=0.493). In the CA1 region there was a main effect of time ($F_{5,24}$=3.021, p=0.03) and a main effect of treatment ($F_{1,24}$=4.471, p=0.045) but no significant interaction ($F_{5,24}$=0.218, p=0.952). In the CA2 region there was no main effect of time ($F_{5,24}$=1.928, p=0.128), a main effect of treatment ($F_{1,24}$=9356, p=0.008) but no interaction ($F_{5,24}$=0.354, p=0.874). In the CA3 region there was no main effect of time ($F_{5,24}$=1.656, p=0.184), treatment ($F_{1,24}$=0.129, p=0.723) or interaction between the variables ($F_{5,24}$=0.996, p=0.441). When analysed as a whole there was a main effect of timepoint ($F_{5,24}$=2.959, p=0.032), and treatment ($F_{1,24}$=6.235, p=0.02) but no interaction effect ($F_{5,24}$=0.334, p=0.887). These results are illustrated in Figure 7.3.

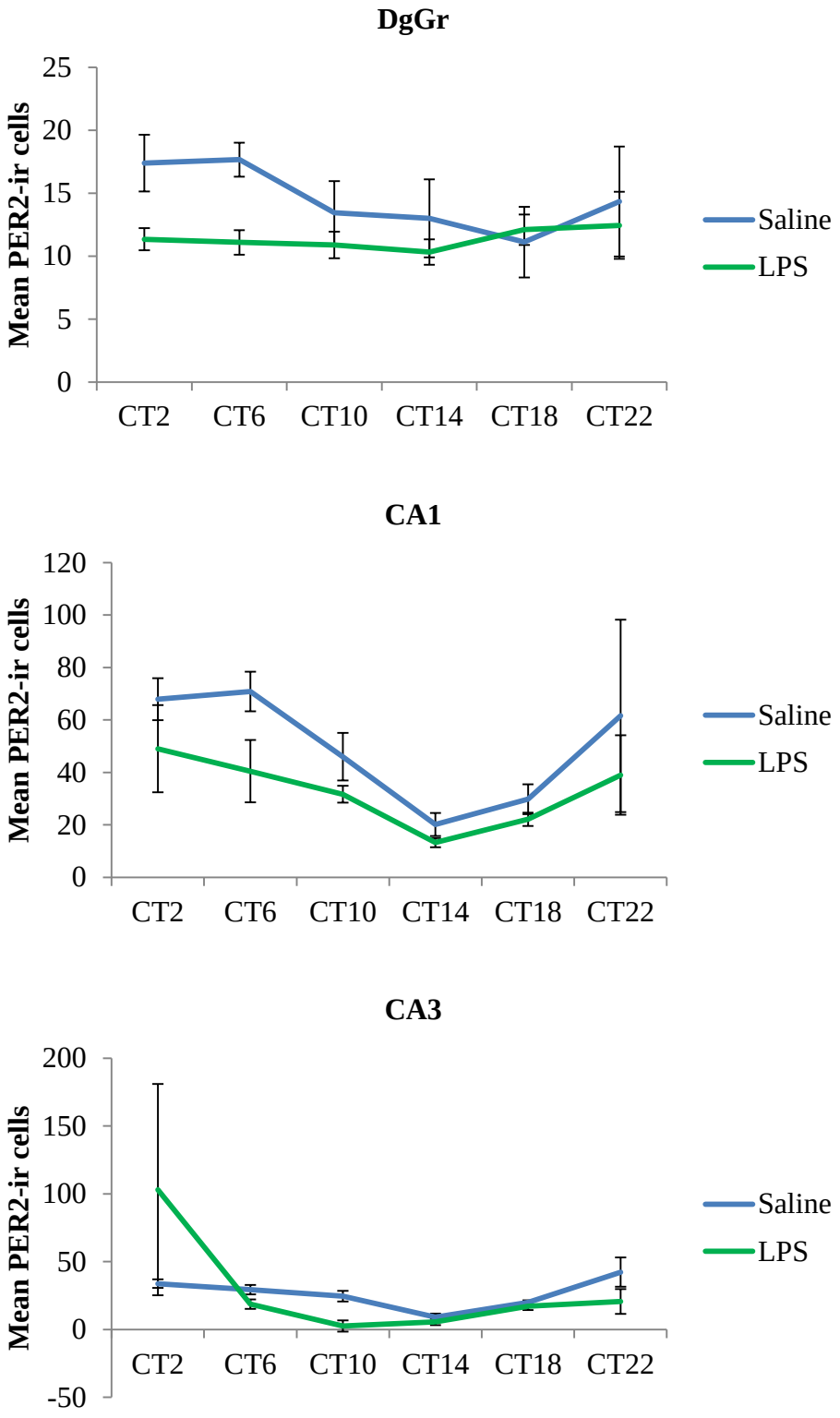



**Figure 7.3 PER2 expression across a 24-hour cycle.** Line graphs illustrating mean PER2 expression in DgGr, CA1 and CA3 at each of the six timepoints. Error bars represent 1xSEM. N=3 per group per timepoint.

*7.3.1.3. CLOCK*

Two-way ANOVAs were conducted for each hippocampal subregion with n=3 mice per group per timepoint for each area. As illustrated in Figure 7.4, there was no significant effect of treatment ($F_{1,24}$=0.401, p=0.533), timepoint ($F_{5,24}$=0.585, p=0.712) or interaction effect ($F_{5,24}$=0.026, p=1.00) in the granule cell layer. In the polymorphic cell layer there was also no main effect of treatment ($F_{1,24}$<0.0005, p=0.992), timepoint, ($F_{5,24}$=0.381, p=0.857) or interaction effect ($F_{5,24}$=0.198, p=0.96). In the CA1 region there was no main effect of treatment ($F_{1,24}$=0.001, p=0.979), timepoint ($F_{5,24}$=0.918, p=0.486) or interaction effect ($F_{5,24}$=0.027, p=1.00). In the CA2 region there was no main effect of treatment ($F_{1,24}$=0.003, p=0.96), timepoint ($F_{5,24}$=0.496, p=0.496) and no interaction effect ($F_{5,24}$=0.321, p=0.895). In the CA3 region there was no main effect of treatment ($F_{1,24}$=0.002, p=0.962), timepoint ($F_{5,24}$=0.253, p=0.934) or interaction effect ($F_{5,24}$=0.146, p=0.979). Analysed as a whole there was also no main effect of treatment ($F_{1,24}$=0.023, p=0.88), timepoint, ($F_{5,24}$=0.569, p=0.723) or interaction effect ($F_{5,24}$=0.114, p=0.988).

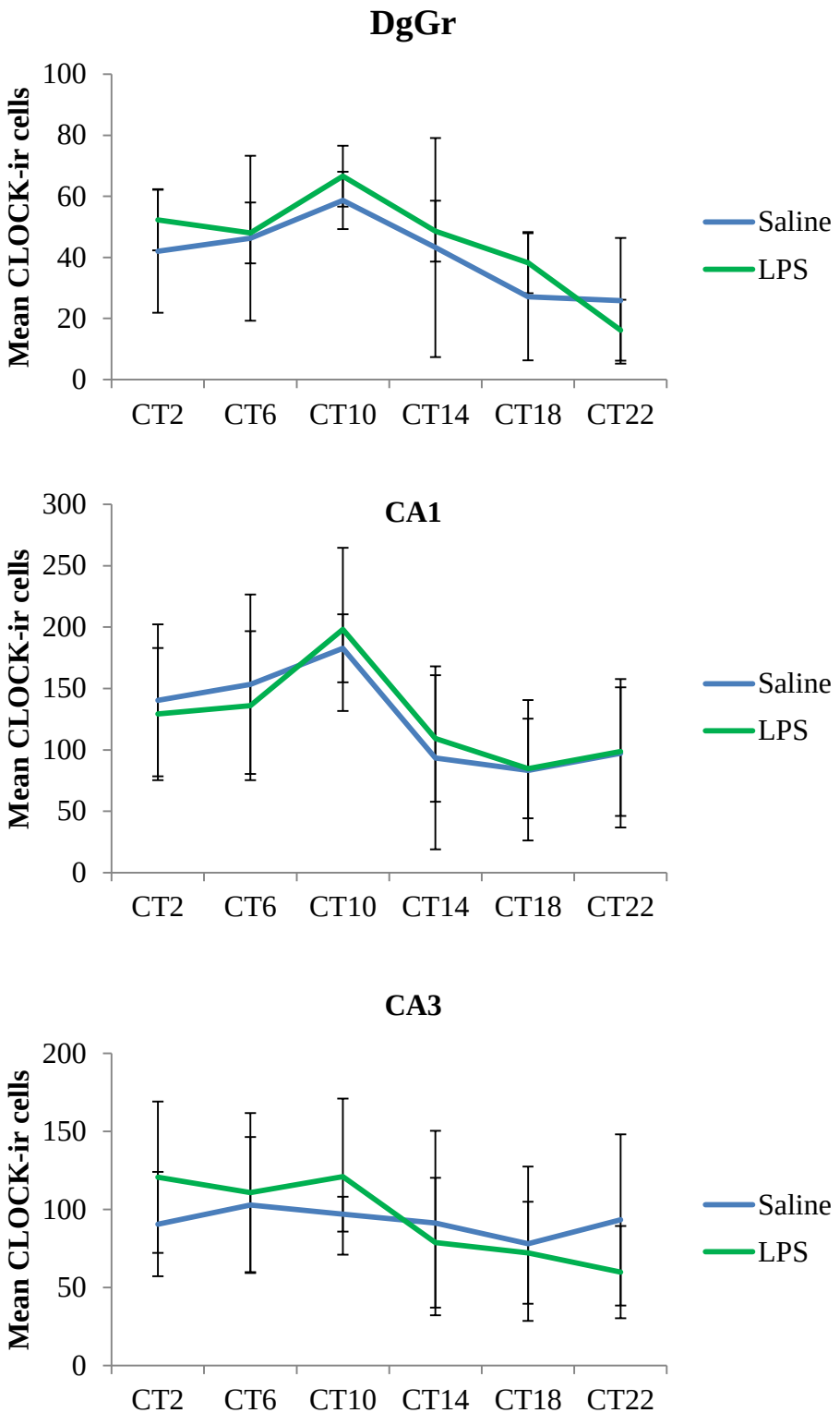



**Figure 7.4 CLOCK expression across a 24-hour cycle.** Line graphs illustrating mean CLOCK expression in DgGr, CA1 and CA3 at each of the six timepoints. Error bars represent 1xSEM. N=3 per group per timepoint.

*7.3.1.4. C-Fos*

Shown in Figure 7.5, two-way ANOVAs showed that there was no main effect of timepoint (n=3/group/timepoint, $F_{5,24}$=0.282, p=0.918), treatment ($F_{1,24}$=0.125, p=0.727) or interaction effect in the granule cell layer ($F_{5,24}$=0.336, p=0.886). In the polymorphic cell layer there was also no main effect of timepoint ($F_{5,24}$=0.882, p=.508), treatment, ($F_{1,24}$=0.076, p=0.786) or interaction effect ($F_{5,24}$=076, p=0.587). In the CA1 region there was no main effect of timepoint ($F_{5,24}$=0.499, p=0.774), treatment ($F_{1,24}$=0.094, p=0.762) or interaction effect ($F_{5,24}$=0.259, p=0.931). In the CA2 region there was no main effect of timepoint ($F_{5,24}$=0.959, p=0.462), treatment ($F_{1,24}$=1.749, p=0.198) or interaction effect ($F_{5,24}$=1.197, p=0.341). In the CA3 region there was also no main effect of timepoint ($F_{5,24}$=0.386, p=0.386), treatment ($F_{1,24}$=0.227, p=0.638) or interaction effect ($F_{5,24}$=0.345, p=0.88). Lastly, when analysed as a whole there was no main effect of timepoint ($F_{5,24}$=0.484, p=0.785), treatment ($F_{1,24}$=0.092, p=0.764) or interaction effect ($F_{5,24}$=0.284, p=0.917).

Figures 7.6 illustrates the rhythmic expression of these proteins in each group while Figure 7.7 features representative micrographs of peak and trough expression for each protein across the entire hippocampus.

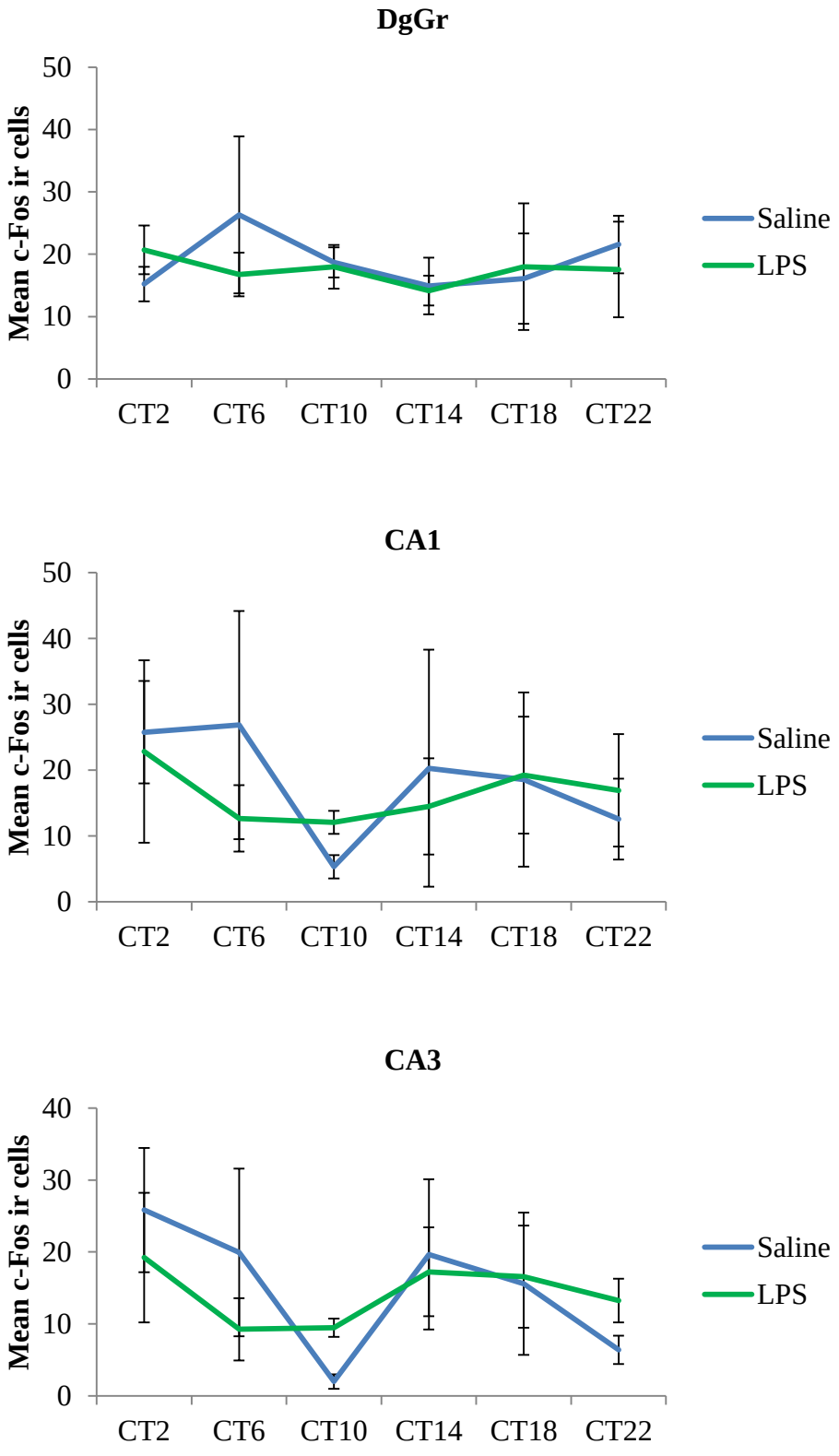



**Figure 7.5 c-Fos expression across a 24-hour cycle.** Line graphs illustrating mean c-Fos expression in DgGr, CA1 and CA3 at each of the six timepoints. Error bars represent 1xSEM. N=3 per group per timepoint.

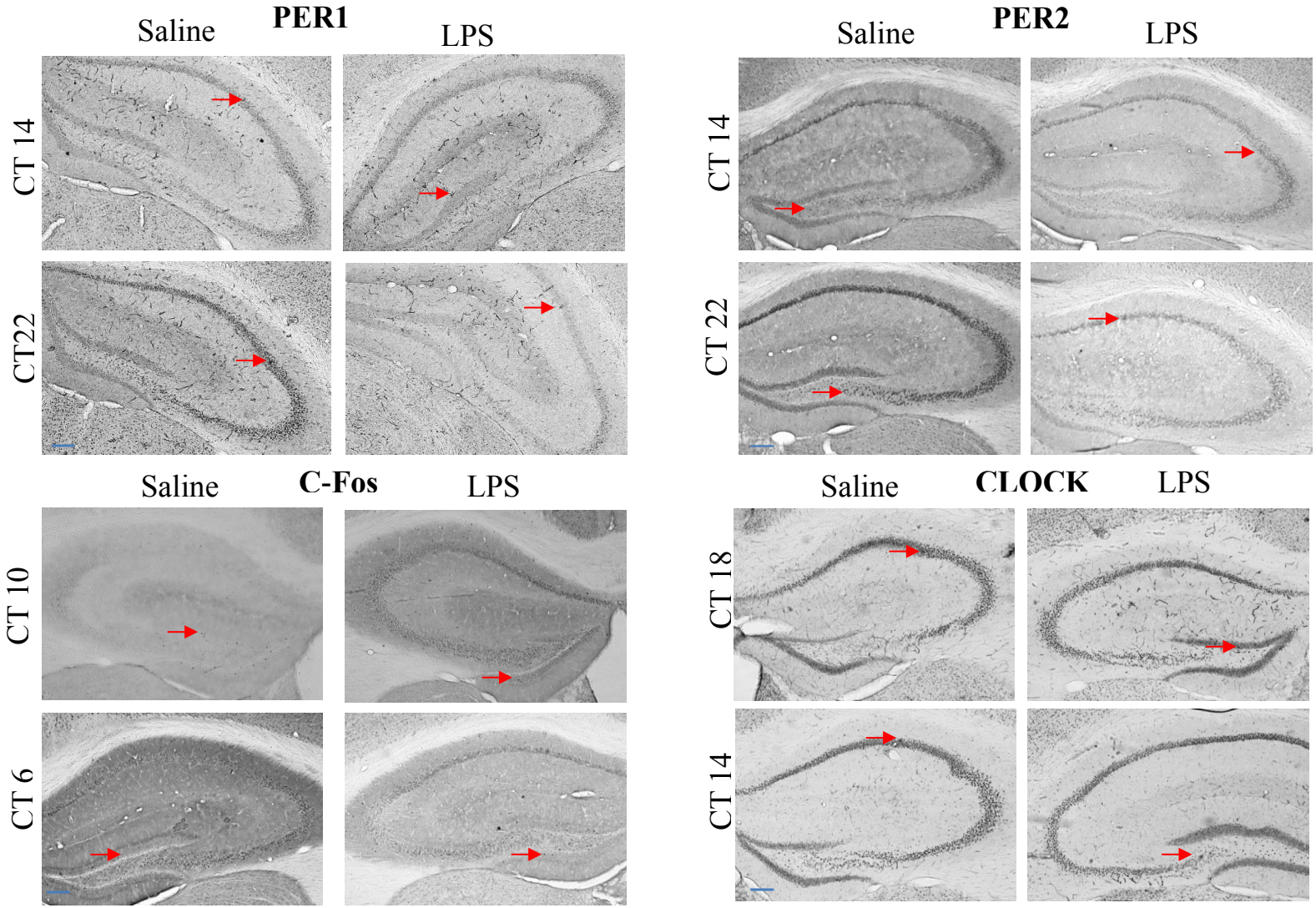



***Figure 7.6 Clock gene expression at peak and trough timepoints in the post-septic hippocampus.***

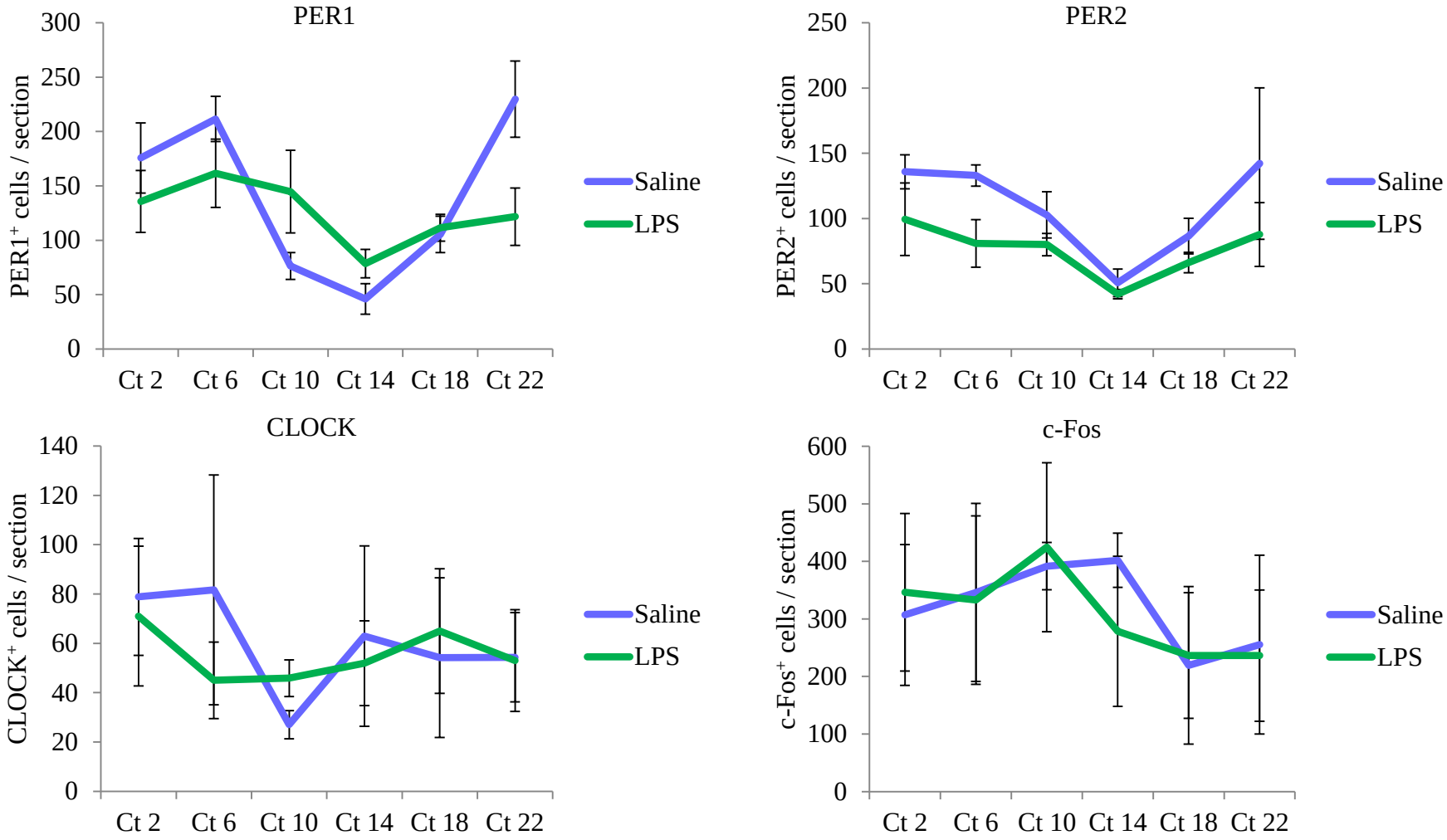



***Figure 7.7 Post-septic mice show alterations in the rhythmic expression of hippocampal clock genes.*** *Values represent mean expression for the total of all five areas. Error bars represent 1x SEM. N=3 per group per timepoint.*

*7.3.2 Experiment Two - Behavioural response of Bmal1$^{+/-}$ mice to LPS*

*7.3.2.1 Open Field Test*

A mixed between-within ANOVA indicated a main effect of LPS treatment (n=21 per treatment) on the distance moved in the open field test ($F_{1,19}$=80.092, p<0.0005) as shown in Figure 7.8. There was no main effect of genotype (n=10 wild-type, 11 BMAL1 $^{+/-}$ mice) $F_{1,19}$=1.005, p=0.329. There was also no treatment x genotype interaction ($F_{1,19}$=0.38, p=0.545). LPS produced a significant reduction in animals' velocity in the arena ($F_{1,19}$=78.955, p<0.0005). There was no main effect of genotype on velocity ($F_{1,19}$=1.054, p=0.318). There was no treatment x genotype interaction ($F_{1,19}$=0.11., p=0.744). LPS also significantly reduced time spent in the inner corridor of the arena ($F_{1,19}$=6.054, p=0.024). There was no main effect of genotype ($F_{1,19}$=2.357, p=0.141). There was no interaction effect between the two variables ($F_{1,19}$=0.183, p=0.674).

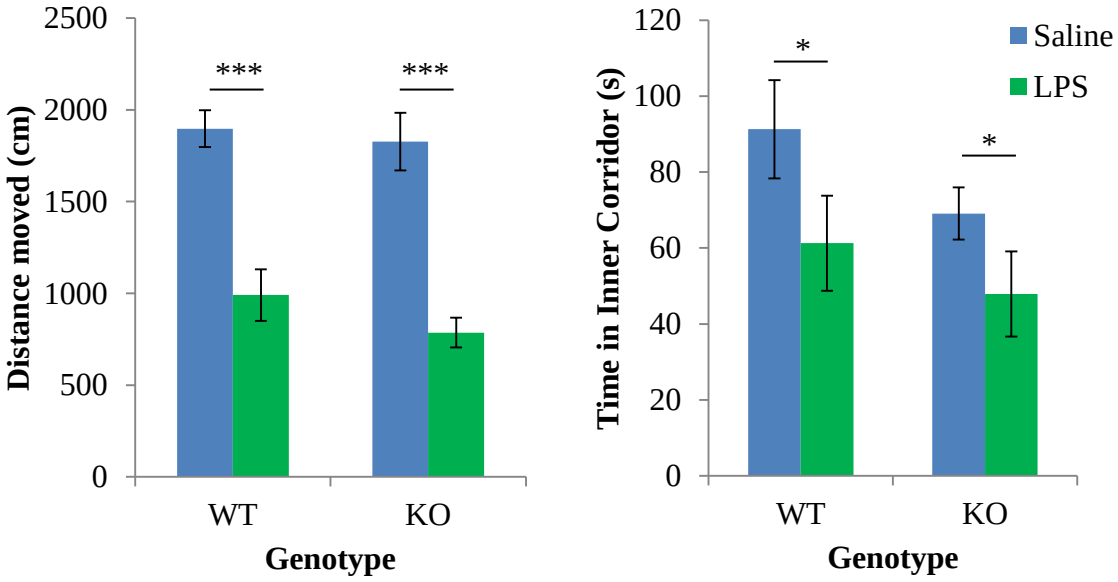



***Figure 7.8 Altered open field exploration following LPS administration in BMAL1$^{+/-}$ mice*** *Bar graphs illustrating distance moved and time spent in the inner portion of the open field test. Error bars represent 1xSEM and */*** represents main effect of LPS administration p<0.05/0/001. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$* mice, n=21 for saline and LPS treatments.

*7.3.1.2 Elevated Plus Maze*

Shown in Figure 7.9, there was a main effect of treatment (n=21 per treatment) on the number of open arm entries in the elevated plus maze ($F_{1,19}$=24.056, p<0.0005). There was no main effect of genotype ($F_{1,19}$= 0.156, p=0.697). There was no interaction effect between treatment and genotype (n=10 wild-type, 11 BMAL1 $^{+/-}$ mice) $F_{1,19}$=0.896, p=0.356. There was also a main effect of treatment on the number of closed arm entries ($F_{1,19}$=37.039, p=0.0092). There was no main effect of genotype ($F_{1,19}$=1.884, p=0.186). There was no interaction effect between the two variables ($F_{1,19}$=3.156, p=0.092). There was a main effect of treatment on time spent in the open arms of the maze ($F_{1,19}$=8.576, p=0.009). There was no main effect of genotype ($F_{1,19}$=0.958, p=0.34). There was no interaction effect between the two variables ($F_{1,19}$=0.073, p=0.789). There was a main effect of treatment on time spent in the closed arms of the maze ($F_{1,19}$=8.318, p=0.01). There was no main effect of genotype ($F_{1,19}$=0.009, p=0.925). There was no interaction effect between the variables ($F_{1,19}$=0.159, p=0.695).

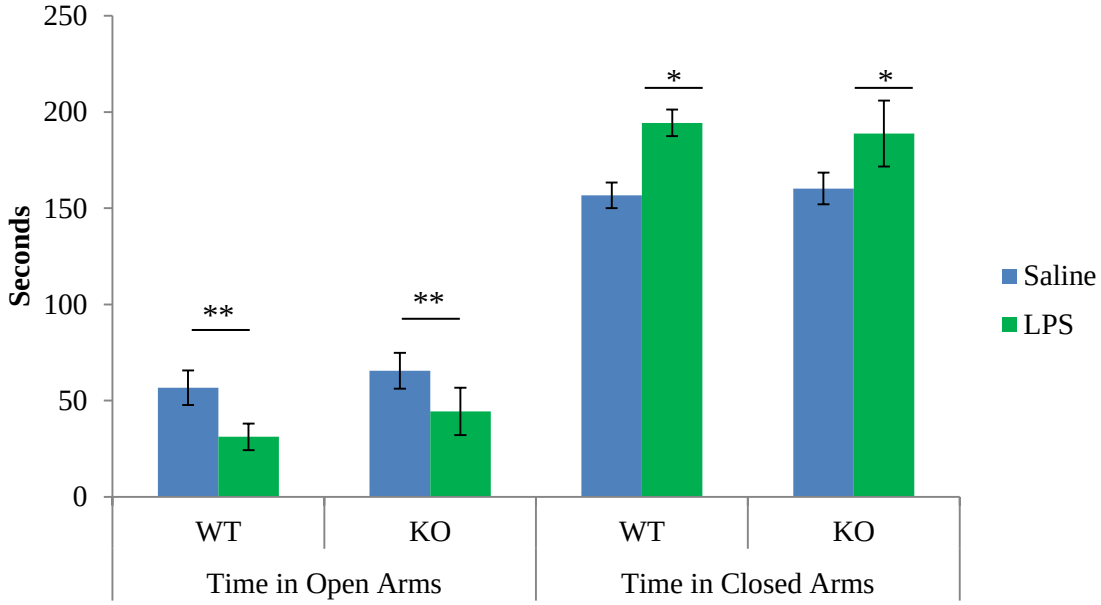



***Figure 75 Altered elevated plus maze exploration following LPS administration in BMAL1$^{+/-}$ mice.*** *Error bars represent 1xSEM and */** represents main effect of LPS*

*administration p<0.05/0/01. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$* mice, n=21 for saline and LPS treatments.

*7.3.1.3 Tail Suspension Test*

In the tail suspension test, as shown in Figure 7.10, there was a significant main effect of LPS treatment (n=20 for saline, 19 for LPS, $F_{1,17}$=5.776, p=0.028). There was no effect of genotype (n=10 for wildtype, n=10 for $^{BMAL1+/-}$, $F_{1,17}$=2.413, p=0.139). There was no interaction effect between the two variables ($F_{1,17}$=1.493, p=0.238). Dependent t-tests revealed a significant effect of treatment in the wild type animals ($t_8$=2.417, p=0.042), but not the KO animals ($t_9$ = 0.886, p = 0.399).

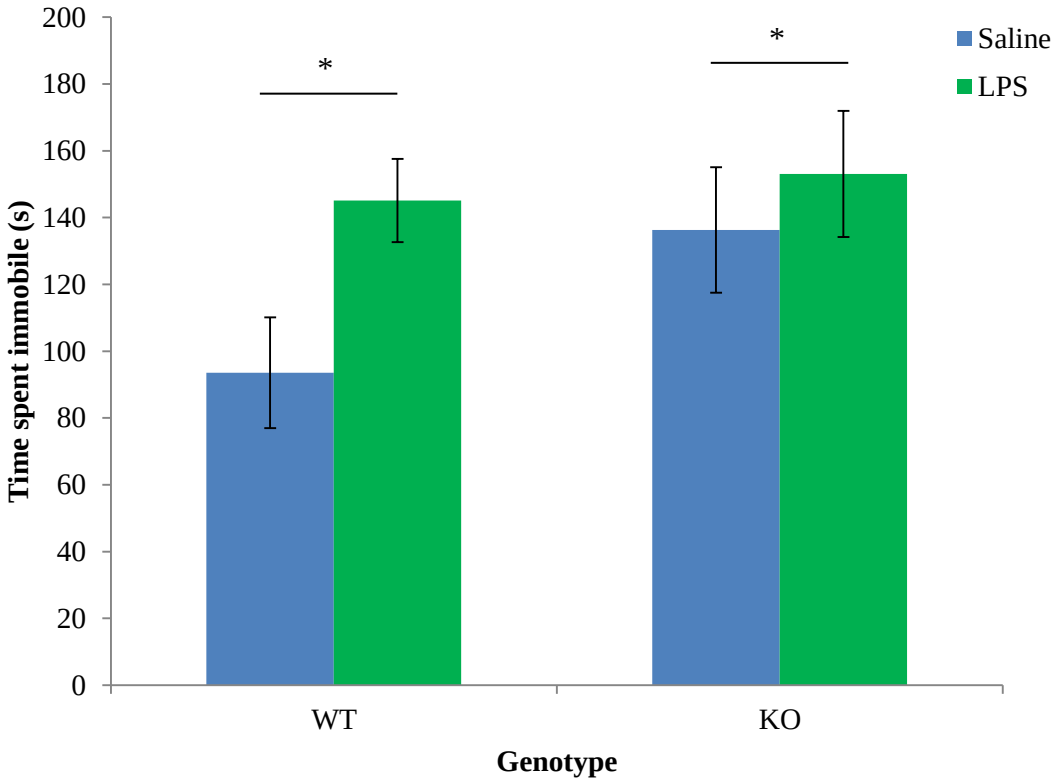



***Figure 7.10 BMAL1$^{+/-}$ mice are less affected by LPS administration in the tail suspension test.*** *Bar graph illustrating time spent immobile by both genotypes following LPS or saline administration. Error bars represent 1xSEM and * represents main effect of LPS administration p<0.05. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$* mice, n=21 for saline and LPS treatments.

### *7.3.3 Baseline behaviour of BMAL1$^{+/-}$ mice*

#### *7.3.3.1 Forced Swim Test*

An independent t-test revealed a significant difference in baseline depressive-like behaviour between wild type (n=10) and *Bmal1$^{+/-}$ (n=11)* mice. As illustrated in Figure 7.11, the wild type mice spent an average of 86±10.50 seconds immobile, while the KO mice spent 119.27±6.27 seconds immobile ($t_{19}$=2.78, p=0.012). This may explain an absence of main effect of LPS on the *Bmal1$^{+/-}$* group in the tail suspension test, since these animals begin with a higher baseline.

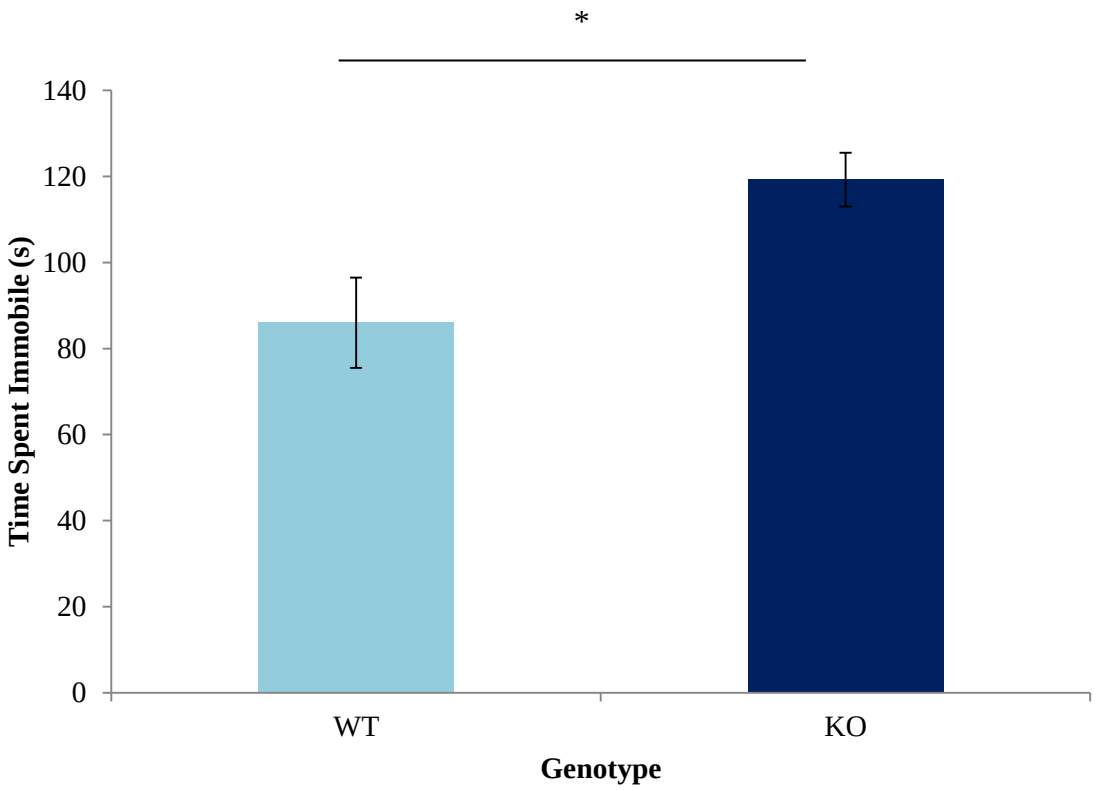



***Figure 7.11 BMAL1$^{+/-}$ mice show increased behavioural despair in the forced swim test.*** *Bar graph illustrating time spent immobile by both genotypes. Error bars represent 1x SEM and * represents main effect of genotype p<0.05. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$* mice.

7.3.3.2 Sucrose Preference Test

An independent t-test, illustrated in Figure 7.8, indicated no difference between wild type (n=10) and knock out (n=11) animals in terms of sucrose preference ($t_{10}$=0.006, p=0.995). As illustrated in Figure 7.12, wild type mice had a mean preference of 72.32±2.98% while *Bmal1*$^{+/-}$ animals had a mean preference of 72.29±4.44%.

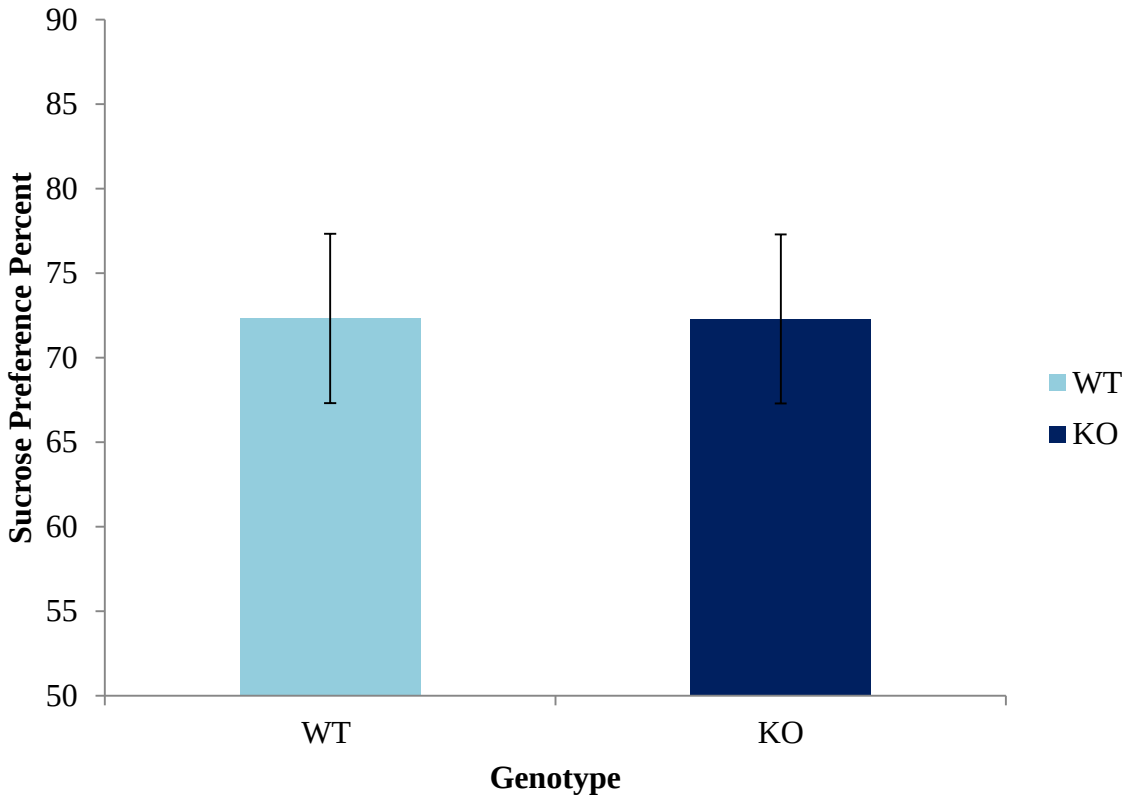



***Figure 7.12 BMAL1$^{+/-}$ mice show no anhedonia in the sucrose preference test***. *Bar graph illustrating sucrose preference percent for both genotypes. Error bars represent 1x SEM and * represents p<0.05. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$.*

*7.3.4 Immunohistochemical analysis*

Immunohistochemical analysis of the number of microglia expressing BMAL1 within the cell nucleus was conducted by double staining with IBA-1 (NovaRED) and BMAL1 (DAB) antibodies. An independent t-test revealed no significant differences in the number of microglia within one field of view of the CA1 region – 40.575±1.5% for WT animals, 38±2.15% for *Bmal1*$^{+/-}$ mice, ($t_{19}$=0.961, p=0.349). Microglial morphology in both groups appeared to be normal. An independent t-test revealed that there was a lower percent of microglia positive for BMAL1 within the nucleus in *Bmal1*$^{+/-}$ mice (n=11, 12.78±1.42%) than WT mice (n=10, 19.51±1.61%), $t_{19}$=3.152, p=0.005. Photomicrographs in Figure 7.13 illustrates the field of view utilised to assess BMAL1 co-localisation with IBA-1 as well as higher magnifications exampling both microglia with and without BMAL1 expression.

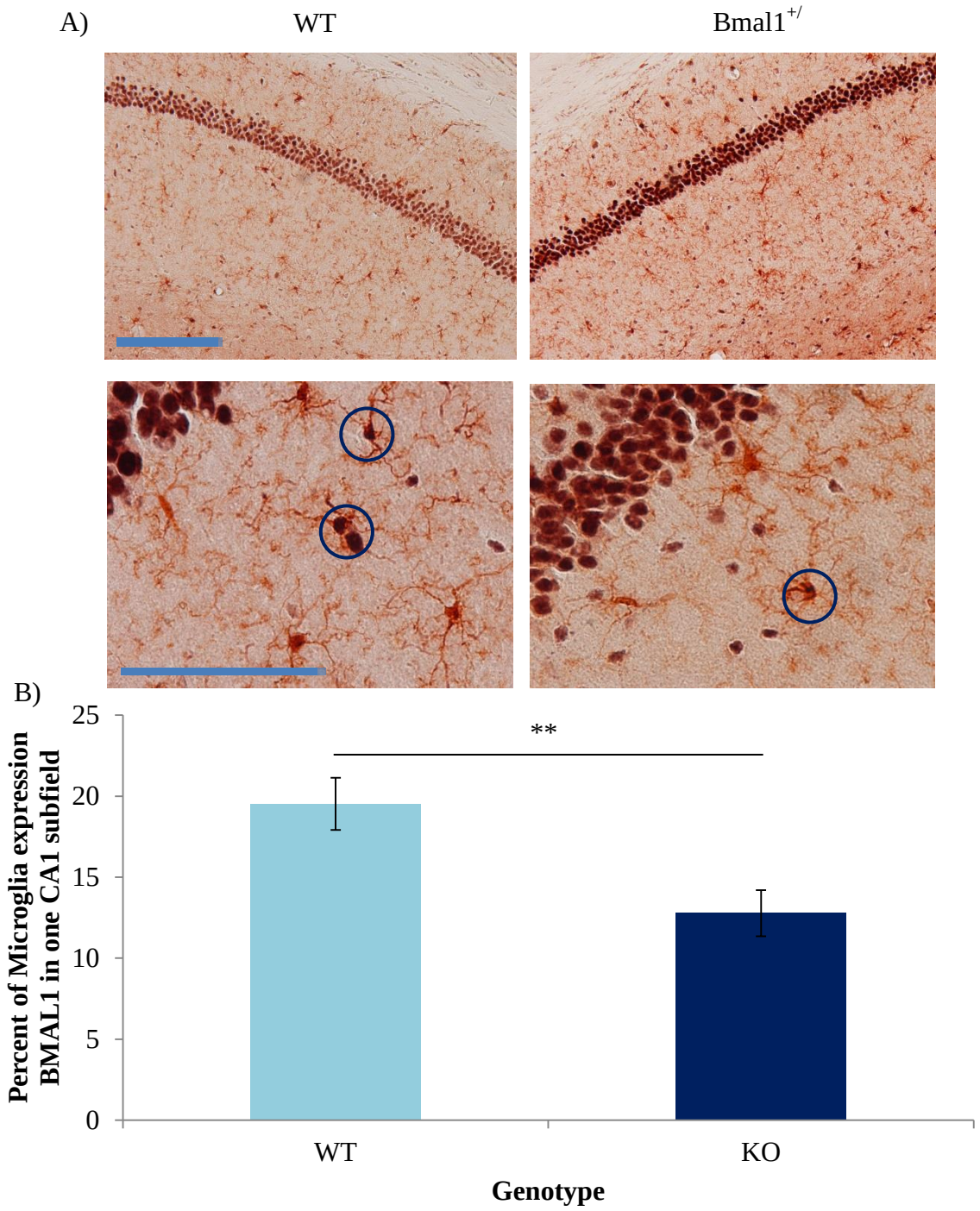



***Figure 7.13 BMAL1 expression in microglia according to genotype*** *A) Photomicrographs (100x, 400x) illustrating co-localised BMAL1 and IBA-1 staining in the CA1 region of WT and Bmal1$^{+/-}$ mice scale bars represent 100µm (100x) and 50µm (400x) B) Bar graph illustrating the percentage of microglia expressing BMAL1 within their cell bodies. Error bars represent 1x SEM, ** represents main effect of genotype p<0.01. N=10 for wild-type mice, n=11 for BMAL1$^{+/-}$* mice.

*7.4 Discussion*

This chapter describes the long-lasting effects of sepsis on circadian rhythmicity within the hippocampus as well as whether genetic disruption of microglial clocks via heterozygous *Bmal1* deletion in myeloid-lineage cells affects the behavioural response to LPS. It is shown that, despite high variation, the clock gene protein products PER1 and PER2 appear to be rhythmically expressed within the hippocampus, while CLOCK and c-Fos are not rhythmic in their expression. In the post-septic animal the magnitude of this rhythmicity is attenuated. In the second set of experiments it is shown that the sickness behaviour exhibited by *Bmal1*$^{+/-}$ mice following acute low dose LPS administration is not significantly different to that of wild type animals, although certain aspects of depression-like behaviour may be elevated at baseline.

*7.4.1 PER1 and PER2 expression in the post-septic hippocampus*

Both PER1 and PER2 expression appeared to be rhythmic in the hippocampus of untreated mice in the present experiment. There were some regional differences in PER1 expression, with the strongest rhythmicity occurring within the CA1 region. Despite variations in the magnitude of rhythmicity among subfields, the temporal pattern of peak and trough expression was relatively even across each area. PER2 on the other hand seemed to be equally rhythmic throughout all hippocampal subfields, again with all areas showing similar temporal patterns. This synchronised expression of clock genes across each area of the hippocampus is in line with other data on hippocampal clock gene expression in C3H/J mice, although the temporal pattern is not (Jilg, et al., 2009; Feillet, Mendoza, Albrecht, Pévet, & Challet, 2008). The differences in rhythmicity between C3H and C57BL/6 mice may be due to the high levels of melatonin production in the former, compared with low levels of production the latter (Dinet, Ansari, Torres-Farfan, & Korf, 2007; von Gall, et al., 2000). These two strains

also show different behavioural patterns of rhythmicity over a 24 hour cycle without intervention. Although even following administration of melatonin to C57BL/6 mice, which express functional melatonin receptors despite not producing the hormone (Dubovich, Hudson, Sumaya, Masana, & Manna, 2005), differences persist (Adamah-Biassi, Stepien, Hudson, & Dubovich, 2013) suggesting that other mechanisms may also contribute to different temporal patterns of clock gene expression within the hippocampus of these strains.

Though the amplitude of both PER1 and PER2 rhythmicity is dampened in the post-septic hippocampus, the phase does not seem to be altered. LPS has previously been shown to elicit short-term suppression of clock gene expression in SCN and liver (Okada, et al., 2008), but the long-lasting effects of sepsis on hippocampal clock gene protein products has not previously been examined. It is possible that this is either a downstream effect of disruption to SCN rhythmicity or output, or that endogenous clock gene production in the hippocampus itself is altered following sepsis-induction. As discussed, it is as of yet unclear precisely which aspects of hippocampal rhythmicity are endogenous and which are under SCN control. Although no definite SCN to hippocampus pathway is known, the SCN does output to areas which in turn affect hippocampal function (Ruby, et al., 2008) – and as the current experiment utilise a whole-system model, in which SCN rhythmicity is affected (O'Callaghan, et al., 2012) it is unlikely that circadian oscillations in the hippocampus are perturbed in isolation.

Disruption of PER1 and PER2 rhythms may have significant consequences for hippocampal functioning in post-septic mice. Bouchard-Cannon et al (2013) showed that *Per2* was essential for gating the entrance of quiescent neural precursor cells to the neurogenesis cycle, thereby controlling the production of newborn hippocampal neurons. *Per2* KO mice also show subtle deficits in LTP, hippocampal phosphorylated-

CREB levels, and long-term, but not short-term, recall of trace fear memories (Wang, et al., 2009). In Bouchard-Cannon et al's (2013) experiment *Per2* KO mice do not however show any deficits in hippocampal-dependent behavioural tasks indicating that *Per2* disruption may not, by itself, be sufficient to impair most hippocampal-dependent function. Similarly to *Per2* ablation, knock out of *Per1* also leads to alterations in a number of parameters within the mouse hippocampus. This includes the cessation of rhythmic pCREB production, as well as altered gene expression and reduced LTP magnitude (Rawashdeh, et al., 2014). *Per1* KO mice also show significant deficits in the hippocampal-dependent radial arm maze (Jilg, et al., 2009). Although mice knocked out completely for *Per1* or *Per2* show these significant changes in hippocampal functioning, disruption of these genes' rhythmic expression may also be sufficient to affect hippocampal neurogenesis and other hippocampal function in post-septic mice. As the expression of BDNF, a key neurotrophin in depressive-like behaviour, and its receptor TrkB are under circadian control (Martin-Fairey & Nunez, 2014), disruption of this rhythmicity due to altered PER1/PER2 expression could represent one mechanism through which hippocampal circadian proteins contribute to affective changes in post-septic mice.

### *7.4.2 CLOCK Expression in the Post-Septic Hippocampus*

CLOCK protein product did not appear to be rhythmically expressed in the hippocampus of either post-septic or control mice. This is in contrast to C3H mice which show rhythmic expression of CLOCK with peaks during the subjective night (Jilg, et al., 2009). Previous examination of CLOCK within the hippocampus of young C57BL/6 mice showed it to be rhythmically expressed in the dentate gyrus but not CA1 or CA3 (Wyse & Coogan, 2010), while BMAL1 was expressed rhythmically in the CA1 region and CA3. A lack of diurnal variation in CLOCK is somewhat surprising since it

is a core part of the activating transcriptional CLOCK:BMAL1 heterodimer. The large degree of inter-animal variation in CLOCK expression, even among non-septic mice, and relatively low number of mice per group/timepoint may have contributed to this lack of effect. Alternatively, if hippocampal circadian rhythmicity is controlled only by synchronization via the SCN rather than endogenous signalling then CLOCK may be functionally redundant, at least in terms of circadian rhythmicity, in the hippocampus. CLOCK may still however fulfil a non-circadian role within the hippocampus (Uz, et al., 2005), such as its action during cocaine sensitization (McClung, et al., 2005), which does not require rhythmic expression. Unlike Per1 and Per2, CLOCK has not been directly implicated in controlling LTP or other aspects of hippocampal functioning, although it is a positive regulator of both Per genes when heterodimerised with BMAL1 (Gerstner, 2012).

*7.4.3. c-Fos Expression in the Post-Septic Hippocampus*

Though c-Fos expression appears rhythmic in the hippocampus of C3H mice (Pantazopoulos, Dolatshad, & Davis, 2011) it did not appear to be rhythmically expressed in any area of the C57BL/6 hippocampus in the present experiment. As c-Fos is most commonly used as a marker of neuronal activation (Benito & Barco, 2014) this likely implies that there is no change in basal activity within hippocampal neurons across the circadian cycle. Although other important hippocampal processes show diurnal oscillations such as adenylyl cyclase activity (Phan, et al., 2011), as well as MAPK and cAMP pathway signalling (Eckel-Mahan, 2012), BDNF expression (Schaaf, Duurland, de Kloet, & Vreugdenhil, 2000), and an increased magnitude in stimulus-induced LTP during the subjective night versus the subjective day (Chaudhury, Wang, & Colwell, 2005) – it is possible that basal neuronal activity in the absence of stimuli does not change significantly over the circadian cycle. Munn and Bilkey (2012) showed

that a majority of 48 CA1 place cells, entrained to environmental entry and food availability, demonstrating circadian oscillations in their firing rate in freely moving Sprague-Dawley rats, although some exhibited antiphase rhythmicity and several were also arrhythmic. These animals also differ from C57BL/6 mice in that they show circadian oscillation in melatonin levels (Price, et al., 2005), and place cells were not examined in isolation in the present experiment. It may be that certain cell populations within the hippocampus show rhythmic activity but when analysed as a whole these areas do not show robust oscillations in c-Fos levels.

Overall, the data from this experiment indicates that both PER1 and PER2 expression oscillate rhythmically within the hippocampus of control animals, although CLOCK does not, and c-Fos production indicates that there is no change in basal neuronal activity across a 24 hour cycle. LPS-induced sepsis appears to reduce the magnitude of rhythmicity in PER1 and PER2 expression although no phase shifting occurs. Post-septic animals also do not show alterations in overall c-Fos expression, in line with results from previous chapters. These changes do not however result in deficits in hippocampal-dependent cognitive tasks suggesting that disruption in Per1/2 rhythmicity may not be necessary for learning and memory – despite their involvement in LTP (Rawashdeh, et al., 2014; Wang, et al., 2009). Zueger et al. (2006) showed, contrary to some other findings, that *Per1* and *Per2* KO mice could still learn normally in the hippocampal-dependent Morris watermaze and contextual fear condition paradigms however showing that rather than being necessary for memory formation *Per1/2* may more subtley modulate hippocampal functioning – for example by controlling diurnal variations in time to learn and successful performance of such tasks.

*7.4.4 Phenotyping an Immune-circadian Mutant*

In the second set of experiments it was examined whether a heterozygous knock out for BMAL1 in myeloid cells would exhibit different responses to LPS treatment than wild-type animals. BMAL1 has been suggested to be one of the most important factor in modulating the circadian aspect of immune responses (Curtis, Bellet, Sassone-Corsi, & O'Neill, 2014). Myeloid-specific deletion of *Bmal1* has previously been shown to alter chemokine expression and predispose mice to pathologies involving both acute and chronic inflammation (Ngyuyen, et al., 2013). *Bmal1* is also the only clock gene whose knockout leads to complete arhythmicity, although this can be remedied in part by artificial promotion of the constitutive expression of *Bmal2* (Shi, et al., 2010). It was therefore hypothesised that these mice may exhibit an exaggerated sickness response to a low dose of LPS. As CLOCK is a driver of NF-κB transcription (Spengler, et al., 2012) and BMAL1:CLOCK dimers suppresses *Clock* transcription, it is possible that the repression of BMAL1 could lead to increased susceptibility to excessive inflammation.

When examining microglia within the hippocampus of these knockout mice, it was found that there were no gross changes to microglia number or morphology. This indicates that despite heterozygous *Bmal1* deletion in myeloid cells, microglia can still develop at a normal rate and without any overt morphological phenotypes. It was found that, despite a significant reduction in the amount of BMAL1 expressed in hippocampal microglia, it was still present in ~10% of cells. This represents roughly half the expression of BMAL1 protein in microglia as in wildtype counterparts. When administered LPS both groups of mice showed similar behavioural changes, indicating that there was no increased susceptibility to LPS-induced sickness behaviour in the KO mice. It was noted however that BMAL1 deficient animals appeared to have higher

baseline depressive and anxious-like behaviour in the tail suspension test and the open field test. It was therefore decided to examined their behaviour in the forced swim test and sucrose preference test without an acute LPS or saline treatment. Here, KO mice showed increased behavioural despair but there was no effect on anhedonia. A lack of exaggerated reaction to LPS may therefore be explained by animals' elevated baseline propensity of some depressive-like behaviours. Another possibility is that these mice may only exhibit altered responses to LPS when examined at specific timepoints. For example, BMAL1 deletion in peritoneal exudates cells, the majority of which are macrophages, leads to a loss of gating in response to LPS, although the magnitude of the response is not more exaggerated overall (Gibbs, et al., 2011). BMAL1 appears to be anti-inflammatory (Curtis, Bellet, Sassone-Corsi, & O'Neill, 2014) and its levels vary according to the circadian cycle. Therefore if LPS is administered at a time when BMAL1 expression is at trough expression in control animals there may appear to be no effect. If LPS were administered when BMAL1 levels are at peak circadian expression in wild-type animals then an effect of BMAL1 knockdown may be more apparent. As this mouse has heterozygous *Bmal1* knockout in all myeloid lineage cells are affected in this mouse any effect, or lack thereof, cannot be attributed specifically to hippocampal microglia but likely involves many other myeloid lineage cells.

That both the *Bmal1*$^{+/-}$ mice and post-septic wildtype C57BL/6 mice show elevated depressive-like behaviours illustrates the interplay between the circadian and immune systems, and the role of both in the pathophysiology of depressive disorders (Salgado-Delgado, Osorio, Saderi, & Escobar, 2011; Patel, 2013). While the link between the circadian and inflammatory systems is bi-directional (Cermakian, et al., 2013), inflammation appears to be associated with pre-disposing, or even causative, factors for depression such as stress and obesity (Berk, et al., 2013) rather than a result

of depressive disorders themselves. The results of the present experiments highlight the ability of sepsis to produce long-lasting changes in hippocampal circadian rhythmicity in addition to prolonged microglial activation. These factors are particularly important for post-septic patients given that circadian disruption is present during severe illness (Verceles, et al., 2012) and long-term sleep impairment, as well as depressive symptoms, are experienced in the aftermath critical illness (Weinhouse & Schwab, 2006).

# Chapter 8. General Discussion

The goal of this thesis has been to characterise to as full an extent as possible the long-lasting effects of a septic dose of LPS in mice. In Chapters 2, and 3 involved an in depth exploration of this model of post-septic encephalopathy – establishing the affective changes present in these animals and the neuroinflammatory state of the hippocampus. In contrast to the intense microglial activation seen at 24 hours post-sepsis, a pattern emerges across Chapters 3, 4 and 5 of a persistent but low-grade microglial activation within the hippocampus which appears to impact on other factors within this region. Despite no increase in cytokine levels, post-septic mice show subtle differences in plasticity-related protein products (ARC, EGR-1) and cell proliferation in the SGZ. There is also a reduction of c-FOS and EGR-1 expression in the amygdala indicating that neuronal activation in this structure may be altered. These post-septic mice do not show any overt changes in locomotion or cognition across any behavioural tasks, yet reliably exhibit depressive-like and anxiety-like behaviours and respond well to anti-depressant treatment.

Building on these findings it was then explored how these changes might be modulated either by disruption of the NF-κB pathway prior to sepsis induction, or by the administration of the SSRI fluoxetine. In light of the findings that post-septic mice appear to show elevated microglial activation without an over-production of cytokines, it was then explored whether these animals might be exhibiting microglial priming. This did not appear to be the case. Also examined were the effects of sepsis on circadian clock gene expression. Circadian parameters are often disturbed in patients with sepsis, and post-septic mice here also appear to show disruption to their circadian system. These later experiments provide insight into some of the processes which may be at play in the post-septic brain and provide further evidence to support the relationship

between excessive microglial activity in the post-septic hippocampus and the behavioural changes observed.

Unexpectedly, our results differed quite significantly both from other experiments modelling post-septic encephalopathy, and from what one might expect given the array of deficits seen in post-septic patients. Despite finding significant neuroinflammatory changes within the dorsal hippocampus of our post-septic mice, these animals did not show the cognitive deficits across a range of tasks that one would expect with this pathology. It could be that the level of inflammation present in our model was not sufficient to produce cognitive deficits – findings from CLP models report more aggressive inflammatory changes with oxidative stress (Comim, et al., 2011) and Aβ accumulation (Schwalm, et al., 2014) which likely lead to more potent disruption of hippocampal processes and resulting cognitive function. The hippocampus is also highly plastic and capable of significant functional recovery after insult (Hoffman, et al., 2011) which may be at play here given the length of time between sepsis-induction and behavioural testing. Although suppressed, adult hippocampal cell proliferation is not absent in post-septic mice, which could contribute to a recovery process (Zhang & Chopp, 2009). This also raises the possibility that the affective changes present post-sepsis are modulated, at least in part, by brain areas outside the hippocampus which have less potential for recovery.

These findings remain valuable, however, and contribute new information to a relatively small field of study regarding a very poorly understood disorder. While it has previously been shown that a single intraperitoneal injection of LPS can induce long lasting inflammation and cognitive symptoms (Semmler, et al., 2007), this is the first time that it has been shown that the same treatment can result in chronic behavioural changes typical of a depressive state. These experiments are also the first to show that

these animals have a deficiency in adult hippocampal cell production – something which may be implicated in both cognitive and affective dysfunction (Christian, Song, & Ming, 2014). This provides a clue to what may underlie cognitive changes in human post-septic patients and suggests an avenue of therapeutic investigation for a syndrome with no treatment currently available. It is shown here that treatment with an SSRI can stimulate cell proliferation in the SGZ as well as reverse depressive-like behaviour in a model of post-septic encephalopathy, and it would be very valuable to investigate if this may also be the case for patients. This is also interesting because fluoxetine's effect on neurogenesis had not previously been studied in the context of a large dose of LPS.

When viewing the present work in its entirety it becomes apparent that systemic LPS administration will not always produce exactly the same effects in every experiment – a factor that is already known in terms of the intraperitoneal administration of other substances (Gaines Das & North, 2007). Reviewing the data from the present experiments it can be seen that there are some cases where null results occur where significant results were present in other cohorts (e.g. the sucrose preference test in Chapter 5 versus Chapters 3 and 4), and in examining the health ratings of animals after LPS administration it can be seen that there is some individual variance in the severity of sepsis experienced. It may therefore be advisable to interpret some findings from this model with caution where they occur in isolation or where sample sises are not large. It is important to note that there is also considerable variability when cecal ligation and puncture is used to model sepsis, particularly when performed by different experimenters (Cuenca, Delano, Kelly-Scumpia, Moldawer, & Efron, 2010). Behavioural testing is also generally prone to high levels of variability, even with inbred mouse strains (Hånell & Marklund, 2014). Of course the human condition of post-septic

encephalopathy is also highly heterogeneous and not all sufferers of sepsis will develop encephalopathy (Papadopoulos, et al., 2000).

Also shown is the importance of considering the hippocampus, as much as possible, in terms of its various subregions rather than as a unitary structure. In several of the comparative studies of post-septic rodents the hippocampus is considered as a homogenous region (Bossù, et al., 2012; Cassol-Jr, et al., 2010; Michels, et al., 2014; Weberpals, et al., 2009). Aside from the functional distinctions proposed between the dorsal and ventral hippocampus (Fanselow & Dong, 2010), it is shown here there is differential susceptibility of the different hippocampus subregions following sepsis. In all three chapters (2, 7 and 8) where IBA1 staining was examined, for example, there seemed to be a greater degree of microglial activation in the CA1 region than CA3 regions. Egr-1 staining throughout these experiments reveals a similar pattern with larger effects in the CA1 region than CA3 – although in this case the difference could also be a function of the sparser staining overall in this subregion. This differential susceptibility is also reflected in the hippocampus' vulnerability to both exogenous and endogenous oxidative stress with the CA1 the most prone to injury (Vornov, Park, & Thomas, 1998). As discussed in Chapter 1, these subregions are different in terms of their cellular anatomy, function and connectivity – and the demonstration of their variation in susceptibility provides further reason for their separate consideration in research on post-septic encephalopathy.

As this work involves the characterisation of a model of a human syndrome, it is also prudent here to evaluate whether this post-septic model is relatable to the human condition of post-septic encephalopathy. Recent work has shed some doubt on the utility of such models, at least in terms of the acute effects of sepsis, with mouse models displaying many differences to the immunological response exhibited by

humans and translatability of clinical findings have generally been poor (Dyson & Singer, 2009; Seok, et al., 2013; Zofaghari, Pinto, Dyson, & Singer, 2013). Specifically the LPS-induced model induces a more rapid and extreme upregulation of pro-inflammatory cytokines than is seen in human sepsis (Rittirsch, Hoesel, & Ward, 2007), although some other aspects of the pathology (e.g. apoptosis of immune cells, lymphocyte gene expression and phenotypic shifting of T cells for example) appear to be better reproduced (Hotchkiss, Monneret, & Payen, 2013). It is difficult to know to what extent this model may reflect the neurological changes present in post-septic patients given the lack of understanding of this condition's aetiology (Ziaja, 2013). In terms of modelling post-septic encephalopathy the most striking difference may be that, as reviewed in the introduction, while post-septic patients often display cognitive difficulties and only sometimes show affective symptoms, our results indicate that following LPS-administration post-septic animals show primarily affective changes. It could therefore be suggested that these post-septic animals may reflect a subset of post-septic patients which experience affective symptoms – or it could be that this model more closely reflects some of the changes present in affective disorders themselves, as has been discussed in Chapter 7. As sepsis in human populations typically occurs either very early or very late in life, one suggestion to increase the relevancy of septic animal models to human conditions is to induce sepsis in aged mice (Fink, 2014).

The human applications of the data from these experiments is therefore limited by our understanding of what specifically drives the lasting cognitive and emotional deficits in post-septic patients. With due caution, however, these results may still inform investigative and therapeutic approaches in post-septic people. The experiments in this thesis have illustrated that post-septic mice reliably show long-lasting microglial activation, and interventions which reduce this activation appear beneficial in terms of

behaviour. New technology is now beginning to allow for the *in vivo* imaging of microglia and macrophages in humans (Venneti, Lopresti & Wiley, 2013). Therefore one avenue of investigation informed by the present results may be to assess whether or not post-septic patients show ongoing microglial activation and whether interventions which target this inflammation are beneficial to these patients' cognitive and emotional states. These treatments would need to be adjusted to specific patients' needs and backgrounds but could include for example and SSRI as used here, or more traditional anti-inflammatories.

The present experiments are limited in certain aspects however. One of the main limitations of these experiments is the use of only immunohistochemistry when analysing the inflammatory state of the hippocampus. Although immunohistochemistry is a powerful technique, allowing for the examination of the spatial, functional and morphological examination of many protein products (Frevert, Johnson, & Stahl, 2014), it is not without pitfalls. The most significant problem with immunohistochemistry is that of antibody specificity – the potential for one's antibody to bind to unintended targets and therefore produce staining which is misleading (Rhodes & Trimmer, 2006). This danger can be mitigated to some extent by verifying antibody specificity by comparison with other techniques such as *in situ* hybridization and testing negative controls which omit the primary or secondary antibody (Rhodes & Trimmer, 2006). It has been shown however that even antibodies which pass these controls may still produce staining in KO models – indicating that true antibody verification may not be possible without examining its staining in a KO animal (Baek, Darlington, Smith, & Ashton, 2013). Although it is not possible within the present study to generate a knockout of every gene examined, analysis of hippocampal tissue with other techniques such as western blotting or ELISAs may help to confirm the present findings. It is also

worth noting that results which were positive in Chapter 3 were generally repeatable in later experiments – although there have been some discrepancies, for example in the margin of effects between hippocampal subregions.

Another limitation which becomes apparent in light of the behavioural results obtained is that analysis of the hipoccampus was done primarily at the dorsal to mid level, rather than at the ventral level. Initially this was done to allow for comparison with other studies of the post-septic brain (Bossù, et al., 2012; Fan, et al., 2012; Qin, et al., 2007; Semmler, et al., 2007; Weberpals, et al., 2009) many of which also reported changes in cognitive measures among post-septic rodents and examine dorsal hippocampal functioning. Here however affective rather than cognitive changes were found when post-septic behaviour was examined. As discussed in the introduction, these changes are usually associated with the ventral hippocampus – as well as other areas such as the amygdala and prefrontal cortex. Given the number of proteins and experimental conditions examined, it was not feasible to quantify the changes in all of these areas – although altered amygdala IEG production is noted. Also, despite the attribution of cognition to the dorsal hippocampus and emotion to the ventral hippocampus, there is evidence that neither function is isolated to a specific area (Beer et al, 2014; Keinath, et al., 2014; Tanti & Belzung, 2012). In addition, the mid region - included for analysis in the present experiments - appears to be more or less equally involved in both types of processing (Kheirbek, et al., 2013), and there is of course a high degree of interconnectivity between different levels of the hippocampus (Amaral & Lavenex, 2007). Therefore, although correlative observations regarding changes in the dorsal hippocampus and related behaviours can be made, it is not prudent to infer causality as other areas are likely also involved.

With these points in mind, future work could evolve from these findings in numerous directions. In order to better understand how this model relates to the human condition, it would be very useful to examine post-mortem brain tissue of post-septic patients. Post-mortem analysis of patients who died during sepsis shows a pathology involving ischaemia, haemorrhaging, as well as occasional involvement of micro-abscesses, microglial and neuronal apoptosis, elevated microglial activation, cytokine levels and vascular iNOS expression (Sharshar, et al., 2002; Sharshar, et al., 2003; Sharshar, et al., 2004). Little work has looked specifically at the hippocampus, although one case study found hippocampal neuronal loss and ischemic lesions in a patient who died while experiencing post-septic encephalopathy (Finelli & Uphoff, 2004). No similar examination has been done in patients who died after recovering from sepsis which would provide data with which to compare neurological findings in animal models. Although such findings may be complicated by comorbidities and medication. Another easier approach would be to further characterise the neurological changes present in post-septic mice – for example, investigating whether the abscesses, hippocampal volume reduction and ischaemia present in human septic encephalopathy are also present in the current model, or investigating electrophysiological changes given the EEG changes present in post-septic patients (Semmler, et al., 2013). There is much still to investigate regarding the microglial activation present within post-septic mice. For example, it is not clear how microglia persist in an activated morphology despite no measurable increase in pro-inflammatory cytokine production. Potential study could examine the time course of microglial morphofunctional change from the acute stages of sepsis to the 2 month timepoint examined here, perhaps with more attention to anti-inflammatory cytokines and microglial “off” signals such as CD200 (Biber, Neumann, Inoue, & Boddeke, 2007). Greater knowledge of how closely this

model mirrors the human condition will lead to improved potential for treatment development.

The other potential direction for future research stemming from this work would be to use the model in the investigation of inflammation and mood disorders. Although the aetiology of the various mood disorders are not much better understood than post-septic encephalopathy, there is a much broader literature, both in terms of human studies and animal models, which provides a context for the interpretation of findings. As has been discussed both acute (Wang Z. , et al., 2014) and chronic (Kubera, et al., 2013) LPS administration are already used to model depression in rodents – although this research provides a different perspective using a single peripheral administration to produce long-lasting effects. Many interesting avenues could be pursued focusing on this aspect of the model. Mechanistic work could be done examining the role of microglia in modulating signalling along hippocampal and amygdala pathways important to mood (Benson, et al., 2014). Other factors implicated in the pathology of mood disorders which have not been investigated in the present model include monoaminergic neurotransmission (Sperner-Untwerger, Kohl, & Fuchs, 2014), glucorticoid signalling (Pandey, Rizavi, Ren, Dwivedi, & Palkovits, 2013) and hippocampal volume (Videbech & Ravnkilde, 2004). As the development of mood disorders is commonly preceded by significant psychological stress (Horesh & Iancu, 2010) another interesting approach could be to combine this inflammatory model with an established behavioural model such as chronic unpredictable mild stress.

In conclusion, this work has provided a detailed characterization of the long-lasting effects of a septic dose of LPS on behaviour, cognition and the neuroinflammatory state of the hippocampus in mice. Further work needs to be done

however to describe the mechanisms which lead to these changes and also to assess how closely this model mirrors the human syndrome.

# References


Abd-El-Fattah, M. A., Abdelakader, N. F., & Zaki, H. F. (2014). Pyrrolidine dithiocarbamate protects against scopolamine-induced cognitive impairment in rats. *European Journal of Pharmacology 273*, 330-338.

Abdel-Salam, O. M., Baiuomy, A. R., & Arbid, M. S. (2004). Studies on the anti-inflammatory effects of fluoxetine in the rat. *Pharmacological Research 29(2)*, 119-131.

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., . . . Block, G. D. (2002). Circadian rhythms in isolated brain regions. *The Journal of Neuroscience 22(1)*, 350-356.

Adamah-Biassi, E. B., Stepien, I., Hudson, R. L., & Dubovich, M. L. (2013). Automated video analysis system reveals distinct diurnal behaviors in C57BL/6 and C3H/HeN mice. *Behavioral Brain Research 243*, 306-312.

Ahmed, S. H., He, Y. Y., Nassief, A., Xu, J., Xu, X. M., Hsu, C. Y., & Faraci, F. M. (2000). Effects of lipopolysaccharide priming on acute ischemic brain injury. *Stroke 31(1)*, 193-199.

Alboni, S., Benatti, C., Montanari, C., Tascedda, F., & Brunello, N. (2013). Chronic antidepressant treatments resulted in altered expression of genes involved in inflammation in the rat hypothalamus. *European Journal of Pharmacology 721(1-3)*, 158-167.

Allen, N. J., & Barres, B. A. (2009). Neuroscience: Glia - more than just brain glue. *Nature 457(5)*, 675-677.

Alme, C. B., Buzzetti, R. A., Marrone, D. F., Leutgeb, J. K., Chawla, M. K., Schaner, M. J., . . . Barnes, C. A. (2010). Hippocampal granule cells opt for early retirement. *Hippocampus 20(10)*, 1109-1123.

Amaral, D. G., Scharfman, H. E., & Lavenex, P. (2007). *The dentate gyrus: A comprehensive guide to structure, function, and clinical implications.* (E. Scharfman, Ed.) Oxford, UK: Elselvier Science.

Amaral, D., & Lavenex, P. (2007). *The Hippocampus Book.* (P. Andersen, R. Morris, D. Amaral, T. Bliss, & J. O'Keefe, Eds.) Oxford, UK: Oxford University Press.

Anacker, C., Zunszain, P. A., Caravalho, L. A., & Pariante, C. M. (2011). The glucocorticoid receptor: Pivot of depression and of antidepressant treatment? *Psychoneuroendocrinology 36(3)*, 415-425.

Anderson, S.T., S. Commins, Moynagh P.N., Coogan A.N. (2014) *Lipopolysaccharide-induced sepsis induces long-lasting affective changes in the mouse.* Brain, Behavior & Immunity (In Print) doi: 10.1016/j.bbi.2014.07.007

Anderson, S. W., Barrash, J., Bechara, A., & Tranel, D. (2006). Impairments of emotion and real-world complex behavior following childhood- or adult-onset damage to ventromedial prefrontal cortex. *Journal of the International Neuropsychological Society 12(2)*, 224-235.

Andreasen, J. T., & Redrobe, J. P. (2009). Antidepressant-like effects of nicotine and mecamylamine in the mouse forced swim and tail suspension tests: role of strain, test and sex. *Behavioral Pharmacology. 20(3)*, 286-295.

Arias-Salvatierra, D; Silbergeld, E. K., Acosta-Saaverdra, L. C., Calderon-Aranda, E. S. (2011) Role of nitric oxide produced by iNOS through NF-κB pathway in migration of cerebellar granule neurons induced by Lipopolysaccharide *Cell Signal 23(2)* 425-435

Arkrout, N., Sharshar, T., Annane, D. (2009) Mechanisms of brain signaling during sepsis. *Current Neuropharmacology 7(4).* 296-301

Arndt, S. S., Laarakker, M. C., van Lith, H. A., van der Staay, J., G, Elise., Salomons, A. R., ban't Klooster, J. & Ohl, F. (2009) Individual housing of mice- Impact on behaviour and stress responses. *Physiology & Behavior. 97(3-4)* 385-393

Arruda-Carvalho, M., Sakaguchi, M., Akers, K. G., Josselyn, S. A., & Frankland, P. W. (2011). Posttraining ablation of adult-generated neurons degrades previously aquired memories. *The Journal of Neuroscience 31(42)*, 15113-15127.

Arszovski, A., Borhegyi, Z., & Klausberger, T. (2014). Three axonal projection routes of individual pyramidal cells in the ventral CA1 hippocampus. *Frontiers in Neuroanatomy 8(53).*

Avi, C. (1997) Neural activity mapping with inducible transcription factors. *Neuroreport 8(13)* 3-7

Aydemir, O., Deveci, A., & Taneli, F. (2005). The effect of chronic antidepressant treatment on serum brain-derived neurotrophic factor levels in depressed patients: a preliminary study. *Progress in neuro=psychopharmacology & biological psychiatry 29(2)*, 261-265.

Babu, H., Ramirez-Rodriguez, G., Fabel, K., Bischofberger, J., & Kempermann, G. (2009). Synaptic network activity induces neuronal differentiation of adult hippocampal precursor cells through BDNF signaling. *Frontiers in Neuroscience 3(49).*

Baek, J. H., Darlington, C. L., Smith, P. F., & Ashton, J. C. (2013). Antibody testing for brain immunohistochemistry: Brain immunolabeling for the cannabinoid CB2 receptor. *Journal of Neuroscience Methods 216(2)*, 87-95.

Baganz, N. L., & Blakely, R. D. (2013). A dialogue between the immune systen and brain, spoken in the language of serotonin. *ACS Chemical Neuroscience 3(1)*, 48-63.

Bai, F., Li, X., Clay, M., Lindstrom, T. & Skolnick, P. (2001) Intra- and interstrain differences in models of "behavioral despair" *Pharmacology Biochemistry and Behavior. 70(2-3)* 187-192

Bailey, T. W.; Hermes, S. M.; Whittier, K. L., Aicher, S. A., Andresen, M. C. (2007) A-type potassium channels differentially tune afferent pathways from rat solitary tract nucleus to caudal ventrolateral medulla or paraventricular hypothalamus. *The Journal of Physiology 582* 613-628

Baj, G., D'Alessandro, V., Musazzi, L., Mallei, A., Sartori, C. R., Sciancalepore, M., . . . Tongiorgi, E. (2012). Physical exercise and antidepressants enhance BDNF targeting in hippocampal CA3 dendrites: Further evidence of a spatial code for BDNF splice variants. *Neuropsychopharmacology 37*, 1600-1611.

Bakirci, S., Kafa, I. M., Uysal, M., & Kurt, M. A. (2011). Increased adult neurogenesis in the subventricular zone in a rat model of sepsis. *Neuroscience Letters 497(1)*, 27-31.

Ball, T., Rahm, B., Eickhoff, S. B., Schulze-Bonhage, A., Speck, O. & Mutschler, I (2007) Response properties of human amygdala subregions: Evidence based on functional MRI combined with probabilistic anatomical maps. *PLoS ONE 2(3)* e307

Balschun, D., Wetzel, W., Del Rey, A., Pitossi, F., Schneider, H., Zuschratter, W., & Besedovsky, H. O. (2004). Interleukin-6: a cytokine to forget. *Federation of American Societies for Experimental Biology 18(14)*, 1788-90.

Banjerjee, R., Ghosh, A. K., Ghosh, B., Bhattacharyya, S., & Mondal, A. C. (2013). Decreased mRNA and protein expression of BDNF, NGF, and their receptors in the hippocampus from suicide: An analysis in human postmortem brain. *Clinical Medicine Insights Pathology 6* , 1-11.

Banke, T. G., & McBain, C. J. (2006). GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development. *The Journal of Neuroscience 26(45)*, 11720-11725.

Banks WA, Kastin AJ, Broadwell RD (1995) Passage of cytokines across the blood-brain barrier. *Neuroimmunomodulation* 2: 241–8

Bannister, N. J., & Larkman, A. U. (1995). Dendritic morphology of CA1 pyramidal neurones from the rat hippocampus: I. Branching patters. . *The Journal of Comparative Neurology 360(1)*, 150-160.

Barichello, T., Machado, R., Constantino, L., Valvassori, S. S., Réus, G. Z., Martins, M. R., . . . Dal-Pizzol, F. (2007). Antioxidant treatment prevented late memory impairment in an animal model of sepsis. *Critical Care Medicine. 35(9)*, 2186-2190.

Barnes, P. J., & Karin, M. (1997). Nuclear Factor-κB - A Pivotal Transcription Factor in Chronic Inflammatory Diseases. *New England Journal of Medicine 336*, 1066-1071.

Barr, A. M., Song, C., Sawada, K., Young, C. E., Honer, W. G., & Phillips, A. G. (2003). Tolerance to the anhedonic effects of lipopolysaccharide is associated with changes in syntaxin immunoreactivity in the nucleus accumbens. *The international journal of neuropsychopharmacology 6(1)*, 23-34.

Bast, T., Wilson, I. A., Witter, M. P., & Morris, R. G. (2009). From rapid place learning to behavioural performance: A key role for the intermediate hippocampus. *PLoS Biology 7(4)*, e1000089.

Bastos, G. N., Moriya, T., Inui, F., Katura, T. & Nakahata, N. (2008) Involvement of cyclooxygenase-2 in lipopolysaccharide-induced impaiment of the newborn cell survival in the adult mouse dentate gyrus. *Neuroscience 155* 454-462

Bauer, M. K., Lieb, K., Schulze-Osthoff, K., Berger, M., Gebicke-Haerter, P. J., Bauer, J., & Fiebich, B. L. (1997). Expression and regulation of cyclooxygenase-2 in rat microglia. *European Journal of Biochemistry 243(3)*, 726-731.

Baune, B. T., Dannlowski, U., Domschke, K., Janssen, D. G., Jordan, M. A., Ohrmann, P., . . . Suslow, T. (2010). The Interleukin 1 Beta (IL1B) Gene is Associated with Failure to Achieve Remission and Impaired Emotion Processing in Major Depression. *Biological Psychiatry. 67(6)*, 543-549.

Baune, B. T., Smith, E., Reppermund, S., Air, T., Samaras, K., Lux, O., . . . Trollor, J. N. (2012). Inflammatory biomarkers predict depressive, but not anxiety symptoms during aging: The prospective Sydney memory and Aging Study. *Psychoneuroendocrinology*.

Beer, Z., Chwiesko, C., & Sauvage, M. M. (2014). Processing of spatial and non-spatial information reveals functional homogeneity along the dorso-ventral axis of CA3, but not CA1. *Neurobiology of Learning and Memory 111*, 56-64.

Bellet, M. M., Zocchi, L., & Sassone-Corsi, P. (2012). The RelB subunit of NFκB acts as a negative regulator of circadian gene expression. *Cell Cycle 11(17)*, 3304-3311.

Benes, F. M., Kwok, E. W., Vincent, S. L., & Todtenkopf, M. S. (1998). A reduction of nonpyramidal cells in sector CA2 of schizophrenics and manic depressives. *Biological Psychiatry 44(2)*, 88-97.

Benito, E., & Barco, A. (2014). The Neuronal Activity-Driven Transcriptome. *Molecular Neurobiology* .

Benros, M. E., Waltoft, B. L., Nordentoft, M., Østergaard, S. D., Eaton, W. W., Krogh, J., & Mortensen, P. B. (2013). Autoimmune Diseases and Severe Infections as Risk Factors for Mood Disorders. *JAMA Psychiatry 70(8)*, 812-820.

Benson, B. E., Willis, M. W., Ketter, T. A., Speer, A., Kimbrell, T. A., Herscovitch, P., . . . Post, R. M. (2014). Differential abnormalities of functional connectivity of the amygdala and hippocampus in unipolar and bipolar affective disorders. *Journal of Affective Disorders 168*, 243-253.

Bergami, M., Rimondini, R., Santi, S., Blum, R., Götz, M., & Canossa, M. (2008). Deletion of TrkB in adult progenitors alters newborn neuron integration into

hippocampal circuits and increases anxiety-like behavior. *Proceedings of the National Academy of Sciences 105(40)*, 15570-15575.

Berk, M., Williams, L. J., Jacka, F. N., O'Neil, A., Pasco, J. A., Moylan, S., . . . Maes, M. (2013). So depression is an inflammatory disease, but where does the inflammation come from? *BMC Medicine 11(200)*, doi:10.1186/1741-7015-11-200.

Bhat, R. V., Cole, A. J., & Baraban, J. M. (1992). Role of monamine systems in activation of zif268 by cocaine. *Journal of Psychiatry & Neuroscience 17(3)*, 94-102.

Biber, K., Neumann, H., Inoue, K., & Boddeke, H. W. (2007). Neuronal 'on' and 'off' signals control microglia. *Trends in Neurosciences 30(11)*, 596-602.

Biff, D., Petronilho, F., Constantino, L., Buolo, F., Zamora-Berridi, G. J., Dall'Igna, D. M., . . . Dal-Pizzol, F. (2013). Correlatio of acute phase inflammatory and oxidative markers with long-term cognitive impairment in sepsis survivors rats. *Shock 40(1)*, doi: 10.1097/SHK.0b013e3182959cfa.

Bizik, G., Bob, P., Raboch, J., Pavlat, J., Uhrova, J., Benakova, H. & Zima, T. (2014) Dissociative symptoms reflect levels of tumor necrosis factor alpha in patients with unipolar depression. *Neuropsychiatric Diseases & Treatments 10* 675-679

Bocchio-Chiavetto, L., Zanardini, R., Bortolomasi, M., Abate, M., Segala, M., Giacopuzzi, M., . . . Perez, J. G. (2006). Electroconvulsive Therapy (ECT) increases serum Brain Derived Neurotrophic Factor (BDNF) in drug resistant depressed patients. *European Neuropsychopharmacology: the Journal of the European College of Neuropsychopharmacology 16(8)*, 620-624.

Boer, K. R., van Ruler, O., van Emmerik, A. A., Sprangers, M. A., de Rooij, S. E., Vroom, M. B., . . . Group, T. D. (2008). Factors associated with posttraumatic stress symptoms in a prospective cohort of patients after abdominal sepsis: a nomogram. *Intensive Care Medicine 34(4)*, 664-674.

Boldrini, M., Santiago, A. N., Hen, R., Dwork, A. J., Rosoklija, G. B., Tamir, H., . . . Mann, J. J. (2013). Hippocampal granule neuron number and dentate gyrus volume in antidepressant-treated and untreated major depression. *Neuropsychopharamacology: Official Publication of the American College of Neuropsychopharmacology 38*, 1068-1077.

Boldrini, M., Underwood, M. D., Hen, R., Rosoklija, G. B., Dwork, A. J., Mann, J. J., & Arango, V. (2009). Antidepressants increase neural progenitor cells in the human hippocampus. *Neuropsychopharmacology 34*, 2376-2389.

Bonaccorso, S., Maier, S. F., Meltzer, H. Y., & Maes, M. (2003). Behavioral changes in rats after acute, chronic and repeated administration of Interleukin-1beta: relevance for affective disorders. *Journal of Affective Disorders. 77(2)*, 143-148.

Bonaccorso, S., Puzella, A., marino, V., Pasquini, M., Biondi, M., Artini, M., . . . Maes, M. (2001). Immunotherapy with interferon-alpha in patients affected by chronic hepatitis C induces an intercorrelated stimulation of the cytokine network and an

increase in depressive and anxiety symptoms. *Psychiatry Research. 105(1-2),* 45-55.

Bonow, R. H., Aïd, S., Zhang, Y., Becker, K. G., & Bosetti, F. (2009). The brain expression of genes involved in inflammatory response, the ribosome, and learning and memory is altereed by centrally injected lipopolysaccharide in mice. Gene expression changes in LPS neuroinflammation. *The Pharmacogenenomics Journal 9,* 116-126.

Bortolotto, V., Cuccurazzu, B., Cononico, P. L. & Grilli, M. (2014) NF-κB Mediated Regulation of Adult Hippocampal Neurogenesis: Relevance to Mood Disorders and Antidepressant Activity. *BioMed Research International*

Bossù, P., Cutuli, D., Palladino, I., Caporali, P., Angelucci, F., Laricchiuta, D., . . . Petrosini, L. (2012). A single intraperitoneal injection of endotoxin in rats induces long-lasting modification in behavior and brain protein levels of TNF-α and IL-18. *Journal of Neuroinflammation 9,* 101.

Bouchard-Cannon, P., Mendoza-Viveros, L., Yuen, A., Kærn, M., & Cheng, H. Y. (2013). The circadian molecular clock regulates adult hippocampal neurogenesis by controlling the timing of cell-cycle entry and exit. *Cell Reports 5(4),* 961-973.

Bourin, M., Petit-Demoulière, B., Dhonnchadha, B. N. & Hascöet, M. (2007) Animal models of anxiety in mice. *Fundamental & Clinical Pharmacology 21(6)* 567-574

Bozon, B., Davis, S. & Laroche, S (2003) A requirement for the Immediate Early Gene zif268 in Reconsolidation of Recognition Memory after Retrieval. *Neuron 40(4)* 695-701

Brenes, J. C., & Fornaguera, J. (2009). The effect of chronic fluoxetine on social isolation-induced changes on sucrose consumption, immobility behavior, and on serotonin and dopamine function in hippocampus and ventral striatum. *Behavioural Brain Research 198(1),* 199-205.

Brinks, V., van der Mark, M., de Kloet, R., & Oitzl, M. (2007). Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57BL/6J mice. *Frontiers in Behavioural Neuroscience 1,* Article 8.

Briones, A., Gagno, S., Martisova, E., Dobarro, M., Aisa, B., Solas, M., . . . Ramírez, M. J. (2012). Stress-induced anhedonia is associated with an increase in Alzheimer's disease-related markers. *British Journal of Pharmacology 165(4),* 897-907.

Brivanlou, I. H., Dantzker, J. L., Stevens, C. F., & Callaway, E. M. (2004). Topographic specificity of functional connections from hippocampal CA3 to CA1. *PNAS 101(8),* 2560-2565.

Bronner, M. B., Knoester, H., Sol, J. J., Bos, A. P., Heymans, H. S., & Grootenhuis, M. A. (2009). An explorative study on quality of life and psychological and

cognitive function in pediatric survivors of septic shock. *Pediatric Critical Care Medicine 10(6)*, 636-642.

Brun, V. H., Leutgeb, S., Wu, H. Q., Schwarcz, R., Witter, M. P., Moser, E. I., & Moser, M. B. (2008). Impaired spatial representation in CA1 after lesion of direct input from entorhinal cortex. *Neuron 57(2)*, 290-302.

Bryan, C. E., Spring, D. R., Gangloff, M., & Gay, N. J. (2010). The molecular basis of the host response to lipopolysaccharide. *Nature Reviews Microbiology 8*, 8-14.

Brynskikh, A., Warren, T., Zhu, J., & Kipnis, J. (2008). Adaptive immunity affects learning behaviour in mice. *Brain, Behavior, and Immunity, 22(6)*, 861-869.

Buchanan, T. W., Driscoll, D., Mowrer, S., III, S., J, J., Thayer, J. F., . . . Tranel, D. (2010). Medial prefrontal cortex damage affects physiological and psychological stress responses differently in men and women. *Psychoneuroendocrinology 35(1)*, 56-66.

Buckmaster, P. S., Strowbridge, B. W., & Schwartzkroin, P. A. (1993). A comparison of rat hippocampal mossy cells and CA3c pyramidal cells. *Journal of Neurophysiology 70(4)*, 1281-1299.

Buiesmans, S. M., Bouwknecht, J. A., Acton, P. D., Davoodi, N., De Haes, P., Kuijlaars, J., . . . Nuydens, R. (2013). Systemic immune activation leads to neuroinflammation and sickness behavior in mice. *Mediators of Inflammation*, http://dx.doi.org/10.1155/2013/271359.

Bunting, K., Rao, S., Hardy, K., Woltring, D., Denyer, G. S., Wang, J., . . . Shannon, M. (2007). Genome-wide analysis of gene expression in T cells to indentify targets of the NF-κB transcription factor c-Rel. *The Journal of Immunology 178(11)*, 7097-7109.

Buras, J. A., Holzmann, B., & Sitkovsky, M. (2005). Animal models of sepsis: setting the stage. *Nature Reviews 4*, 854-865.

Burgdorf, J., Kroes, R. A., Weiss, C., Oh, M. M., Disterhoft, J. F., Brudzynski, S. M., . . . Moskal, J. R. (2011). Positive emotional learning is regulated in the medial prefrontral cortex by GluN2B-containing NMDA receptors. *Neuroscience 192*, 515-523.

Butler, M. P., O'Connor, J. J., & Moynagh, P. N. (2004). Dissection of tumor-necrosis factor-alpha inhibition of long-term potentiation (LTP) reveals a p38 mitogen-activated protein kinase-dependent mechanism which maps to early-but not late-phase LTP. *Neuroscience 124(2)*, 319-26.

Calogero, A., Arcella, A., De Gregorio, G., Porcellini, A., Mercola, D., Liu, C., . . . Ragona, G. (2001). The early growth response gene EGR-1 behaves as a suppressor gene that is down-regulated independent of ARF/Mdm2 but not p53 alterations in Fresh Human Gliomas. *Clinical Cancer Research 7*, 2788-2796.

Calsavara, A. C., Rodrigues, D. H., Miranda, A. S., Costa, P. A., Lima, C. X., Vielela, M. C., . . . Teixeira, A. L. (2013). Late anxiety-like behavior and

neuroinflammation in mice subjected to sublethal polymicrobial sepsis. *Neurotoxicity Research 24(2)*, 103-108.

Cameron, H. A., & McKay, R. D. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *Journal of Comparative Neurology 435(4)*, 406-417.

Cao, S., Zhang, X., Edwards, J. P., & Mosser, D. M. (2006). NF-κB1 (p50) Homodimers Differentially Regulate Pro- and Anti-inflammatory Cytokines in Macrophages. *The Jounral of Biologolical Chemistry 281*, 26041-26050.

Capuron, L., Gumnick, J. F., Musselman, D. L., Lawson, D. H., Reemsnyder, A., & Nemeroff, C. B. (2002). Neurobehavioral Effects of Interferon-α in Cancer Patients: Phenomenology and Paroxetine Responsiveness of Symptom Dimensions. *Neuropsychopharmacology. 26(5)*, 643-652.

Carlini, V. P., Poretti, M. B., Rask-Anderse, M., Chavan, R. A., Ponzio, M. F., Sawant, R. S., . . . de Cuneo, M. F. (2012). Deifferential effects of fluoxetine and venlafaxine on memory recognition: possible mechanisms of action. *Progress in Neuropsychopharmacology & Biological Psychaitry 38(2)*, 159-167.

Caruana, D. A., Alexander, G. M., & Dudek, S. M. (2012). New insights into the regulation of synaptic plasticity from and unexprected place: Hippocampal area CA2. *Learning & Memory 19*, 391-400.

Carvalho, L. A., Bergink, V., Sumaski, L., Wijkhuijs, J., Hoogendijk, W. J., Birkenhager, T. K., & Drexhage, H. A. (2014). Inflammatory activation is associated with a reduced glucocorticoid receptor alpha/beta expression ratio in monocytes of inpatients with melancholic major depressive disorder. *Translational Psychiatry 4*, e344.

Casassus, P., Caillat-Vigneron, N., Martin, A., Simon, J., Gallais, V., Beaudry, P., . . . Lortholary, O. (2002). Treatment of adult systemic mastocytosis with interferon-α: results of a multicentre phase II trial on 20 patients. *British Journal of Haematology. 119*, 1090-1097.

Cassol-Jr, O. J., Comim, C. M., Constantino, L. S., Rosa, D. V., Mango, L. A., Stertz, L., . . . Dal-Pizzol, F. (2011). Acute low dose of MK-801 prevents memory deficits without altering hippocampal DARPP-32 expression and BDNF levels in sepsis survivor rats. *Journal of Neuroimmunology. 230(1-2)*, 48-51.

Cassol-Jr, O. J., Comim, C. M., Silva, B. R., Hermani, F. V., Constantino, L. S., Felisberto, F., . . . Dal-Pizzol, F. (2010). Treatment with cannabidiol reverses oxidative stress paramaters, cognitive impairment and mortality in rats submitted to sepsis by cecal ligation and puncture. *Brain Research. 1348*, 128-138.

Castanon-Cervantes, O., Wu, M., Ehlen, J. C., Paul, K., Gamble, K. L., Johnson, R. L., Besing, R. C., Menaker, M., Gewirtz, A. T. & Davidson, A. J. (2010) Dysregulation of Inflammatory Responses by Chronic Circadian Disruption. The *Journal of Immunology 185(10)* 5796-5805

Cavaillon, J. M., Adrie, C., Fitting, C., & Adib-Conguy, M. (2003). Endotoxin tolerance: is there a clinical relevance? *Journal of Endotoxin Research 9(2)*, 101-107.

Ceranik, K., Bender, R., Geiger, J. R., Monyer, H., Jonas, P., Frotscher, M., & Lübke, J. (1997). A Novel Type of GABAergic Interneuron Connecting the Input and the Output Regions of the Hippocampus. *The Journal of Neuroscience 17(14)*, 5380-5394.

Cerbai, F., Lana, D., Nosi, D., Petkova-Kirova, P., Zecchi, S., Brothers, H. M., . . . Giovannini, M. G. (2012). The neuron-astrocyte-microglia triad in normal brain ageing and in a model of neuroinflammation in the rat hippocampus. *PLOS One 7(9)*, e45250.

Cermakian, N., Lamont, E. W., Boudreau, P., & Boivin, D. B. (2011). Circadian clock gene expression in brain regions of Alzheimer's disease patients and control subjects. *Journal of Biological Rhythms 26(2)*, 160-170.

Cermakian, N., Lange, T., Golombek, D., Sarkar, D., Nakao, A., Shibata, S., & Mazzoccoli, G. (2013). Crosstalk between the circadian clock circuitry and the immune system. *Chronobiology interational 30(7)*, 870-888.

Chabicovsky, M., Prieschl-Grassauer, E., Seipelt, J., Muster, T., Szolar, O. H., Hebar, A., & Doblhoff-Dier, O. (2010). Pre-clinical safety evaluation of pyrrolidine dithiocarbamate. *Basic & Clinical Pharmacology & Toxicology*, 758-767.

Chang, Y-, C., Tsai, M-, H., Sheu, W., H-; Hsieh, S-., C., Chiang, A-, N. (2013) The therapeutic potential and mechanisms of action of quercetin in relation to lipopolysaccharide-induced sepsis in vitro and in vivo. *PLoS One8(11) e80744*

Cahpman, N. R., & Perkins, N. D. (2000) Inhibition of the RelA(p65) NF-kappaB subunit by Egr-1. *Journal of Biological Chemistry 275(7)* 4719-4725

Chaudhury, D., Wang, L. M., & Colwell, C. S. (2005). Circadian Regulation of Hippocampal Long-Term Potentiation. *Journal of Biological Rhythms 20(3)*, 225-236.

Chawla, M. K., Guzowski, J. F., Ramirez-Amaya, V., Lipa, P., Hoffman, K. L., Marriott, L. K., . . . Barnes, C. A. (2005). Spare, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. *Hippocampus 15(5)*, 579-586.

Chelazzi, C., Consales, G., & De Gaudio, A. R. (2008). Sepsis associated encephalopathy. *Current Anaesthesia & Critical Care. 19(1)*, 15-21.

Chen, B., Dowlatshahi, D., MacQueen, G. M., Wang, J. F., & Young, L. T. (2001). Increased hippocampal bdnf immunoreactivity in subjects treated with antidepressant medication . *Biological Psychiatry 50(4)*, 260-265.

Chen, J., Buchanan, J. B., Sparkman, N. L., Godbout, J. P., Freund, G. G., & Johnson, R. W. (2008). Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain, Behavior, and Immunity 22(3)*, 301-311.

Chen, Q., Yu, W., Shi, J., Shen, J., Gao, T., Zhang, J., . . . Li, N. (2014). Insulin alleviates the inflammatory response and oxidative stress injury in cerebral tissues in septic rats. *Journal of Inflamation 11(18).*

Chen, S. H., Liu, S. H., Liang, Y. C., Lin, J. K., & Lin-Shiau, S. Y. (2000). Death signaling pathway induced by pyrrolidine dithiocarbamate-Cu(2+) complex in the cultured rat cortical astrocytes. *Glia 31(3),* 249-261.

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., Kidd, G. J., Bergmann, C. C., Stohlman, S. A. & Trapp, B. D. (2012) Lipopolysaccharide-Induced Microglial Activation and Neuroprotection against Experimental Brain Injury is Independent of Hematogenous TLR4. *Neurobiology of Disease 32(34)* 11706-11715

Cheng, G. Z., Zhang, Z., Ghassemi, M., Silva, I., Ainslie, P., & Celi, L. A. (2014). Modeling circadian rhythm variations during sepsis. *American Journal of Respiratory and Critical Care medicine 189*, A3795.

Chevaleyre, V., & Siegelbaum, S. A. (2010). Strong CA2 pyramidal neuron synapses define a powerful disynaptic cortico-hippocampal loop. *Neuron 66(4),* 560-572.

Cho, C. H. (2012). Molecular mechanism of circadian rhythmicity of seizures in temporal lobe epilepsy. *Frontiers in Cellular Neuroscience 6(55).*

Choi, H Y; Park, S; Do, K; Yang, H; Moon, Y (2012) Pro-inflammatory NF-κB and early growth response gene 1 regulate epithelial barrier disruption by food additive carrageenan in human intestinal epithelial cells *Toxicology Letters 211(3)* 289-295

Choi, J. H. & Won, M. (2011) Microglia in the aged hippocampus. *Laboratory Animal Research 27(3)* 181-187

Chourbaji, S., Zacher, C., Sanchis-Segura, C., Spanagel, R., & Gass, P. (2005). Social and structural housing conditions influence the development of a depressive-like phenotype in the learned helplessness paradigm. *Behavioural Brain Research. 164(1),* 100-106.

Christian, K. M., Song, H., & Ming, G.-L. (2014). Functions and dysfunctions of adult hippocampal neurogenesis. *Annual Reviews of Neuroscience 37*, 243-262.

Chung, E. S., Chung, Y. C., Bok, E., Baik, H. H., Park, E. S., Park, J. Y., . . . Jin, B. K. (2010). Fluoxetine prevents LPS-induced degeneration of nigral dopaminergic neurons by inhibiting microglia-mediated oxidative stress. *Brain Research 1363*, 143-150.

Chung, Y. C., Kim, S. R., Park, J. Y., Chung, E. S., Park, K. W., Won, S. Y., . . . Jin, B. K. (2011). Fluoxetine prevents MPTP-induced loss of dopaminergic neurons by inhibiting microglial activation. *Neuropharmacology 60(6),* 963-974.

Clark, D. B., Birmaher, B., Axelson, D., Monk, K., Kalas, C., Ehmann, R., . . . Brent, D. (2005). Fluoxetine for the treatment of childhood anxiety disorders: open-label,

long-term extension to a controlled trial. *Journal of the American Academy of Child & Adolescent Psychiatry 44(12)*, 1263-1270.

Coleman, D. L., Bartiss, A. H., Sukhatme, V. P., Liu, J., & Rupprecht, H. D. (1992). Lipopolysaccharide induces Egr-1 mRNA and protein in murine peritoneal macrophages. *The Journal of Immunology 149(9)*, 3045-3051.

Collingridge, G. L., Peineau, S., Howland, J. G., & Wang, Y. T. (2010). Long-term depression in the CNS. *Nature Reviews Neuroscience 11*, 459-472.

Collins, L. M., Toulouse, A., Connor, T. J., & Nolan, Y. (2012). Contributions of central and systemic inflammation to the pathophysiology of Parkinson's disease. *Neuropharmacology 62*, 2154-2168.

Comim, C. M., Cassol-Jr, O. J., Constantino, L. C., Petronilho, F., Constantino, L. S., Stertz, L., . . . Dal-Pizzol. (2010). Depressive-Like Parameters in Sepsis Survivor Rats. *Neurotoxicity Research. 17*, 279-286.

Comim, C. M., Cassol-Jr, O. J., Constantino, L. S., Felisberto, F., Petronilho, F., Rezin, G. T., . . . Dal-Pizzol, F. (2011). Alterations in inflammatory mediators, oxidative stress parameters and energetic metabolism in the brain of sepsis survivor rats. *Neurochemical Research 36(2)*, 304-311.

Comim, C. M., Silva, N. C., Mina, F., Dominguini, D. S., Morais, M. O., Rosa, D. V., . . . Dal-Pizzol, F. (2014). Evaluation of NCS-1, DARPP-32 and neurotrophins in hippocampus and prefrontal cortex in rats submitted to sepsis. *Synapse*, doi: 10.1002/syn.21760.

Conti, B., Maier, R., Barr, A. M., Morale, M. C., Lu, X., Sanna, P. P., . . . Bartfai, T. (2007). Region-specific transcriptional changes following the three antidepressant treatments electro convulsive therapy, sleep deprivation and fluoxetine. *Molecular Psychiatry 17*, 167-189.

Conway-Campbell, B. L., Sarabdjitsingh, R. A., McKenna, M. A., Pooley, J. R., Kershaw, Y. M., Meijer, O. C., . . . Lightman, S. L. (2010). Glucorticoid ultradian rhythmicity directs cyclical gene pulsing of the clock gene period 1 in rat hippocampus. *Journal of Neuroendocrinology 22(10)*, 1093-1100.

Cornonata, A., Englert, J. A., Lederer, J., Choi, A. M., Fredenburgh, L. E., & Haspel, J. A. (2014). Circadian rhythm disruption via Bmal1 deletion increases sepsis severity. *American Journal of Respiratory and Critical Care Medicine 189*, A5746.

Cornwell, B. R., Salvadore, G., Colon-Rosario, D. R., Latov, T. H., Carve, F. W., Coppola, R., . . . Grillon, C. (2010). Abnormal hippocampal functioning and impaired spatial navigation in depressed individuals: evidence from whole-head magnetoencephalography. *American Journal of Psychiatry 167*, 836-844.

Corona, A. W., Norden, D. M., Skendelas, J. P., Huang, Y., O'Connor, J. C., Lawson, M., . . . Godbout, J. P. (2013). Indoleamine 2,3-dioxygenase inhibition attenuates lipopolysaccharide induced persistent microglial activation and depressive-like complications in fractalkine receptor (CX(3)CR1)-deficient mice. *Brain, Behavior, and Immunity* , 134-42.

Cuenca, A. G., Delano, M. J., Kelly-Scumpia, K. M., Moldawer, L. L., & Efron, P. A. (2010). Current protocols in immunology: Cecal ligation and puncture. *Current Protocols in Immunology 19(13)*.

Cruz, F. C., Koya, E., Guez-Barber, D. H., Bossert, J. M., Lupica, C. R., Shaham, Y. & Hope, B. T. (2013) New technologies for examining the role of neuronal ensembles in drug addiction and fear. *Nature Reviews Neuroscience 14* 743-754

Cui, C., Jin, D., Yoo, K H, Lee, C., Hwang, J K, Ha, I., Han, J. (2008) Macelignan attenuates LPS-induced inflammation and reduces LPS-induced spatial learning impairments in rats. *Neuroscience Letters 1(19)* 110-114

Cunha, A. B., Frey, B. N., Andreazza, A. C., Goi, J. D., Rosa, A. R., Gonçalves, C. A., . . . Kapczinski, F. (2006). Serum brain-derived neurotrophic factor is decreased in bipolar disorder during depressive and manic episodes. *Neuroscience Letters 398(3)*, 215-9.

Cunningham, C. (2012). Microglia and neurodegeneration: The role of systemic inflammation. *Glia 61(1)*, 71-90.

Cunningham, C., Wilcockson, D. C., Campion, S., Lunnon, K. & Perry, V. H. (2005) Central and systemic endotoxin challenges exacerbate the local inflammatory response and increase neuronal death during chronic neurodegeneration. *The Journal of Neuroscience 25(40)* 9275-9284

Curtis, A. M., Bellet, M. M., Sassone-Corsi, P., & O'Neill, L. A. (2014). Circadian clock proteins and immunity . *Immunity 40(2)*, 178-186.

Cuthbertson, B. H., Elders, A., Hall, S., Taylor, J., Maclennan, G., Mackirdy, F., . . . Group, t. S. (2013). Mortality and quality of life in the five years after severe sepsis. *Critical Care 17(2)*, R70.

Daniels, R. (2011). Surviving the first hours in sepsis: getting the basics right (an intensivist's perspective). *Journal of Antimicrobrial Chemotherapy 66(s2)*, ii11-ii23.

Dantzer, R. (2001). Cytokine-induced sickness behavior: mechanisms and implications. *Annals of the New York Academy of Sciences 933*, 222-234.

Dantzer, R. (2004). Cytokine-induced sickness behavior: a neuroimmune response to activation of innate immunity. *European Journal of Pharmacology 500(1-3)*, 399-411.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., & Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nature Reviews Neuroscience 9*, 46-56.

Daumas, S., Halley, H., Francés, B., & Lassalle, J. M. (2005). Encoding, consolidation, and retrieval of contextual memory: Differential involvement of dorsal CA3 and CA1 hippocampal subregions. *Learning & Memory 12*, 375-382.

David, D. J., Samuels, B. A., Rainer, Q., Wang, J. W., Marsteller, D., Mendez, I., . . . Hen, R. (2009). Behavioral effects of fluoxetine in an animal model of

anxiety/depression are mediated by both neurogenesis-dependent and independent mechanisms. *Neuron 62(4)*, 479-493.

Davydow, D. S., Hough, C. L., Langa, K. M., & Iwashyna, T. J. (2013). Symptoms of depression in survivors of severe sepsis: a prospective cohort study of older Americans. *The American Journal of Geriatric Psychiatry 21(9)*, 887-897.

Dawson, D. A., Furuya, K., Gotoh, J., Nakao, Y., & Hallenbeck, J. M. (1999). Cerebrovascular hemodynamics and ischemic tolerance: lipopolysaccharide-induced resistance to focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. *Journal of cerebral blood flow and metabolism 19(6)*, 616-623.

De La Garza II, R., Fabrizio, K. R., Radoi, G.-E., Tudor, V., & Asnis, G. M. (2004). The non-steroidal anti-inflammatory drug diclofena attenuates lipopolysaccharide-induced alterations to reward behavior and corticosterone release. *Behavioural Brain Research. 149(1)*, 77-85.

Deacon, R. M. (2006). Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nature Protocols. 1(1)*, 122-124.

Deacon, R. M. (2011). Hyponeophagia: A measure of Anxiety in the Mouse. *J. Vis. Exp. (51), e 2613, DOI: 10.3791/2613*.

Debruyne, J. P., Weaver, D. R., & Reppert, S. M. (2007). CLOCK and NPAS2 have overlapping roles in the supreachiasmatic circadian clock. *Nature Neuroscience 10*, 543-545.

Del Rey, A., Balschun, D., Wetzel, W., Randolf, A., & Besedovsky, H. O. (2013). A cytokine network involving brain-borne IL-1β, IL-1ra, IL-18, IL-6, and TNFα operates during long-term potentiation and learning. *Brain, Behavior, and Immunity 33*, 15-23.

Della Ragione, F., Cucciolla, V., Criniti, V., Indaco, S., Borriello, A., & Zappia, V. (2002). Antioxidants induce different phenotypes by a distinct modulation of signal transductioin. *FEBS Letters 532(3)*, 289-294.

Deng, H., Maitra, U., Morris, M., & Li, L. (2013). Molecular mechanism responsible for the priming of macrophage activation. *The Journal of Biological Chemistry 288*, 3897-3906.

Denis-Donini, S., Dellarole, A., Crociara, P., Francese, M. T., Bortolotto, V., Quadrato, G., Canonico, P. L., Orsetti, M., Ghi, P., Memo, M., Bonini, S. A., Ferrari-Toninelli, G. & Grilli, M. (2008) Impaired adult neurogenesis associated with short-term memory defects in NF-κB p50-deficient mice. *The Journal of Neuroscience 28(5)* 3911-3919

Dent, J. A., Galvin, N. J., Stanfield, B. B., & Cowan, W. M. (1983). The mode of termination of the hypothalamic projection to the dentate gyrus: An EM autoradiographic study. *Brain Research 258(1)*, 1-10.

DeSantis, A. S., DiezRoux, V., A., Hajat, A., Aiello, A. E., Golden, S. H., . . . Shea, S. (2012). Associations of salivary cortisol levels with inflammatory markers: The

Multi-Ethnic Study of Atherosclerosis. *Psychoneuroendocrinology 37(7)*, 1009-1018.

Deuschle, M., Gilles, M., Scharnholz, B., Lederbogen, F., Lang, U. E., & Hellweg, R. (2013). Changes of serum concentrations of brain-derivedneurotrophic factor (BDNF) during treatment with venlafaxine and mirtazapine: role of medication and response to treatment. *Pharmacopsychiatry 46*, 54-58.

DeVito, L. M., Konigsberg, R., Lykken, C., Sauvage, M., III, W. S., & Eichenbaum, H. (2009). Vasopressin 1b receptor knock-out impairs memory for temporal order. *The Journal of Neuroscience 29(9)*, 2676-2683.

Dhingra, D., & Bhankher, A. (2014). Behavioral and biochemical evidences for antidepressant-like activity of pamatine in mice subjected to chronic unpredictable mild stress. *Pharmacologyical Reports 66(1)*, 1-9.

Di Filippo, M., Chiasserini, D., Gardoni, F., Viviani, B., Tozzi, A., Giampà, C., . . . Calabresi, P. (2013). Effects of central and peripheral inflammation on hippocampal synaptic plasticity. *Neurobiology of Disease 52*, 229-236.

Diamond, D. M., Park, C. R., Campnell, A. M., & Woodson, J. C. (2005). Competitive interactions between endogenous LTD and LTP in the hippocampus underlie the storage of emotional memories and stress-induced amnesia. *Hippocampus 15(8)*, 1006-1025.

Dinet, V., Ansari, N., Torres-Farfan, C., & Korf, H. W. (2007). Clock gene expression in the retina of melatonin-proficient (C3H) and melatonin-deficient (C57BL6) mice. *Journal of Pineal Research 42(1)*, 83-91.

Diniz, B. S., Teixeira, A. L., Talib, L., Gattaz, W. F., & Forlenza, O. V. (2010). Interleukin-1beta serum levels is increased in antidepressant-free elderly depressed patients. *The American Journal of Geriatric Psychiatry 18(2)*, 172-176.

Diz-Chavez, Y., Astiz, M., Bellini, M. J., & Garcia-Segura, L. M. (2013). Prenatal stress increases the expression of proinflammatory cytokines and exacerbates the inflammatory response to LPS in the hippocampal formation of adult male mice. *Brain, Behavior, and Immunity 28*, 196-206.

Doi, K., Leelahavanichkul, A., Yuen, P. S., & Star, R. A. (2009). Animal models of sepsis and sepsis-induced kidney injury. *Journal of Clinical Investigation. 119(10)*, 3868-3878.

Dong, H. W., Swanson, L. W., Chen, L., Fanselow, M. S., & Toga, A. W. (2009). Genomic-anatomic evidence for distinct functional domains in hippocampal field CA1. *PNAS 106(28)*, 11794-11799.

Doosti, M. H., Bakhtiari, A., Zare, P., Amani, M., Majidi-Zolbanin, N., Babri, S., & Salari, A. A. (2013). Impacts of early intervention with fluoxetine following early neonatal immune activation on depression-like behaviors and body weight in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry 43*, 55-65.

Duan, X., Kang, E., Liu, C. Y., Ming, G. I., & Song, H. (2008). Development of neural stem cell in the adult brain 18(1). *Current Opinion in Neurobiology*, 108-115.

Dubovich, M. L., Hudson, R. L., Sumaya, I. C., Masana, M. I., & Manna, E. (2005). Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. *Journal of Pineal Research 39(2)*, 113-120.

Dowlati, Y., Herrmann, N., Swardfager, W., Liu, H., Sham, L., Reim, E. K., Lancôt, K. L. (2010) A Meta-Analysis of Cytokines in Major Depression. *Biological Psychiatry, 67* 446-457

Duncan, G. R., Knapp, D. J., & Breese, G. R. (1996). Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Research 713*, 79-91.

Dunn, A. J., & Swiergiel, A. H. (2008). Effects of acute and chronic stressors and CRF in Rat and Mouse Tests for Depression. *Annals of the New York Academy of Sciences 1148(1)*, 118-126.

Duman, R. S. & Monteggia, L. M. (2006) A Neurotrophic Model for Stress-Related Mood Disorders. *Biological Psychiatry 59* 1116 - 1127

Duran, M., Berton, O., Aguerre, S., Edno, A. L., Combourieu, I., Mormède, P., & Chaouloff, F. (1999). Effects of repeated fluoxetine on anxiety-related behaviours, central serotonergic systems, and the corticotropic axis in SHR and WKY rats. *Neuropharmacology 38*, 893-907.

Dwivedi, Y., S, R. H., Conley, R. R., Roberts, R. C., Tamminga, C. A., & Pandey, G. N. (2003). Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Archives of General Psychiatry 60(8)*, 804-815.

Dyson, A., & Singer, M. (2009). Animal models of sepsis: why does preclinical efficacy fail to translate to the clinical setting? *Critical Care Medicine 37(S1)*, S30-37.

Eckel-Mahan, K. L. (2012). Circadian oscillations within the hippocampus support memory formation and persistence. *Frontiers in Molecular Neuroscience 5(46)*.

Ekdahl, C. T., Claasen, J. H., Bonde, S., Kokaia, Z., & Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. *PNAS 100(23)*, 13632-13637.

Ekdahl, C. T., Kokaia, Z., & Lindvall, O. (2009). Brain inflammation and adult neurogenesis: The dual roe of microglia. *Neuroscience 158(3)*, 1021-1029.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology 35(4)*, 495-516.

Encinas, J. M., Vaahtokari, A., & Enikolopov, G. (2006). Fluoxetine targets early progenitor cells in the adults brain. *PNAS 103(21)*, 8233-8238.

Enciu, A. M., & Popescu, B. O. (2013). Is there a causal link between inflammation and dementia? *BioMed Research International 2013*.

Engblom, D., Ek, M., Saha, S., Ericsson-Dahlstrand, A., Jakobsson, P., & Blomqvist, A. (2002). Prostaglandins as inflammatory messengers across the blood-brain barrier. *Journal of Molecular Medicine 80(1)*, 5-15.

Ennaceur, A., Michalikova, S., van Rensburg, R., Chazot, P. L. (2006) Models of anxiety: Responses of mice to novelty and open spaces in a 3D maze. *Behavioural Brain Research 174* 9-38

Erl, W., Weber, C., & Hansson, G. K. (2000) Pyrrolidine dithiocarbamate-induced apoptosis depends on cell type, density, and the presence of Cu(2+) and Zn(2+). American Journal of Physiology. *Cell Physiology 278(6)* 1116-1125

Espósito, S. M., Piatti, V. C., Laplagne, D. A., Morgenstern, N. A., Ferrari, C. C., Pitossi, F. J., & Schinder, A. F. (2005). Neuronal differentiation in the adult hippocampus recapitulates embryonic development. *The Journal of Neuroscience 25(44)*, 10074-10086.

Etkin, A., Egner, T., & Kalisch, R. (2011). Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends in Cognitive Science 15(2)*, 85-93.

Fahey, B., Hickey, B., Kelleher, D., O'Dwyer, A. -M., & O'Mara, S. (2007). The widely-used anti-viral drug interferon-alpha induces depressive- and anxiogenic-like effects in healthy rats. *Behavioural Brain Research. 182(1)*, 80-87.

Fan, K., Wu, X., Fan, B., Li, N., Lin, Y., Yao, Y., & Ma, J. (2012). Up-regulation of microglial cathepsin C expression and activity in lipopolysaccharide-induced neuroinflammation. *Journal of Neuroinflammation 9(96)*.

Fanselow, M. S., & Dong, H. W. (2010). Are the dorsal and ventral hippocampus functional distinct structures? *Neuron 65(1)*, 7.

Faramarzi, M., Alipor, A., Esmaelzadeh, S., Kheirkhah, F., poladi, K., & Pash, H. (2008). Treatment of depression and anxiety in infertile women: Cognitive behavioral therapy versus fluoxetine. *Journal of Affective Disorders 108(1-2)*, 159-164.

Faraut, B., Boudjeltia, K. Z., Vanhamme, L., & Kerkhofs, M. (2012). Immune, inflammatory and cardiovascular consequences of sleep restriction and recovery. *Sleep Medicine Reviews 16(2)*, 137-149.

Feillet, C. A., Mendoza, J., Albrecht, U., Pévet, P., & Challet, E. (2008). Forebrain oscillators ticking with different clock hands. *Molecular and Cellular Neuroscience 37(2)*, 209-221.

Felger, J. C., & Miller, A. H. (2012). Cytokines effects on the basal ganglia and dopamine function: The subcortical source of inflammatory malaise. *Frontiers in Neuroendocrinology 33(3)*, 315-327.

Felix-Ortiz, A. C., & Tye, K. M. (2014). Amygdala inputes to the ventral hippocampus bidirectionally modulate social behavior. *The Journal of Neuronscience 34(2)*, 586-595.

Felix-Ortiz, A. C., Beyeler, A., Seo, C., Leppla, C. A., Wildes, C. P., & Tye, K. M. (2013). BLA to vHPC inputs modulate anxiety-related behaviours. *Neuron 79(4)*, 658-664.

Feng, K. J., Wang, S. P., Wu, Y. S., Lin, P. Y., Tai, M. Y., & Tsai, Y. F. (2010). Brief spatial experiences increase granule cell survival in the dentate gyrus of adult rats. *Behavioural Brain Research 210(1)*, 143-146.

Ferrier, C. M., de Witte, H. H., Straatman, H., van Tienoven, D. H., van Geloof, W. L., Rietveld, F. J., . . . van Muijen, G. N. (1999). Comparison of immunohistochemistry with immunoassay (ELISA) for the detection of components of plaminogen activation system in human tumour tissue. *British Journal of Cancer 79(9-10)*, 1534-1541.

Festjens, N., Vanden Berghe, T., Cornelis, S., & Vandenabeele, P. (2007). RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death and Differentiation 14*, 400-410.

Feterowski, C., Weighardt, H., Emmanuilidis, K., Hartung, T., & Holzmann, B. (2001). Immune protection against septic peritonitis in endotoxin-primed mice is related to reduced neurtophil apoptosis. *European Journal of Immunology 31(4)*, 1268-1277.

Finelli, P., & Uphoff, D. (2004). Magnetic resonance imaging abnormalities with septic encephalopathy. *Journal of Neurology, Neurosurgery & Psychiatry with Practical Neurology 75(8)*, 1189-1191.

Fink, M. P. (2014). Animal models of sepsis. *Virulence 5(1)* 145-153

Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Bisintin, A., Latz, E., . . . Golenbock, D. T. (2003). LPS-TLR4 Signaling to IRF-3/7 and NFκB Involves the Toll Adapters TRAM and TRIF. *The Journal of Experimental Medicine 198(7)*, 1043-1055.

Frank, M. G., Barrientos, R. M., Hein, A. M., Biedenkapp, J. C., Watkins, L. R., & Maier, S. F. (2010). IL-1RA blocks E. coli-induced suppression of Arc and long-term memory in aged F344 x BN F1 rats. *Brain, Behavior, and Immunity 24*, 254-262.

Franklin, K., & Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* San Diego: Academic Press.

Frenois, F., Moreau, M., O'Connor, J., Lawson, M., Micon, C., Lestage, J., . . . Castanon, N. (2007). Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. *Psychoneuroendocrinology 32(5)*, 516-531.

Frevert, C. W., Johnson, B., & Stahl, W. L. (2014). Immunohistochemistry: Antibody Specificity. *Pathobiology of Human Disease*, 3087-3816.

Fujise, N., Liu, Y., Hori, N., & Kosaka, T. (1998). Distribution of calretinin immunoreactivity in the mouse dentate gyrus: II. Mossy cells, with special

reference to their dorsoventral difference in calrentin immunoreactivity. *Neuroscience 82(1)*, 181-200.

Fukioka, H. & Akema, T. (2010) Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus. *Brain Research 1352* 35-42

Furuya, K., Zhu, L., Kawahara, N., Abe, O., & Kirino, T. (2005). Differences in infarct evolution between lipopolysaccharide-induced tolerant and nontolerant conditions to focal cerevral ischemia . *Journal of neurosurgery 103(4)*, 715-723.

Gaines Das, R., & North, D. (2007). Implications of experimental technique for analysis and interpretation of data from animal experiments: outliers and increased variability resulting from failure of intraperitoneal injection procedures. *Laboratory Animals 41(3)*, 312-320.

Gallagher, P .J., Castro, V., Fava, M., Weillburg, J. B., Murphy, S. N., Gainer, V. S., Churchill, S. E., Kohane, I. S., Iosifescu, D. V., Smoller, J. W. & Perlis, R. H. (2012) Antidepressant response in patients with major depression exposed to NSAIDs: A pharmacovigilance study. *The American Journal of Psychiatry 169(10)* 1065-1072

Gavins, F. N., Hughes, E. L., Buss, N. A., Holloway, P. M., Getting, S. J., & Buckinghan, J. C. (2012). Leukocyte recruitment in the brain in sepsis: involvement of the annexin 1-FPR2/ALX anti-inflammatory system. *The FASEB Journal 26(12)*, 4977-4989.

Gebara, E., Sultan, S., Kocher-Braissant, J., & Toni, N. (2013). Adult hippocampal neurogenesis inversely correlates with microglia in conditions of voluntary running and aging. *Frontiers in Neuroscience 7(145)*, 1-9.

Gerstner, J. R. (2012). On the evolution of memory: a time for clocks. *Frontiers in Molecular Neuroscience 5(23)*, 10.3389/fnmol.2012.00023 .

Gibbs, J., Blaikley, J., Beesley, S., Matthews, L., Simpson, K. D., Boyce, S. H., . . . Loudon, A. S. (2011). The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *PNAS 109(2)*, 582-587.

Gilbert, P. E., & Brushfield, A. M. (2009). The role of the CA3 hippocampal subregion in spatial memory: A process oriented behavioral assessment . *Progress in Neuro-Psychopharmacology & Biological Psychiatry 33(5)*, 774-781.

Gilbert, P. E., & Kesner, R. P. (2003). Localization of function within the dorsal hippocampus: the role of the CA3 subregion in paired-associate learning. *Behavioural Neuroscience 117(6)*, 1385-1394.

Gilhooley, M. J., Pinnok, S. B., & Herbert, J. (2011). Rhythmic expression of per1 in the dentate gyrus is suppressed by corticosterone: Implications for neurogenesis. *Neuroscience Letters 489*, 177-181.

Gitto, E., Karbownik, M., Reiter, R. J., Tan, D. X., Cuzzocrea, S., Chiurazzi, P., . . . Barberi, I. (2001). Effects of melatonin treatment in septic newborns. *Pediatric Research 50(6)*, 756-760.

Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., & Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice after activation of he peripheral innate immune system. *The FASEB Journal 19(10)*, 1329-1331.

Godbout, J. P., Moreau, M., Lestage, J., Chen, J., Sparkman, N. L., O'Connor, J., . . . Johnson, R. W. (2007). Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. *Neuropsychopharmacology 33(10)*, 2341-2351.

Goldberg, J. O., & Schmidt, L. A. (2001). Shyness, sociability, and social dysfunction in shizophrenia. *Schizophrenia Research 48(2-3)*, 343-349.

Gonul, A. S., Akdeniz, F., Taneli, F., Donat, O., Eker, Ç., & Vahip, S. (2005). Effect of treatment on serum brain-derived neurotrophic factor levels in depressed patients. *European Archives of Psychiatry and Clinical Neuroscience 255(6)*, 381-386.

González-Navajas, J. M., Lee, J., David, M., & Raz, E. (2012). Immunomodulatory functions of type I interferons. *Nature Reviews Immunology 12*, 125-135.

Gould, N. F., Holmes, F. B., Luckenbaugh, D. A., Pine, D. S., Gould, T. D., Burgess, N., . . . Zarate Jr, C. A. (2007). Performance on a virtual reality spatial memory navigation task in depressed patients . *American Journal of Psychiatry 164*, 516-519.

Granja, C., Dias, C., Costa-Pereira, A., & Sarmento, A. (2004). Quality of life of survivors from severe sepsis and septic shock may be similar to that of others who survive critical illness. *Critical Care 8(2)*, R91-R98.

Grant, L. D., & Jarrard, L. E. (1968). Functional dissociation within hippocampus. *Brain Research 10(3)*, 392-401.

Greisman, S. E., & Hornick, R. B. (1975). The nature of endotoxin tolerance. *Transactions of the American Clinical and Climatological Association 86*, 43-50.

Greten, F. R., Arkan, M. C., Bollrath, J., Hsu, L-., C., Goode, J., Miething, C. … Karin, M. (2008) NF-κB is a negative regulator of IL-1β secretion as revealed by genetic and pharmacological inhibition of IKKβ *Cell 130(5)* 918-31

Griebel, G., Cohen, C., Perrault, G., & Sanger, D. J. (1999). Behavioral effects of acute and chronic fluoxetine in Wistar-Kyoto rats. *Physiology & behaviour 67*, 315-320.

Guera, C., Linde-Zwirble, W. T., & Wunsch, H. (2012). Risk factors for dementia after critical illness in elderly medicare beneficiaries. *Critical Care 16(6)*, R233.

Guerrero-Vargas, N. N., Salgado-Delgado, R., Basualdo Mdel, C., García, J., Guzmán-Ruiz, M., Carrero, J. C., . . . Buijis, R. M. (2014). Reciprocal interaction between the suprachiasmatic nucleus and the immune system tunes down the inflammatory response to lipopolysaccharide. *Journal of Neuroimmunology 273(1-2)*, 22-30.

Guo, W., Patzlaff, N. E., Jobe, E. M., & Zhao, X. (2012). Isolation of multipotent neural stem/progenitor cells from both the dentate gyrus and subventricular zone of a single adult mouse. *Nature Protocols 7(11)*, 2005-2012.

Guo, J. & Friedman, S. L. (2010) Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis *Fibrogenesis Tissue Repair 3(21)*

Guthrie, K. M., Tran, A., Baratta, J., Yu, J., & Robertson, R. T. (2005). Patterns of afferent projections to the dentate gyrus studied in organotypic co-cultures. *Developmental Brain Research 157(2)*, 162-171.

Guzowski, J. F., Setlow, B., Wagner, E. K., & McGaugh, J. L. (2001). Experience-Dependent Gene Expression in the Rat Hippocampus after Spatial Learning: A comparison of the Immediate-Early Genes Arc, c-fos, and zif268. *The Journal of Neuroscience 21(14)*, 5089-5098.

Ha, E., Jung, K. H., Choe, B. K., Bae, J. H., Shin, D. H., & Baik, H. H. (2006). Fluoxetine increases the nitric oxide production via nuclear factor kappa B-mediated pathway in BV2 murine microglial cells. *Neuroscience Letters 397(3)*, 185-189.

Haba, R., Shintani, N., Onaka, Y., Kanoh, T., Wang, H., Takenaga, R., . . . Baba, A. (2014). Central CRTH2, a Second Prostaglandin D2 Receptor, Mediates Emotional Impairment in the Lipopolysaccharide and Tumor-Induced Sickness Behavior Model. *The Journal of Neuroscience 34(7)*, 2514-2523.

Haba, R., Shintani, N., Onaka, Y., Wang, H., Takenaga, R., Hayata, A., . . . Hashimoto, H. (2012). Lipopolysaccharide affects exploratory behaviors toward novel objects by impairing cognition and/or motivation in mice: Possible role of activation of the central amygdala. *Behavioural Brain Research. 228(2)*, 423-431.

Hack, C. E., De Groot, E. R., Felt-Bersma, R. J., Nuijens, J. H., Strack Van Schijndel, R. J., Erenberg-Belmer, A. J., . . . Aarden, L. A. (1989). Increased Plasma Levels of Interleukin-6 in Sepsis. *Blood 74(5)*, 1704-1710.

Hagenal, H., & Manahan-Vaughan, D. (2011). Learning-facilitated synaptic plasticity at CA3 mossy fiber and commissural-associational synapses reveals different roles in information processing. *Cerebral Cortex 21(11)*, 2442-2449.

Hahn, A., Stein, P., Windischberger, C., Weisenbacher, A., Spindelegger, C., Moser, E., . . . Lanzenberger, R. (2011). Reduced resting-state functional connectivity between amygdala and orbitofrontal cortex in social anxiety disorder. *NeuroImage 56(3)*, 881-889.

Hamer, M., Batty, G. D., Marmot, M. G., Singh-Manoux, A., & Kivimäki, M. (2011). Anti-depressant medication use and C-reactive protein: Results from two population-based studies. *Brain, Behavior, and Immunity. 25(1)*, 168-173.

Hånell, A., & Marklund, N. (2014). Structured evaluation of rodent behavioral tests used in drug discovery research. *Frontiers in Behavioural Neuroscience 8*, 252.

Hart, B. L. (1988) Biological basis of the behavior of sick animals. *Neuroscience & Biobehavioral Reviews 12(2)* 123-137

Hascoët, M., Bourin, M., & Bradwej, J. (1991). Behavioral models in mice. Implication of the alpha noradrenergic system. *Progress in Neuro-Psychopharmacology and Biological Psychiatry. 15(6),* 825-840.

Hauss-Wegrzyniak, B., Vannucchi, M. G., & Wenk, G. L. (2000). Behavioral and ultrastructural changes induced by chronic neuroinflammation in young rats. *Braing Research. 859(1),* 157-166.

Hayashi, Y., Koyanagi, S., Kusunose, N., Okada, R., Wu, Z., Tozaki-Saitoh, H., . . . Nakanishi, H. (2013). The intrinsic microglial molecular clock controls synaptic strength via the circadian expression of cathepsin S. *Scientific Reports 3*.

Hein, A. H., Stasko, M. R., Matousek, S. B., Scott-McKean, J. J., Maier, S. F., Olschowka, J. A., . . . O'Banion, M. K. (2010). Sustained IL-1β overexpression impairs contextual and spatial memory in transgenic mice. *Brain, Behavior, and Immunity. 24(2),* 243-253.

Hein, A. M., Stutzman, D. L., Bland, S. T., Barrientos, R. M., Watkins, L. R., Rudy, J. W., & Maier, S. F. (2007). Prostaglandings are necessary and sufficient to induce contextual fear learning impairments after interleukin-1 beta injections into the dorsal hippocampus. *Neuroscience. 150(4),* 754-763.

Hellweg, R., Ziegenhorn, A., Heuser, I., & Deuschle, M. (2008). Serum concentrations of nerve growth factor in depressed patients before and after antidepressant treatment. *Pharmacopsychiatry 41,* 66-71.

Henry, C. J., Huang, Y., Wynne, A. M., & Godbout, J. P. (2009). Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1β and anti-inflammatory IL-10 cytokines. *Brain, Behavior, and Immunity 23(3),* 309-317.

Henze, D. A., & Buzsáki, G. (2007). Hilar mossy cells: functional identification and activity in vivo. *Progress in Brain Research 163*, 199-216.

Hernandes, M. S., Avila, J. C., Trevelin, S. C., Reis, P. A., Kinjo, E. R., Lopes, L. R., . . . Bozza, F. A. (2014). The role of Nox2-dervied ROS in the development of cognitive impairment after sepsis. *Journal of Neuroinflammation 11(36)*, doi:10.1186/1742-2094-11-36.

Hersh, E. V., Pinto, A., & Moore, P. A. (2007). Adverse drug interactions involving common prescription and over-the-counter analgesic agents. *Clinical Therapeutics 29,* 2477-2497.

Hiscott, J., Marois, J., Garoufalis, J., D'Addario, M., Roulston, A., Kwan, I., Pepin, N., Lacoste, J., Nguyen, H., Bensi, G. (1993) Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop *Molecular Cell Biology 13(10)* 6231-6240

Hitti, F. L., & Siegelbaum, S. A. (2014). The hippocampal CA2 region is essential for social memory. *Nature 508`*, 88-92.

Höcherl, K., Schmidt, C., Birgül, K., & Bucher, M. (2010). Inhibition of NF-κB ameliorates sepsis-induced downregulation of aquaporin-2/V2 receptor expression and acute renal failure. *Americal Journal of Physiology - Renal Physiology 298,* 196-204.

Hoesel, B., & Schmid, J. A. (2013). The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer 12(86).*

Hofjuis, J. G., Spronk, P. E., van Stel, H. F., Schrijvers, A., J., Rommes, J., H., Bakker, J. (2008) The impact of severe sepsis on health-related quality of life: a long-term follow-up study. *Anasthesis and Analgesia 107(6)* 1957-1964

Hoffman, A. N., Krigbaum, A., Ortiz, J. B., Mika, A., Hutchinson, K. M., Bimonte-Nelson, H. A., & Conrad, C. D. (2011). Recovery after chronic stress within spatial reference and working memory domains: correspondence with hippocampal morphology. *European Journal of Neuroscience 34(6),* 1023-1030.

Hoge, E. A., Brandstetter, K., Moshier, S., Pollack, M. H., Wong, K. K., & Simon, N. M. (2009). Broad spectrum of cytokine abormalities in panic disorder and posttraumatic stress disorder. *Depression and Anxiety 26(5),* 447-455.

Hoge, J., & Kesner, R. P. (2007). Role of CA3 and CA1 subregions of the dorsal hippocampus on temporal processing of objects. *Neurobiology of Learning and Memory 88(2),* 225-231.

Holmes, A., Li, Q., Koenig, E. A., Gold, E., Stephenson, D., Yang, R. J., . . . Crawley, J. N. (2005). Phenotypic assessment of galanin overexpressing and galanin receptor R1 knockout mice in the tail suspension test for depression-related behavior. *Psychopharmacology, 178(2-3),* 276-285.

Hopkins, R. O., Ely, E. W., & Jackson, J. C. (2007). The role of future longitudinal studies in ICU survivors: understanding determinants and pathophyiology of brain dysfunction. *Current Opinions in Critical Care. 13(5),* 497-502.

Horesh, N., & Iancu, I. (2010). A comparison of life events in patients with unipolar disorder or bipolar disorder and controls. *Comprehensive Psychiatry 51(2),* 157-164.

Hotchkiss, R. S., Monneret, G., & Payen, D. (2013). Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nature Reviews Immunology 13*, 862-874.

Hsieh, J. (2012). Orchestrating transcriptional control of adult neurogenesis. *Genes & Development 26*, 1010-1021.

Hu, S., Chao, C. C., Ehrlich, L. C., Sheng, W. S., Sutton, R. L., Rockswold, G. L., & Peterson, P. K. (1999). Inhibition of microglial cell RANTES production by IL-10 and TGF-beta. *Journal of Leukocyte Biology 65(6),* 815-821.

Huang, C., He, J., Chen, Y., Zhang, Y., & Chen, C. (2013). Penehyclidine hydrochloride inhibits the LPS-induced inflammatory response in microglia. *Journal of Surgical Research*.

Huang, G. J., Bannerman, D., & Flint, J. (2008). Chronic fluoxetine treatment alters behavior, but not adult hippocampal neurogenesis, in BALB/cJ mice. *Molecular Psychiatry 13*, 119-121.

Humblot, N., Thiriet, N., Gobaille, S., Aunis, D., & Zwiller, J. (1998). The serotonergic system modulates the cocaine-induced expression of the immediate early genes egr-1 and c-fos in rat brain. *Annals of the New York Academy of Sciences 844*, 7-20.

Hunsaker, M. R., Rosenberg, J. S., & Kesner, R. P. (2008). The role of the dentate gyrus, CA3a, b, and CA3c for detecting spatial and environmental novelty. *Hippocampus 18*, 1064-1073.

Husse, J., Leliavski, A., Tsang, A. H., Oster, H., & Eichele, G. (2014). The light-dark cycle controls peripheral rhythmicity in mice with a genetically ablated suprachiasmatic nucleus clock. *FASEB J* , pii: fj.14-256594.

Hutchins, N A; Unsinger, J; Hotchkiss, R S; Ayala, A (2014) The new normal: immunomodulatory agents againist sepsis immune sepression. *Trends in Molecular Medicine*

Igarashi, K. M., Ito, H. T., Moser, E. I., & Moser, M. B. (2014). Functional diversity along the transverse axis of the hippocampal area CA1. *FEBS letters 588(15)*, 2470-2476.

Imamura, Y., Wang, H., Matsumoto, N., Muroya, T., Shimazaki, J., Ogura, H., & Shimazu, T. (2011). Interleukin-1β causes long-term potentiation deficiency in a mouse model of septic encephalopathy. *Neuroscience. 187*, 63-69.

Irwin, M., McClintick, J., Costlow, C., Fortner, M., White, J., & Gillin, J. C. (1996). Partial night sleep depreivation reduces natural killer and cellular immune responses in humans. *The FASEB Journal 10(5)*, 643-653.

Ishikawa, A., & Nakamura, S. (2003). Convergence and interaction of hippocampal and amygdalar rojections within the prefrontal cortex in the rat. *The Journal of Neuroscience 23(31)*, 9987-9995.

Ishiyama, K., Ohdan, H., Tokita, D., Shishida, M., Tanaka, Y., Irei, T., & Asahara, T. (2006). Induction of endotoxin tolerance inhivits alloimmune responses. *Transpolant Immunology 16(3-4)*, 158-165.

Ishizuka, N., Weber, J., & Amaral, D. G. (1990). Organization of intrahippocampal projections originating from CA3 pyramidal cells in the rat. *Journal of Comparative Neurology 295(4)*, 580-623.

Islam, M. R., Moriguchi, S., Tagashira, H., & Fukunaga, K. (2014). Rivastigmine improves hippocampal neurogenesis and depression-like behaviors via 5-HT1A receptor stimulation in ofactory bulbectomised mice. *Neuroscience 272*, 116-130.

Islam, Z. & Pestka, J. J. (2006) LPS priming potentiates and prolongs proinflammatory cytokine response to the trichothecene deoxynivalenol. *Toxicology and Applied Pharmacology 211(1)* 53-63

Iwashyna, T. J., Ely, W., Smith, D. M., & Langa, K. M. (2010). Long-term cognitive impairment and functional disability among survivors of severe sepsis. *The Journal of the American Medical Association 304(16)*, 1787-1794.

James, A. B., Conway, A. & Morris, B. J. (2006) Regulation of the Neuronal Proteasome by Zif268 (Egr1*). The Journal of Neuroscience 26(5)* 1624-1634

Janitzky, K., Schwegler, H., Kröber, A., Roskoden, T., Yanagawa, Y., Linke, R. (2011) Species-relevant inescapable stress differently influences memory consolidation and retrieval of mice in a spatial radial arm maze. *Behavioural Brain Research. 219(1)* 142-148

Jaako-Movits, K., Zharkovsky, T., Pedersen, M., & Zharkovsky, A. (2006). Decreased hippocampal neurogenesis following olfactory bulbectomy is reversed by repeated citalopram administration. *Cellular and Molecular Neurobiology 26(7-8),* 1559-1570.

Jackson, J. C., Pandharipande, P. P., Girard, T. D., Brummel, N. E., Thompson, J. L., Hughes, C. G., . . . Ely, E. W. (2014). Depression, post-traumatic stress disorder, and functional disability in survivors of critical illness in the BRAIN-ICU study: a longitudinal cohort study. *The Lancet Respiratory Medicine 2(5),* 369-379.

Jacob, A., Brorson, J. R., & Alexander, J. J. (2011). Septic encephalopathy: Inflammation in man and mouse. *Neurochemistry International, 58(4),* 472-476.

Jaenichen, D., Brunkhorst, F. M., Strauss, B., & Rosendahl, J. (2012). Physicaly and mental long-term sequelae following intensive care of sepsis in patients and relatives. *Psychotherapie, Psychosomatik, medizinishe Psychologie 62(9-10),* 335-343.

Jagannath, A., Peirson, S. N., & Foster, R. G. (2013). Sleep and circadian rhythm disruption in neuropsychiatric illness. *Current Opinion in Neurobiology 23(5),* 888-894.

Jang, S., & Lee, K. (2000). p38 MAPK and NF-κB are required for LPS-induced RANTES production in immortalised murine microglia (BV-2). *Korean Journal of Physiology and Pharmacology 4(5),* 339-346.

Jangula, A., & Murphy, E. J. (2013). Lipopolysachharide-induced blood-brain barrier permeability is enhanced by alpha-synuclein expression. *Neuroscience Letters 551*, 23-27.

Jarcho, M. R., Slavich, G. M., Tylova-Stein, H., Wolkowitz, O. M., & Burke, H. M. (2013). Dysregulated diurnal cortisol pattern is associated with glucocorticoid resistance in women with major depressive disorder. *Biological Psychiatry 93(1),* 150-158.

Jedynak, P., Kos, T., Sandi, C., Kaczmarek, L., & Filipkowski, R. K. (2014). Mice with ablated adult brain neurogenesis are not impaired in antidepressant responses to chronic fluoxetine. *Journal fo Psychiatric Research 56,* 106-111.

Ji, W. W., Wang, S. Y., Ma, Z. Q., Li, R. P., Li, S. S., Xue, J. S., . . . Ma, S. P. (2014). Effects of perillaldehyde on alternations in serum cytokines and depressive-like

behavior in mice after lipopolysaccharide administration. *Pharmacology Biochemistry and Behaviour 116*, 1-8.

Jiang, C., & Salton, S. R. (2013). The role of neurotrophins in major depressive disorder. *Translational Neuroscience 4(1)*, 46-58.

Jilg, A., Lesny, S., Peruzki, N., Schwegler, H., Selbach, O., Dehghani, F., & Stehle, J. H. (2009). Temporal dynamics of mouse hippocampal clock gene expression support memory processing. *Hippocampus 20*, 337-388.

Jin, D., Sung, J., Hwang, Y. K., Kwon, K. J., Han, S., S, M. S., & Han, J. (2008). Dexibuprofen (S(+)-isomer ibuprofen) reduces microglial activation and impairments of spatial working memory induced by chronic lipopolysacchardie infusion. *Pharmacology Biochemistry and Behaviour. 89(3)*, 404-411.

Jinde, S., Zsiros, V., & Nakazawa, K. (2013). Hilar mossy cell circuitry controlling dentate granule cell excitability. *Frontiers in Neural Circuits 7(14)*, 14.

Johansson, D., Falk, A., Marcus, M. M., & Svensson, T. H. (2012). Celecoxib enhances the effect of reboxetine and fluoxetine on cortical noradrenaline and serotonin output in the rat. *Progress in Neuro-Psychopharmacology and Biological Psychiatry 39(1)*, 143-148.

Jones, C., & Griffiths, R. D. (2013). Mental and physical disability after sepsis. *Minerva Anestesiological 79(11)*, 1306-1312.

Jones, M. W., & McHugh, T. J. (2011). Updating hippocampal representations: CA2 joins the circuit. *Trends in Neuroscience 34(10)*, 526-535.

Joshi, A. R., Chung, C. S., Song, G. Y., Lomas, J., Priester, R. A., & Ayala, A. (2002). NF-kappaB activation has tissue-specific effects on immune cell apoptosis during polymicrobial sepsis. *Shock 18(4)*, 380-386.

Jung, M. W., & McNaughton, B. L. (1993). Spatial selectivity of unit activity in the hippocampal granular layer. *Hippocampus 3(2)*, 165-182.

Juskewitch, J. E., Knudsen, B. E., Platt, J. L., Nath, K. A., Knutson, K. L., Brunn, G., J., Grande, J., P. 2(012) LPS-induced murine systemic inflammation is driven by parenchymal cell activation and exclusively predicted by early MCP-1 plasma levels. *American Journal of Pathology 180(1*) 32-40

Kacimi, R., Giffard, R. G., & Yenari, M. A. (2011). Endotoxin-activated microglia injure brain derived endothelial cells via NF-κB, JAK-STAT and JNK stress kinase pathways. *Journal of Neuroinflammation 8(7)*, doi:10.1186/1476-9255-8-7.

Kadmiel, M., & Cidlowski, J. A. (2013). Glucocorticoid receptor signaling in health and disease. *Trends in Pharmacological Sciences 34(9)*, 518-530.

Kalin, N. H., Shelton, S. E. & Davidson (2004) The role of the central nucleus of the amygdala in mediating dear and anxiety in the primate. *The Journal of Neuroscience 24(24)* 5506-5515

Kao, T. O., Lin, S., Pan, H., Song, P., Raung, S., lai, C., . . . Chen, C. (2011). Luteolin inhibits cytokine expression in endotoxin/cytokine-stimulated microglia. *The Journal of Nutriional Biochemistry 22(7)*, 612-624.

Kaplan, M. S., & Hinds, J. W. (1977). Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. *Science 197(4308)*, 1092-1094.

Karege, F., Perret, G., bondolfi, G., Schwald, M., bertschy, G., & Aubry, J. M. (2002). Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Research 109(2)*, 143-148.

Karege, F., Vaudan, G., Schwald, M., Perroud, N., & La Harpe, R. (2005). Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. *Molecular Brain Research 136(1-2)*, 29-37.

Karin, M., & Ben-Neriah, Y. (2000). Phosphorylation Meets Ubiquitination: The Control of NF-kB Activity. *Annual Review of Immunology 18*, 621-663.

Karolewicz, B. & Paul, I. A. (2001) Group housing of mice increases immobility and antidepressant sensitivity in the forced swim and tail suspension tests. *European Journal of Pharmacology 415* 197-201

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., & Green, D. R. (1998). DNA damaging agents induce expression of Fas Ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. *Molecular Cell 1(4)*, 543-551.

Katona, I., Acsády, L., & Freund, T. F. (1999). Postsynaptic targets of somatostatin-immunoreactive interneurons in the rat hippocampus. *Neuroscience 88(1)*, 37-55.

Kaushik, D K; Basu, A (2013) A Friend in Need may not be a Friend Indeed: Role of Microglia in Neurodegenrative Diseases *CNS & neurological disorders drug targets*

Keinath, A. T., Wang, M. E., Wann, E. G., Yuan, R. K., Dudman, J. T., & Muzzio, I. A. (2014). Precise spatial coding is preserved along the longitudinal hippocampal axis. *Hippocampus* .

Keller, M., Mazuch, J., Abraham, U., Eom, G. D., Herzog, E. D., Volk, H. D., . . . Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. *PNAS 106(50)*, 21407-21412.

Kempermann, G. (2012). New neurons for 'survival of the fittest[. *Nature Reviews Neuroscience 13*, 727-736.

Keshavarzi, S; Sullivan, R. K. P., Ianno, D. J. & Sah, P. (2014) Functional properties and rojections of neurons in the medial amygdala. *The Journal of Neuroscience 34(26)* 8699-8715

Kesner, R. P., Hunsaker, M. R., & Ziegler, W. (2011). The role of the dorsal and ventral hippocampus in olfactory working memory. *Neurobiology of Learning and Memory 96*, 361-366.

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K. R., . . . Wang, P. S. (2003). The epidemiology of major depressive disorder: Results from the national comorbidity survey replication(NCS-R). *The Journal of the American Medical Assocation 289(23)*, 3095-3105.

Kettenmann, H., Hanisch, U., Noda, M., & Verkhratsky, A. (2011). Physiology of Microglia . *Physiological Reviews 91(2)*, 461-553.

Kheirbek, M. A., Drew, L. J., Burghardt, N. S., Costantini, D. O., Tannenholz, L., Ahmari, S. E., . . . Hen, R. (2013). Differential control of learning and anxiety along the dorsovental axis of the dentate gyrus. *Neuron 77(5)*, 955-968.

Kiecolt-Glaser, J. K., Belury, M. A., Andridge, R., Malarkey, W. B., & Glaser, R. (2011). Omega-3 supplementation lowers inflammation and anxiety in medical students: A randomised controlled trial. *Brain, Behavior, and Immunity. 25(8)*, 1725-1734.

Kim, J. B., Han, A. R., Park, E. Y., Kim, J. Y., Cho, W., Lee, J., Seo, E. K., Lee, K. T. (2007) Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. *Biological & Pharmaceutical Bulletin 30(12)* 2345-2351

Kim, Y. S., Choi, M. Y., Lee, D. H., Jeon, B. T., Roh, G. S., Kim, H. J., . . . Choi, W. S. (2014). Decreased interaction between Fox03a and Akt correlates with seizure-induced neuronal death. *Epilepsy Researach 108(3)*, 367-378.

Kipnis, J., Cohen, H., Cardon, M., Ziv, Y., Schwartz, M. (2004) T cell deficincy leads to cognitive dysfunction: Implications for therapeutic vaccination for schizophrenia and other psychiatric conditions *PNAS 101(21)* 8180-8185

Knable, M. B., Barci, B. M., Webster, M. J.-W., Torrey, E. F., & Consortium, S. N. (2004). Molecular abnormalities of the hippocampus in severe psychiatric illness: postmortem findings from the Stanley Neuropathology Consortium. *Molecular Psychiatry 9(6)*, 609-620.

Kohara, K., Pignatelli, M., Rivest, A. J., Jung, H. Y., Kitamura, T., Suh, J., . . . Tonegawa, S. (2014). Cell type-specific genetic and optogenetic tools reveal novel hippocampal CA2 circuits. *Nature Neuroscience 17(2)*, 269-279.

Konda, S., Kohsaka, S., & Okabe, S. (2011). Long-term changes of spine dynamics and microglia after transient peripheral immune response triggered by LPS in vivo. *Molecular Brain 4*, 27.

Kong, L., Chen, K., Tang, Y., Wu, F., Driesen, N., Womer, F. … Wang, F. (2013) Functional connectivity between the amygdala and prefrontal cortex in medication-naïve individuals with major depressive disorder. *Journal of Psychiatry & Neuroscience 38(6)* 417-422

Kovács, K. J, (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochemisty International 33(4)* 287-297

Kubera, M., Curzytek, K., Duda, W., Leskiewicz, M., Basta-Kaim, A., Budziszewska, B., . . . Maes, M. (2013). A new animal model of (chronic) depression induced

by repeated and intermittent lipopolysaccharide administration for 4 months. *Brain, Behavior, and Immunity 31*, 96-104.

Kumar, A., Chen, S., Kadiiska, M. B., Hong, J., Zielonka, J., Kalyanaraman, B., & Mason, R. P. (2014). Inducible nitric oxide synthase is key to peroxynitrite-mediated, LPS-induced protein radical formation in murine microglia BV2 cells. *Free Radical Biology and Medicine* , in press.

Kurt, M., Arik, A. C., & Celik, S. (2000). The effects of sertraline and fluoxetine on anxiety in the elevated plus-maze test in mice. *Journal of Basical Clinical Physiology and Pharmacology 11*, 173-180.

Kwon, I., Choe, H. K., Son, G. H., & Kim, K. (2011). Mammalian Molecular Clocks. *Experimental Neurobiology 20*, 18-28.

Lai, A. Y., Swayze, R. D., El-Husseini, A., & Song, C. (2006). Interleukin-1 beta modulates AMPA receptor expression and phosphorylation in hippocampal neurons. *Journal of Neuroimmunology 175(1-2)*, 97-106.

Lamar, C. D., Hurley, R. A., Hayman, L. A., & Taber, K. H. (2011). Sepsis-Associated Encephalopathy: Review of the Neuropsychiatric Manifestations and Cognitive Outcome. *The Journal of Neuropsychiatry and Clinical Neuroscience 23(3)*, 237-241.

Lamont, E. W., Robinson, B., Stewart, J., & Amir, S. (2005). The central and basolateral nuclei of the amygdala exhibit opposite diurnal rhythms of expression of the clock protein Period2. *PNAS 102(11)*, 4180-4184.

Lange, T., Dimitrov, S., & Born, J. (2010). Effects of sleep and circadian rhythm on the human immune system. *Annals of the New York Academy of Sciences 1193*, 48-59.

Lanquillon, S., Krieg, J. C., Bening-Abu-Shach, U., & Vedder, H. (2000). Cytokine production and treatment response in major depressive disorder. *Neuropsychopharmacology 22*, 370-379.

Lapmanee, S., Charoenphandhu, J., & Charoenphandhu, N. (2013). Beneficial effects of fluoxetine, reboxetine, venlafaxine, and voluntary running exercise in stressed male rats with anxiety- and depression-like behaviors. *Behavioral Brain Research 250*, 316-325.

Lauzurica, P., Martínez-Martínez, S., Marazuela, M., Gómez del Arco, P., Martínez-A, C., Sánchez-Madrid, F. & Redondo, J M (1999) Pyrrolidine dithiocarbamate protects mice from lethal shock induced by LPS or TNF-α *European Journal of Immunology 29(6)* 1890-1900

Lawrence, T. (2009). The Nuclear Factor NF-κB Pathway in Inflammation. *Cold Spring Harbor Perspectives in Biology 1(6)*, a001651.

Lazosky, A., Young, G. B., Zirul, S., & Philips, R. (2010). Quality of life after septic illness. *Journal of Critical Care. 25(3)*, 406-412.

LeGates, T. A., Altimus, C. M., Wang, H., Lee, H. K., Yang, S., Zhao, H., . . . Hattar, S. (2012). Aberarrant light directly impairs mood and learning through melanopsin-expressing neurons. *Nature 491*, 594-598.

Lehner, B., Sandner, B., Marschallinger, J., Lehner, C., Furtner, T., Couillard-Despres, S., . . . Aigner, L. (2011). The dark side of BrdU in neural stem cell biology: detrimental effects on cell cycle, differentiation and survival. *Cell Tissue Research 345(3)*, 313-328.

Lemstra, A. W., Groen in't Woud, J. C., Hoozemans, J. J., van Haastert, E. S., Rozemuller, A. J., Eikelenboom, P., & van Good, W. A. (2007). Microglia activation in sepsis: a case-control study. *Journal of Neuroinflammation 4(4)*.

Levin, E. D., Petro, A., Rezvani, A. H., Pollard, N., Christopher, N. C., Strauss, M., . . . Rose, J. E. (2009). Nicotinic α7- or β2-containing receptor knockout: Effects on radial-armmaze learning and long-term nicotine consumption in mice. *Behavioural Brain Research 196(2)*, 207-213.

Lewejohann, L., Zipser, B., & Sachser, N. (2011). "Personality" in laboratory mice used for biomedical research: a way of understanding variability. *Developmental Psychobiology 53(6)*, 624-630.

Li, J. Z., Bunney, B. G., Meng, F., Hagenauer, M. H., Walsh, D. M., Vawter, M. P., . . . Bunney, W. E. (2013). Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *PNAS*, 10.1073/pnas.1305814110.

Li, S., Strelow, A., Fontana, E. J., & Wesche, H. (2002). IRAK-4: A novel member of the IRAK family with the properties of and IRAK-kinase. *PNAS 99(8)*, 5567-5572.

Li, X. G., Somogyi, P., Ylinen, A., & Buzsáki, G. (1994). The hippocampal CA3 network: an in vivo intracellular labeling study. *The Journal of Comparative Neurology 339(2)*, 181-208.

Li, X., Morrow, D., & Witkin, J. M. (2005). Decreases in nestlet shredding of mice by serotonin uptake inhibitors: Comparison with marble burying. *Life Sciences. 78(17)*, 1933-1939.

Li, Y., Stam, F. J., Aimone, J. B., Goulding, M., Callaway, E. M., & Gage, F. H. (2013). Molecular layer perforant path-associated cells contribute to feed-forward inhibition in the adult dentate gyrus. *PNAS 110(22)*, 9106-9111.

Libermann, T. A., & Baltimore, D. (1990). Activation of interleukin-6 gene expression through the NF-κB transcritption factor. *Molecular and Cellular Biology 10(5)*, 2327-2334.

Lichtblau, N; Schmidt, F M; Schumann, R; Kirkby, K C; Himmerich, H. (2013) Cytokines as biomarkers in depressive disorder: Current standing and prospects. *International Review of Psychiatry 25(5)* 592-603

Lijia, Z., Zhao, S., Wang, X., Wu, C., & Yang, J. (2012). A self-propelling cycle mediated by reactive oxide species and nitric oxide exists in LPS-activated microglia. *Neurochemistry International 61(7)*, 1220-1230.

Lin, L., Chen, Y., Lee, W., Chen, H. & Yang, R (2010) Heat shock pretreatment attenuates sepsis-associated encephalopathy in LPS-induced septic rats. *Brain and Development 32(5)* 371-377

Lim, C., Kim, S., Park, J., Kim, C., Yoon, S. H., & Lee, J. (2009). Fluoxetine affords robust neuroprotection in the postischemic brain via its anti-inflammatory effect. *Journal of Neuroscience Research 87(4)*, 1037-1045.

Lin, Y. L., & Wang, S. (2014). Prenatal lipopolysacharide exposure increases dpression-like behaviors and reduces hippocampal neurogenesis in adults rats. *Behavioral Brain Research 259*, 24-34.

Liston, C., Cichon, J. M., Jeanneteau, F., Jia, Z., Chao, M. V., & Gan, W. B. (2013). Circadian glucocorticoid oscillations promote learning-dependent synapse formation and maintencance. *Nature Neuroscience 16*, 698-705.

Liu, A. C., Tran, H. G., Zhang, E. E., Priest, A. A., Welsh, D. K., & Kay, S. A. (2008). Redundant Function of REV-ERBα and β and Non-Essential Role for Bmal1 Cycling in Transcriptional Regulation of Intracellular Circadian Rhythms. *PLoS Genetics 4(2)*, e1000023.

Liu, D., Wang, Z., Liu, S., Wang, F., Zhao, S., & Hao, A. (2011). Anti-inflammatory effects of fluoxetine in lipopolysaccharide(LPS)-stimulated microglial cells. *Neuropharmacology 61(4)*, 592-599.

Liu, Q. Y., Wang, C. Y., Cai, Z. L., Xu, S. T., Liu, W. X., Xiao, P., & Li, C. H. (2014). Effects of intrahippocampal GABAB receptor antagonist treatment on the behavioral long-term potentiation and Y-maze learning performance. *Neurobiology of Learning and Memory 114*, 26-31.

Liu, S. F., Ye, X., & Malik, A. B. (1999). Pyrrolidine dithiocarbamate prevents I-kappaB degradation and reduces microvascular injury induced by lipopolysaccharide in multiple organs. *Molecular Pharmacology 55(4)*, 658-667.

Liu, X., Wu, Z., Hayashi, Y., & Nakanishi, H. (2012). Age-dependent neuroinflammatory responses and deficits in long-term potentiation in the hippocampus during system inflammation. *Neuroscience 216*, 133-142.

Liukkonen, T., Räsänen, P., Jokelainen, J., Leionen, M., Järvelin, M. -R.-R., & Timonen, M. (2011). The association between anxiety and C-reactive protein (CRP) levels: Results from the Northern Finland 1966 Birth Cohort Study. *European Psychiatry. 26(6)*, 363-369.

Loftis, J. M., & Hauser, P. (2004). The phenomenology and treatment of interferon-induced depression. *Journal of Affective Disorders. 82(2)*, 175-190.

Logan, R. W., Zhang, C., Murugan, S., O'Connell, S., Levit, D., Rosenwasser, A. M., & Sarkar, D. K. (2012). Chronic shift-lag alters the circadian clock of NK cells and promotes lung cancer growth in rats. *The Journal of Immunology 188(6)*, 2583-2591.

Lokensgard, J. R., Hu, S., van Fenema, E. M., Sheng, W. S., Peterson, P. K. (2000) Effect of thalidomide on chemokine production by human microglia *Journal of Infectious Diseases 182(3)* 983-987

Londei, T., Valentini, A. M., Leone, V. G. (1998) Investigative burying by laboratory mice may involve non-functional, compulsive, behaviour. *Behavioural Brain Research 94* 249-254

Lonergan, M. E., Gafford, G. M., Jarome, T. J., & Helmstetter, F. J. (2010). Time-dependent expression of Arc and Zif268 after acquisition of fear conditioning. *Neural Plasticity* , doi: 10.1155/2010/139891.

Lu, Y., Yeh, W., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine 42(2)*, 145-151.

Lugbert, S., Basak, O., Knuckles, P., Haussler, U., Fabel, K., Götz, M., . . . Giachino, C. (2010). Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell 6(5)*, 445-456.

Lund, S., Christensen, K. V., Hedtjärn, M., Mortensen, A. L., Hagberg, H., Falsig, J., Hasseldam, H., Schrattenholz, A., Pörzgen, P. & Leist, M (2006) The dynamics of the LPS triggered inflammatory response of murine microglia under different culture and in vivo conditions. *Journal of Neuroimmunology 180(1-2)* 71-87

Luo, A. H., Tahsili-Fahadan, P., Wise, R. A., Lupica, C. R., & Aston-Jones, G. (2011). Linking context with reward: a functional circuit from hippocampal CA3 to ventral tegmental area. *Science 336(6040)*, 353-357.

Lüscher, C., & Malenka, R. C. (2012). NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). *Cold Spring Habour Perspectives in Biology 4(6)*.

Lyons, A., Downer, E. J., Crotty, S., Nolan, Y. M., Mills, K. H., & Lynch, M. A. (2007). CD200 ligand-receptor interaction modulates microglial activation in vivo and in vitro: A role for IL-4. *The Journal of Neuroscience 27(31)*, 8309-8313.

Ma, Y., Matsuwaki, T., Yamanouchi, K., & Nishihara, M. (2014). Differential roles of cyclooxygenase-2-related signaling in regulating hypothalamic neuronal activity under various acute stresses. *Journal of Veterinary Medical Science 76(2)*, 219-227.

MacDougall, M. J., & Howland, J. G. (2013). Acute stress and hippocampal output: exploring dorsal CA1 and subicular synaptic plasticity simultaneously in anesthetised rats. *Physiological Reports 1(2)*.

Maciel, I. S., Silva, R. B., Morrone, F. B., Calixto, J. B., & Campos, M. M. (2013). Synergistic effects of celecoxib and bupropion in a model of chronic inflammation-related depression in mice. *PLOS One* , DOI: 10.1371/journal.pone.0077227.

MacQueen, G. M., Campbell, S., McEwen, B. S., Macdonald, K., Amano, S., Joffe, R. T., . . . Young, L. T. (2003). Course of illness, hippocampal function, and hippocampal volume in major depression. *PNAS 100(3)*, 1387-1392.

Maes, M., Meltzer, H. Y., Bosmans, E., Bergmans, R., Vandoolaughe, E., Ranjan, R., & Desnyder, R. (1995). Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression. *Journal of Affective Disorders 34(4)*, 301-309.

Mages, J., Dietrich, H., & Lang, R. (2008). A genome-wide analysis of LPS tolerance in macrophages. *Immunobiology 212(9-10)*, 723-737.

Majidi-Zolbanin, J., Azarfarin, M., Samadi, H., Enayati, M., & Salari, A. A. (2013). Adolescent fluoxetine treatment decreases the effects of neonatal immune activation on anxiety-like behavior in mice. *Behavioural Brain Research 250*, 123-132.

Malberg, J. E., Eisch, A. J., Nestler, E. J., & Duman, R. S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *The Journal of Neuroscience 20(24)*, 9104-9110.

Malkani, S., & Rosen, J. B. (2000). Specific induction of early growth response gene 1 in the lateral nucleus of the amygdala following contextual fear conditioning in rats. *Neuroscience 97(4)*, 693-702.

Malm, T. M., Iivonen, H., Goldsteins, G., Keksa-Goldsteine, V., Ahtoniemi, T., Kanninen, K., . . . Koistinaho, J. (2007). Pyrrolidine dithiocarbamate activates Akt and improves spatial learning in APP/PS1 mice without affecting β-amyloid burded. *The Journal of Neuroscience 27(14)*, 3712-3721.

Manning, B. H., Martin, W. J., & Meng, I. D. (2003). The rodent amygdala contributes to the production of cannabinoid-induced antinociception. *Neuroscience 120(4)*, 1157-1170.

Marcelin, B., Liu, Z., Chen, Y., Lewis, A. S., Becker, A., McClelland, S., . . . Bernard, C. (2012). Dorsoventral differences in intrinsic properites in developing CA1 pyramidal cells. *The Journal of Neuroscience 32(11)*, 3736-3747.

Marek, R., Strobel, C., Bredy, T. W. & Sah, P. (2013) The amygdala and medial prefrontal cortex: partners in the fear circuit. *The Journal of Phsiology 591(10)* 2381-2391

Marin, I. & Kipnis, J (2013) Learning and memory … and the immune system. *Learning & Memory 20(10)* 601-606

Marlatt, M. W., Lucassen, P. J., & van Praag, H. (2010). Comparison of neurogenic effects of fluoxetine, duloxetine and running in mice. *Brain Research 1341*, 93-99.

Marpegán, L., Bekinschtein, T. A., Costas, M. A., & Golombek, D. A. (2005). Circadian responses to endotoxin treatment in mice . *Journal of Neuroimmunology 160(1-2)*, 102-109.

Marpegan, L., Bekinschtein, T. A., Freudenthal, R., Rubio, M. F., Gerreyra, G. A., Romano, A., & Golombek, D. A. (2004). Participation of transcription factors from the Rel/NF-kappa B familiy in the circadian system in hamsters. *Neuroscience Letters 358*, 9-12.

Marpegan, L., Leone, M. J., Katz, M. E., Sobrero, P. M., Bekinstein, T. A. & Golombek, D. A. (2009) Diurnal variation in endotoxin-induced mortality in mice: correlations with proinflammatory factors. *Chronbiology International 26(7)* 1430-1442

Martin, T., Cardarelli, P. M., Parry, G. C., Felts, K. A., Cobb, R. R. (1997) Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1 *Europoean Journal of Immunology 27(5)* 1091-1097

Martin-Fairey, C. A., & Nunez, A. A. (2014). Circadian modulation of memory and plasticity gene products in a diurnal species. *Brain Research*.

McCarthy, M. J., & Welsh, D. K. (2012). Cellular circadian clocks in mood disorders. *Journal of Biological Rhythms 27(5)*, 339-352.

McClung, C. A., Sidiropoulou, K., Vitaterna, M., Takahashi, J. S., White, F. J., Cooper, D. C., & Nestler, E. J. (2005). Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *PNAS 102(26)*, 9377-9381.

McCusker, R. H., & Kelley, K. W. (2013). Immune-neural connections: how the immune system's response to inefctious agents influences behavior. *The Journal of Experimental Biology 216*, 84-98.

Meisner, M., Schmidt, J., Schywalsky, M., & Tschaikowsky, K (2000) Influence of pyrrolidine dithiocarbamate on the inflammatory response in macrophages and mouse endotoxin shock. International *Journal of Immunopharmacology 22(1)* 83-90

Mercer, A., Trigg, H. L., & Thomson, A. M. (2007). Characterization of neurons in the CA2 region subfield of the adult rat hippocampus. *The Journal of Neuroscience 27(27)*, 7329-7338.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J. (2006) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. *J Neurosci. 26* 4752–4762

Michels, M., Vieira, A. S., Vuolo, F., Zapelini, H. G., Mendonça, B., Mina, F., . . . Dal-Pizzol, F. (2014). The role of microglia activation in the development of sepsis-induced long-term cognitive impairment. *Brain, Behavior, and Immunity*.

Michelucci, A., Heurtaux, T., Grandbarbe, L., Morga, E., & Heuschling, P. (2009). Characterization of the microglial phenotype under specific pro-inflammatory and anti-inflammatory conditions: Effects of oligomeric and fibrillar amyboid-β. *Journal of Neuroimmunology 210*, 3-12.

Miller, A. H., Maletic, V., & Raison, C. L. (2009). Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. *Biol Psychiatry. 65(9),* 732-741.

Miller, B. H., Schultz, L. A., Gulati, A., & Cameron, M. D. (2008). Genetic regulation of behavioral and neuronal responses to fluoxetine. *Neuropsychopharmacology 33*, 1312-1322.

Milner, R., & Campbell, I. L. (2003). The extracellular matrix and cytokines regulate microglial integrin expression and activation. *The Journal of Immunology 170*, 3850-3856.

Ming, G. L., & Song, H. (2011). Adult neurogenesis in the mammalian brain: signficant answers and significant questions. *Neuron 70(4)*, 687-702.

Mirescu, C., & Gould, E. (2006). Stress and Adult Neurogenesis. *Hippocampus 16(3)*, 223-238.

Mizuseki, K., Diba, K., Pastalkova, E., & Buzsáki, G. (2011). Hippocampal CA1 pyramidal cells form functionally distinct sublayers. *Nature Neuroscience 14*, 1174-1181.

Mizuseki, K., Royer, S., Diba, K., & Buzsáki, G. (2012). Activity dynamics and behavioral correlates of CA3 and CA1 hippocampal pyramidal neurons. *Hippocampus 22(8)*, 1659-1680.

Moellering, D., McAndrew, J., Jo, H., & Darley-Usmar, V. M. (1999). Effects of pyrrolidine dithiocarbamate on endothelial cells: protection against oxidative stress. *Free Radical Biology and Medicine 26(9-10)*, 1138-1145.

Moita, M. A., Rosis, S., Zhou, Y., LeDoux, J. E., & Blair, H. T. (2004). Putting fear in its place: remapping of hippocampal place cells during fear conditioning. *The Journal of Neuroscience 24(31)*, 7015-7023.

Mokin, M., & Keifer, J. (2005). Expression of the immediate-early gene-encoded protein Egr-1 (zif268) during classical conditioning. *Learning & Memory 12(2)*, 144-148.

Monje, M. L., Toda, H., & Palmer, T. D. (2003). Inflammatory Blockade Restores Adult Hippocampal Neurogenesis. *Science 302(5651)*, 1760-1765.

Monnet, F. P. (2002). Melatonin Modulates [3H]Serotonin Release in the Rat Hippocampus: Effects of Circadian Rhythm. *Journal of Neuroendocrinology 14(3)*, 194-199.

Monti, J. M., & Monti, D. (2000). Sleep disturbance in generalised anxiety disorder and its treatment. *Sleep Medicine Reviews 4(3)*, 263-276.

Montkowski, A., Landgraf, R., Yassouridis, A., Holsboer, F., & Schöbitz, B. (1997). Central Administration of IL-1 Reduces Anxiety and Induces Sickness Behaviour in Rats. *Pharmacology Biochemistry and Behavior. 58(2)*, 329-336.

Moore, A. H., Wu, M., Shafter, S. S., Graham, K. A., & O'Banion, M. K. (2009). Sustained expression of interleukin-1β in mouse hippocampus impairs spatial memory. *Neuroscience, 164(4),* 1484-1495.

Moustafa, A. A., Gilbertson, M. W., Orr, S. P., Herzallah, M. M., Seratius, R. J. & Myers, C. E. (2013) A model of amygdala-hippocampal-prefrontal interaction in fear conditioning and extinction in animals. *Brain and Cognition81(1).* 29-43

Morris, G. P., Clark, I. A., Zinn, R., & Vissel, B. (2013). Microglia: A new frontier for synaptic plasticity, learning and memory, and neurodegenerative disease research. *Neurobiology of Learning and Memory 105*, 40-53.

Morris, R. G. (1981). Spatial Localization Does Not Require the Presence of Local Cues. *Learning and Motivation, 12*, 239-260.

Mort, J. R., Aparasu, R. R., & Baer, R. K. (2012). Interaction between selective serotonin reuptake inhibitors and nonsteroidal antiinflammatory drugs: review of the literature. *Pharmacoptherapy: The Journal of Human Pharmacology and Drug Therapy 26(9),* 1307-1313.

Moser, M. B., & Moser, E. I. (1998). Functional differentiation in the hippocampus. *Hippocampus 8(6),* 608-619.

Mössner, R., Mikova, O., Koutsilieri, E., Saoud, M., Ehlis, A., Müller, N., Fallgatter, A. J., Riederer, P. (2007) Consensus paper of the WFSBP Task Force on Biological Markers: Biological Markers in Depression. *The World Journal of Biological Psychiatry, 8(3)* 141-174

Motzkin, J. C., Philippi, C. L., Wolf, R. C., Baskaya, M. K., & Koenigs, M. (2014). Ventromedial prefrontal cortex is critical for the regulation of amygdala activity in humans . *Biological Psychiatry* .

Mundigler, G., Delle-Karth, G., Koreny, M., Zehetgruber, M., Steindl-Munda, P., Marktl, W., Ferti, L. & Siostrzonek, P. (2002) Impaired circadian rhythm of melatonin secretion in sedated critically ill

patients with sever sepsis. *Critical Care Medicine 30(3)* 536-540

Müller, N. (2009). The impact of neuroimmune dysregulation on neuroprotection and neurotoxicity in psychiatric disorders - relation to drug treatment. *Dialogues in Clinical Neuroscience 11*, 319-332.

Müller, N., Schwarz, M. J., Dehning, S., Douhe, A., Cerovecki, A., Goldstein-Müller, B., . . . Riedel, M. (2006). The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomised, placebo controlled, add-on pilot study. *Molecular Psychiatry 11(7),* 680-684.

Munhoz, C. D., Lepsch, L. B., Kawamoto, E. M., Malta, M. B., Lima Ide, S., Avellar, M. C., . . . Scavone, C. (2006). Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal conrtex and hippocampus via glucorticoid secretion. *The Journal of Neuroscience 26*, 3813-3820.

Munn, R. G. K. & Bilkey, D. K. (2012) The firing rate of hippocampal CA1 place cells is modulated with a circadian period. *Hippocampus 22(6)* 1325-1337

Muñoz, C., Pascual-Salcedo, D., Castellanos, M. C., Alfranca, A., Argonés, J., Vara, A., . . . de Landázuri, M. O. (1996). Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediatiors. *Blood 88(9)*, 3482-3490.

Murray, A. J., Sauer, J. F., Riedel, G., McClure, C., Ansel, L., Cheyne, L., . . . Wulff, P. (2011). Parvalbumin-positive CA1 interneurons are required for spatial working but not for reference memory. *Nature Neuroscience 14*, 297-299.

Murray, C., Sanderson, D. J., Barkus, C., Deacon, R. M., Rawlins, J. N., Bannerman, D. M., & Cunningham, C. (2012). Systemic inflammation induces acute working memory deficits in the primed brain: relevance for delirium. *Neurobiology of Aging 33(3)*, 603-616.

Murray, P. S., & Holmes, P. V. (2011). An overview of brain-derived neurotrophic factor and implications for excitotoxic vulnerability in the hippocampus. *International Journal of Peptides* , Article ID 654085.

Musselman, D. L., Lawson, D. H., Gumnick, J. F., Manatunga, A. K., Penna, S., Goodkin, R. S., . . . Miller, A. H. (2001). Paroxetine for the prevention of depression induced by high-dose interferon alfa. *New England Journal of Medicine. 344(13)*, 961-966.

Mutlu, O., Gumuslu, E., Ulak, G., Celikyurt, I. K., Kokturk, S., Kir, H. M., . . . Erden, F. (2012). Effects of fluoxetine, tianeptine and olanzapine on unpredictable chronic mild stress-induced depression-like behavior in mice. *Life Sciences 91(25-26)*, 1252-1262.

Myers, C. E., & Scharfman, H. E. (2011). Pattern separation in the dentate gyrus: A role for the CA3 backprojection. *Hippocampus 21(11)*, 1190-1215.

Na, K., Jung, H., & Kim, Y. (2012). The role of pro-inflammatory cytokines in the neuroinflammation and neurogenesis of schizophrenia. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* .

Nadjar, A., Tridon, V., May, M. J., Ghosh, S., Dantzer,R., Amemdee, T., Partnet, P (2005) NFkappaB activates in vivo the synthesis of iniducible Cox-2 in the brain. *Journal of Cerebral Blood Flow and Metabolism 25(8)*, 1047-1059

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., . . . Miyake, K. (2002). Essential role of MD02 in LPS responsiveness and TLR4 distribution. *Nature Immunology 3*, 667-672.

Najafi, M. R., Toghianifar, N., Etemadifar, M., Haghighi, S., Maghzi, A. H., & Akbari, M. (2013). Circadian rhythm sleep disorders in patients with multiple sclerosis and its association with fatigue: A case-control study. *Journal of Research in Medical Sciences 18(S1)*, S71-S73.

Najjar, S., & Pearlman, D. M. (2014). Neuroinflammation and white matter pathology in schizophrenia: systematic review. *Schizophrenia Research* .

Nakatsuka, H. & Natsume, K. (2014) Circadian rhythm modulates long-term potentiation induced at CA1 in rat hippocampal slices. *Neuroscience Research 80*. 1-9

Nakajima, K., Matsushita, Y., Tohyama, K. S., & Kurihara, T. (2006). Differential suppression of endotoxin-inducible inflammatory cytokines by nuclear factor kappa B (NFκB) inhibitor in rat microglia. *Neuroscience Letters 301(3)*, 199-202.

Naudon, L., Yacoubi, M. E., Vaugeois, J. M., Leroux-Nicollet, I., & Costentin, J. (2002). A chronic treatment with fluoxetine decreases 5-HT1A receptors labeling in mice selected as a genetic model of helplessness. *Brain Research 936(1-2)*, 68-75.

Navigatore-Fonzo, L. S., Delgado, S. M., Golini, R. S., & Anzulovich, A. C. (2014). Circadian rhythms of locomotor activity and hippocampal clock genes expression are dampened in vitamin A-deficient rats. *Nutrition Research 34(4)*, 326-335.

Navratilova, Z., Hoang, L. T., Schwindel, C. D., Tatsuno, M., & McNaughton, B. L. (2012). Experience-dependent firing rate remapping generates directional selectivity in hippocampal place cells. *Frontiers in Neural Circuits 6(6)*.

Nelson, G., Fike, J., Limoli, C., Obenaus, A., Raber, J., Soltessz, I., & Vlkolinský, R. (2014). Responses of the central nervous system to high linear energy transfer radiation: NSCOR project highlights. *Journal of Radiation Research 55 (suppl 1): i22-i23*, doi:10.1093/jrr/rrt214.

Németh, Z. H., Haskó, G., & Viz, i. E. (1998). Pyrrolidine dithiocarbamate augments IL-10, inhibits TNF-alpha, MIP-1alpha, IL-12, and nitric oxide production and protects from the lethal effect of endotoxin. *Shock 10(1)*, 49-53.

Neunuebel, J. P., & Knierim, J. J. (2014). CA3 retrieves coherent representations from degraded input: direct evidence for CA3 pattern completion and dentate gyrus pattern separation. *Neuron 81(2)*, 416-427.

Neves, G., Cooke, S. F., & Bliss, T. V. (2008). Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nature Reviews Neuroscience 9*, 65-75.

Newman, E. L., & Hasselmo, M. E. (2014). CA3 sees the big picture while dentate gyrus splits hairs. *Neuron 81(2)*, 226-228.

Ngyuyen, K. D., Fentress, S. J., Qiu, Y., Yun, K., Cox, J. S., & Chawla, A. (2013). Circadian gene BMAL1 regulates diurnal osciallations of Ly6Chi inflammatory monocytes. *Science 341(6153)*, 1483-1488.

Nicholls, R. E., Alarcon, J. M., Malleret, G., Carroll, R. C., Grody, M., Vronskaya, S., & Kandel, E. R. (2008). Transgenic mice lacking NMDAR-dependent LTD exhibit deficits in behavioral flexibility. *Neuron 58(1)*, 104-117.

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science 308(5726)*, 1314-1318.

Ninan, P T; Shelton, R C; Bao, W; Guico-Pabia, C J (2014) BDNF, interleukin-6, and salivary cortisol levels in depressed patients treated with desvenlafaxine. *Progress in Neuro-psychopharmacology & Biological Psychiatry 48* 86-91

Nisticò, R., Mango, D., Mandolesi, G., Piccinin, S., Berretta, N., Pignatelli, M., . . . Centonze, D. (2013). Inflammation subverts hippocampal synaptic plasticity in experimental multpiple sclerosis. *PLOS One 8(1)*, e54666.

Niu, W., Zang, T., Zou, Y., Fang, S., Smith, D. K., Bachoo, R., & Zhang, C. L. (2013). In vivo reprogramming of astrocytes to neuroblasts in the adult brain . *Nature Cell Biology* , 1164-1175.

Noh, H., Jeon, J., & Seo, H. (2014). Systemic injection of LPS induces region-specific neuroinflammation and mitochondrial dysfunction in normal mice brain. *Neurochemistry International*, http://dx.doi.org/10.1016/j.neuint.2014.02.008.

Nollet, M., Gaillard, P., Tanti, A., Girault, V., Belzung, C., & Leman, S. (2012). Neurogenesis-independent antidepressant-like effects on behavior and stress axis response of a dual orexin receptor antagonist in a rodent model of depression. *Neuropsychopharmacology 37(1)*, 2210-2221.

Nurmi, A., Perttu, J. L., Koistinaho, M., Zhang, W., Juettler, E., Karjalainen-Lindsberg, M-.L., Weih, F., Frank, N., Schwaninger, M. & Koistinaho, J. (2004) Nuclear Factor-κB Contributes to Infarction After Permanent Focal Ischemia. *Stoke (35)* 987-991

Nurmi, A., Vartiainen, N., Pihlaja, R., Godlsteins, G., Yrjänheikki, J., Koistinaho, J. (2004) Pyrrolidine dithiocarbamate inhibits translocation of nuclear factor kappa-B in neurons and protects against brain

ischaemia with a wide therapeutic time window. *Journal of Neurochemistry 91(3)* 755-765

Obuchowicz, E., Bielecka, A. M., Paul-Samojedny, M., Pudełko, A., & Kowalski, J. (2014). Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. *Pharmacological Reports 66(1)*, 34-43.

O'Callaghan, E. K., Anderson, S. T., Moynagh, P. N., & Coogan, A. N. (2012). Long-lasting effects of sepsis on circadian rhythms in the mouse. *PLOS One 7(10)*, e47087.

Oddo, S., Lux, S., Weis, P. H., Schwab, A., Welzer, H., Markowitsch, H. J., & Fink, G. R. (2010). Specific role of medial prefrontal cortex in retrieving recent autobiographical memories: An fMRI study of young female subjects. *Cortex 46(1)*, 29-39.

O'Donovan, A., Hughes, B. M., Slavich, G. M., Lynch, L., Cronin, M. -T., O'Farrelly, C., & Malone, K. M. (2010). Clinical anxiety, cortisol and interleukin-6: Evidence for specificity in emotion-biology relationships. *Brain, Behavior, and Immunity*, 1074-1077.

Ognjanovski, N., Maruyama, D., Lashner, N., Zochowski, M., & Aton, S. J. (2014). CA1 hippocampal network activity changes during sleep-dependent memory consolidation. *Frontiers in Systems Neuroscience 8*, 61.

Ohgi, Y., Futamura, T., Kikuchi, T., & Hashimoto, K. (2013). Effects of antidepressants on alternations in serum cytokines and depressive-like behavior in mice after lipopolysaccharide administration. *Pharmacology Biochemistry and Behavior 103(4)*, 853-859.

Ohira, K., & Miyakawa, T. (2011). Chronic treatment with fluoxetine for more than 6 weeks decreases neurogenesis in the subventricular zone of adult mice. *Molecular Brain 4(10)*, doi:10.1186/1756-6606-4-10.

Ohsawa, K., Irino, S. T., Nakamura, Y., Suzuki, E., Inoue, K., & Kohsaka, S. (2010). P2Y12 receptor-mediated integrin-β1 activatuib regulates microglial process extension induced by ATP. *Glia 58*, 790-801.

Okada, K., yano, M., Doki, Y., Azama, T., Iwanaga, H., Miki, H., . . . Monden, M. (2008). Injection of LPS causes transient suppresion of biological clock genes in rats. *Journal of Surgical Research 145*, 5-12.

Okazaki, S; Tachibana, Y; Koga-Ogawa, Y; Takeshita, K. (2014) Redox evaluation in sepsis model mice by the invivo ESR technique using acyl-protected hydroxylamine. *Free Radical Biology & Medicine 68* 72-79

O'Keefe, J. & Dostrovsky, J. (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Research 34(1)*, 171-175

Omerod, B. K., Hanft, S. J., Asokan, A., Haditsch, U., Lee, S. W., & Palmer, T. D. (2013). PPARγ activation prevents impairments in spatial memory and neurogenesis following transient illness. *Brain Behavior & Immunity 29*, 28-38.

Önder, E., & Tural, Ü. (2003). Faster response in depressive patients treated with fluoxetine alone than in combination with buspirone. *Journal of Affective Disorders. 76(1-3)*, 223-227.

O'Neill, J., Senior, T., & Csicsvari, J. (2006). Place-selective firing of CA1 pyramidal cells during sharp wave/ripple network patterns in exploratory behavior. *Neuron 49(1)*, 143-155.

O'Neill, L. A., & Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature Reviews Immunology 7*, 353-364.

Oomen, C. A., Soeters, H., Audureau, N., Vermunt, L., van Hasselt, F. N., Manders, E. M., . . . Lucassen, P. J. (2011). Early maternal deprivation affects dentate gyrus

structure and emotional learning in adult female rats. *Psychopharmacology 214(1)*, 249-260.

Ota, Y; Zanetti, A T; Hallock, R M (2013) The role of astrocytes in the regulation of synaptic plasticity and memory formation *Neural Plasticity*

Otte, C. (2008). Incomplete remission in depression: role of psychiatric and somatic comorbidity. *Dialogues in Clinical Neuroscience 10(4)*, 453-460.

Paladino, N., Leone, M. J., Plano, S. A., & Golombek, D. A. (2010). Paying the circadian toll: the circadian response to LPS injection is dependent on the Toll-like receptor 4. *Journal of Neuroimmunology 225(1-2)*, 62-67.

Paladino, N., Mul Fedele, M. L., Duhart, J. M., Marpegan, L., & Golombek, D. A. (2014). Modulation of mammalian circadian rhythms by tumor necrosis factor-α. *Chronobiology International 31(5)*, 668-679.

Pan, W., & Kastin, A. J. (2002) TNFα Transport across the blood-brain barrier is abolished in receptor knockout mice *Experimental Neurology 174(2)* 193-200

Pandey, G. N., Dwivedi, Y., Rizavi, H. S., Ren, X., Zhang, H., & Pavuluri, M. N. (2010). Brain-derived neurotrophic factor gene and protein expression in pediatric and adult depressed subjects. *Progress in neuro-psychopharmacology & biological psychiatry 34(4)*, 645-651.

Pandey, G. N., Rizavi, H. S., Ren, X., Dwivedi, Y., & Palkovits, M. (2013). Region-specific alterations in glucorticoid receptor expression in the postmortem brain of teenage suicide victims. *Psychoneuroendocrinology 38(11)*, 2628-2639.

Pandey, G. N., Rizavi, H. S., Ren, X., Fareed, J., Hoppensteadt, D. A., Roberts, R. C., . . . Dwivedi, Y. (2012). Proinflammatory cytokines in the prefrontal cortex of teenage suicide victims. *Journal of Psychiatric Research 46(1)*, 57-63.

Pantazopoulos, H., Dolatshad, H., & Davis, F. C. (2011). A fear-inducing odor alters PER2 and c-Fos expression in brain regions involved in fear memory. *PLoS ONE 6(5)*, e20658.

Papadimitriou, G. N., & Linkowski, P. (2005). Sleep disturbance in anxiety disorders . *International Review of Psychiatry 17(4)*, 229-236.

Papadopoulos, M. C., Davies, D. C., Moss, R. F., Tighe, D., & Bennett, E. D. (2000). Pathophysiology of septic encephalopathy: A review. *Ciritical Care Medicine. 28(8)*, 3019-3024.

Pape, H. C., & Pare, D. (2010). Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of coditioned fear. *Physiological reviews 90(2)*, 419-463.

Parent, M. A., Wang, L. Su, J., Netoff, T. & Yuan, L-L. (2010) Idntification of the hippocampal input to medial prefrontal cortex in vitro. *Cerebral Cortex 20(2)* 393-403

Park, E., Dvorak, D., & Fenton, A. A. (2011). Ensemble place codes in hippocampus: CA1, CA3, and dentate gyrus place cells have multiple place fields in large environments. *PLoS ONE 6(7)*, e22349.

Partch, C. L., Green, C. B., & Takahashi, J. S. (2014). Molecular architecture of the mammalian circadian clock. *Trends in Cell Biology 24(2)*, 90-99.

Patel, A. (2013). Review: The role of inflammation in depression. *Psychiatria Danubina 25(S2)*, S216-223.

Paxinos, G., & Watson, C. (2007). *The rat brain in stereotaxic coordinates.* Amsterdam: Elsevier/AP.

Pei, H., Zhang, Y., Ren, L., Jia, X., Zhang, Y., & Chen, M. (2013). Relationship between iNOS expression and apoptosis in cerebral tissue, and the effects of sini injection in endotoxin shock rats. *Journal of Traditional Chinese Medicine 33(4)*, 486-491.

Penke, Z., Chagneau, C., & Laroche, S. (2011). Contribution of Egr1/zig268 to Activity-Dependent Arc/Arg3.1 Transcription in the Dentate Gyrus and Area CA1 of the Hippocampus. *Frontiers in Behavioral Neuroscience 5(48)*.

Perez-Caballero, L., Torres-Sanchez, S., Bravo, L., Mico, J. A., & Berrocoso, E. (2014). Fluoxetine: a case history of its discovery and preclinical development. *Expert Opinion on Drug Discovery 9(5)*, 567-578.

Pérez-Cadahía, B., Drobic, B., & Davie, J. R. (2011). Activation and function of immediate-early genes in the nervous system. *Biochemistry and Cell Biology 89(1)*, 61-73.

Perras, B., Kurowski, V., & Dodt, C. (2006). Nocturnal melatonin concentration is correlated with illness severity in patients with septic disease. *Intensive Care Medicine 32(4)*, 624-625.

Perreau-Lenz, S., Kalsbeek, A., Garidou, M. L., Wortel, J., van der Vliet, J., van Heijningen, C., . . . Buijs, R. M. (2003). Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms. *European Journal of Neuroscience 17(2)*, 221-228.

Perry, V. H. & Teeling, J. (2013) Microglia and macrophages of the central nervous system: the contribution of microglia priming and systemic inflammation to chronic neurodegeneration. *Seminars in Immunopathology 35(5)* 601-612

Pestka, J. & Zhou, H. (2006) Toll-like receptor priming sensitises macrophages to proinflammatory cytokine gene induction by deoxynivalenol and other toxicants. *Toxicological Sciences 92(2)* 445-455

Petrik, D., Lagace, D. C., & Eisch, A. J. (2012). The neurogenesis hypothesis of affective and anxiety disorders: are we mistaking the scaffolding for the building? *Neuropharmacology 62(1)*, 21-34.

Petronilho, F., Périco, S. R., Vuolo, F., Mina, F., Constantino, L. C., Quevedo, J., . . . Dal-Pizzol, F. (2012). Protective effects of guanosine against sepsis-induced damage in rat brain and cognitive impairment. *Brain, Behavior, and Immunity 26(6)*, 904-910.

Phan, T. H., Chan, G. C. K., Sindreu, C. B., Eckel-Mahan, K. L. & Storm, D. R. (2011) The Diurnal Oscillation of MAP (Mitogen-Activated Protein) Kinase and Adenylyl Cyclase Activities in the Hippocampus Depends on the Suprachiasmatic Nucleus. *The Journal of Neuroscience 31(29)* 10640-10647

Pikkarainen, M., Rönkkö, S., Savander, V., Insausti, R., & Pitkänen, A. (1999). Projections from the lateral, basal and accessory basal nuclei of the amygdala to the hippocampal formation. *Journal of Computational Neurology 403(2),* 229-260.

Piskorowski, R. A., & Chevaleyre, V. (2013). Delta-Opioid Receptors Mediate Unique Plasticity onto Parvalbumin-Expressing Interneurons in Area CA2 of the Hippocampus. *The Journal of Neuroscience 33(36),* 14567-14578.

Pitsavos, C., Panagiotakos, D. B., Papageorgiou, C., E, T., Soldatos, C., & Stefanadis, C. (2006). Anxiety in relation to inflammation and coagulation markers, among healthy adults: The ATTICA Study. *Athlerosclerosis, 185(2),* 320-326.

Plaschke, K., Bent, F., Wagner, S., Zorn, M., & Kopitz, J. (2014). In contrast to its anti-inflammatory and anti-apoptotic peripheral effect, levosimendan failed to induce a long-term neuroprotective effect in a rat model of mild septic encephalopathy: A pilot study. *Neuroscience Letters 560*, 117-121.

Poltorak, A., He, X., Smirnova, I., Liu, M., Van Huffel, C., Du, X., . . . Beutler, B. (1998). Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science 282(5396),* 2085-2088.

Porsolt, R. D., Bertin, A., & Jalfre, M. (1997). Behavioral despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther., 229*, 327-336.

Pribaiag, H., & Stellwagen, D. (2014). Neuroimmune regulation of homeostatic synaptic plasticity. *Neuropharmacology 78*, 13-22.

Price, M. R., Kruse, J. A., Galvez, M. E., Lorincz, A. M., Avigdor, M., & Heideman, P. D. (2005). Failure to respond to endogenous or exogenous melatonin may cause nonphotoresponsiveness in Harlan Sprague Dawley rats. *Journal fo Circadian Rhythms 3(12).*

Pugh, C. R., Kumagawa, K., Fleshner, M., R, W. L., Maier, S. F., & W, R. J. (1998). Conditioning, Selective Effects of Peripheral Lipopolysaccharide Administration on Contextual and Auditory-Cue Fear. *Brain, Behaviour, and Immunity. (12),* 212-229.

Purves, D., Augustine, G. J., Fitzpatrick, D., Katz, L. C., LaMantia, A. S., McNamara, J. O., & Williams, S. M. (2001). *Neuroscience (2nd Edition).* Sunderland (MA): Sinauer Associates.

Qin, L., Wu, X., Block, M. L., Breese, G. R., Hong, J. S., Knapp, D. J., & Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia. 55(5)*, 453-462.

Qiutang, L. & Verma, I. M. (2002) NF-kB regulation in the immune system. *Nature Reviews Immunology 2* 725-734

Quan, N., Banks, W. A. (2007) Brain-immune communication pathways. *Brain, Behavior & Immunity 21(6)* 727-735

Rakai, B. D., Chrusch, M. J., Spanswick, S. C., Dyck, R. H., & Antle, M. C. (2014). Survival of adult generated hippocampal neurons is altered in circadian arrhythmic mice. *PLOS One 9(6)*, e99527.

Ramaglia, V., Hughes, T. R., Donev, R. M., Ruseva, M. M., Wu, X., Huitinga, I., . . . Morgan, B. P. (2012). C3-dependent mechanism of microglial priming relevant to multiple sclerosis. *PNAS 109(3)*, 965-970.

Ransome, M. I., Renoir, T., & Hannan, A. J. (2012). Hippocampal neurogenesis, cognitive deficits and affective disorder in huntington's disease. *Neural Plasticity vol2012*, Article ID 874387.

Raspé, C., Höcherl, K., Rath, S., Sauvant, C., & Bucher, M. (2013). NF-κB-mediated inverse regulation of fractalkine and CXCR1 during CLP-induced sepsis. *Cytokine 61(1)*, 97-103.

Rawashdeh, O., Jilg, A., Jedlicka, P., Slawska, J., Thomas, L., Saade, A., . . . Stehle, J. H. (2014). PERIOD1 coordinates hippocampal rhythms and memory processing with daytime. *Hippocampus 24(6)*, 712-723.

Reisinger, E. C., Kern, P., Dietrich, M., Ernst, M., Flad, H. D., & Bock, P. (1990). Inhibition of HIV progression by dithiocarbamates. German DTC Study Group. . *Lancet 335(8691)*, 679-682.

Remick, D. G., Newcomb, D. E., Bolgos, G. L., & Call, D. R. (2000). Comparison of the mortality and inflammatory response of two models of sepsis: lipopolysaccharide vs. cecal ligation and puncture. *SHOCK 13(2)*, 110-116.

Renard, C. E., Dailly, E., David, D. J., Hascoet, M., & Bourin, M. (2003). Monoamine metabolism changes following the mouse forced swim test but not the tail suspension test. *Fundamental & Clinical Pharmacology. 17*, 449-455.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature 418*, 935-941.

Rhodes, K. J., & Trimmer, J. S. (2006). Antibodies as valuable neuroscience research tools versus reagents of mass distraction. *The Journal of Neuroscience 26(31)* , 8017-8020.

Ribak, C. E. (1992). Local circuitry of GABAergic basket cells in the dentate gyrus. *Epilepsy Research Supplement 7*, 29-47.

Ribak, C. E., & Seress, L. (1983). Five types of basket cell in the hippocampal dentate gyrus: a combined golgi and electron microscopic study. *Journal of Neurocytology 12(4),* 577-597.

Risold, P. Y., & Swanson, L. W. (1997). Connections of the rat lateral septal complex. *Brain Research. Brain Research Reviews 24(2-3),* 115-195.

Ritter, C., Miranda, A. S., Giombelli, V. R., Tomasi, C. D., Comim, C. M., Teixeira, A. L., . . . Dal-Pizzol, F. (2012). Brain-derived neurotrophic factor plasma levels are associated with mortality in critically ill patients even in the absence of brain injury. *Critical Care 16,* R234.

Rittirsch, D., Hoesel, L. M., & Ward, P. A. (2007). The disconnect between animal models of sepsis and human sepsis. *Journal of Leukocyte Biology 81(1),* 137-143.

Rittirsch, D., Huber-Lang, M. S., Flierl, M. A., & Ward, P. A. (2008). Immunodesign of experimental sepsis by cecal ligation and puncture. *Nature Protocols. 4,* 31-36.

Rivest, S. (2009). Regulation of innate immune responses in the brain . *Nature Reviews Immunology 9,* 429-439.

Robert, G., Drapier, D., Bentué-Ferre, D., Renault, A., & Reymann, J. M. (2011). Acute and chronic anxiogenic-like response to fluoxetine in rats in the elevated plus-maze: Modulation by stressful handling. *Behavioural Brain Research 220(2),* 344-348.

Robertson, C. M., & Coopersmith, C. M. (2006). The systemic inflammatory response syndrome. *Microbes and infection. 8(5),* 1382-1389.

Roche, M., Diamond, M., Kelly, J. P., & Finn, D. P. (2007). In vivo modulation of LPS-induced alterations in brain and peripheral cytokines and HPA axis activity by cannabinoids. *Journal of Neuroimmunology 181(1-2),* 57-67.

Rolls, E. T. (2013). The mechanisms for pattern completion and pattern separation in the hippocampus. *Frontiers in Systems Neuroscience 7,* 74.

Rolls, E. T., & Kesner, R. P. (2006). A computational theory of hippocampal function, and empirical tests of the theory. *Progress in Neurobiology 79(1),* 1-48.

Ropireddy, D., Bachus, S. E., & Ascoli, G. A. (2012). Non-homogeneous stereological properties of the rat hippocampus from hig-resolution 3D serial reconstruction of thin histological sections. *Neuroscience 205,* 91-111.

Ropireddy, D., Scorcioni, R., Lasher, B., Buzsáki, G., & Ascoli, G. A. (2011). Axonal morphometry of hippocampal pyramidal neurons semi-automatically reconstructed after in-vivo labeling in different CA3 locations. *Brain Sturcture & Function 216(1),* 1-15.

Rosen, J. B. & Donley, M. P. (2006) Animal studies of amygdala function in fear and uncertainty: Relevance to human research. *Biological Psychology 73(1)* 49-60

Rosen, J. B., Adamec, R. E., & Adamec, B. L.. (2005). Expression of egr-1 (zif268) mRNA in select fear-related brain regions following exposure to a predator. *Behavioral Brain Research 162(2)*, 279-288.

Rosenblat, J. D., Cha, D. S., Mansur, R. B., & McIntyre, R. S. (2014). Inflamed moods: A review of the interactions between inflammation and mood disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry 53*, 23-34.

Rosenzweig, H. L., Lessov, N. S., Henshall, D. C., Minami, M., Simon, R. P., & Stenzel-Poole, M. P. (2004). Endotoxin preconditioning prevents cellular inflammatory response during ischemic neuroprotection in mice . *Stroke 35*, 2576-2581.

Rosi, S. (2011). Neuroinflammation and the plasticity-related immediate-early gene Arc. *Brain, Behavior, and Immunity 25(1)*, S39-S49.

Rosi, S., Ramirez-Amaya, V., Vazdarjanova, A., Worley, P. F., Barnes, C. A. & Wenk, G. L. (2005) Neuroinflammation alters the hippocampal pattern of behaviourally induced Arc expression. *The Journal of Neuroscience 25(3)* 723-73

Rosi, S., Ramirez-Amaya, V., Hauss-Wegrzyniak, B., & Wenk, G. L. (2004). Chronic brain inflammation leads to a decline in hippocampal NMDA-R1 receptors. *Journal of Neuroinflammation 1(12)*, doi:10.1186/1742-2094-1-12.

Rosi, S., Vazdarjanova, A., Ramirez-Amaya, V., Worley, P. F., Barnes, C. A., Wenk, G. A. (2006) Memantine protects against LPS-induced neuroinflammation, restores behaviorally-induced gene expression and spatial learning in the rat *Neuroscience, 142(4)* 1303-1315

Ross, B. M. (2009). Omega-3 polyunsaturated fatty acids and anxiety disorders. *Protaglandins, Leukotrienes and Essential Fatty Acids. 81*, 309-312.

Roumestan, C., Michel, A., Bicho, F., Portet, K., Detoc, M., Henriquet, C., . . . Mathieu, M. (2007). Anti-inflammatory properties of desipramine and fluoxetine. *Respiratory Research 8(35)*, doi:10.1186/1465-9921-8-35.

Roy, A., Jana, A., Yatish, K., Feidt, M. B., Fung, Y. K., Martinson, J. A., & Pahan, K. (2008). Reactive oxygen species yup-regulate CD11b in mircoglia via nitric oxide: implications for neurodegenerative diseases. *Free Radical Biuology and Medicine 45(5)*, 686-699.

Ruby, N. F., Hwang, C. E., Wessells, C., Fernandez, F., Zhang, P., & Sapolsky, R. H. (2008). Hippocampal-dependent learning requires a functional circadian system. *PNAS 105(40)*, 15593-15598.

Sacai, H., Sasaki-Hamada, S., Sugiyama, A., Saitoh, A., Mori, K., Yamada, M., & Oka, J. I. (2014). The impairment in spatial learning and hippocampal LTD induced through the PKA pathway in juvenile-onset diabetes rats are rescued by modulating NMDA receptor function. *Neuroscience Research 81-82*, 55-63.

Salgado-Delgado, R., Osorio, A. T., Saderi, N., & Escobar, C. (2011). Disruption of circadian rhythms: A crucial factor in the etiology of depression. *Depression Research and Treatment* , Article ID 839743.

Sammut, S., Bethus, I., Goodall, G., & Muscat, R. (2002). Antidepressant reversal of interferon-alpha-induced anhedonia. *Physiology & Behaviour 75(5)*, 765-772.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battagli, F., Dulawa, S., . . . Hen, R. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science 301(5634)*, 805-9.

Sato, S., Sakurai, T., Ogasawara, J., Takahashi, M., Imaizumi, K., Taniguchi, N., . . . Kizaki, T. (2014). A circadian clock gene, Rev-erbα, modulates the inflammatory function of macrophages through the negative regulation of Ccl2 expression. *Journal of Immunology 192(1)*, 407-417.

Satoh, A., Shimosegawa, T., Fujita, M., Kimura, K., Masamune, A., Koizumi, M. & Toyota, T. (1999) Inhibition of nuclear factor-κB activation improves the survival of rats with taurocholate pancreatitis. *Gut 44* 253-258

Sauro, M. D., Jorgensen, R. S., & Pedlow, C. T. (2003). Stress, glucocorticoids, and memory: A meta-analytic review. *Stress 6(4)*, 235-245.

Schaaf, M. J., Duurland, R., de Kloet, E. R., & Vreugdenhil, E. (2000). Circadian variation in BDNF mRNA expression in the rat hippocampus. *Molecular Brain Research 75(2)*, 342-344.

Scharfman, H. E. (1991). Dentate hilar cells with dendrites in the molecular layer have lower thresholds for synaptic activation by perforant path than granule cells. *The Journal of Neuroscience 11(6)*, 1660-1673.

Scharfman, H. E. (1995). Electrophysiological evidence that dentate hilar mossy cells are excitatory and innervate both granule cells and interneurons. *Journal of Neurophysiology 74(1)*, 179-194.

Scharfman, H. E. (2007). The CA3 "backprojection" to the dentate gyrus. *Progress in Brain Research 163*, 627-637.

Scharfman, H. E., & Myers, C. E. (2013). Hilar mossy cells of the dentate gyrus: a historical perspective. *Frontiers in Neuronal Circuits 6(106)*.

Scharfman, H., Goodman, J., Macleod, A. P., Antonelli, C., & Croll, S. (2005). Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Experimental Neurology 192(2)*, 348-356.

Schefold, J. C., Hasper, D., Volk, H. D. & Reinke, P. (2008) Sepsis: Time has come to focus on the later stages. *Medical Hypotheses 81(2)* 203-208

Scheiermann, C., Kunisaki, Y., & Frenette, P. S. (2013). Circadian control of the immune system. *Nature Reviews Immunology 13*, 190-198.

Schneider, P., Ho, Y., Spanagel, R. & Pawlak, C. R. (2011) A Novel Elevated Plus-Maze Procedure to Avoid the One-Trial Tolerance Problem. *Frontiers in Behavioural Neuroscience 5(43)* 1-8

Schulz, K., & Korz, V. (2010). Emotional and cognitive information processing: Relations to behavioral performance and hippocampal long-term potentiation in

vivo during a spatial water maze training in rats. *Learning & Memory 17*, 552-560.

Schumann R., R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., Ulevitch, R. J. (2009) Structure and function of lipopolysaccharide binding protein. *Science 249*: 1429–1431

Schütze, S., Wiegmann, K., Machleidt, T., & Krönke, M. (1995). TNF-induced activation of NF-kappaB. *Immunobiology 193(2-4)*, 193-203.

Schwalm, M. T., Psaquali, M., Miguel, S. P., dos Santos, J. P., Vuolo, F., Comim, C. M., . . . Dal-Pizzol, F. (2014). Acute brain inflammation and oxidative damage are related to long-term cognitive deficits and markers of neurodegeneration in sepsis-survivor rats. *Molecular Neurobiology 49(1)*, 380-385.

Scorcioni, R., Bouteiller, J. M., & Ascoli, G. A. (2002). A real-scale anatomical model of the dentate gyrus based on single cell reconstructions and 3D rendering of a brain atlas. *Neurocomputing 44-46*, 629-634.

Segall, L. A., Milet, A., Tronche, F., & Amir, S. (2009). Brain glucocorticoid receptors are necessary for the rhythmic expression of the clock protein, PERIOD2, in the central extended amygdala in mice. *Neuroscience Letters 457(1)*, 58-60.

Semmler, A., Frisch, C., Debeir, T., Ramanathan, M., Okulla, T., Klockgether, T., & Heneka, M. T. (2007). Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model. *Experimental Neurology 204(2)*, 733-740.

Semmler, A., Hermann, S., Mormann, F., Weberpals, M., Paxian, S. A., Okulla, T., . . . Heneka, M. T. (2008). Sepsis causes neuroinflammation and concomitant decrease of cerebral metabolism. *Journal of Neuroinflammation 5*, 38.

Semmler, A., Okulla, T., Sastre, M., Dumitrescu-Ozimek, L., & Heneka, M. T. (2005). Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *Journal of Chemical Neuroanatomy 30(2-3)*, 144-157.

Semmler, A., Widmann, C. N., Okulla, T., Urbach, H., Kaiser, M., Widman, G., . . . Heneka, M. T. (2013). Persistent cognitive impairment, hippocampal atrophy and EEG changes in sepsis survivors. *Journal of Neurology, Neurosurgery, and Psychiatry 84(1)*, 62-69.

Seok, J., Warren, H. S., Mindrinos, M. N., Baker, H. V., Xu, W., & ... the Inflammation and Host Response to Injury, L. S. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *PNAS 110(9)*, 3507-3512.

Seress, L., & Pokorny, J. (1981). Structure of the granular layer of the rat dentate gyrus. A light microscopic and golgi study. *Journal of Anatomy 133(pt2)*, 181-195.

Sesack, S. R., Deutch, A. Y., Roth, R. H., & Bunney, B. S. (1989). Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: An anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *The Journal of Comparative Neurology 290(2)*, 213-242.

Shah, F. A., Pike, F., Alvarez, K., Angus, D., Newman, A. B., Lopez, A. B., . . . Yende, S. (2013). Bidirectional relationship between cognitive function and pneumonia. *American Journal of Respiratory and Critical Care Medicine 188(5),* 586-592.

Shakhov, A. N., Collart, M. A.. Vassakkum P., Nedospasov, S. A., Jongeneel, C. V. (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *Journal of Experimental Medicine 171(1)* 35-47

Shao, D. Z., Lee, J. J., Huang, W. T., Liao, J. F., & Lin, M. T. (2004). Inhibition of nuclear factor-kappa B prevents staphylococcal enterotoxin A-induced fever. *MOlecular and cellular biochemistry. 262(1-2),* 177-185.

Sharma, V., Gilhotra, R., Dhingra, D., & Gilhotra, N. (2011). Possible underlying influence of p38MAPK and NF-κB in the diminished anti-anxiety effect of diazepam in stressed mice. *Journal of Pharmacological Sciences 116(3),* 257-263.

Sharshar, T., Annane, D., de la Grandmaison, G. L., Brouland, J. P., Hopkinson, N. S., & Françoise, G. (2004). The neuropathology of septic shock. *Brain Pathology 14(1),* 21-33.

Sharshar, T., Gray, F., de la Grandmaison, G. L., Hopkinson, N. S., Dorandeu, A., Orlikowksi, D., . . . Annane, D. (2003). Apoptosis of neurons in cardiovascular autonomic centres triggered by inducible nitric oxide synthase after death from septic shock. *The Lancet 362(9398),* 1799-1805.

Sharshar, T., Gray, F., Poron, F., Raphael, J. C., Gajdos, P., & Annane, D. (2002). Multifocal necrotizing leukoencephalpathy in septic shock . *Critical Care Medicine 30,* 2371-2375.

Shaulian, E., & Karin, M. (2002). AP-1 as a regulator of cell life and death. *Nature Cell Biology 4,* 131-136.

Shelton, R. C. (2012) Does concomitant use of NSAIDs reduce the effectiveness of antidepressants? *The American Journal of Psychiatry 169(10)* 102-105

Shi, S., Hida, A., McGuinness, O. P., Wasserman, D. H., Yamazki, S., & Johnson, C. H. (2010). Circadian Clock Gene Bmal1 Is Not Essential After All; Functional Replacement with its Paralog, Bmal2. *Current Biology 20(4),* 316-321.

Shi, Y., Ikrar, T., Olivas, N. D., & Xu, X. (2014). Bidirectional global spontaneous network activity preceds the canonical unidirectional circuit organization in the developing hippocampus. *The Journal of Comparative Neurology 522(9),* 2191-2208.

Shin, L. M., Rauch, S. L. & Pitman, R. K. (2006) Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Annals of the New York Academy of Science 1071* 67-79

Shirayama, Y., Chen, A. C., Nakagawa, S., Russell, D. S., & Duman, R. S. (2002). Brain-Derived Neurotrophic Factor produces antidepressant effects in behavioral models of depression. *The Journal of Neuroscience 22(8),* 3251-3261.

Shpargel, K., Jalabi, W., Jin, Y., Dadabayev, A., Penn, M. S., & Trapp, B. D. (2008). Preconditionging paradigms and pathways in the brain. *Cleveland Clinic journal of medicine 75(Suppl 2),* S77-82.

Shu, L. S., Huang, Y. S., Fones Soon Leng, C., Strijckmans Crutel, V., & Kim, Y. S. (2014). Comparable efficacy and safety of 8 weeks treatment with agomelatine 25-50mg or fluoxetine 20-40mg in Asian out-patients with major depressive disorder. *Asian Journal of Psychiatry 8*, 26-32.

Sierra, A., Encinas, J. M., Deudero, J. J., Chancey, J. H., Enikolopov, G., Overstreet-Wadiche, L. S., . . . Maletic-Savatic, M. (2010). Microglia shape adult hippocampal neurogensis through apoptosis-coupled phagocytosis. *Cell Stem Cell 7(4)*, 483-495.

Sik, A., Penttonen, M., & Buzsáki, G. (1997). Interneurons in the Hippocampal Dentate Gyrus: an in vivo intracellular study. *European Journal of Neuroscience*, 573-588.

Sik, A., Penttonen, M., Ylinen, A., & Buzsáki, G. (1995). Hippocampal CA1 interneurons: An in vivo intracellular study. *The Journal of Neuroscience 15(10)*, 6651-6665.

Silva, M. R., Alves, C. R., & Santarem, E. M. (1999). Anxiogenic-like effect of acute and chronic fluoxetine on rats tested on the elevated plus-maze. *Brazilian Journal of Medical and Biological Research 32*, 333-339.

Silva, R. C., & Brandão, M. L. (2000). Acute and chronic effects of gepirone and fluoxetine in rats tested in the elevated plus-maze: an ehtological analysis. *Pharmacology Biochemistry & behavior 65*, 209-216.

Silver, A. C., Arjona, A., Walker, W. E., & Fikrig, E. (2012). The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity 36(2)*, 251-261.

Silvia, B., Lucia, C., Roberto, A., Simon, B., Gerrard, P. A., & Maria, R. (2009). Differential behavioral, physiological, and hormonal sensitivity to LPS challenge in rats. *International Journal of Interferon, Cytokine and Mediator Research 1*, 1-13.

Simon, D., Adler, N., Kaufmann, C., & Kathmann, N. (2014). Amygdala hyperactivation during symptom provocation in obsessive–compulsive disorder and its modulation by distraction. *NeuroImage: Clinical 4*, 549-557.

Singh, A. K., Jiang, Y. (2004) How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology 201*. 197-207

Slattery, D. A., Morrow, J. A., Hudson, A. L., Hill, D. R., Nutt, D. J., & Henry, B. (2005). Comparison of alterations in c-fos and Egr-1 (zif268) expression throughout the rat brain following acute administration of different classes of antidepressant compounds. *Neuropsychopharmacology 30*, 1278-1287.

Smith, J. A., Das, A., Ray, S. K., & Banik, N. L. (2012). Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Research Bulletin 87(1),* 10-20.

Snyder, J. S., & Cameron, H. A. (2012). Could adult hippocampal neurogenesis be relevant for human behavior? *Behavioural Brain Research 227(2),* 384-390.

Solstad, T., Yousif, H. N., & Sejnowski, T. J. (2014). Place cell rate remapping by CA3 recurrent collaterals. *PLoS Computational Biology 10(6),* e1003648.

Soma, M., Aizawa, H., Ito, Y., Maekawa, M., Osumi, N., Nakahira, E., . . . Yuasa, S. (2009). Development of the mouse amygdala as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation. *Journal of Comparative Neurology 513(1),* 113-128.

Somogyi, P., & Klausberger, T. (2005). Defined types of cortical interneurone structure space and spike timing in the hippocampus. *The Journal of Pysiology 562(1),* 9-26.

Song, S., Abdelmohsen, K., Zhang, Y., Becker, K. G., Gorospe, M., & Bernier, M. (2011). Impact of Pyrrolidine Dithiocarbamate and Interleukin-6 on Mammalian Target of Rapamycin Complex 1 Regulation and Global Protein Translation. *The Journal of Pharmacology and Experimental Therapeutics 339(3),* 905-913.

Song, X., Li, W., Yang, Y., Zhao, J., Jiang, C., Li, W., & Lv, L. (2011). The nuclear factor-κB inhibitor pyrrolidine dithiocarbamate reduces polyinosinic-polycytidilic acid-induced immune response in pregnant rats and the behavioral defects of their adult offspring. *Behavioral and Brain Functions 7(50),* doi:10.1186/1744-9081-7-50.

Sossin, W. S., & Barker, P. A. (2007). Something old, something new: BDNF-induced neuron survival requires TRPC channel function. *Nature Neuroscience 10,* 537-538.

Soszynski, D. (2002). Inhibition of nitric oxide synthase delays the development of tolerance to LPS in rats. *Physiology & Behavior 76(1),* 159-169.

Soulé, J., Penke, Z., Kanhema, T., Alme, M. N., Laroche, S. & Bramham, C. R. (2008) Object-Place Recognition Learning Triggers Rapid Induction of Plasticity-Related Immediate Early Genes and Synaptic Proteins in the Rat Dentate Gyrus. *Neural Plasticity*

Souêtre, E. S., Belugou, J. L., Pringuey, D., Candito, M., Krebs, B., Ardisson, J. L., & Darcourt, G. (1989). Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. *Psychiatric Research 28(3),* 263-278.

Spengler, M. L., Kuropatwinski, K. K., Comas, M., Gasparian, A. V., Fedtsova, N., Gleiberman, A. S., . . . Antoch, M. P. (2012). Core circadian protein CLOCK is a positive regulator of NF-κB–mediated transcription. *PNAS 109(37),* E2457-E2465.

Sperner-Untwerger, B., Kohl, C., & Fuchs, D. (2014). Immune changes and neurotransmitters: Possible interactions in depression? *Progress in Neuro-Psychopharmacology and Biological Psychiatry 48*, 268-276.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J. A., Binder, B. R., & Lipp, J. (1998). Nuclear Factor (NF)-κB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor α-induced apoptosis. *The Journal of Experimental Medicine 188(1)*, 211-216.

Stellwagen, D., & Malenka, R. C. (2006). Synaptic scaling mediated by glial TNF-alpha. *Nature 440*, 1054-1059.

Steru, L., Chermat, R., Thierry, B., & Simon, P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology, 367*, 367-370.

Stevens, S. L., Leung, P. Y., Vartanian, K. B., Gopalan, B., Yang, T., Simon, R. P., & Stenzel-Poore, M. P. (2011). Multiple preconditioning paradigms converge on interferon regulatory factor-dependent signaling to promote tolerance to ischemic brain injury. *The Journal of Neuroscience 31(23)*, 8456-8463.

Stipursky, J., Romão, L., Tortelli, V., Neto, V. M., & Gomes, F. C. (2011). Neuron-glia signaling: Implications for astocyte differentiation and synapse formation. *Life Sciences 89*, 524-531.

Stow, J. L., & Murray, R. Z. (2013). Intracellular trafficking and secretion of inflammatory cytokines. *Cytokine & Growth Factor Reviews 24(3)*, 227-239.

Streit, W. J. (2002). Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia 40(2)*, 133-139.

Strekalova, T., Spanagel, R., Bartsch, D., Henn, F. A., & Gass, P. (2004). Stress-Induced Anhedonia in Mice is Associated with Deficits in Forced Swimming and Exploration. *Neuropsychopharmacology. 29*, 2007-2017.

Stubbs, D. J., Yamamoto, A. K., & Menon, D. K. (2013). Imaging in sepsis-associated encephalopathy - insights and opportunities. *Nature Reviews Neurology 9*, 551-561.

Sun, J., & Kapur, J. (2012). M-type potassium channels modulate Schaffer collateral-CA1 glutamatergic synaptic transmission. *The Journal of Physiology 590(16)*, 3953-3964.

Swiergiel, A. H., & Dunn, A. J. (2007). Effects of interleukin-1β and lipopolysaccharide on behavior of mice in the elevated plus-maze and open field tests. *Pharmacology, Biochemistry and Behavior. 86*, 651-659.

Szabadics, J., & Soltesz, I. (2009). Functional specificity of mossy fiber innervation of GABAergic cells in the hippocampus. *The Journal of Neuroscience 29(13)*, 4239-4251.

Tahara, Y., Kuroda, H., Saito, K., Nakajima, Y., Kubo, Y., Ohnishi, N., . . . Shibata, S. (2012). In vivo monitoring of peripheral circadian clocks in the mouse. *Current Biology 22(11)*, 1029-1034.

Takagi, T., Inada, Y., & Naito, Y. (2013). Circadian rhythm and inflammatory bowel disease. *Nihon rinsho. Japanese journal of clinical medicine 71(12)*, 2165-2170.

Tamamaki, N., Abe, K., & Nojyo, Y. (1988). Three-dimensional analysis of the whole axonal arbors originating from single CA2 pyramidal neurons in the rat hippocampus with the aid of a computer graphic technique. *Brain Research 452(1-2)*, 255-272.

Tang, M., He, T., meng, Q. Y., Broussard, J. I., Yao, L., Diao, Y., . . . Zhao, S. (2014). Immobility responses between mouse strains correlate with distinct hippocampal serotonin transporter protein expression and function. *The International Journal of Neuropsychopharmacology I*, 1-14.

Tanti, A., & Belzung, C. (2012). Neurogenesis along the septo-temporal axis of the hippocampus: Are depression and the action of antidepressants region-specific? *Neuroscience 252*, 234-252.

Tasaki, K., Ruetzler, C. A., Ohtsuki, T., Martin, D., Nawashiro, H., & Hallenbeck, J. M. (1997). Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. *Brain research 748(1-2)*, 267-270.

Tashiro, A., Sandler, V. M., Toni, N., Zhao, C., & Gage, F. H. (2006). NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. *Nature 442*, 929-933.

Tellez, R., Rocha, L., Castillo, C., & Meneses, A. (2010). Autoradiographic study of serotonin transporter during memory formantion. *Behavioural Brain Research 212(1)*, 12-26.

Thomas, A. J., Davis, S., Morris, C., Jackson, E., Harrison, R. & O'Brien, J. T. (2005) Increase in interleukin-1ß in late-life depression. *The American Journal of Psychiatry 162(1)* 175-177

Thompson, C. (2002). Onset of action of antidepressants: resutls of different analyses. *Human Psychopharmacology: Clinical and Experimental 17(S1)*, S27-32.

Thompson, C. L., Pathak, S. D., Jeromin, A., Ng, L. L., MacPherson, C. R., Mortrud, M. T., . . . Lein, E. S. (2008). Genomic anatomy of the hippocampus. *Neuron 60(6)*, 1010-1021.

Tissières, P., Dunn-Siegrist, I., Schäppi, M., Elson, G., Comte, R., Nobre, V., Pugin, J.Soluble MD-2 is an acute-phase protein and an opsonin for Gram-negative bacteria *Blood 111(4) 2122-2131*

Tsao, C. W., Lin, Y. S., Chen, C. C., Bai, C. H., & Wu, S. R. (2006). Cytokines and serotonin transporter in patients with major depression. *Progress in Neuro-Psychopharmacology & Biological Psychiatry 30*, 899-905.

Tukker, J. J., Lasztóczi, B., Katona, L., Roberts, J. D., Pissadaki, E. K., Dalezios, Y., . . . Somogyi, P. (2013). Distinct dentritic arborization and in vivo firing patters of parvalbumin-expressing basket cells in the hippocampal area CA3. *The Journal of Neuroscience 33(16)*, 6809-6825.

Tuon, L., Comim, C. M., Antunes, M. M., Constantino, L. S., Machado, R. A., Izquierdo, I., . . . Dal-Pizzol, F. (2007). Imipramine reverses the depressive symptoms in sepsis survivor rats. *Intensive Care Medicine. 33(22),* 2165-2167.

Tuon, L., Comim, C. M., Barichello, T., Izquierdo, I., Quevedo, J., & Dal-Pizzol, F. (2008). Time-dependent behavioral recovery after sepsis in rats. *Intensive Care Medicine. 34,* 1724-1731.

Tyler, C. R. (2014). Fluoxetine treatment ameliorates depression induced by perinatal arsenic exposure via a neurogenic mechanism. *Neurotoxicity 44,* 98-109.

Tynan, R. J., Weidenhofer, J., Hinwood, M., Cairns, M. J., Day, T. A., & Walker, F. R. (2012). A comparative examination of the anti-inflammatory effects of SSRI and SNRI antidepressants on LPS stimulated microglia. *Brain, Behavior, and Immunity 26(3),* 469-479.

Uesugi, M., Nakajima, K., Tohyama, Y., Kohsaka, S., & Kurihara, T. (2006). Nonparticipation of nuclear factor kappa B (NFkappaB) in the signaling cascade of c-Jun N-terminal kinase (JNK)- and p38 mitogen-activated protein kinase (p38MAPK)-dependent tumor necrosis factor alpha induction in LPS-stimulated microglia. *Brain Research 1073-4,* 1073-1074.

Utsuyama, M., & Hirokawa, K. (2002). Differential expression of various cytokine receptors in the brain after stimulation with LPS in young and old mice. *Experimental Gerontology 37(2-3),* 411-420.

Uz, T., Ahmed, R., Akhisaroglu, M., Kurtuncu, M., Imbesi, M., Dirim Arslan, A., & Manev, H. (2005). Effect of fluoxetine and cocaine on the expression of clock genes in the mouse hippocampus and striatum. *Neuroscience 134(4),* 1309-1316.

Valluzzi, J. A., & Chan, K. (2007). Effects of fluoxetine on hippocampal-dependent and hippocampal-independent learning tasks. *Behavioural Pharmacology 18(5-6),* 507-513.

Van Dam, A. M., Bol, J. G., Gaykema, R. P., Goehler, L. E., Maier, S. F., Watkins, L. R., & Tilders, F. J. (2000). Vagotomy does not inhibit high dose lipopolysaccharide-induced interleukin-1β immunoreactivity in rat brain and pituitary gland. *Neuroscience Letters 285,* 169-172.

Van Waes, V., Beverley, J., Marinelli, M., & Steiner, H. (2010). Selective serotonin reuptake inhibitor antidepressants potentiate methylphenidate (Ritalin)-induced gene regulation in the adolescent striatum. *European Journal of Neuroscience 32(3),* 435-447.

Venet, F., Lukaszewicz, A., Payen, D., Hotchkiss, R. & Monneret, G. (2013) Monitoring the immune response in sepsis: a rational approach to administration of immunoadjuvant therapies. *Current Opinion in Immunology 25(4)* 477-483

Venetti, S, Lopresti, B. J., & Wiley C. A. (2013) Molecular imaging of microglia/macgrophages in the brain. *Glia 61(6)* 10-23

Verma S, Nakaoke R, Dohgu S, Banks WA (2006) Release of cytokines by brain endothelial cells: A polarised response to lipopolysaccharide. *Brain Behav Immun* 20: 449–455

Verceles, A. S., Silhan, L., Terrin, M., netzer, G., Shanholtz, C., & Scharf, S. M. (2012). Circadian rhythm disruption in severe sepsis: the effect of ambient light on urinary 6-sulfatoxymelatonin secretion. *Intensive Care Medicine 38(5)*, 804-810.

Videbech, P., & Ravnkilde, B. (2004). Hippocampal volume and depresion: a meta-analysis of MRI studies. *The American Journal of Psychiatry 161(11)*, 1957-1966.

Viney, T. J., Lasztoczi, B., Katona, L. C., Tukker, J. J., Klausberger, T., & Somogyi, P. (2013). Network state-dependent inhibition of identified hippocampal CA3 axo-axonic cells in vivo. *Nature Neuroscience 16*, 1802-1811.

von Gall, C., Lewy, A., Schomerus, C., Vivien-Roels, B., Pevét. P, K. H., & Stehle, J. H. (2000). Transcription factor dynamics and neuroendocrine signalling in the mouse pineal gland: a comparative analysis of melatonin-deficient C57BL mice and melatonin-proficient C3H mice. *The European Journal of Neuroscience 12(3)*, 964-972.

Vorhees, C. V., & Williams, M. T. (2006). Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nature Protocols. 1(2)*, 848-858.

Vornov, J. J., Park, J., & Thomas, A. J. (1998). Regional vulnerability to endogenous and exogenous oxidative stress in organotypic hippocampal culture. *Experimental Neurology 149(1)*, 109-122.

Vouimba, R. M., & Richter-Levin, G. (2013). Different patterns of amygdala priming differentially affect dentate gyrus plasticity and corticosterone, but not CA1 plasticity. *Frontiers in Neural Circuits 7*, 80.

Vukovic, J., Colditz, M. J., Blackmore, D. G., Ruitenberg, M. J., & Bartlett, P. F. (2012). Microglia modulate hippocampal neural precursor activity in response to exercise and aging. *The Journal of Neuroscience 32(19)*, 6435-6443.

Wake, H., Moorhouse, A. J., Miyamoto, A. & Nabekura, J. (2013) Microglia: actively surveying and shaping neuronal circuit structure and function. *Trends in Neurosciences 36(4)* 209-217

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature Protocols, 2*, 322-328.

Walker, F. R., Beynon, S. B., Jones, K. A., Zhao, Z., Kongsui, R., Cairns, M., & Nilsson, M. (2014). Dynamic structural remodelling of microglia in health and disease: A review of the models, the signals and the mechanisms. *Brain, Behavior, and Immunity 37*, 1-14.

Walker, M. C., Pavlov, I., & Kullmann, D. M. (2010). A 'sustain pedal' in the hippocampus. *Nature Neuroscience 13*, 146-148.

Walker, W., Bozzi, A., & Fikrig, E. (2011). Circadian rhythms influence disease severity in a mouse model of sepsis. *The Journal of Immunology 186*, 110.9.

Wan, W., Wetmore, L., Sorensen, C. M., Greenberg, A. H., & Nance, D. M. (1994). Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain. *Brain Research Bulletin 34(1)*, 7-14.

Wang, L. M., Dragich, J. M., Kudo, T., Odom, I. H., Welsh, D. K., O'Dell, T. J., & Colwell, C. S. (2009). Expression of the circadian clock gene period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. *ASN Neuro 1(3)*.

Wang, M. E., Wann, E. G., Yuan, R. K., Ramos Álvarez, M. M., Stead, S. M., & Muzzio, I. A. (2012). Long-term stabilization of place cell remapping produced by a fearful experience. *The Journal of Neuroscience 32(45)*, 15802-15814.

Wang, X., Zhang, D., & Lu, X. Y. (2014). Dentate gyrus-CA3 glutamate release/NMDA transmission mediates behavioral despair and anti-depressant-like responses to leptin. *Molecular Psychiatry* , 1-11.

Wang, Y. Z., Plane, J. M., Jiang, P., Zhou, C. J., & Deng, W. (2012). Concise review: Quiescent and active states of endogenous adult neural stem cells: Identification and Characterization. *Stem Cells 29(6)*, 907-912.

Wang, Y., Cui, X. L., Liu, Y. F., Gao, F., Wei, D., Li, X. W., . . . Jiang, W. (2011). LPS inhibits the effects of fluoxetine on depression-like behavior and hippocampal neurogenesis in rats. *Progress in Neuro-Psychopharmacology and Biological Psychiatry 35(8)*, 1831-1835.

Wang, Z., Gu, J., Wang, X., Xie, K., Luan, Q., Wan, N., . . . Liu, D. (2013). Antidepressant-like activity of resveratol treatment in the forced swim test and tail suspension test in mice: the HPA axis, BDNF expression and phosphorylation of ERK. *Pharmacology Biochemistry and Behavior 112*, 104-110.

Wang, Z., Zhang, Q., Yuan, L., Wang, S., Liu, L., Yang, X., . . . Liu, D. (2014). The effects of curcumin on depressive-like behavior in mice after lipopolysaccharide administration. *Behavioural Brain Research 274*, 282-290.

Wang, Z., Zhao, H., Peng, S., & Zuo, Z. (2013). Intranasal pyrrolidine dithiocarbamate decreases brain inflammatory mediators and provides neuroprotection after brain hypoxia-ischemia in neonatal rats. *Experimental neurology 249*, 74-82.

Wanner, S. P., Yoshida, K., Kulchitsky, V. A., Ivanov, A. I., Kanosue, K., & Romanovsky, A. A. (2013). Lipopolysaccharide-induced neuronal actiavtion in the paraventricular and dorsomedial hypothalamus depends on ambient termperature. *PLoS ONE 8(9)*, DOI: 10.1371/journal.pone.0075733.

Warner-Schmidt, J. L., Vanover, K. E., Chen, E. Y., Marshall, J. J., & Greengard, P. (2011). Antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) are attenuated by antiinflammatory drugs in mice and humans. *PNAS 108(22)*, 9262-9267.

Warren, B. L., Iñiguez, S. D., Alcantara, L. F., Wright, K. N., Parise, E. M., Weakley, S. K., & Bolaños-Guzmán, C. A. (2011). Juvenile administration of concomitant methylphenidate and fluoxetine alters behavioral reactivity to reward- and mood-related stimuli and disrupts ventral tegmantal area gene expression in adulthood. *The Journal of Neuroscience 31(28)*, 10347-10358.

Weber, M. D., Frank, M. G., Sobesky, J. L., Watkins, L. R. & Maier, S F. (2013) Blocking toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge. *Brain, Behavior, and Immunity 32* 112-121

Weberpals, M., Hermes, M., Hermann, S., Kummer, M. P., Terwel, D., Semmler, A., . . . Heneka, M. T. (2009). NOS2 Gene Deficiency Protects from Sepsis-Induced Long-Term Cognitive Deficits. *The Journal of Neuroscience 29(45)*, 14177-14184.

Weinhouse, G. L., & Schwab, R. J. (2006). Sleep in the critically ill patient. *SLEEP 29(5)*, 707-716.

Whitlock, J. R., Heynen, A. J., Shuler, M. G., & Bear, M. F. (2006). Learning induces long-term potentiation in the hippocampus. *Science 313(5790)*, 1093-1097.

Widmann, C. N., & Heneka, M. T. (2014). Long-term cerebral consequences of sepsis. *The Lancet Neurology 13(6)*, 630-636.

Wilhelm, C. J., Murphy-Crews, A., Menasco, D. J., Huckans, M. S., & Loftis, M. J. (2012). Corticotropin releasing factor-1 receptor antagonism alters the biochemical, but not behavioral effects of repeated interleukin-1β administration. *Neuropharmacology. 62(1)*, 313-321.

Wilms, H., Hartmann, D., & Sievers, J. (1997). Ramification of microglia, monocytes and macrophages in vitro: influences of various epithelial and mesenchymal cells and their conditioned media. *Cell & Tissue Research 287*, 447-458.

Wilson, J. X., & Young, G. B. (2003). Progress in clinical neurosciences: Sepsis-associated encephalopathy: Evolving concepts. *Canadian Journal of Neurological Sciences. 30(2)*, 98-105.

Winters, B. D., Eberlein, M. E., Leung, J., Needham, D. M., Pronovost, P. J., & Sevransky, J. E. (2010). Long-term mortality and quality of life in sepsis: A systematic review. *Critical Care Medicine 38(5)*, 1276-1283.

Wintzer, M. E., Boehringer, R., Polygalov, D., & McHugh, T. J. (2014). The hippocampal CA2 ensemble is sensitive to contextual change. *The Journal of Neuroscience 34(8)*, 3056-3066.

Witcher, M R; Kirov, S A; Harris, K M (2007) Plasticity of perisynaptic astroglia during synaptogenesis in the mature rat hippocampus *Glia 55(1)* 13-23

Witter, M. P. (2007). Intrinsic and extrinsic wiring of CA3: Indications of connectional heterogeneity. *Learning & Memory 14*, 705-713.

Wong, D. T., Horng, J. S., Bymaster, F. P., Hauser, K. L., & Molloy, B. B. (1974). A selective inhibitor of serotonin uptake: Lilly 110140, 3-(p-

Trifluoromethylphenoxy)-n-methyl-3-phenylpropylamine. *Life Science 15(3)*, 471-479.

Wong, M. -L., & Licinio, J. (2001). Research and treatment approaches to depression. *Nature Reviews Neuroscience. 2*, 343-351.

Wong, M.-L., Dong, C., Maestre-Mesa, J., & Licinio, J. (2008). Polymorphisms in inflammation-related genes are associated with susceptibility to major depression and antidepressant response. *Molecular Psychiatry. 13*, 800-812.

Wosiski-Kuhn, M., & Stranahan, A. M. (2013). From pattern separation to mood regulation: multiple roles for developmental signals in the adult dentate gyrus. *Frontiers in Cellular Neuroscience 7(96).*

Wright, K. P., Lowry, C. A., & LeBourgeois, M. K. (2012). Circadian and wakefulness-sleep modulation of cognition in humans. *Frotiers in Molecular Neuroscience 5*.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., Mathison, J. C. (1990) CD13, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249(4975)* 1431-1433

Wu, J., Dong, L., Zhang, M., Jia, M., Zhang, G., Qiu, L., . . . Yang, J. (2013). Class I histone deacetylase inhibitor valproic acid reverses cognitive deficits in a mouse model of septic encephalopathy. *Neurochemical Research 38(11)*, 2440-2449.

Wulff, K., Dijk, D. J., Middleton, B., Foster, R. G., & Joyce, E. M. (2012). Sleep and circadian rhythm disruption in schizophrenia. *The British Journal of Psychiatry 200*, 308-316.

Wynne, A. M., Henry, C. J. & Godbout, J. P. (2009) Immune and behavioral consequences of microglial reactivity in the aged brain. *Integrative and Comparative Biology 49(3)* 254-266

Wyse, C. A., & Coogan, A. N. (2010). Impact of aging on diurnal expression patterns of CLOCK and BMAL1 in the mouse brain. *Brain Research 1337*, 21-31.

Yao, J., Mackman, N., Edgington, T. S., & Fan, S. (1997). Lipopolysaccharide INduction of the Tumor Necrosis Factor-α Promoter in Human Monocytic Cells Regulation By Egr-1, c-Jun and NF-κB Transcription Factors. *The Journal of Biological Chemistry, 272*, 17795-17801.

Yao, Y. M., Xu, C. L., Yao, F. H., Yu, Y. & Sheng, Z. Y. (2006) The pattern of nuclear factor-kappaB activation in rats with endotoxin shock and its role in biopterin-mediated nictric oxide induction. *Chinese Journal of Burns 22(6)* 405-410

Yassa, M. A., Hazlett, R. L., Stark, C. E., & Hoehn-Saric, R. (2012). Functional MRI of the amygdala and bed nucleus of the stria terminalis during conditions of uncertainty in generalised anxiety disorder. *Journal of Psychiatric Research 46(8)*, 1045-1052.

Yi, C. H., & Yuan, J. (2009). The Jekyll and Hyde Functions of Caspases. *Developmental Cell 16*, 21-34.

Yi, J., Park, S., Kapadia, R., & Vemuganti, R. (2007). Role of transcription factors in mediating post-ischemic cerebral inflammation and brain damage. *Neurochemistry International 50(7-8)*, 1014-1027.

Yirmiya, R., & Goshen, I. (2011). Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brian, Behaviour, and Immunity 25(2)*, 181-213.

Yirmiya, R., Pollak, Y., Barak, O., Avitsur, R., Ovadia, H., better, M., . . . Weidenfeld, J. (2001). Effects of Antidepressant Drugs on the Behavioral and Physiological Responses to Lipopolysaccharide(LPS) in Rodents. *Neuropsychopharmacology. 24(5)*, 531-544.

Yoon, T., Okada, J., Jung, M. W. & Kim, J. J. (2008) Prefrontal cortex and hippocampus subserve different components of working memory in rats. *Learning & Memory 15(3)* 97-105

Yoshida, K., Hashimoto, T., Sakai, Y., & Hashiramoto, A. (2014). Involvement of the circadian rhythm and inflammatory cytokines in the pathogenesis of rheumatoid arthritis . *Journal of Immunology Research* , e282495.

Yoshimura, R., Mitoma, M., Sugita, A., Hori, H., Okamoto, T., Umene, W., . . . Nakamura, J. (2007). Effects of paroxetine or milnacipran on serum brain-derived neurotrophic factor in depressed patients. *Progress in Neuro-Psychopharmacology and Biological Psychiatry 31(5)*, 1034-1037.

You, Z., Luo, C., Zhang, W., Chen, Y., He, J., Zhao, Q., . . . Wu, Y. (2011). Pro- and anti-inflammatory cytokines expression in rat's brain and spleen exposed to chronic mild stress: Involvement in depression. *Behavioural Brain Research. 225(1)*, 135-141.

Young, L., Bechara, A., Tranel, D., Damasio, H., Hauser, M., & Damasio, A. (2010). Damage to ventromedial prefrontal cortex impairs judgement of harmful intent. *Neuron 65(6)*, 845-851.

Yu, J. T., Lee, C. H., Yoo, K., Choi, J. H., Li, H., Park, O., . . . Won, M. (2010). Maintenance of anti-inflammatory cytokines and reduction of glial activation in the ischemic hippocampal CA1 region preconditioned with lipopolysaccharide. *Journal of the Neurological Sciences 296(1-2)*, 69-78.

Yuan, L. W., Ren, X., Liu, Q., Wang, J., & Liu, X. (2014). Isoorientntin attenuates lipopolysaccharide-induced pro-inflammatory responses through down-regulation of ROS-related MAPK/NF-κB signaling pathway in BV-2 microglia. *Molecular and Cellular Biochemistry 386(1-2)*, 153-165.

Zhang, F., Zhou, H., Wilson, B. C., Shi, J. S., Hong, J. S., & Gao, H. M. (2012). Fluoxetine protects neurons against microglial activation-mediated neurotoxicity. *Parkinsonism & Related Disorders 18(S1)*, S213-S217.

Zhang, J., Jiang, W., & Zuo, Z. (2014). Pyrrolidine dithiocarbamate attenuates surgery-induced neuroinflammation and cognitive dysfunction possibly via inhibition of nuclear factor κB. *Neuroscience 261*, 1-10.

Zhang, Z. G., & Chopp, M. (2009). Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. *Lancet Neurology 8(5),* 491-500.

Zhao, M., Zhou, A., Xu, L., & Zhang, X. (2014). The role of TLR4-mediated PTEN/PI3K/AKT/NF-κB signaling pathway in neuroinflammation in hippocampal neurons. *Neuroscience 269,* 93-101.

Zhao, Z., Huang, G., Wang, B., & Zhong, Y. (2013). Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate partially attenuates hippocampal MMP-9 and improves cognitive deficits in streptozotocin-induced diabetic rats. *Behavioural Brain Research 238,* 44-47.

Zheng, Y., Guo, Z., He, W., Yang, Y., Li, Y., Zheng, A., . . . Yu, Y. (2012). Ephedrine hydrochloride protects mice from LPS challenge by promoting IL-10 secretion and inhibiting proinflammatory cytokines. *International Immunopharmacology 13(1),* 46-53.

Ziaja, M. (2013). Septic Encephalopathy. *Current Neurology and Neuroscience Reports 13:383*.

Zofaghari, P. S., Pinto, B. B., Dyson, A., & Singer, M. (2013). The metabolic phenotype of rodent sepsis: cause for concern? *Intensive Care Medicine Experimental 1(6)*.

Zueger, M., Urani, A., Chourbaji, S., Zacher, C., Lipp, H. P., Albrecht, U., Spanagel, R., Wolfer, D. P. & Gass, P. (2006) mPER1 and mPER2 mutant mice show regular spatial and contextual learning in standardised tests for hippocampus-dependent learning. *Journal of Neural Transmission 113* 347-356

Zujovic, V., Schussler, S., Jourdain, D., Duverger, D., & Taupin, V. (2001). In vivo neutralization of endogenous brain fractalkine increases hippocampal TNFα and 8-isoprostane production induced by intracerebroventricular injection of LPS. *Journal of Neuroimmunology 115(1-2),* 135-143.