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Examining the Acute and Chronic Effects of Sepsis on the Circadian Clock in the Mouse.

A thesis submitted to the Department of Psychology, National University of Ireland Maynooth for the degree of Doctor of Philosophy in the Faculty of Science and Engineering.

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Abstract

Circadian rhythms are recurring patterns (~24hrs) in behaviour and physiology that are driven primarily by an endogenous biological timekeeping system, with the master pacemaker located in the suprachiasmatic nucleus. Studies have indicated bidirectional relationships between the circadian and the immune systems, however while there is much evidence regarding the regulation of immune function by the circadian system, information regarding the impact of immune processes on the timekeeping system is more limited, including that regarding the long-term modulation of the circadian system following immune challenge. The current set of studies address this gap in the literature by examining the long-term impact of sepsis, a substantial immune challenge, on circadian timekeeping processes, following sepsis induction by peripheral treatment with lipopolysaccharide (5mg/kg). Following recovery, post-septic circadian behaviour, SCN molecular oscillations and SCN responsiveness were assessed. SCN neurochemistry was also assessed both in the acute phase and in the long-term post LPS treatment.

LPS induced sepsis did not affect core circadian locomotor rhythmicity parameters, but did result in long-term attenuations in post-septic resetting in response to phase advancing photic stimulation, and alterations in re-entrainment to advances of the photoperiod. Perturbations were observed in SCN neurochemistry in the acute phase following septic LPS treatment, and chronic attenuations were also found in post-septic SCN clock gene protein product expression. LPS induced sepsis caused attenuations in SCN functional activation in response to both photic and immune stimulation, as well as alterations in circadian resetting in response to phase resetting immune stimuli.

Overall, these data provide further insight into immune circadian communication, and the long-term impact of immune challenge on timekeeping processes, and describe a previously unknown impact of the chronic effects of experimental sepsis on the circadian timekeeping system.

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Abbreviations

3’ UTR – three prime untranslated region

3V - third ventricle

5-HT - serotonin

8-OH-DPAT - 8-hydroxy-2-dipropylaminotetralin hydrobromide

ABC - avidin-biotin-peroxidase complex

Ach - Acetylcholine

ACTH - Adrenocorticotropic hormone

AD - Alzheimer’s Disease

ADR - adrenals

ADX - adrenalectomy

AII - angiotensin II

ANS - autonomic nervous system

anti-CII - anti-type II collagen

AP - antigen presentation

APC - antigen presenting cell

APP - amyloid precursor protein

Arc - activity-regulated cytoskeleton-associated protein

ARC - arcuate nuclei

ARNTL2 - aryl hydrocarbon receptor nuclear translocator-like 2

AVP - arginine vasopressin

BBB - blood brain barrier

bHLH - basic helix loop helix

BMAL1- brain muscle arnt like protein 1

BNST-OV - bed nucleus of the stria terminalis

C. burnetii – *Coxiella burnetii*

Ca^{2+} - calcium

CalB – calbindin

CaMKII - Calmodulin-dependent Protein Kinase II

CAR - calretinin

Ccg- clock controlled gene

cGMP - cyclic guanosine monophosphate

CBT – core body temperature

CJL - chronic jet lag

CK - Casein Kinase

CLC - cardiotrophin like cytokine

CLOCK- circadian locomotor output cycle kaput

CLP- cecal ligation and puncture

cNOS- constitutive nitric oxide synthase

CNS- central nervous system

CRE- cAMP-responsive elements

CREB- cAMP response element binding protein

CRF- corticotropin releasing factor

CRH - corticotropin releasing hormone

Cry - *Cryptochrome*

CSF – cerebrospinal fluid

CT – circadian time

Cxcl1 - Chemokine (C-X-C motif) ligand 1

DAB - diaminobenzidine

DBP - D-Binding Protein

dbt - doubletime

DC-dendritic cell

DD-constant darkness

Dec- differentiated embryo chondrocytes

DM - dorsomedial

DMEM- Dulbeccos Modified Eagles Medium

DMH - dorsomedial hypothalamus

dmSCN - dorsomedial SCN

dSPZ - dorsal subparaventricular zone

E. coli - *Escherichia coli*

EAE - experimental autoimmune encephalomyelitis

EC- entrohinal cortex

EEG - electroencephalographic

EGR-1 – early growth response 1

ELISA - enzyme-linked immuno sorbent assay

Elk-1 - ETS domain-containing protein 1

EPSP - excitatory postsynaptic potential

ERK - extracellular signal related kinase

FAA - food-anticipatory activity

FASPS - Familial Advanced Sleep Phase Syndrome

FRP- free running period

GABA - gamma amino butyric acid

GAD - glutamic acid decarboxylase

GAL - galanin
GC - glucocorticoid
GFAP – glial fibrillary acidic protein
GFP - green fluorescent protein
GHT - geniculohypothalamic tract
GluR - glutamate receptor
GR - glucocorticoid receptors
GRP - gastrin-releasing peptide
GZMB - granzyme B
H_20_2 – hydrogen peroxide
HAT - Human African trypanosomiasis
HBSS - Hank's Balanced Salt Solution
Hcrt - hypocretin
HLF - Hepatocyte Leukemia Factor
HPA – hypothalamic pituitary adrenal
HRP - Horseradish peroxidase
i.p. - intra-peritoneal
ICAM - intracellular adhesion molecule
IEG - immediate-early response gene
IFN - interferon
IRF - interferon regulatory factor
IGL - intergeniculate leaflet
IHC - Immunohistochemical
IL - interleukin
iNOS – inducible nitric oxide synthase
IOD - integrated optical density
i.r. - immunoreative
IκB – I-KappaB's
IκK- IkappaB kinase
LD - light dark cycle
LGN - lateral geniculate nucleus
LH - lateral hypothalamus
LL - constant light
LPS - lipopolysaccharide
LRR - leucine-rich repeat region
LTP – long-term potentiation
MAC-1 – macrophage antigen complex 1 (also known as integrin CD11b)

MAP - mitogen-activated protein kinase

MCP-1 - Monocyte chemotactic protein-1

MEK - MAP kinase kinase

mENK - met-enkephalin

MET - metyrapone

MHC - major histocompatibility complex

MIP – macrophage inflammatory protein

MK-801 - (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate

MnPO - median preoptic nucleus

mRNA - messenger ribonucleic acid

MyD88 - myeloid differentiation primary response 88

NaCl - saline

NADPH - Nicotinamide adenine dinucleotide phosphate

NBM - nucleus basalis magnocellularis

NE - norepinephrine

NF-κB – nuclear factor kappa B

NGS - normal goat serum

NHS – normal horse serum

NIH3T3 - mouse embryonic fibroblast cells

NK - natural killer

NMDA - *N*-methyl-D-aspartic acid

nNOS – nitric oxide synthase

NO - nitric oxide

NOS2 – nitric oxide synthase 2

NPAS2 - neuronal PAS domain protein 2

NPY – neuropeptide Y

NREM - non-rapid eye movement

OC - optic chiasm

PACAP - pituitary adenylate cyclase activating peptide

PAMP - pathogen-associated molecular patterns

PAS - PER-ARNT-SIM

PB - phosphate buffer

PBL - peripheral blood leukocytes

PD - Parkinson’s Disease

PDTC - ammonium pyrrolidinedithiocarbamate

Per - period

p-ERK- phosphorylated ERK

PFA - paraformaldehyde

PGE2 – prostaglandin E2

PHI - peptide histidine isoleucine

PK2 - prokineticin-2

PKC - protein kinase C

PKG - protein kinase G

POMC - proopiomelanocortin.

PRC - Phase Response Curve

PRR - Pathogen recognition receptor

PSL - prednisolone

PTX3 - pentraxin 3

PVN - paraventricular nucleus PVN

RA - Rheumatoid Arthritis

RBC – red blood cell

RelB - v-rel reticuloendotheliosis viral oncogene homolog B

REM - rapid eye movement;

RHT - retinohypothalamic tract

RORE - retinoic acid-related orphan receptor response element

Rorα - retinoic acid receptor-related orphan receptor α

ROS - reactive oxygen species

rTDT - Terminal Deoxynucleotidyl Transferase Recombinant

RU486 – glucocorticoid antagonist

SCN - suprachiasmatic nucleus

SE - Septic Encephalopathy

SIRS - systemic inflammatory response syndrome

SN - substantia nigra

SOCS - Suppressors of cytokine signalling

SP - substance-P

SPF - specific pathogen free

sPVZ - subparaventricular zone

SRE - Serum response element

Stat - signal transducer and activation of transcription

SWA – slow-wave activity

Tb. brucei - *Trypanosoma brucei*

TEF - Thyroid Embryonic Factor

TGF - transforming growth factor

TH - tyroxine hydroxylase

TLR - Toll like receptor

TNF- tumour necrosis factor

TOD – time of day

TRIF - TIR-domain-containing adapter-inducing interferon-β

TTX - Tetrodotoxin

TUNEL - Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling

U0126 - 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (MEK inhibitor)

Usp - Ubiquitin Specific Protease

UV - ultraviolet

VIP - Vasoactive Intestinal Polypeptide

VLPO - ventrolateral preoptic nucleus

vlSCN - ventrolateral SCN

VPAC2 - Vasoactive intestinal peptide receptor 2

vSPZ - ventral SPZ

WBC – white blood cell

ZT - Zeitgeber

β-EP – peptide beta endorphin

Chapter One

General Introduction

The term Chronobiology, derived from Greek (*chronos* meaning time, *bios* meaning life, *logos* meaning study) refers to the study of biological rhythms and timekeeping mechanisms. The term circadian coined by Franz Halberg in 1959, comes from the two Latin words 'circa' meaning about, and 'dies' meaning day, literally translated, circadian then means 'about a day', and describes the endogenous ~24hr recurring rhythms in behavioural, endocrine and physiological parameters (Levi and Schibler, 2007) which serve to synchronize the internal state with the external environment, and ensure that internal processes do not occur all at once, but at specific times. Circadian rhythms have been conserved throughout evolution and are seen to occur in all eukaryotic organisms including plants and animals, along with certain fungi and cyanobacteria. These biological rhythms enable organisms to react to and not merely adapt to changes in their surroundings, conferring advantages onto organisms and increasing fitness. Mammalian circadian rhythms are generated by endogenous oscillators and entrained daily by environmental time cues or "Zeitgebers", of which environmental light is the most potent, in order to keep the rhythm in phase with the environment (Golombek and Rosenstein, 2010). In order for a rhythm to be circadian, there are three properties that it must exhibit. The rhythm must be self-sustaining and persist in the absence of external environmental cues, be capable of being reset or synchronized by external cues, and must be temperature compensated.

While there are many early documented observations of circadian patterns in physiology in both plants and animals, it was not until the 17th century that much of the investigations into chronobiology began, and human circadian rhythm studies did not begin until the late 1930's. In 1938, Nathaniel Kleitman and Bruce Richardson utilised the Mammoth Caves in Kentucky, isolated from the external environment and maintained under constant environmental conditions of temperature, humidity and darkness. At this time, artificial light was not recognised as an "entraining" stimulus or "Zeitgeber" for circadian rhythms and hence subjects were allowed access to artificial lighting. Studies by Jurgen Aschoff (Aschoff, 1960) utilizing an underground bunker in Germany, similarly shielded from external environmental

cues and maintained under constant environmental conditions concluded that circadian rhythms regulate various mammalian behavioural and physiological parameters (Aschoff, 1960). These studies and those that followed in the proceeding 20 years were key in the development of chronobiological studies.

1.1 The Master pacemaker

It was first reported by Richter (1967) that anterior hypothalamic lesions disrupted behavioural circadian rhythm expression in the rat, and it was then found that loss of the Suprachiasmatic Nucleus (SCN) specifically, impaired neuroendocrine and behavioural circadian rhythmicity in the hamster (Rusak, 1979) and in the rat (Moore and Eichler, 1972; Moore and Klein, 1974; Stephan and Zucker, 1972). The SCN is bilaterally distributed in the anterior hypothalamus at the base of the third ventricle, above the optic chiasm (Klein et. al, 1991) (Figure 1.1).

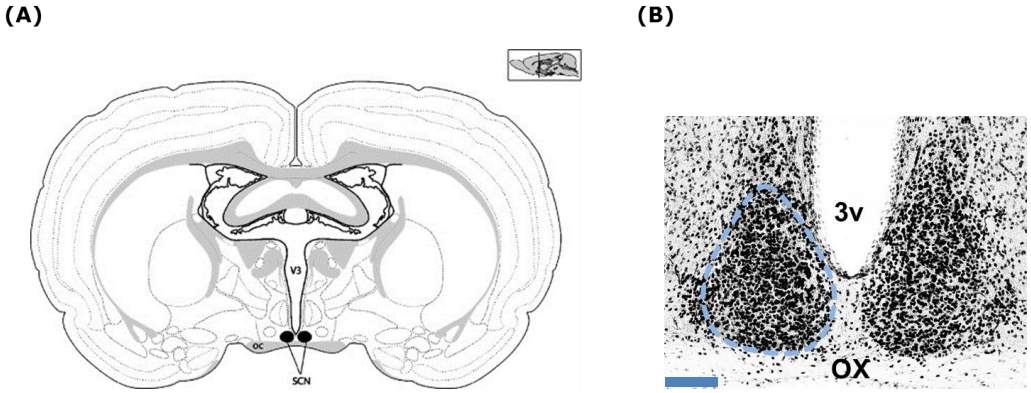


Figure 1.1: The location of the mammalian circadian clock*. (A) Coronal section through a rodent brain showing the location of the SCN. The SCN of the anterior hypothalamus sits directly above the optic chiasm (OC) and surrounding the third ventricle (3v) (Kriegsfeld and Silver, 2006). Dashed line outlines the SCN. The approximate rostral-caudal location depicted in the coronal section is depicted by the sagittal schematic in the upper right corner. (B) Pictomicrogaph of the SCN immunostained for the clock gene protein CLOCK (scale bar approx 100µm).*

A biological timekeeping role of the SCN was then indicated by a variety of studies. When SCN connections were severed to the rest of the brain, SCN neurons retained their rhythmic electrical activity but those outside of the SCN were abolished (Inouye and Kawamura, 1979), highlighting that SCN projections convey rhythmicity to neurons outside of the SCN. Further, foetal SCN transplants from

wild-type hamsters to *tau* mutant hamsters which show shortened circadian free running periods (FRP), showed the donor phenotype to be expressed by the mutant host (Ralph et. al 1990). The restoration of drinking, eating and motor activity circadian rhythms was also shown following transplantation of foetal SCN tissue to SCN lesioned ahrrythmic rodents (Aguilar-Roblero et. al 1992; Aguilar-Roblero et. al 1994; DeCoursey and Buggy, 1989; Griffioen et. al., 1993; Lehman et. al 1987). These studies and those that followed established the SCN as the location of the principal circadian oscillator in the body. The SCN is now known to serve as the master oscillator, coordinating peripheral circadian clocks in individual cells and organs throughout the body and orchestrating coherent circadian rhythms. The SCN is positioned so as to receive both direct and indirect retinal input serving to convey photic input for entrainment to the environmental photoperiod (Reppert and Weaver, 2001). The SCN contain about 20,000 neurons, 10,000 neurons in each nucleus (Reppert and Weaver, 2001), which generate coherent circadian rhythms of neuronal firing (Meijer and Schwartz, 2003). SCN neurons have cell body diameters of 7-12µm, are of high density, and show a broad phenotypic heterogeneity, expressing a wide and differential variety of neuroactive compounds, however GABA is expressed by almost all neurons (Okamura et al., 1989; Moore & Speh, 1993; Shirakawa et al., 2001; Guilding and Piggins, 2007).

1.1.1. Subdivisions of the SCN

The SCN can be subdivided into two distinct anatomical regions, the dorsomedial SCN (dmSCN) or "shell" and the ventrolateral SCN (vlSCN) or "core" (Van den Pol, 1980), with each subregion containing neurons expressing different populations of neuropeptides. The core contributes 43% to the total neuronal population of the SCN, with the shell contributing 57% (Moore et al., 2002). The different neuropeptide and neuronal compositions of the subdivisions of the SCN indicate regional differences in circadian rhythm generation and regulation.

The dorsomedial "shell" receives input from non-visual cortical and subcortical regions, receiving projections from the limbic forebrain and the hypothalamus, and the neurons in the shell express arginine vasopressin (AVP) or somatostatin expressing neurons, as well as gamma amino butyric acid (GABA) and calretinin (CAR) (Moore et. al., 2002). Further, the SCN shell also contains neurons

that synthesize angiotensin II (AII) and met-enkephalin (mENK), and the shell receives input from Vasoactive Intestinal Polypeptide (VIP) immunoreactive fibres and galanin (GAL) (Abrahamson and Moore, 2001).

The retinorecipient "core" receives dense visual input from the retina and from the intergeniculate leaflet and midbrain raphe afferents and contains neurons synthesizing VIP, calbindin (CalB), Gastrin Releasing Peptide (GRP) (Card and Moore, 1984) colocalized with GABA and neuromedin S (Miyazato et. al., 2008; Mori et. al., 2005) and neurotensin, and receive input from fibres containing 5-hydroxytryptamine (5-HT) and neuropeptide Y (NPY) (Abrahamson and Moore, 2001). (Figure 1.2). RHT fibres are seen throughout the SCN, and the RHT projections terminate upon all VIP and GRP neurons, as well as the AVP, CalR, CalB and mENK neurons, except those that are most medial (Morin et al., 2006).

The different subregions also show variation in clock gene and immediate early gene (IEG) expression. The SCN core is retinorecipient, and in response to photic stimulation the VIP, GRP and CalB neurons of the core are thought to display robust short latency electrical responses, and the induction of various IEGs and photically sensitive clock genes occurs in the SCN core, but not in the shell (Hamada et al., 2001; Kornhauser et al., 1990; Rea, 1992). The AVP neurons of the SCN shell are thought to show robust spontaneous rhythmicity in gene expression, neurotransmitter release and electrical activity (Antle and Silver, 2005; Lee et. al., 2003). The cells of the core show relatively low amplitude expression of clock genes which may allow environmental signals to more easily reset these cells (Lakin-Thomas et al., 1991; Pulivarthy et al., 2007; Colwell, 2011).

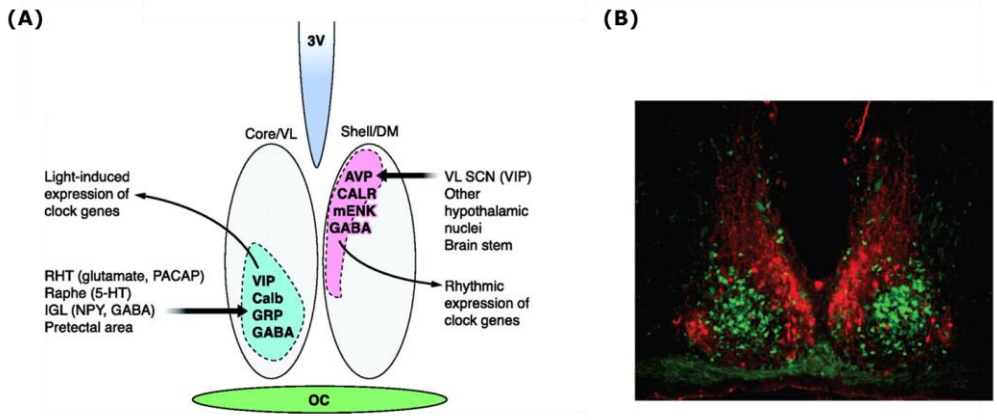


Figure 1.2: SCN functional and neurochemical subdivisions. *(A) Neurochemicals of the SCN core are illustrated in the left SCN while those of the SCN shell are*

illustrated in the right SCN. The SCN core is directly innervated by the RHT, the raphe nuclei, and the IGL. The SCN shell is innervated by ventrolateral SCN and other hypothalamic areas. RHT, retinohypothalamic tract; 3V, third ventricle; OC, optic chiasm; IGL, intergeniculate leaflet; VL, ventrolateral; DM, dorsomedial; other abbreviations as in text (adapted from Golombek and Rosenstein, 2010). (B) Immunofluorescent staining in the mouse SCN, with the SCN shell region delineated by immunofluorescent labeling for AVP (red) and the SCN core region delineated by green fluorescent protein (GFP) expressed in GRP expressing neurons (green) (Welsh et al., 2010).

1.1.2. SCN Coupling and Electrical Activity

A core to shell signalling model exists (Moore, 1996; Speh et al., 2002), and studies have shown the SCN core to show numerous efferents to the SCN shell while there are fewer projections innervating the core from the shell region (Moore et al., 2002). The light sensitive neurons within the core convey light information to the SCN shell which in turn projects to efferent regions, and it is thought that projections from the SCN to other brain regions are the initial steps involved in the regulation of rhythmicity both in the CNS and the periphery (Bartness et al., 2001; Aston-Jones et al., 2001; Kalsbeek and Buijs, 2002; Buijs et al., 2003; Deurveilher and Semba, 2005).

The two SCN nuclei also appear to be coupled, since unilateral SCN lesions disrupt behavioural circadian rhythms, altering the FRP (Pickard and Turek, 1982, 1983), and projections between the two nuclei have been identified (Abrahamson and Moore, 2001; Kalsbeek et al., 1993), with each core and shell of an individual SCN nucleus projecting contralaterally to the core or shell of the other nucleus (Leak et al., 1999). Rhythmic electrical activity of similar period with small phase differences is found in each nucleus (Inouye and Kawamura, 1982), and each can produce rhythmic circadian behavioural activity (Pickard and Turek, 1982, 1983; Davis and Gorski, 1984).

The presence of multiple circadian oscillators is supported by the observation of locomotor activity rhythm ‘splitting’ in the golden hamster (Pittendrigh and Daan 1976a) and in the ground squirrel (Pittendrigh 1960) following prolonged housing under constant conditions, and it is suggested that each nucleus may be oscillating independently, each oscillation producing a behavioural rhythm (Pittendrigh and Daan, 1976a). In animals with split behavioural activity the left and right SCN

display antiphase c-Fos and clock gene expression (de la Iglesia et al., 2000, 2003; Mendoza et al., 2004; Ohta et al., 2005). Furthermore, rodent housing under constant light (LL) results in lengthening of the FRP and can result in circadian arrhythmicity, which is probably due to the decoupling of neurons resulting in oscillator desynchrony within the SCN (Aschoff, 1960; Pittendrigh and Daan 1976a; Ohta et. al., 2005).

The circadian timing mechanism is expressed autonomously within individual SCN neurons, and these independent oscillators are 'coupled' to eachother creating a network of oscillators, enabling the generation of coherent, robust circadian outputs when they oscillate in synchrony (Welsh, 2009). The SCN has been shown to display a circadian pattern of electrical activity which is high during the light phase and low during the dark phase, and is in antiphase to neuronal activity patterns outside of the SCN (Inouye and Kawamura, 1979; Inouye and Kawamura, 1982), and is seen to persist in constant lighting conditions. The electrical activity pattern has been shown to be an endogenous property of the pacemaker (Inouye and Kawamura, 1979), and to be maintained and persist *in vitro*, and the day night rhythm in electrical SCN firing has been demonstrated in *in vitro* slice preparations, confirming peak electrical firing in the middle of the subjective day, and a nadir approximately 12 hours later in the subjective night (Green and Gillette, 1982; Shibata et al., 1982; Groos and Hendricks, 1982; Brown and Piggins, 2007). Individual SCN neurons dissociated in culture have been shown to display independent circadian rhythms in firing, showing a range of phases (Welsh et al., 1995). Communication between individual SCN cells is essential for accurate pacemaker function, and individual neurons adapt their amplitude in response to feedback from other SCN cells. The individual oscillators must then be synchronized together, resulting in rhythmic electrical activity and the generation of rhythmic, coherent circadian output rhythms such as behavioural outputs (Schwartz et al., 1987).

Ionic currents drive spontaneous electrical firing rhythms of SCN neurons. A number of voltage sensitive currents play roles in the electrical firing rate of neurons within the SCN, including those that supply the excitatory drive necessary for spontaneously active neurons, currents that convert the excitatory drive into a regular pattern of action potentials, and those that hyperpolarize the membrane and underlie the silencing of electrical firing at night (Colwell, 2011). Studies by Wheal and

Thompson (1984) and Kim and Dudek (1991, 1992, 1993), have shown $GABA_A$ receptors to be involved in modulating inhibitory input on rodent SCN neurons, while the major excitatory inputs to SCN neurons are from glutamatergic sources. Optic nerve stimulation was seen to induce an excitatory postsynaptic potential (EPSP) from rodent SCN neurons which was mediated via both N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors.

SCN neuronal firing rhythms are crucial in transmitting circadian timing information to the brain and periphery (Schwartz et. al., 1987). In *Clock* mutant mice, individual oscillators do not show rhythmic electrical activity, correlating with the arrhythmic locomotor behaviour exhibited by these animals (Herzog et al., 1998), while in *tau* mutant hamsters exhibiting shortened circadian free running periods, the period of the electrical activity rhythm is seen to reflect this in *in vitro* recordings of SCN neurons (Liu et al., 1997).

Neural activity may be required for the generation of circadian oscillations in the core clock genes in the SCN, since various studies have shown that perturbations in these lead to alterations in molecular oscillations. In transgenic mice and Drosophila models where clock cells are hyperpolarized, perturbations have been observed in behavioural and molecular rhythms (Nitabach et al., 2002; Pakhotin et al., 2006), and further, molecular rhythms have been shown to be attenuated by hyperpolarizing Ca^{2+} neurons in culture or by blocking Ca^{2+} channels (Lundkvist et al., 2005). Other studies by Yamaguchi and colleagues (2003) have shown electrical firing rhythms to be involved in the generation of robust rhythmic clock gene expression in individual neurons in SCN cultures. Studies by Belle and colleagues (2009) have shown a dissociation between clock gene expression and action potential generation. These authors show that in the middle of the day, at the supposed peak of electrical firing, *Per1* expressing SCN neurons show depolarized membrane states but do not generate action potentials, while at this time, *Per1* mRNA expression as measured by GFP fluorescence signal in *Per1*::GFP mice was shown to be high.

There are a number of ways in which SCN neurons are coupled. Synaptic transmission between cells is the principal mechanism involved in coupling of SCN cells (Welsh, 2009). Both calcium dependent GABA release (Shirakawa et al., 2000) and sodium-dependent action potentials (Honma et. al., 2000) have been shown to play roles in synchronization of circadian and spike to spike firing patterns, and

GABA transmits information between the core and shell regions of the SCN (Albus et al., 2005). Tetrodotoxin (TTX) has been used to block neuronal firing rates in cultured SCN neurons causing cells to lose rhythm synchrony (Yamaguchi et. al 2003; Honma et al., 2000). Gap junctions play a role in intercellular communication within the SCN, as shown by application of the gap junction blocker halothane (Colwell, 2000), and further in mice lacking the gap junction protein Connexin36 electrical synapses were blocked and perturbations in circadian locomotor activity rhythms were observed (Long et al., 2005).

This coupled system is synchronized to the environmental light dark cycle via signalling from light sensitive retinal ganglion cells allowing environmental entrainment (Morin and Allen, 2006; Berson 2003). The retinorecipient core plays a crucial role in SCN coupling (Welsh, 2009), and circadian rhythms have been shown to be abolished at the entire animal level as a result of SCN core lesions, consistent with loss of SCN output signals (LeSauter and Silver 1999). In rostral SCN slices lacking a core, SCN coupling has been shown to be disrupted (Yan et al., 2007), while separation of the SCN subregions has shown loss of coupling in shell neurons, and those in the core remain coupled (Yamaguchi et. al 2003). In knockout mice lacking VIP or its receptor, coordination between SCN neurons has been shown to be severely disrupted (Aton et. al. 2005; Ciarlegio et. al 2009; Maywood et. al 2006), and these animals show altered circadian locomotor activity rhythms (Colwell et al., 2003; Harmar et al., 2002).

1.2 Afferent input to the SCN

Input to the SCN is via three main projections, the Retinohypothalamic Tract (RHT), the Geniculohypothalamic Tract (GHT) and the raphe nuclei.

1.2.1 The Retinohypothalamic Tract

The importance of the RHT for circadian rhythm regulation was established through ablation studies which highlighted that the RHT is both sufficient and necessary for the photic entrainment of the master pacemaker in the SCN (Johnson et al., 1988a). The retina transmits information to the brain via the optic nerve formed by the axons of retinal ganglion cells (Foster and Hankins, 2002). The RHT is formed from a small number of retinal ganglion cells dispersed throughout the retina,

distinct from those involved in primary visual pathways (Moore et al., 1995), and these cells send a random projection to the SCN and contain the photosensitive protein melanopsin which functions as the circadian photoreceptor (Berson et al., 2002; Gooley et al., 2001; Hannibal et al., 2002; Hattar et al., 2002; Provencio et al., 2000) and is involved in photic entrainment (Freedman et al., 1999). Monosynaptic RHT projections terminate in the SCN (Moore and Lenn, 1972), and are seen to project to the vast majority of the SCN in both the rat and the mouse, with approximately equal contralateral and ipsilateral retinal projections seen to the mouse SCN, while in the rat, these projections are seen to be predominantly contralateral (Morin et al., 2006). Bilateral RHT innervation is received by the entire SCN in the hamster (Muscat et al., 2003). These projections are coextensive with GRP and VIP immunoreactive perikarya in the SCN.

The RHT terminates in other brain regions including the subparaventricular zone (sPVZ), the anterolateral hypothalamus and the supraoptic region (Abrahamson and Moore, 2001; Johnson et al., 1988b; Levine et al., 1991). The RHT also projects to specific regions of the thalamus, including the intergeniculate leaflet (IGL) which subsequently projects to the SCN (Morin, 1994; Moore and Card, 1994).

The main neurotransmitters involved in the RHT are the excitatory amino acid neurotransmitter glutamate, and pituitary adenylate cyclase-activating polypeptide (PACAP). Glutamate is released by RHT terminals in response to photic stimulation into the VIP containing SCN core (Mikkelsen et al., 1995; Ding et al., 1994; De Vries et al., 1994). *In vivo* and *in vitro* studies indicate that glutamate acts through NMDA and non-NMDA receptors, activating various intracellular signalling molecules including, among others, nitric oxide (NO), protein kinase G (PKG), protein kinase C (PKC), calmodulin, cAMP-responsive element binding protein (CREB) and calcium (Gillette 1996; Gillette and Mitchel, 2002) as well as inducing the expression of immediate early genes (Kornhauser et al., 1996b) subsequently leading to increased transcription of certain clock genes (Shigeyoshi et al., 1997; Moriya et al., 2000). Application of glutamate receptor (GluR) antagonists *in vivo* has been shown to inhibit both photically induced Fos in the SCN as well as photic phase responses (Abe et al., 1992; Colwell and Menaker, 1992), while application of glutamate receptor agonists causes excitation of SCN neurons (Bos and Mirmiran, 1993; Scott and Rusak, 1996). PACAP colocalizes extensively with glutamate and melanopsin in the RHT and the retina (Hannibal, 2002), and may be

involved in mediating photic information, as well as potentiating the effects of glutamate release on the SCN (Harrington et al., 1999; Minami et al., 2002). The effects of PACAP on the circadian gene expression and phase-shifting are both dose and phase dependent, with PACAP administration blocking the effects of glutamate at high doses, as well as inducing non-photic like phase advances during the subjective day, and at low does, potentiating and or mimicking the effects of glutamate on rhythmic phase-shifting (Hannibal et al., 1997, 2001; Chen et al., 1999; Harrington et al., 1999; Nielsen et al., 2001; Piggins et al., 2001; Bergstrom et al., 2003).

Substance-P (SP) is also thought to be released via RHT terminals, and plays a role in RHT transmission, and its administration mimics photic phase shifts and appears to be dependent on SP induced glutamate release, since administration of the NMDA channel blocker, MK-801, blocks phase-shifting effects (Hamada et al., 1999; Piggins and Rusak, 1997; Kim et al., 2001). Light and glutamate induced phase-shifting and gene expression are blocked by selective SP antagonists both *in vitro* and *in vivo* (Abe et al., 1996; Challet et al., 1998, 2001; Kim et al., 2001).

1.2.2 The Intergeniculate Leaflet

The IGL located between the dorsal lateral and ventral lateral geniculate complex (LGN) is a retinorecipient region (Harrington, 1997; Moore and Card, 1994; Morin, 1994; Rosenwasser, 2009) involved in the indirect integration of photic information to the SCN. The IGL consists of geniculate neurons that contain the inhibitory neurotransmitters GABA and NPY that project to the SCN via the geniculohypothalamic tract (Morin and Allen, 2006). Terminal fields of the GHT are seen in the SCN core (Moga and Moore, 1997). These IGL neurons are sensitive to alterations in overall radiance levels and receive direct binocular input from the retina (Harrington and Rusak, 1989; Pickhard, 1982).

The IGL has widespread connections, extending from the optic chiasm through the lateroanterior hypothalamus to the midline, the SCN included, and to the subparaventricular zone and adjacent perifornical anterior hypothalamus (Morin and Allen, 2006). The IGL is involved in the integration of both photic and non-photic phase-shifting events (Harrington et al., 1985; Pickard et al., 1987; Morin and Pace, 2002). The IGL has been shown to modulate photic phase-shifting effects by lesion

studies, altering photic phase-shifting responses and contributing to the effects of constant lighting conditions on the circadian system, with IGL lesions attenuating the period lengthening response (Edelstein and Amir, 1999; Harrington and Rusak, 1986, 1988; Morin and Pace, 2002; Pickard et al., 1987; Pickard, 1989; Rosenwasser, 2009).

The IGL is thought to be involved in mediating the effects of non-photic stimuli on the circadian system, and NPY and GABA release from the GHT in the SCN may play a role, since non-photic phase-shifting effects have been mimicked by NPY and GABAergic drugs, as well as serotonergic drugs (Rosenwasser, 2009). Under constant dark (DD) conditions, lesions of the IGL have been shown to either shorten (Harrington and Rusak, 1986; Harrington and Rusak, 1988; Pickard et al., 1987) or lengthen (Lewandowski and Usarek, 2002; Pickard, 1994) the FRP. Lesion studies targeting the non-photic phase-shifting responses such as induced wheel running activity, and the benzodiazepine, triazolam, have further highlighted that the GHT is necessary to communicate non-photic information to the SCN (Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Johnson et al., 1988c; Marchant et al., 1997). Further, IGL lesions have been shown to compromise phasic effects following saline injection (Maywood et al., 1997). NPY antiserum infused onto the SCN *in vivo* has also been shown to block the phase-shifting effects of novel wheel running (Biello et. al., 1994). Additionally, the IGL is thought to play a role in sleep and arousal since it projects to areas believed to modulate sleep and arousal processes (Saper et al., 2001; Morin and Blanchard 2005).

1.2.3. Midbrain raphe nuclei projection

Serotonergic projection to the SCN via the midbrain raphe nuclei, provides another source of major input to the SCN core (Moga and Moore, 1997), where the projection is seen to extensively terminate upon core VIP neurons (Reghunandanan and Reghunandanan, 2006). A projection is also seen from the SCN to the raphe nuclei (Bons et al., 1983). The IGL is also innervated by serotonergic projections extending from the dorsal raphe nucleus, which may serve as another route by which the SCN receives serotonergic input (Rosenwasser, 2009). Serotonergic projections to the SCN and the IGL play a role in the subjective day non-photic effects on circadian timing, and subjective night photic effects (Mistlberger et al., 2000; Morin

and Allen, 2006; Rea and Pickard, 2000). Serotonin (5HT) receptor agonists cause phase shifts in the SCN during the portion of the PRC where photic phase-shifting does not occur (Medanic and Gillette 1992; Edgar et al., 1993). Further, non-photic phase shifts in the subjective day are seen in response to stimulation of the midbrain raphe nuclei (Meyer-Bernstain and Morin, 1999).

Serotonergic projections may modulate SCN photic responses, and serotonergic mechanisms have been suggested to positively modulate RHT glutamate release and phase-shifting effects (Graff et al., 2005, Graff et al., 2007; Kennaway and Moyer, 1998; Kennaway et al., 2001; Kohler et al., 1999; Rosenwasser, 2009). Photic phase-shifting and gene expression in the SCN have also been shown to be negatively regulated by mechanisms increasing serotonergic tone such as inhibition of serotonin reuptake (Gannon and Millan, 2007) and the application of serotonin agonists (Glass et al., 1995; Pickard et al., 1996; Pickard and Rea, 1997; Rea et al., 1994). 5HT antagonists enhance photically induced phase shifts (Rea et al., 1995) and photically induced increases in SCN electrical activity (Ying et al., 1994).

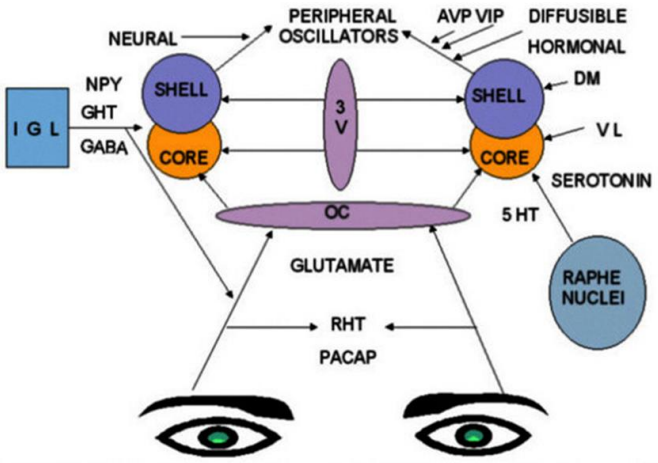


Figure 1.3.: Afferent inputs and efferent pathways of the SCN. (Reghunandanan and Reghunandanan, 2006).

1.3 Neurotransmitters of the SCN

A large number of neurotransmitters have been shown to be present in the SCN (Card and Moore, 1984; Van den Pol, 1986, Rusak and Bina, 1990;

Reghunandanan et al., 1991; Abrahamson and Moore, 2001), and are implicated in the functioning of the pacemaker, with individual neurotransmitters playing various roles in timekeeping processes, and may be involved in afferent projections to the SCN and in entrainment of the pacemaker, within the pacemaker itself for integration and synchronized output, or in efferent projections of the SCN and in the regulation of overt rhythmicity (Reghunandanan and Reghunandanan, 2006). Additional neurotransmitters in the SCN include neuromedin S, neurotensin, histamine, acetylcholine, somatostatin, peptide histidine isoleucine (PHI) calretinin, galanin, angiotensin II, Met-Enkephalin and prokineticin 2 (Reghunandanan and Reghunandanan, 2006).

The neurotransmitter GABA plays important roles in the regulation of SCN function (Reghunandanan and Reghunandanan, 2006). GABA is synthesized from glutamic acid decarboxylase (GAD), and both GAD and GABA are known to be present in the majority of SCN neurons (Moore and Speh, 1993), and the SCN expresses GABA receptors and receptor subunits (Moore and Speh, 1993; Castel and Morris, 2000; Naum et al., 2001). GABA has been shown to have both inhibitory and excitatory effects on SCN neurons (Wagner et al., 1997), and may play roles in synchronization of SCN cells (Shirakawa et al., 2000; Liu and Reppert, 2000). Direct application of GABA to SCN slices in culture promotes intercellular synchrony and phase shifts electrical activity rhythms of neurons (Liu and Reppert, 2000). Application of a GABA receptor antagonist continuously inhibits SCN neuronal resynchronization following a phase shift of the photoperiod (Albus et al., 2005). GABA has been suggested to modulate photic input to the SCN, since administration of GABA receptor agonsists and antagonists impacts upon SCN photically induced c-Fos (Gillespie et al., 1999) and photically induced phase shifts (Gillespie et al., 1996; Gillespie et al., 1997). GABAergic neurotransmission in the SCN has been shown to exert differential modulation during the subjective day and night, which may serve to provide a time dependent gating mechanism, promoting the potentiation of excitatory signals throughout the SCN at night and inhibiting daytime excitation (de Jeu and Pennartz, 2002).

Vasoactive intestinal polypeptide is another major neurotransmitter involved in the function of the SCN, and is expressed by 9-24% of SCN neurons and shows circadian expression (Shirakawa et al., 2001; Herzog et al., 2004; Reghunandanan

and Reghunandanan, 2006). This polypeptide in retinorecipient and activated by photic stimulation and found in the core of the SCN. Exogenous application of VIP both *in vivo* and *in vitro* resets the circadian clock in a manner similar to photic stimulation (Piggins and Cutler, 2003), and VIP induces *Per1* and *Per2* expression in the SCN in a phase dependent manner (Nielsen et al., 2002). The action of VIP with its receptor VPAC2, which is present in approximately 60% of SCN neurons and responsive to alterations in the firing rate of the polypeptide (Reed et al., 2002; Cutler et al., 2003), is seen to be involved in photic resetting and in the maintenance of SCN rhythmicity (Piggins and Cutler, 2003; Reghunandanan and Reghunandanan, 2006). VIP plays important roles in behaviour and physiology. Alterations in VIP signalling cause a significant reduction in rhythmic neuronal populations, and those that continue to show rhythmicity display a decrease in rhythm amplitude (Aton et al., 2006; Brown et al., 2007). Coherent clock gene expression rhythms, wheel running and SCN neuronal activity are not observed in *Vipr2*$^{-/-}$ mice, lacking the VPAC2 receptor gene (Harmar et al., 2002; Cutler et al., 2003). Further, these *Vipr2*$^{-/-}$ mice show perturbations in gating of photic input to the SCN (Hughes et al., 2004). Additionally, perturbations are seen in sleep parameters (Hu et al., 2011) and cardiovascular and body temperature rhythms in *Vipr2*$^{-/-}$ or VIP deficient mice (Hu et al., 2011; Sheward et al., 2010; Hannibal et al., 2011), as well as disrupted metabolic and endocrine rhythms (Asnicar et al., 2002; Bechtold et al., 2008; Loh et al., 2008; Fahrenkrug et al., 2012), highlighting the importance of VIP signalling in physiological function.

The ventrolateral region of the hamster SCN is seen to be densely packed with CalB containing cells in its caudal aspect (Silver et al., 1996a; Hamada et al., 2001). CalB cells receive direct retinal input (Bryant et al., 2000) and show photic induced c-Fos expression (Silver et al., 1996a). *Per1* and *Per2* mRNA are induced in response to photic stimulation in the CalB subregion (Hamada et al., 2001). CalB cells colocalize with GRP, SP and VIP (LeSauter et al., 2002), and do not show rhythmic expression of clock genes (Hamada et al., 2001; Hamada et al., 2004), or rhythmic electrical activity (Jobust and Allen, 2002), but may play roles in the maintenance of locomotor rhythmicity, since half-SCN transplants that contain cells of the CalB subregion restore rhythmic locomotor behaviour in SCN lesioned hosts, while transplantation of SCN tissue lacking these cells does not restore rhythmic behaviour (LeSauter and Silver, 1999). Further, CalB cells have been suggested to be

involved in the gating of photic input to the SCN, since administration of CalB antisense oligodeoxynuclotides blocks subjective night photically induced phase shifts and *Per* expression normally, and these are instead enhanced during the subjective day (Hamada et al., 2003). CalB deficient mice show altered photic induced locomotor phase delays and *Per2* expression in the SCN, indicating a role in resetting (Stadler et al., 2010).

Rodent SCN neurons synthesize GRP and the GRP receptor BB_2 (Battey and Wada, 1991; Ladenheim et al., 1992) and GRP has been suggested to play roles in photic entrainment (Tanaka et al., 1997; Aioun et al., 1988). GRP application *in vitro* phase shifts rhythmic electrical activity, causing early subjective night phase delays and a phase advance in the late subjective night, and further, application of a GRP receptor antagonist inhibits phase-shifting effects (McArthur et al., 2000; Reghunandanan and Reghunandanan, 2006).

Arginine vasopressin is expressed in cells of the SCN shell, co-localized with somatostatin and GABA, and is thought to be one of the major transmitters of the SCN (Ingram et al., 1998), and may play roles in SCN output. The synthesis and release of AVP shows circadian variation and approximately one third of SCN neurons are seen to synthesize AVP in rats (Reghunandanan and Reghunandanan, 2006). AVP has been shown to play an excitatory role through its activation of V1a receptors (Ingram et al., 1998) and to be involved in electrical firing acitivity in the SCN (Mihai et al., 1994). A reduction in SCN AVP content and AVP neurons has been shown to accompany perturbations in the sleep wake cycle, a reduction in the amplitude of rhythmic activity and an increase in rhythm fragmentation (Hofman et al., 1994; Hofman et al., 1995; Lucassen et al., 1995). AVP deficient rats show reduced amplitude of circadian sleep rhythms (Brown and Nunez, 1989), highlighting the roles this neuropeptide plays in output rhythms.

Prokineticin 2 (PK2) is involved in output from the SCN, and may play roles in the timing of locomotor behaviour in nocturnal animals (Cheng et al., 2002), and PK2 SCN expression is regulated by the circadian clock and by light (Cheng et al., 2005).

1.4 Other sources of input to the SCN

Other SCN afferent systems provide input the circadian system, including noradrenergic input from the brainstem (Cagampang et al., 1994; Moga and Moore, 1997, Rosenwasser, 2009), cholinergic projections arising from the pontine tegmentum and basal forebrain (Bina et al., 1993; Rosenwasser, 2009), and input from the posterior hypothalamus via histaminergic projections (Panula et al., 1989; Wada et al., 1991; Rosenwasser et al., 2009). These afferents target the SCN shell and may be involved in the regulation of the circadian clock (Moga and Moore, 1997; Moore, 1996; Rosenwasser, 2009). Further, the IGL also projects via noradrenergic and cholinergic inputs. Studies have suggested that histamine may play roles in photic effects on the circadian system (Cote and Harrigton, 1993; Harrington et al., 2000; Eaton et al., 1995; Meyer et al., 1998) or possibly be involved in entrainment downstream from glutamate release (Jacobs et al., 2000; Rosenwasser et al., 2009). Various cholinergic effects on the circadian system have been described. Application of the cholinergic agonist cabechol has mimicked both photic and non-photic phase-shifting effects (Bina and Rusak, 1996), while the free running period is perturbed upon near SCN implantation of a cabechol secreting pellet (Furukawa et al., 1987). Studies inhibiting cholinergic transmission have shown SCN gene expression and photic induced phase shifts to be attenuated (Keefe et al., 1987; Zhang et al., 1993; Beaule and Amir, 2002; Erhardt et al., 2004). The noradrenergic system may play roles in the modulation of photic input, and has been shown to alter the FRP under constant conditions (Dwyer and Rosenwasser, 2000; Rosenwasser, 1996; Rosenwasser et al., 1995; Rosenwasser et al., 2009).

1.5. The Molecular Circadian Clock

The circadian clock system is composed of three integral components, input pathways receiving and subsequently conveying environmental information to the pacemaker and adjusting the time, a central pacemaker which generates the circadian rhythm and output pathways affecting circadian physiology and behaviour (Balsalobre et. al 1998).

The molecular mechanisms governing the clock were unknown until the early 1970's, when the first circadian rhythm mutants were isolated, the *period* mutants in Drosophila, fruitfly (Konopka and Benzer, 1971) and *frequency* in Neurospora,

fungi, (Feldman and Hoyle, 1973), with the isolation of the period and frequency genes occurring later. In the years that followed several other clock genes involved in the molecular clockworks were identified in both flies and mammals, and there is a general conservation in the molecular clock mechanism between the organisms (Reppert and Weaver, 2001). "Clock genes" of the molecular clock are involved in the generation and maintenance of circadian rhythms and must display certain properties in order to be considered a core clock gene. Clock genes are usually rhythmically transcribed and if this gene is deleted or mutated circadian rhythms are disturbed. Further, clock proteins feedback and regulate their own transcription and in order to generate circadian oscillations of expression, there must be a delay between when a gene is activated and when it is inhibited or a steady state will result (Allada et al., 2001). Clock genes are expressed both in the SCN, in oscillators in other regions of the brain and in peripheral tissues, and the molecular clock mechanism is similar for the master circadian pacemaker in the SCN and in peripheral oscillators (Balsalobre et al., 2000b; Nagoshi et al., 2004; Welsh et al., 2004; Brown et al., 2005; Ko and Takahashi, 2006).

At a molecular level, the set of clock genes and proteins are interconnected to form complex transcription-translation autoregulatory loops that produce accurate and robust circadian rhythms. These transcription translation feedback loops that underpin the molecular circadian clock drive expression of the core clock genes (Lowrey and Takahashi 2004; Reppert and Weaver, 2002), and the oscillations of clock gene protein products are required for the generation and regulation of circadian rhythms in single cells throughout the organism (Takahashi, 2004).

1.5.1 Transcription/Translation Feedback loop in mammals

The Transcription/translation clock feedback loop is comprised of both positive and negative limbs. Three basic helix-loop-helix (bHLH)-PER-ARNT-SIM (PAS) protein-containing transcription factors, "circadian locomotor output cycle kaput" (CLOCK or its homolog NPAS2) and "brain-muscle-arnt-like protein 1" (BMAL1) comprise the positive elements limb of the transcription/translation feedback loop. CLOCK and BMAL1 bind to each other forming heterodimers, and initiate transcription of target genes containing E-box *cis*-regulatory enhancer sequences (CACGTG) which comprise the negative feedback loop, the *Period* genes

(*Per 1, Per2 and Per3*) and the *Cryptochrome* genes (*Cry1* and *Cry2*) (Gekakis et al., 1998; Zheng et. al 2001; Bunger et al., 2000; Kume et al., 1999). Once transcribed, PER and CRY bind each other forming PER:CRY heterodimers which then translocate back to the nucleus and act on the CLOCK:BMAL1 complex, repressing their own transcription, comprising the negative component of the feedback loop (Lee et. al 2001; Kume et. al 1999; Okamura et al.,1999; Shearman et al., 2000b; Sato et al., 2006). While the CRY proteins turn off CLOCK:BMAL1-mediated transcription, PER2 may contribute to *Bmal1* transcription, promoting CLOCK:BMAL1 heterodimerization, restarting the transcriptional cycle (Reppert and Weaver, 2001).

Another regulatory loop is induced by CLOCK:BMAL1 heterodimers activating the retinoic acid-related orphan nuclear receptors transcription of *Rev-erbα* and *Rorα* (Preitner et. al 2002; Sato et. al 2004; Akashi et. al 2005; Ko and Takahashi, 2006), the protein products of which then compete to bind retinoic acid-related orphan receptor response elements (ROREs) located in the *Bmal1* promoter, and form an auxillary or stabilizing feedback loop regulating *Bmal1* transcription levels (Preitner et al., 2002; Sato et al., 2004; Son et al., 2011). The circadian oscillation of *Bmal1* is regulated by RORs and REV-ERBs, with REV-ERBα/β repressing the transcription of *Bmal1* (Guillamound et al., 2005; Ko and Takahashi, 2006) and RORα activating *Bmal1* transcription (Guillamound et. al 2005, Akashi et. al 2005). As previously stated, CLOCK:BMAL1 heterodimers activate the gene encoding REV-ERBα, which in turn represses *Bmal1* transcription. The inhibition of CLOCK:BMAL1 heterodimers by the action of PER:CRY in the nucleus indirectly activates *Bmal1* through inhibition of *Rev-erbα* gene expression (Cermakian and Boivin, 2003; Preitner et. al., 2002). The renewal of BMAL1 levels at the end of the night may increase CLOCK:BMAL1 heterodimers at the appropriate circadian time driving *Per/Cry* transcription and restarting the oscillation, therefore BMAL1 availability is required at the start of a new circadian day (CT 0) to renew the transcription/translation feedback loops (Reppert and Weaver, 2001). (Figure 1.4).

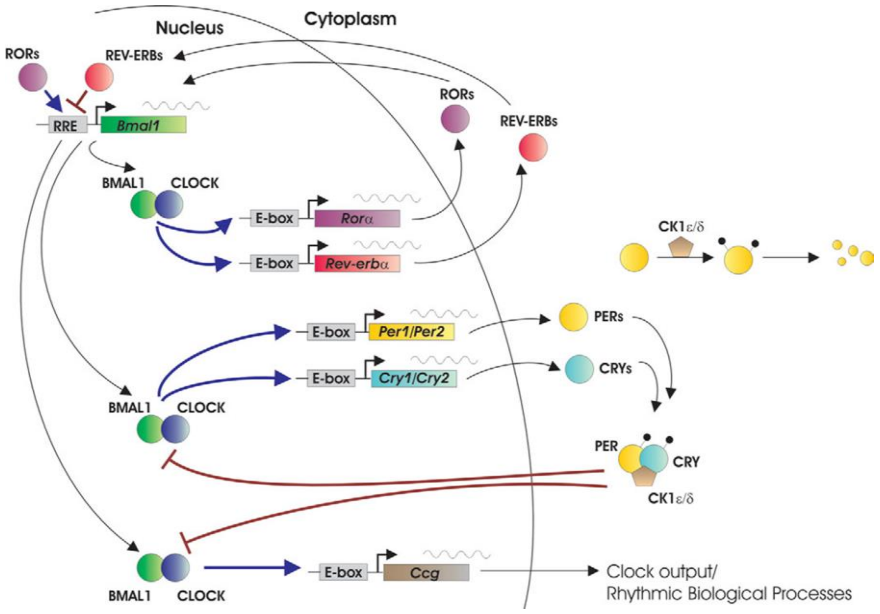


Figure 1.4.: A network of transcriptional–translational feedback loops constitutes the mammalian circadian clock *(Ko and Takahashi, 2006).*

The majority of the components of the molecular clock show rhythmic circadian expression in the SCN both under constant conditions and in LD (Guilding and Piggins, 2007). *Per1* mRNA shows peak expression in the SCN between ZT/CT 4 and 6 (where ZT0 is the time at lights on under a 12:12 LD cycle and where CT0 is the start of the subjective day in constant conditions (Thresher et al., 1998; Shearman et al., 1997; Zheng et al., 1999; Bunger et al., 2000; Guilding and Piggins, 2007). Both *Cry1* mRNA (Miyamoto and Sancar, 1998; Thresher et al., 1998) and *Per2* mRNA expression peak at approximately ZT/CT10 (Albrecht et al., 1997; Shearman et al., 1997; Bunger et al., 2000; Bae et al., 2001; Oster et al., 2003). Peak *Bmal1* mRNA expression is seen in the SCN at approximately ZT/CT18 (Abe et al., 1998; Honma et al., 1998; Reppert and Weaver, 2002). *Rev-erbα* mRNA shows peak expression at approximately ZT/CT4 (Onishi et al., 2002). *Cry2* is not expressed in rhythmic fashion in the SCN, while *Clock* is expressed constitutively across the 24hr circadian cycle (Oster et al., 2003; Gekakis et al., 1998). With regard to mRNA levels, the expression of protein products is seen to be delayed by 4-6 hours (Guilding and Piggins, 2007).

1.5.2. Post-translational modifications and clock control

The precise ~24hour period of the molecular clock is governed by posttranslational modifications of the clock gene transcripts involved in the feedback loops, creating time lags between transcription, translation and translocation of proteins from the cytoplasm to the nucleus (Lee et al., 2001; Reppert & Weaver, 2002; Ko and Takahashi, 2006; Guilding and Piggins, 2007). Post-translational modifications involved in the localization, degradation and activity of the core circadian clock components include phosphorylation, ubiquitination, methylation, sumoylation and histone acetylation (reviewed in Gallego and Vishrup, 2007). Evidence of posttranscriptional/posttranslational control in the molecular clock is seen in Drosophila, where peak levels of PER proteins are seen 4-6 hours following peak *Per* RNA levels (Harms et al., 2004).

Various key processes are tuned to generate a 24hr cycle including the transcription rate of PER and CRY proteins and protein stability (Hasting et al., 2007; Gallego and Virshup, 2007). *Casein kinase 1 epsilon* (*CKIε*) and *Casein kinase 1 delta* (*CK1δ*) are accepted as components of the core molecular clock (Akashi et al., 2002) and are essential in regulation of mammalian circadian protein turnover (Ko and Takahashi, 2006; Lowrey et al., 2000; Eide et al., 2002; Akashi et al., 2002). Casein Kinase 1 (CK1) plays roles in regulating the nuclear entry of PER in mammals, and CKIε and CK1δ phosphorylate PER1 and PER2 to modulate nuclear translocation times and target proteins for degradation. CKIε is constitutively expressed in the SCN, and has been shown to bind and phosphorylate PER regulating its subcellular localization, impacting on its ability to repress transcription and targeting it for ubiquitination and subsequent degradation through a proteasomal pathway involving the F-box proteins β-TrCP1 and β-TrCP2 (Etchegaray et al., 2009; Vielhaber et al., 2000; Vanselow et al., 2006). CK1δ is also seen to phosphorylate PER proteins, reducing their stability *in vitro* (Camacho et al., 2001; Xu et al., 2005).

CKIε and *CK1δ* mutations highlight the importance of post translational modifications in the molecular clock mechanism. A mutation in *CK1ε* in hamsters known as the *tau* mutation is seen to cause a 20% decrease in the FRP and an altered phase angle of entrainment (Ralph and Menaker, 1988). Furthermore, this mutation results in decreased PER stability and their accelerated proteasomal degradation

(Lowrey et al., 2000) and enhanced clearance from the nucleus sufficient to explain the shortened FRP phenotype (Dey et al., 2005). Mutations in *CK1ε* and *CK1δ* are implicated in Familial Advanced Sleep Phase Syndrome (FASPS) in humans (Xu et al., 2005). Currently a model based on the *doubletime* (*Dbt)* mutation in Drosophila, for the *tau* mutant and FASPS, suggests that reduced efficacy of PER phosphorylation results in prevention of PER degradation, allowing PER to rapidly enter the nucleus, leading to acceleration of the clock (Lowrey et al., 2000). The absence of CK1δ has been shown to reduce PER protein turnover, and this possibly underlies the increase in period length that has been observed in CK1δ-deficient fibroblasts and CK1δ-deficient liver explants *in vitro* (Etchegaray et. al, 2009)

In vitro studies have shown CLOCK and BMAL1 to be subject to post-translational modifications. Casein Kinase (CK)2α has been shown to phosphorylate BMAL1 and is important in the entry of BMAL1 to the nucleus and disruption of this phosphorylation attenuates clock gene expression in cultured fibroblasts *in vitro* (Tamaru et al., 2009). In cells in culture, PKC mediated phosphorylation of CLOCK followed by the recruitment of co-factors of the CLOCK:BMAL1 heterodimer have been shown to be important in resetting clock gene mRNA oscillations (Jung et al., 2003; Shim et al., 2007; Lee et al., 2010; Son et al., 2011).

Following phosphorylation, proteins to be degraded by the proteasomal pathway are poly ubiquitinlyated (Hastings et al., 2007). PER proteins are thought to be targeted for ubiquitination and subsequent degradation through a proteasomal pathway involving the F-box proteins βTrCP (Hastings et al., 2007). The CRY proteins are also regulated by phosphorylation and degradation events to impact upon circadian period length, and CRY1 stability has been shown to be regulated by the F-box protein FBXL3, (Busino et al., 2007; Siepka et al., 2007). The F-Box protein's interaction with the ubiquitin ligase complex and the leucine-rich repeat region (LRR) of its targets reduces affinity for CRY proteins, slowing down their clearance from the cells, prolonging the negative feedback phase, reducing the expression of *Per* mRNA required, thereby lengthening the period of circadian oscillations (Hastings et al., 2007)

A self-sustaining cycle of the core clock components which takes approximately 24hrs to complete one oscillation is produced as a result of the processes outlined above (Guilding and Piggins, 2007).

1.5.3. Mutations or deletions of the core clock genes

Mice with targeted disruptions or deletions in the circadian clock genes exhibit attenuations in circadian behaviour and altered expression profiles of circadian genes (Lowrey and Takahashi, 2004).

1.5.3.1. *Cry* mutations

In mammals, cryptochromes are involved in photoreception for circadian entrainment, and are essential components of the negative feedback loop of the circadian timekeeping mechanism and are crucial for the maintenance of circadian rhythmicity. Deletion of *Cry2* lengthens the circadian period (Thresher et.al 1998; Van der Horst et. al 1999), while deletion of *Cry1* shortens the circadian period (Van der Horst et.al 1999; Vitaterna et al. 1999). While rhythmicity can be maintained through the presence of either CRY1 or CRY2, double knockout mice for *Cry1* and *Cry2* show complete arrhythmicity in DD (Van der Horst et.al 1999; Vitaterna et al. 1999) necessitating the functional importance of the proteins for circadian timekeeping processes.

1.5.3.2. *Per* mutations

Mice with a null mutation in the *Per1* gene show a shorter circadian period along with an attenuation in the precision and stability of the period, indicating that PER1 plays a role in functionally regulating the circadian clock and in maintenance of the circadian period. The circadian clock in *Per1* mutant mice still appears to be partially functional with no perisistant loss of circadian rhythmicity (Zheng et al., 2001). During prolonged housing in DD, *Per1* deficient mice and *mPer2*^{ldc} mutant mice display perturbed locomotor activity rhythms (Bae et al., 2001). Locomotor activity rhythms of *Per1/Per3* double-mutant mice resemble activity rhythms of mice with disruption of *Per1,* while *Per2/Per3* double-mutant mice have a phenotype identical to *Per2* knockout mice, indicating that *Per3* functions outside the molecular clock mechanism (Cermakian and Boivin, 2003). *Per1/Per2* double-mutant mice have been shown to exhibit immediate arrhythmia (Bae et al., 2001), and *Per1/Per2* mutants display complete loss of a functional circadian clock in DD (Zheng et al., 2001).

PER2 is thought to play a critical role in mammals in the maintenance of circadian behavioural rhythmicity in both mice and humans. *Per2* mutant mice show delayed loss of circadian rhythmicity in mice and unstable circadian rhythms (Zheng et al., 1999), and exposure to a light pulse during arrhythmia in DD re-establishes circadian rhythmicity (Zheng et al., 2001). *Per2* mutant mice show reduced levels of SCN gene expression (Bae et al., 2001; Shearman et al., 2000b; Zheng et al., 1999). A *Per2* mutation in humans affecting the phosphorylation of hPER2 by casein kinase 1epsilon is implicated in Familial Advanced Sleep Phase Syndrome (Toh et al., 2001). *Per3* knockout mice display only subtle circadian locomotor activity alterations, with slight shortening of the FRP under constant conditions (Shearman et al., 2000a).

1.5.3.3. *Clock* and *Npas2* mutations

CLOCK has been shown to be necessary for rhythm maintenance in peripheral tissues since $Clock^{-/-}$ mice show loss of functional peripheral oscillators (DeBruyne et al., 2007b). Heterozygous *Clock* mutant mice, in DD display abnormally long free running periods (~24.5 hours) while homozygotes, $Clock^{\Delta 19/\Delta 19}$ show free running periods of ~28 hours initially, followed by degeneration into arrhythmia (Vitaterna et al., 1994). $Clock^{-/-}$ mutants display altered photic behavioural phase-shifting, with decreased phase delays and exaggerated magnitudes of phase advances, and an altered phase angle of entrainment under LD (DeBruyne et al., 2006), indicating that CLOCK plays roles in conveying photic information to the SCN or in regulating SCN sensitivity to photic stimulation. $Clock^{-/-}$ mice also defy Aschoff's Rule which states that nocturnal rodents lengthen their FRP under LL (Dallman et al., 2011).

NPAS2 (also known as MOP4) is a CLOCK related protein, both being paralogs of Drosophila CLOCK, and shares sequence homology within the b-H-L-H PAS domain (King et al., 1997) and heterodimerizes with BMAL1 to drive gene expression (Kume et al., 1999; Hogenesch et al., 1998). NPAS2 deficient mice show strong circadian rhythms and slight shortening of circadian period (Dudley et al., 2003; DeBruyne et al., 2007a, Dallman et al., 2011). Mice missing both CLOCK and NPAS2 show arrhythmia, indicating functional redundancy of NPAS2 in the SCN (DeBruyne et al., 2007a). NPAS2 deficient mice show attenuations in *Per2*

(Reick et al., 2001) and accelerated re-entrainment to a 4 hour phase advance of the photoperiod, and therefore show altered photic responses, similar to *Clock* mutant mice (Dudley et al., 2003), however, *Npas2*$^{m/m}$ mice show normal phase angles of entrainment under LD (Dallman et al., 2011).

The expression of several core clock genes and clock controlled genes are downregulated in *Clock* mutant mice, implicating CLOCK as a critical transcriptional activator in the core clock mechanism (Kume et al., 1999; Shearman et al., 2000b, Zylka et al., 1998; DeBruyne et al., 2006). *Npas2* mRNA levels and nuclear protein levels are elevated in the livers of CLOCK deficient mice, however, bioluminescence recordings of liver and lung rhythms exhibit arrhythmicy, indicating that NPAS2 is unable to maintain rhythmicity in peripheral tissues in *Clock*$^{-/-}$ animals, highlighting the necessity of CLOCK in peripheral oscillations (DeBruyne et al., 2006).

1.5.3.4. *Bmal1* mutations

BMAL1 (MOP3) plays roles in both the master circadian pacemaker and in peripheral clocks and is involved in the generation and maintenance of behavioural rhythms and is suggested to function in behavioural output. *Mop3*$^{-/-}$ mutant mice, show arrhythmicity in DD, attenuations in activity distribution in LD and DD, altered entrainment to the LD cycle, and perturbations in peripheral expression of *Dbp,* and impaired *Per1* and *Per2* rhythmicity in the SCN (Bunger et al., 2000). *Bmal1*$^{-/-}$ mice also show signs of premature aging and reduced lifespan (Kondratov et al., 2006), impaired glucose homeostasis (Rudic et al., 2004), progressive arthropathy (Bunger et al., 2005) and infertility (Kennaway et al., 2005).

1.5.3.5. Other components of the molecular clock mechanism and their mutations

Dec1 and *Dec2* are two basic helix-loop-helix factors that have also been shown to be involved in the regulation of the molecular clock (Honma et al., 2002). *Dec1* and *Dec2* are expressed in the SCN in a circadian manner, and *Dec1* is induced in a phase dependent manner in the SCN in response to photic stimulation (Honma et al., 2002). These factors suppress the activity of CLOCK:BMAL1 dimers through association with them and or competition with the E-box elements in target promoters, and have been shown to suppress the transactivation of the mouse *Per1*

promotor (Honma et al., 2002). Recent studies have suggested DEC1 and DEC2 to be involved in the finer regulation and robustness of the molecular clock (Nakashima et al., 2008). *In vitro*, alterations in *Dec1* affect the oscillations of clock genes, and *Dec2* knock down is seen to affect the phase of expression of certain clock genes, while double knockout of *Dec1* and *Dec2* was seen to decrease the amplitude of circadian expression of several clock genes (Nakashima et al., 2008). Further, mice deficient in *Dec1* show longer free running periods in DD as well as accelerated reentrainemnt to an LD phase advance (Nakashima et al., 2008). Rossner et al (2008) have also shown that *Dec* mutant mice display attenuations in the FRP and in photic entrainment. More recently, studies have shown interactions of *Dec1/2* genes with *Per1* to be important for accurate entrainment of activity to the LD cycle (Bode et al., 2011).

Dbp is a clock controlled gene (Ripperger et al., 2000), and its product, the basic leucine zipper (bZip) transcription factor, albumin site D-Binding Protein (DBP) is shown to oscillate in peripheral tissues (Wuarin et al., 1992; Fonjallaz et al., 1996) in the SCN (Lopez-Molina, 1997), and regulates a number of genes (Mueller et al., 1990; Lopez-Molina, 1997). The Thyroid Embryonic Factor (TEF) and the Hepatocyte Leukemia Factor (HLF) which share extensive sequence similarity with DBP also show circadian expression in several tissues (Falvey et al., 1995; Fonjallaz et al., 1996; Lopez-Molina et al., 1997). Mice homozygous for a *Dbp*-null allele show perturbations in locomotor activity, with reduced levels of activity and a shortened free running period (Lopez-Molina et al., 1997).

Rev-erbα deficient mice show perturbations in phase-shifting of locomotor behaviour and a reduction in the free running period in constant conditions (Preitner et al., 2002), however these studies show that REV-ERBα is not required for circadian rhythm generation. As previously mentioned, mutations in *CK1ε* (*tau* mutation) also causes a shortened free running period and altered phase angle of entrainment (Ralph and Menaker, 1988).

Together, these studies highlight the impact of perturbations in components of the molecular clock mechanism on circadian output rhythms.

1.6 Entrainment

1.6.1 Phase Response Curves (PRC)

PRCs outline the time across the circadian cycle at which a stimulus will impact upon circadian rhythmicity, resulting in phase shifts of circadian locomotor activity, thereby resetting the phase of the oscillator. The PRC is an intrinsic species specific property, with species variation seen in the shape and amplitude of the PRC (Golombek and Rosenstein, 2010). Two general categories of PRCs exist, those to photic stimuli and those to non-photic stimuli, and the sensitivity of the clock to phase resetting stimuli varies across the circadian cycle.

1.6.1.1. Non-photic stimuli and the non-photic PRC

A variety of non-photic Zeitgebers have been shown to cause phase advances during the subjective day when the clock does not show responses to photic stimulation (Hastings et al., 1998). In rodents, locomotor activity rhythms can be entrained by scheduled feeding (Mistlberger, 1994; White and Timberlake, 1995; Abe et al., 1989; Mistlberger, 1993) and restricted exercise (Edgar and Dement, 1991; Marchant and Mistlberger, 1996; Hastings et al., 1998), acute exposure to sexual odours and social interaction (Mrovosky et al., 1989). Phase advances are seen in response to behavioural arousal or saline injection in the subjective day (Hastings et al., 1992), as well as single exposure to novel running wheels (Mrovosky et al., 1996; Turek, 1989; Wickland and Turek, 1994). The IGL and NPY neurotransmission in the SCN, and serotonergic projection from median raphe to the SCN are involved in non-photic phase-shifting (Van Esseveldt et al., 2000; Meyer-Bernstein and Morin, 1996; Mintz et al., 1997). (Figure 1.5).

1.6.1.2. The Photic PRC

The photic PRC describes where photic stimulation at different circadian phases will induce a phase delay, a phase advance or no alteration in circadian phase. In nocturnal animals, photic PRC's display phase shifts of locomotor behaviour during the subjective night, while the subjective day comprises a "dead zone" whereby light falling on this phase of the circadian cycle causes no alterations in behaviour (Daan and Pittendrigh, 1976). In response to photic stimuli, the internal

oscillator resets rapidly within one cycle, however overt behaviour exhibits transient cycles until steady state entrainment is achieved (Johnson et al., 2003). Pittendrigh and Daan (1976a) modelled the circadian oscillator of rodents as an intricate timekeeper comprised of two mutually coupled oscillators, a morning oscillator which is accelerated by light and synchronized to dawn, and an evening oscillator decelerated by light and synchronized to dusk (Pittendrigh and Daan, 1976a; Meijer and Schwartz, 2003). Phase advancing photic stimulation applied during the late subjective night advances the morning oscillator with a later shift of the evening oscillator and the morning peak of SCN electrical activity and the offset of locomotor behaviour would also be advanced. (Pittendrigh and Daan, 1976a; Meijer and Schwartz, 2003). At the behavioural level, these phase advances following light application in the late portion of the subjective night cause locomotor activity to begin earlier on the next cycle (Daan and Pittendrigh, 1976; Rusak and Boulos, 1981). Phase delaying photic stimulation would delay the evening oscillator with a later shift of the morning oscillator (Pittendrigh and Daan, 1976a; Meijer and Schwartz, 2003). These phase delays occur in response to light in the early part of the subjective night, delaying the phase of locomotion, causing activity onset of the subsequent cycle to occur later (Daan and Pittendrigh, 1976; Rusak and Boulos, 1981). Coupled with the delay in the evening oscillator would be a delay in SCN electrical activity and the offset of locomotor activity offset (Pittendrigh and Daan, 1976a; Meijer and Schwartz, 2003). (Figure 1.5).

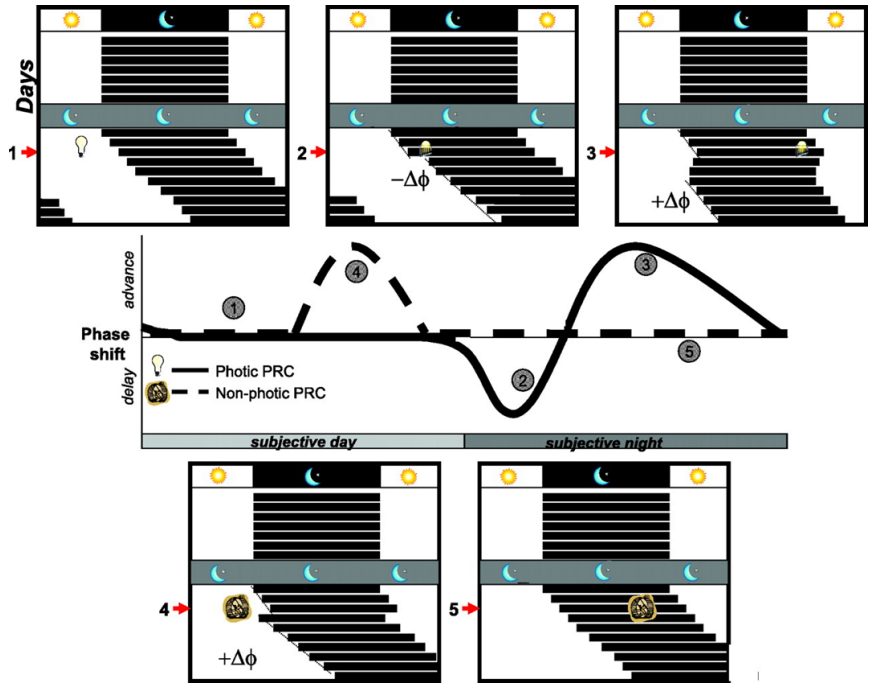


Figure 1.5: Phase response curves of circadian rhythms. *In the top panel, actograms represent behavioural responses to photic stimulation during 1) the subjective day, 2) the early subjective night (inducing a phase delay of the rhythm), and 3) the late subjective night (inducing a phase advance). The bottom panel represents the phase-shifting effects of non-photic stimuli (e.g. novel running wheel) presented 4) in the middle of the subjective day (inducing a phase advance) or 5) during the subjective night (no response). The central graphs illustrate both the photic PRC (solid line), and the non-photic PRC (dashed line), with number corresponding to acrogram respresentations (Golombek and Rosenstein, 2010).*

1.6.2 Photic Entrainment

Environmental light is the principal Zeitgeber, entraining or synchronizing an organism's activity to the 24hr solar day. The linear model of entrainment of the circadian system starts with environmental light stimulating specialized retinal ganglion cells (Rollag et al., 2003) which in turn causes synaptic transmission and innervation of the SCN's core clock cells, resulting in the activation of the proteins involved in the resetting of the transcription-translation feedback loop underpinning circadian oscillations (Meijer and Schwartz, 2003).

1.6.2.1. Intracellular Signalling Pathways underlying photic entrainment

The molecular mechanisms that underpin entrainment of the circadian clock are thought to begin with glutamate activating SCN NMDA receptors, which subsequently leads to an influx of Ca^{2+} into SCN neurons (Ding et al. 1997; Schurov et al. 1999; Gau et al., 2002). The excitatory glutamatergic input activates various intracellular signalling molecules and their signalling pathways including, among others, the activation of NO, PKG, PKC, calmodulin, CREB and calcium (Gillette 1996; Gillette and Mitchel, 2002). These signalling pathways underlying photic entrainment converge on CREB which, following its phosphorylation becomes activated, resulting in its translocation to the nucleus and binds to cAMP-responsive elements (CRE) on promotor regions of photically inducible genes (Ding et al., 1997; Gau et al., 2002; Ginty et al., 1993; Obrietan et al., 1999; Travnickova-Bendova et al., 2002) and IEGs (Rusak et al., 2002). *Per1* and *Per2* are known to be induced in response to photic stimulation and to be involved in photic phase-shifting (Yan and Silver, 2002), as are a variety of immediate early genes (Rusak et al., 2002).

The extracellular signal related kinases (ERKs) are members of the mitogen-activated protein kinase (MAP) kinase superfamily. The ERK pathway is implicated in the phase-shifting response following photic stimulation. Active, phosphorylated ERK (p-ERK) shows variation across the circadian cycle (Obrietan et al., 1998) and phase dependent photic induction, being rapidly upregulated in the SCN upon exposure to light during the subjective night, but not during the subjective day (Obrietan et al. 1998; Butcher et al. 2003; Coogan and Piggins 2003; Nakaya et al. 2003). This photic regulation of p-ERK mirrors the pattern of photic phase-shifting of circadian locomotor behaviour (Pittendrigh and Daan, 1976b; Schwartz and Zimmerman, 1990).

Activation of ERK results in the phosphorylation of the cAMP response element binding protein (CREB) (Kornhauser et al., 1996a; Obrietan et al., 1998; Coogan & Piggins, 2003; Coogan and Piggins, 2004; Dziema et al., 2003; Lee et al., 2003; Antle et al., 2005), subsequently leading to the transcription of photically inducible genes (Grewal et al., 1999; West et al., 2001). The phorphorylation of CREB in the SCN is regulated by photic and circadian stimuli (Ginty et al. 1993; Obrietan et al. 1999; Gau et al. 2002) and further, CRE mediated transcription shows

both photic and circadian regulation (Obrietan et al., 1999). As well as containing CRE in their promotor regions, both the *Per1* and *Per2* gene promotor regions also contain Serum response element (SRE) consensus sequences (Wilsbacher et al. 2002), and these are known to be targets for phosphorylated Elk-1, which is a substrate for ERK (Coogan and Piggins, 2004), and is phosphorylated in response to photic stimulation in the hamster (Coogan and Piggins, 2003). Photically inducible IEGs contain both CREs and SREs in their promotor regions (Coogan and Piggins, 2003) and these include Egr-1 junB, c-Fos and fosB (Greenberg et al., 1992; Perez-Albuerne et al., 1993) and are induced in response to subjective night photic stimulation (Rusak et al., 2002).

The activation of ERK has been shown to be necessary for photic entrainment of the circadian timekeeper (Obrietan et al., 1998; Wang and Sehgal, 2002; Coogan and Piggins, 2004) and ERK plays roles in the molecular events that underlie photic phase-shifting. In NIH3T3 fibroblast cells in culture *in vitro*, the oscillations of both *Per1* and *Per2* are perturbed following blockade of the ERK cascade (Akashi and Nishida 2000; Oh-Hashi et al. 2002). Administration of the MEK (MAP kinase kinase) inhibitor U0126 attenuates the induction of photically induced IEGs in the SCN in mice during the delay portion of the PRC (Dziema et al. 2003). Inhibition of ERK signalling attenuates the phosphorylation of CREB by glutamate (Obrietan et al., 1999). Further, mice lacking the CREB phosphorylation site show perturbations in photic induced phase shifts of locomotor behaviour, as well as attenuations in *Per1* in the SCN and c-Fos (Gau et al., 2002). MEK inhibitors also impact upon photically induced behavioural responses (Coogan and Piggins, 2003; Butcher et al., 2002).

Additionally, other members of the MAP kinase superfamily have been shown to be under circadian and photic control, including P38 and c-jun-N-terminal kinase (JNK) (Pizzio et al., 2003; Coogan and Piggins, 2004). More recently, MAPK has been shown to be autonomously activated in the SCN, and its inhibition attenuates SCN autonomous neuronal firing rhythms and further, impacts upon circadian clock gene oscillations in individual neurons in culture, suggesting that MAPK signalling contributes to the robustness of the SCN autonomous circadian system (Akashi et al., 2008) and highlighting the importance of these signalling cascades in the circadian timekeeping system.

1.6.3 Photically induced Per genes

Photic resetting of the circadian clock in mammals involves the acute induction of the clock genes *Per1* and *Per2* (Dunlap, 1999; Lowrey and Takahashi, 2000), by a mechanism independent of the CLOCK:BMAL1 controlled E-box activation (Travnickova-Bendova et al., 2002; Guilding and Piggins, 2007). The protein products of these genes then function in the core molecular feedback loop to reset the circadian oscillator (Yan and Silver, 2004). The protein products of these genes show phase dependent photic induction across the circadian cycle, induced upon exposure during subjective night, but not in response to subjective day photic stimulation, and their pattern of photic induction is similar to the pattern of photic phase-shifting of circadian locomotor behaviour (Pittendrigh and Daan, 1976b; Schwartz and Zimmerman, 1990) as well as the pattern of p-ERK photic induction (Obrietan et al. 1998; Butcher et al. 2003; Coogan and Piggins 2003; Nakaya et al. 2003). Phase-shifting of the molecular clock is seen to result in photically induced *Per1* gene expression in the SCN shell following a phase advancing light pulse, while *Per2* gene expression is seen to be induced in the SCN shell in association with phase delays (Yan and Silver, 2002). The PER proteins have been shown to be differentially increased in the different SCN subregions in response to phase resetting photic stimulation depending on the portion of the PRC at which photic stimulation occurs, and PER2 induction has been suggested to be important for phase delays, while PER1 induction is important for phase advances (Yan and Silver, 2004). Therefore, the induction of PER1 and PER2 in response to light may play roles in photic entrainment (Yan and Silver, 2004)

1.6.4 Photically induced immediate early genes

Light induced phase shifts are marked by the induction of photically inducible IEGs, including *egr-1 jun-B, c-fos* and *fosB*, which contain cAMP-responsive elements (CRE) in their promotor regions, and as previously described, bind CREB and are subsequently transcribed in response to photic stimulation in the subjective night (Greenberg et al., 1992; Perez-Albuerne et al., 1993; Rusak et al., 2002). Inhibition of ERK signalling attenuates photic IEG induction in the SCN (Dziema et al. 2003), highlighting the importance of this signalling pathway in IEG induction. c-Fos is the most extensively studied IEG in response to photic

stimulation, and a pattern of c-Fos mRNA and immunoreactive Fos protein are photically elevated in response to a light pulse during the subjective night and in response to lights on at dawn in the light:dark cycle. Photic stimulation during the subjective night causes rapid induction of c-Fos in the SCN within an hour of exposure and is associated with photic induced phase shifts of free running locomotor activity (Kornhauser et al., 1990; Rusak et al., 1990; Ebling et al., 1991; Mead et al., 1992). Light pulses as short as 5 minutes have been shown to cause c-Fos induction, while a saturating light pulse in mice shows an upper limit of c-Fos photically induced cells to be approximatey 20% of the SCN's cellular population (Castel et. al., 1997). c-Fos expression is observed in the SCN core in response to photic stimulation (Cai et al., 1997; Guido et al., 1999b; Schwartz et al., 1995; Schwartz et al., 2000; Takahashi, 1993) and is suggested to be involved in circadian function and photic entrainment (Aronin and Schwartz, 1991; Schwartz et al., 2000; Kornhauser et al., 1990).

1.7 SCN Pacemaker Connectivity

Both humoral and neuronal SCN signalling are involved in output from SCN to target sites (Kalsbeek et al., 2006).

1.7.1 SCN Efferent Projections

SCN efferent projections travel short distances, and it is mainly other hypothalamic regions that receive output information from the SCN (Abrahamson and Moore, 2001). These regions serve as relay stations, projecting SCN timing information throughout the nervous and endocrine systems, thereby allowing SCN communication to the CNS and the periphery (Dibner et al., 2010; Deurveilher and Semba, 2005; Kalsbeek et al., 2006). The SCN's principal efferent pathway extends to the subparaventricular zone (SPZ) and dorsomedial hypothalamus (DMH) which then extends to the hypothalamic paraventricular nucleus (PVN) (Watts, 1991). The SPZ and DMH receive input from the dorsomedial shell region of the SCN, while efferents from the SCN core terminate in the lateral SPZ (Leak et al., 1999). These SCN outputs must communicate SCN timing information to regions of the CNS involved in the regulation of various behavioural and physiological parameters including neuroendocrine and autonomic systems, body temperature, sleep wake

behaviour, locomotor activity and feeding behaviour (Rosenwasser et al., 2009). The dorsal SPZ (dSPZ) regulates body temperature (Lu et al., 2001), while the ventral SPZ (vSPZ) is involved in the regulation of locomotor activity and circadian rhythms of sleep and wakefulness (Saper et al., 2005). The DMH is involved in the regulation of circadian rhythms of sleep and wakefulness, locomotor activity, feeding and corticosterone secretion (Chou et al., 2003). Additionally, independent of the DMH and SPZ relay stations, the SCN is also seen to show direct but sparse projections to target areas of the DMH and SPZ involved in the circadian regulation of behaviour and physiology including, sleep and wakefulness rhythm regulation via the ventrolateral preoptic nucleus (VLPO), rhythmic hormonal release via the PVN and feeding rhythms via projections to orexin neurons (reviewed in Saper et al., 2005).

1.7.2 Humoral SCN Outputs

Signalling independent of neural projections has also been shown to convey circadian timing information. It has been shown that locomotor rhythmicity can be restored within SCN lesioned hamsters upon transplantation of viable SCN tissue encapsulated within a membrane and therefore preventing neural outgrowth and therefore neural communication with the adjacent hypothalamus (Silver et al., 1996b), indicating the presence of a secretory signal. Various neurochemical outputs from the SCN have been proposed, and these include signalling via AVP, VIP, PK2, cardiotrophin like cytokine (CLC) and TGF-α, which act upon their targets both synaptically and humorally (Cheng et al., 2002; Gerhold et al., 2005; Kalsbeek et al., 1992; Kramer et al., 2001; Kriegsfeld et al., 2002; Kraves and Weitz, 2006; Son et al., 2011). Further glucocorticoid (GC) signalling may serve to communicate information between the SCN and the periphery (Son et al., 2011), since GC's are seen to impact upon clock gene expression in peripheral tissues *in vivo* (Koyanagi et al., 2006; Son et al., 2008) and in mammalian cells *in vitro* (Balsalobre et al., 2000a).

1.8 Non-SCN oscillators and Pacemaker Hierarchy

1.8.1 Non-SCN neural oscillators

While many brain regions display SCN-dependent rhythms, fewer regions within the CNS have been shown to display SCN independent rhythms (Rosenwasser et al., 2009; Guilding and Piggins, 2007). The olfactory bulb shows persisting rhythmicity *in vivo* in the absence of the SCN and further, individual olfactory bulb neurons show autonomous rhythmicity in the expression of clock genes and in electrical activity in culture *in vitro* (Abraham et al., 2005; Granados-Fuentes et al., 2004a; Granados-Fuentes et al., 2004b). These oscillators are involved in the regulation of the free running period of SCN dependent locomotor activity behaviour and in the regulation of olfactory sensitivity with time of day (Granados-Fuentes et al., 2006). The lateral habenula and the arcuate nucleus have shown rhythms independent from the SCN (Guilding and Piggins, 2007; Rosenwasser et al., 2009). The retina also displays autonomous oscillators that function in the regulation of retinal function and visual sensitivity with time of day (Tosini and Menaker, 1996; Tosini et al., 2008). These oscillators then signal to the SCN regulating behaviour (Yamazaki et al., 2002; Tosini et al., 2007).

1.8.2 Oscillators in the periphery

Peripheral clocks are now known to be located in most tissues and organs. The peripheral clocks require synchronization from the SCN in order to display robust circadian oscillations among the oscillator populations, since in non-SCN cells, dampening of circadian molecular oscillations occurs readily as a result of gradual phasing out of individual rhythms between the cells (Guo et al., 2006; Nagoshi et al., 2004; Son et al., 2011). SCN communication to peripheral tissues ensures that peripheral oscillators are entrained to the environmental cycle via circadian regulation of gene transcription, enabling peripheral tissues to execute appropriate functions at the appropriate phase (Desai et al., 2004; Yamamoto et al., 2004; Kita et al., 2002; Storch et al., 2002). Clock genes within the SCN generate circadian rhythms in physiology and behaviour by synchronizing peripheral oscillators and their expression of clock genes via neural and endocrine signalling (Terazono et al., 2003; McNamara et al., 2001; Balsalobre et al., 2000a). Peripheral

oscillators have also been seen to be directly entrained by specific Zeitgebers including body temperature alterations and feeding-fasting schedules (Buhr et al., 2010; Damiola et al., 2000; Son et al., 2011).

1.10. The Immune Response

The mammalian immune system has evolved to defend the body against infection. The function of the immune system is to distinguish between "self" and "non-self" antigens and in the case of non-self antigens, to launch an appropriate immune response resulting in protection of the body against this microorganism. In this way, the immune system protects the body against bacteria, viruses, parasites, fungus etc. The immune system is comprised of two interdependent subdivisions, the innate immune system and the adaptive immune system which act in synergy forming a complex system. The non-specific innate immune system provides the first line of defence to pathogen invasion and comprises the physical barriers of the mammalian body such as the skin epithelial layer, and the non-specific protective cells. Cells of the immune system originate from the bone marrow, including those of myeloid and lymphoid origin. The myeloid cells include the phagocytic cells, the macrophages, neutrophils, eosinophils, basophils and dendritic cells, while the B lymphocytes, T lymphocytes and Natural Killer (NK) cells are of lymphoid origin. The adaptive immune system is antigen specific, and comprises the second set of host responses to invading microorganisms, and demonstrates immunological memory.

Inflammation followed by activation of the complement cascade are the first steps in the immune response. In response to infection and or tissue damage, the cells of the innate immune system react rapidly, adopting a complex pathophysiological state known as inflammation (Nathan, 2002; Foster and Medzhitov, 2009; Biswas and Lopez-Collazo, 2009). Pathogen recognition receptors (PRRs) are expressed in innate immune cells such as dendritic cells and macrophages (Medzhitov, 2001), and recognise pathogen-associated molecular patterns (PAMPs), structures that are present on the surface of invading microbes, leading to the activation of the innate immune response. PRR signalling following microbial recognition causes the production of inflammatory cytokines and type 1 interferon, leading to DC maturation and antigen presentation and consequently

adaptive immunity (Akira et al., 2001). The PRR Toll like receptor 4 (TLR4) is the primary PRR that detects Gram-negative bacteria and the endotoxins associated with them, such as lipolysaccharide (LPS) and Lipid A (Beutler, 2004; O'Neill and Bowie, 2007; Biswas and Lopez-Callazo, 2009). TLR signalling occurs via 2 pathways, an MyD88 dependent pathway resulting in the the early phase activation of the NF-κB signalling pathway (Akira and Takeda, 2004) and the production of proinflammatory cytokines, and an MyD88-independent pathway leading to late phase NF-κB activation as well as the activation of interferon regulatory factor (IRF3), resulting in the induction of IFN-inducible genes and maturation of dendritic cells (Yamamoto et al., 2003; Akira and Takeda, 2004). The TLR4 pathway signals through the adaptors MyD88, and TRIF, which is involved in the MyD88-independent pathway (O'Neill and Bowie, 2007; Akira, 2009; Biswas and Tergaonkar, 2007). (Figure 1.6).

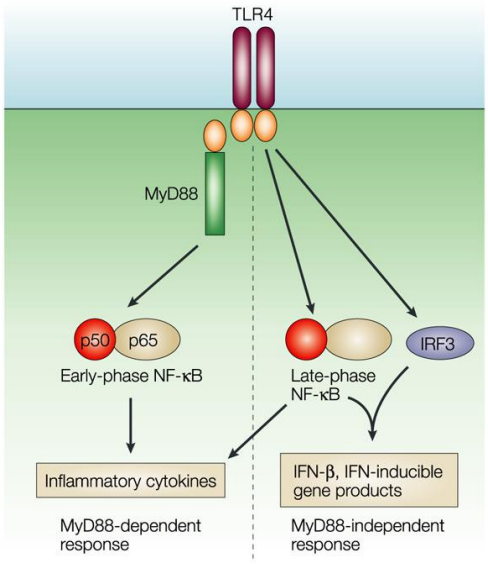


Figure 1.6: TLR4 signalling: MyD88-dependent and MyD88-independent pathways. *(Akira and Takeda, 2004).*

Under normal conditions, the translocation of NF-κB to the nucleus from the cytosol is prevented by tight association with inhibitors known as the I-KappaB's (IκBs) (Baeuerle and Baltimore 1988), and in response to signals that activate NF-κB, the enzyme IkappaB kinase (IκK) becomes activated and subsequently phosphorylates IκB which then dissociates from NF-κB and becomes degraded, allowing NF-κB to translocate to the nucleus and subsequent NF-κB DNA binding

and the induction of the transcription of different components of the inflammatory response including cytokines such as IL-6, NOS, IL-1β, TNF-α, immunoglobulins, Rel family proteins, IκB and neurotransmitters, as well as various other genes (Beg et al., 1993; Verma et al., 1995; Nomura, 2001; Marpegan et al., 2004; Yamamoto et al., 2003).

1.11. Cytokines and the CNS

The Blood Brain Barrier (BBB) forms a tight separation between the central nervous system (CNS) and the peripheral immune system and this lead to the historic view of the brain as an immunologically privileged organ, not susceptible to immune activation or inflammation. Far from now being considered an immunoprivileged site, the brain is known to contain a variety of immune components and is sensitive to inflammatory mediators (Schmidt et al., 2005). Immune mediators of the peripheral immune system such as cytokines, chemokines and complement activated proteins are synthesized in the CNS by neurons, microglia and astrocytes, and furthermore, these brain cells express receptors for these immune mediators (Schmidt et al., 2005). Cytokine receptors expressed in the brain are functionally and structurally similar to those in the periphery (Dantzer, 2004).

1.11.1. Neuroinflammation ensues following a systemic immune challenge.

Systemic cytokines transmit information to the brain following an immune insult, altering cytokine expression in regions of the CNS (Dantzer et al., 2004; Turrin et al., 2001). Cytokines can be either pro or anti-inflammatory and belong to five basic families, the chemokines, the growth and cell stimulating factors, tumour necrosis factors (TNF), interleukins (IL) and interferons (IFN). Activated macrophages release the proinflammatory cytokines TNF-α, IL-1β and IL-6, while T-cells release the anti-inflammatory cytokines IL-10 and IL-4 (Jacob et al., 2011). The CNS carries out immune surveillance of the local environment and in response to an insult following infection, disease or injury, a neuroinflammatory response is initiated, whereby the CNS cells produce inflammatory mediators including prostaglandins, free radicals, proinflammatory cytokines, and complement, subsequently inducing chemokines, adhesion molecules, immune cell recruitment and glial activation (Lucas et al., 2006; Schmidt et al., 2005). The brain contains a

cytokine network, whereby cytokines stimulate their own synthesis and that of others, subsequently leading to a cascade of proinflammatory mediator release (Taylor and Grossberg, 1998). The peripheral cytokines produced in response to an immune insult communicate with, and exert their effects on the CNS by a number of mechanisms including neural and humoral mechanisms and by peripherally activated immune cells (Capuron and Millar, 2011). Cytokine action at the brain endothelium, which contains saturable cytokine specific transport molecules and can actively transport cytokines across may be one mechanism, cytokines can cross the blood brain barrier at areas where it has become compromised, cytokine information may be communicated to the CNS via afferent nerves, for example, the vagus nerve which is seen to communicate various aspects of the immune response following LPS treatment, and peripherally activated monocytes may access the brain parenchyma causing the release of secondary messengers such as nitric oxide and prostaglandins following the activation of endothelial cells (Rivest et al., 2000; Konsman et al., 2002; Watkins et al.,1995; Plotkin et al., 1996; Quan and Banks, 2007; D'Mello et al., 2009; Capuron and Miller, 2011). (Figure 1.7).

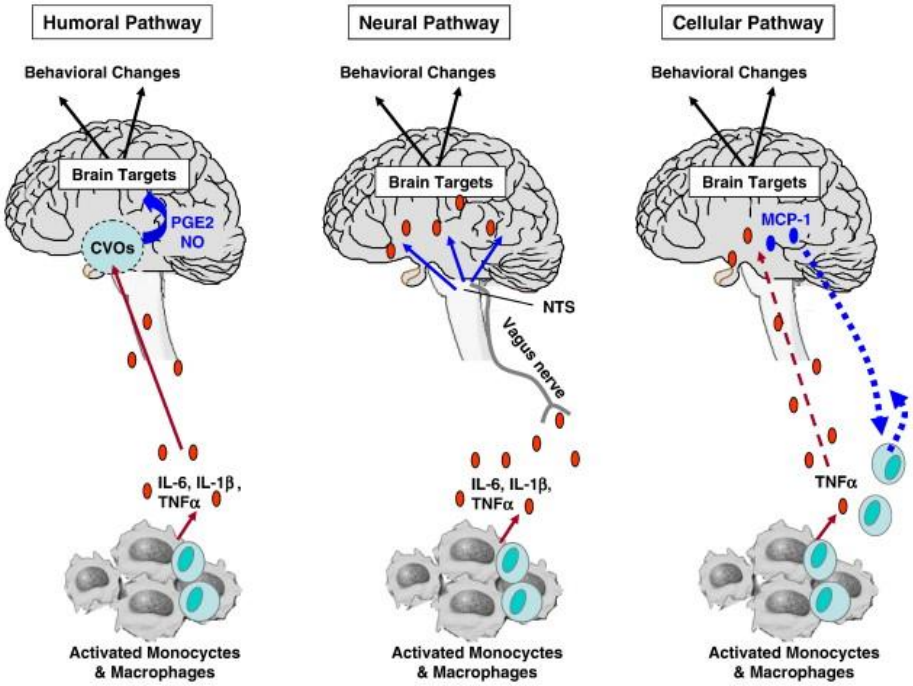


Figure 1.7: Depiction of the routes by which the systemic immune response may be communicated to the CNS. *(Capuron and Millar, 2011).*

1.11.2. Microglia in the CNS

Microglia are the resident innate immune cells of the CNS and comprise approximately 12% of cells in the CNS (del Rio-Hortega, 1932). Microglia are of mesodermal/mesenchymal origin, and are derived from progenitors that migrated from the periphery, entering the brain early in development (Chan et al., 2007; Kettenmann et al., 2011). These microglial cells migrate throughout the CNS and adopt a ramified "resting" morphology (Kettenmann et al., 2011). A stable population of microglial cells exist in the healthy CNS, however it has been shown that following BBB damage, a subpopulation of monocytes enter the CNS and transform into microglial cells (Kettenmann et al., 2011; Mildner et al., 2007). In response to an immune challenge within the CNS, microglia become activated, and activated microglia are phenotypically and functionally distinct from those in the resting state. The activation of microglia sees the cells progress through a series of morphological phenotypes, the end stage of which they appear hypertrophic and are seen to resemble phagocytic cells (Kreutzberg, 1996). In the healthy mature CNS, the resting ramified morphology of microglia is characterized by a small soma with fine cellular processes that are motile and actively scan the environment of the CNS (Kettenmann et al., 2011). Compared to resting microglia, activated microglia exhibit an amoeboid morphology with a larger cell body and processes that are shorter and denser than those seen to project from resting microglia (Dheen et al. 2007; Kaur and Ling 1991; Kaur et al. 1985). Reactive microglia are generally evidenced as small, spherical cells with no processes, however an ameboid-like or rod-shaped morphology may also be observed (Davis et al. 1994). (Figure 1.8).

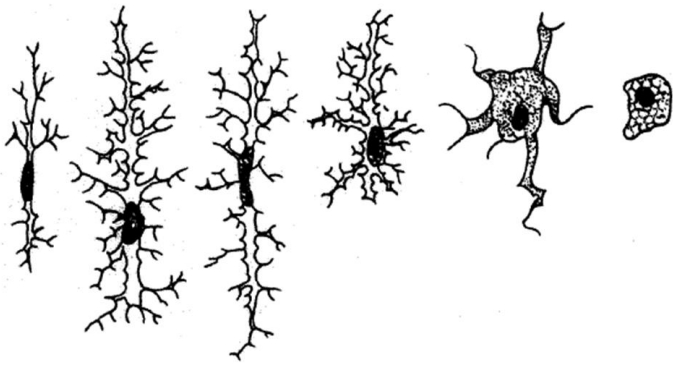


Figure 1.8: Morphology of microglia. *Images from left to right illustrate the transformation of microglia from a resting state into an activated state. These*

activated microglia resemble phagocytic cells at the end stage (adapted from Kreutzberg, 1996).

Microglia can communicate with neurons and cells of the immune system, and contain receptors for immune mediators such as cytokines and chemokines, as well as receptors for a variety of neuroactive compounds (reviewed in Kettenmann et al., 2011). Microglial activation in the CNS parenchyma seems to be vital for communication between the immune system and the brain (Konsman et al., 2002; Combrinck et al., 2002), playing either harmful or beneficial roles. Following CNS insult, the activation of microglia is essential for host defence and neuronal survival. Activated microglial cells can become motile and follow a chemotactic gradient migrating to the site of injury, as well as provide additional defence and protection by increasing microglial numbers through proliferation of local microglial densities, and further, can phagocytose cells and cellular compartments (Kettenmann et al., 2011). Microglial activation may also play a beneficial role by the release of neurotrophic molecules, promoting aspects of regeneration (Streit et al., 1999; Schwartz, 2003; Ekdahl et al., 2003).

However in response to a diverse range of stimuli such as neuronal injury or environmental toxins (Block et al., 2007), the overactivation or dysregulation of these cells can be harmful and have neurotoxic consequences (Polazzi and Contestabile, 2002), releasing a variety of inflammatory mediators which have been shown to be neurotoxic *in vitro* (Ekdahl et al., 2003), such as IL-1β, IL-6, TNF-α, NO, and reactive oxygen species (ROS) (Pocock and Liddle, 2001; Hanisch, 2002; Gebicke-Haerter, 2001; Vallieres et al., 2002), as well as induce the activation of the complement cascade (Jacob et al., 2007). Overactivation of microglia can contribute to neuronal damage during neurodegenerative disease (reviewed in Block et al., 2007).

Following microglial activation, microglial cells can go through an intermediate "primed" state (Perry, 2004). On-going pathology within the CNS primes microglia, whereby these are excessively reactive to subsequent immune challenges including those in the periphery (Cunningham et al., 2005; Lunnon et al., 2011). In the CNS, studies indicate the presence of "primed" microglia in chronic inflammatory conditions such as in aging, in obesity and in Alzheimer's Disease patients (Dilger and Johnson, 2008; Bilbo and Tsang, 2010; Holmes et al., 2009; Holmes et al., 2011) and the stimulation of these cells may have detrimental

consequences, such as impact upon cognitive decline or result in delerium (Perry et al., 2003).

1.11.3. The presence of inflammatory cytokines in the brain under normal conditions

As well as their role in immunomodulations, cytokines play roles within the brain in the absence of immune challenge. In the brain under normal physiological conditions, proinflammatory cytokines may play roles in sleep processes. Various studies show that non-REM sleep is increased following the activation of TNF and/or IL-1 systems, while NREM sleep is decreased following inhibition of these (Opp, 2005). REM sleep is seen to be diminished while non-REM sleep is increased following injection of TNF-α into the preoptic area of the hypothalamus (Kubota et al., 2002). Non-REM sleep is increased following injection of IL-1β into the dorsal raphe nucleus of rats (Manfridi et al., 2003). Double knockout mice for the IL-1 type 1 receptor and the TNF receptor show attenuations in REM and non-REM sleep following sleep deprivation studies (Baracchi and Opp, 2008). Additionally, administration of an NF-κB inhibitor peptide is seen to inhibit spontaneous sleep, supporting the involvement of a brain-cytokine network in sleep regulation (Kubota et al., 2000).

1.12. Immune-Circadian Communication

A bidirectional communication is suggested to exist between the immune system and the circadian system (Coogan and Wyse, 2008), with circadian variation and regulation seen in components of the immune system and immune cells themselves acting on peripheral oscillators and on the master pacemaker in the SCN.

1.12.1 Circadian regulation of immune function

Immune function is thought to be strongly influenced by the circadian timekeeping system and light:dark cycles (Roberts, 2000), and disruption of the circadian system is seen to impact upon immune processes as evidenced through jetlag and sleep deprivation studies (Silver et al., 2012), and alterations in cellular adhesion molecule expression on monocytes and lymphocytes have been shown in studies of acute sleep disruption (Redwine et al., 2004), while studies in humans

have shown that early night sleep deprivation reduces natural killer and cellular immune responses (Irwin et al., 1996).

Furthermore, some cells of the immune system contain molecular clocks which play roles in the regulation of immune function (Logan and Sarkar, 2012). Circadian variation of immune components is seen in the blood in hormone levels, cytokines and numbers of circulating haematopoietic cells (Haus and Smolensky, 1999), and these oscillate in accordance to the rest activity pattern of the species, i.e. whether they are diurnal or nocturnal (Scheiermann et al., 2013). Both mice and humans have shown circadian variations in different immune parameters such as diurnal variations in cytokine levels (Scheff et al., 2010; Petrovsky et al., 1998; Petrovsky and Harrison, 1998; Holzheimer et al., 2002), and circadian regulation of natural killer cell activity, antigen presentation and lymphocyte proliferation has also been shown (Esquifino et al., 1996; Arjona and Sarkar, 2005; Silver et al., 2012). Further, cytokine production, apoptosis and leukocyte trafficking and proliferation are known to be modulated by the neuroendocrine and autonomic nervous systems, both of which show circadian oscillations (Petrovsy, 2001). The proinflammatory cytokines IL-1β and TNF, as well as levels of glucocorticoids, noradrenaline and adrenaline show circadian variation in expression which peaks at the onset of the activity phase (Haus and Smolensky, 1999; Haus et al., 1983; Scheiermann et al., 2013), and it is thought that the circadian regulation of immune pathway components may serve to enhance immune responsiveness at the time of the circadian cycle at which infection is most likely to occur.

1.12.2 Communication of circadian information to the immune system

Circadian timing signals are transmitted to immune cells through endocrine and neural signals (Logan and Sarkar, 2012) and the SCN communicates with the PVN and arcuate nuclei through axonal connections to regulate stress and immune function (Saeb-Parsy et al., 2000; Kalsbeek and Buijs, 2002).

The SCN and the arcuate nuclei (ARC) are thought to comprise a neuroimmune axis. The ARC and the SCN exhibit bidirectional inhibitory and excitatory connectivity allowing for feedback control and regulation of circadian networks (Saeb-Parsy et al., 2000; Logan and Sarkar, 2012). Opioids are thought to be involved in the SCN-ARC circadian regulation of the immune system, and

perikarya producing the endogenous opioid peptide beta endorphin (β-EP) are found in the ARC, and project to the hypothalamus along with other regions of the brain (O'Donohue and Dorsa, 1982; Wilcox et al., 1986). β-EP is derived from precursor proopiomelanocortin (POMC) (Nakanishi et al., 1979), and POMC peptides are also found in the pituitary gland (Rossier et al., 1977), in macrophages (Mousa et al., 2004) and in lymphocytes (Sitte et al., 2007; Labuz et al., 2010; Logan and Sarkar, 2012). The ARC are thought capable of generating circadian oscillations in electrical activity and hormonal output, including POMC oscillations, independent of the SCN (Wyse and Coogan, 2010; Guilding and Piggins, 2007; Chen et al., 2004). Oscillations in NK cytokine expression and cytolytic activity have been shown to mirror those in ARC POMC (Chen et al., 2004) and abolishing or dampening ARC POMC oscillations similarly affects oscillations in NK cells (Arjona et al., 2004; Arjona and Sarkar, 2006a,b; Chen et al., 2004; Logan and Sarkar, 2012). Hypothalamic β-EP stimulation of NK cytotoxicity appears to involve corticotrophin releasing hormone (CRH) neurons in the PVN that regulate sympathetic signalling, since blocking the function of these neurons inhibits this stimulation (Boyadjieva et al., 2006). As well as NK cytotoxicity enhancement (Boyadjieva et al., 2006), β-EP is also known to enhance the production of granzyme B (GZMB) and interferon gamma (IFN-γ) (Dokur et al., 2004; Boyadjieva et al., 2001; Logan and Sarkar, 2012) and to stimulate the proliferation of spleenic lymphocytes (Boyadjieva et al., 2002), and it has been suggested that the SCN, ARC oscillators, and peripheral β-EP may regulate the rhythmicity of immunocompetent cells, in particular NK cells (Logan and Sarkar, 2012). (Figure 1.9).

The SCN, PVN and sympathetic nervous system are thought to form a network via which the circadian system regulates stress and the immune system (Logan and Sarkar, 2012), with the SCN regulating sympathetic and parasympathetic outflow to peripheral tissues, thereby modulating this neuroimmune system (Buijs et al., 2003, 2008). Circadian information is passed to the immune system through SCN regulation of the PVN's endocrine and autonomic neuronal activity (Arjona and Sarkar, 2008; Boyadjieva et al., 2001). Inhibitory and excitatory SCN efferents regulate sympathetic and parasympathetic neurons in the SCN (Kalsbeek et al., 2008). CRH and oxytocin producing neurons in the PVN govern autonomic inputs to the periphery by modulating sympathetic preganglion neurons and parasympathetic neurons in the dorsal nucleus of the vagus (Buijs et al., 2003;

Dibner et al., 2010; Logan and Sarkar, 2012) and are thought to be the PVN's neuronal targets for SCN projections (Stanley et al., 2010). The lymph nodes, spleen and thymus receive autonomic input (Bellinger et al., 1993). The spleen releases norepinephrine (NE) upon sympathetic noradrenergic innervation by hypothalamic neurons, mediating the activity of various lymphocytes including macrophages and NK cells (Bellinger et al., 1993; Dokur et al., 2004; Elenkov et al., 2000; Nance and Burns, 1989; Logan and Sarkar, 2012). A circadian pattern of expression is observed in NE input to the spleen, and abolishing this has been shown to attenuate expression patterns of certain cytokines and cytolytic factors in splenocytes and NK cells (Logan et al., 2011). The liver which contains large quantities of lymphocytes, also receives clock regulated sympathetic input (Cailotto et al., 2005, Gao et al., 2009; Terazono et al., 2003). (Figure 1.9).

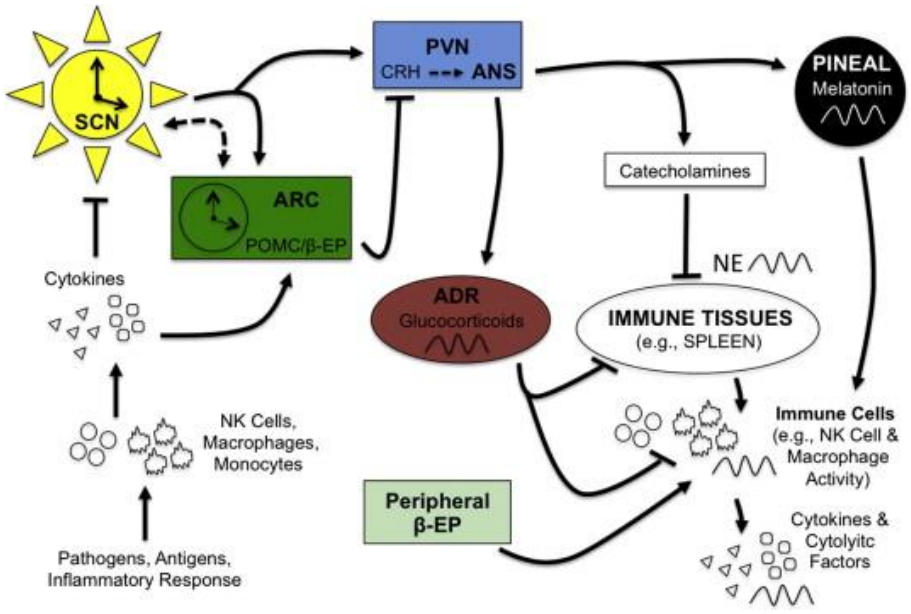


Figure 1.9: Conceptual framework of biological clocks regulating immune function. *Oscillators in the SCN, ARC, and immune cells regulate circadian rhythms of immune function. The SCN and ARC project to the PVN of the hypothalamus modulating autonomic nervous system (ANS) endocrine and neural signalling to immune tissues in the periphery. When activated, cytokines and immune mediators modulate clocks in the SCN and ARC. ADR, adrenals; β-EP, beta-endorphin; CRH, corticotropin releasing hormone; NK, natural-killer cells; POMC, proopiomelanocortin. (Logan and Sarkar, 2012)*

Immune function can also be influenced by endocrine hormones which act on circadian oscillations of immune cells. Pineal melatonin and glucocorticoid which show antiphase rhythms play strong roles in the circadian regulation of the immune

response (Logan and Sarkar, 2012). The SCN modulation of autonomic input to adrenal glands and PVN neurons producing CRH regulates the rhythmic expression of peripheral glucocorticoid (Dibner et al., 2010), and further, local oscillators in the adrenal gland regulate the sensitivity of the gland to Adrenocorticotropic hormone (ACTH) and adrenal GC release (Dickmeis, 2009). Peripheral glucocorticoids show peak expression levels during the day and are known to regulate immune processes such as the production of cytokines, cellular proliferation, leukocyte distribution and apoptosis (Fu and Lee, 2003; Logan and Sarkar, 2012) and may then be important in communicating circadian information to immune cells (Haus and Smolensky, 1999). SCN autonomic outputs generate circadian oscillations in pineal melatonin which exhibits night-time peaks (Logan and Sarkar, 2012), and rhythmic melatonin plays roles in the regulation of pro and anti-inflammatory cytokines in the spleen (Naidu et al., 2010), as well as in the regulation of expression patterns of NK cell activity, cytokine production and leukocyte proliferation in bone marrow cells (del Gobbo et al., 1989; Drazen et al., 2001; Haldar et al., 1992; Matsumoto et al., 2001; Logan and Sarkar, 2012).

Circadian regulation of immune system transcription factors has been shown, including that of signal transducer and activation of transcription 3 and 5 (STAT 3 and 5) which play roles in the adaptive and innate immune response in cytokine signalling pathways (Alexander and Hilton, 2004; Fu, 2006; Logan and Sarkar, 2012), and studies have suggested *Cry2* to regulate STAT3 (Hoffman et al., 2009). Further, both Egr-1 and NF-κB pathways which play roles in the immune system show circadian regulation (Bozek et al., 2009, Logan and Sarkar, 2012). A direct molecular link has previously been shown between the circadian system and the innate immune system with circadian control seen in the expression and function of Toll-like receptor 9 (TLR9), an innate immune pathogen-recognition receptor (Silver et al., 2012). *Per2*^{brdm1} transgenic mice which exhibit a defective molecular clock show altered TLR9 expression and function (Silver et al., 2012). Studies by Spengler and colleagues (Spengler et al., 2012) have shown the clock gene protein product CLOCK to be a positive regulator of NF-κB mediated transcription independent of BMAL1, and stimulation of NF-κB is seen to be reduced in cell lines isolated from *Clock* deficient mice compared to wild type controls. Further, these studies show that CLOCK is found in protein complexes with the NF-κB subunit p65.

Circadian variations in aspects of the LPS-TLR4 response pathway, including FOS, JUN, RELA and IκBα, have been shown in peritoneal macrophages, indicating a role for circadian regulation in macrophage LPS activated pathways (Keller et al., 2009). Further, these studies show macrophages to be important in the circadian variation in responsiveness to LPS, and macrophage expression of TNF-α and IL-6 resulting from LPS stimulation is known to depend on circadian clock phase (Keller et al., 2009). Endotoxin treatment shows phase dependent variation in the induction of inflammatory mediators, showing markedly increased levels in serum at the beginning of the active phase compared to at the beginning of the resting phase, and this temporal gating has been shown to be dependent on clock genes, in particular *Rev-erbα,* implicating the circadian clockwork, in the regulation of innate immune responses (Gibbs et al., 2012).

Many inflammatory diseases display time of day variation in symptom severity and these circadian patterns are outlined in the table below adapted from a recent review by Scheiermann and colleagues (2013).

Table 1: Influence of circadian timing on disease manifestations. *(Scheiermann et al., 2013).*

Species	Disease or model	Oscillating parameter	Acrophase	Refs
Human	Rheumatoid arthritis	Stiffness, pain, serum TNF, serum IL-6	Time of Day (TOD) 5–8	Cutulo, 2012.
	Allergic rhinitis	Sneezing, nasal congestion	TOD 6	Smolensky et al., 2007.
	Bronchial asthma	Bronchoconstriction	TOD 6	Smolensky et al., 2007.
		Sputum eosinophils, serum IL-5	TOD 7	Panzer et al., 2003.
	Myocardial infarction	Pain	TOD 9	Muller et al., 1985.
	Ischaemic stroke	Hypertension	TOD 6–CT12	Gupta & Shetty, 2005.
	Sickle cell vaso-occlusive crisis	Hospital admission	TOD 18	Auvil-Novak et al., 1996.
Mouse	LPS-induced inflammation	Lethality	CT16	Halberg et al., 1960.
		Lethality, leukocyte recruitment, ICAM1	ZT13	Scheiermann et al., 2012.
	Pneumococcal infection	Lethality	CT16	Feigin et al., 1969 Shackelford & Feigin, 1973.
	CLP-induced sepsis	Lethality, TLR9 on splenic macrophages	ZT19	Silver et al., 2012.
	Coxsackie B3	Gross myocardial lesions	CT16	Feigin et al.,

Species	Disease or model	Oscillating parameter	Acrophase	Refs
	virus infection			1972.
	TNF-induced inflammation	Lethality	ZT10	Hrushesky et al., 1994.
	Sickle cell vaso-occlusion	Lethality, leukocyte adhesion, RBC–WBC interactions	ZT13	Scheiermann et al., 2012.

1.12.3 The effects of circadian misalignment on the immune system

Given the proportion of immune processes regulated by the circadian system, it is not surprising that dysfunction of timekeeping activities perturbs immune system function. Chronic disruption of the circadian system is known to impact on health and can lead to a higher risk of many pathologies.

Human studies of those working rotating shifts highlight an increased risk for developing certain cancers, including prostate (Conlon et al., 2007; Kubo et al., 2006), breast (Davis et al., 2001; Hansen, 2001; Schernhammer et al., 2001), non-Hodgkins Lymphoma (Lahti et al., 2008) endometrial (Viswanathan and Schernhammer, 2009) and colon cancer (Kloog et al., 2009; Schernhammer et al., 2003). Furthmore, shift workers show a higher prevalence for diseases such as diabetes, obesity and cardiovascular disease (Tuchsen et al., 2006; Morikawa et al., 2005; Chaput et al., 2006). Inflammation is a key risk factor in the development of many of the pathologies associated with chronic circadian misalignment. The robustness of circadian oscillations in salivary cortisol and in sleep wake cycles strongly predict survival in colon and breast cancer patients (Mormont et al., 2000; Sephton et al., 2000), pointing to attenuations of the circadian system during the pathogenesis of the disease.

Murine studies using chronic jet lag (CJL) paradigms or SCN ablation to induce impairment of molecular clock function to assess the effects of circadian disruption on immune processes have further highlighted the importance of circadian regulation of immune function in health. Clock genes are known to regulate certain tumour suppressor proteins, and disruption of the molecular clock through downregulation of clock gene expression in affected tissues and tumour cells is involved in cancer progression (Filipski et al., 2006; Fu and Lee, 2003), and further, restoration of clock gene expression phase in peripheral tissues slows the rate of tumour growth (Yasuniwa et al., 2010). CJL subjected mice and *Clock* mutant mice show attenuations in DNA repair, apoptosis and cellular proliferation, linked with an

increase in tumour growth (Filipski et al., 2006; Fu and Lee, 2003; Miyazaki et al., 2010; Sukumaran et al., 2010). An increase in the oncogenic activation of tumour cell proliferation pathways has been shown in another study (Lee et al., 2010) where sympathetic output to peripheral tissues is impacted upon by CJL paradigms. CJL mice show altered clock gene expression in the SCN, liver, thymus and peritoneal macrophages, and is associated with inflammatory response perturbations (Castanon-Cervantes et al., 2010). These studies show an enhanced sensitivity to endotoxin administration both *in vitro* in macrophages isolated from chronically shifted mice, and *in vivo*, with an increase in LPS induced mortality observed in chronically shifted mice compared to controls. Chronically phase shifted aged mice also show an increase in mortality rates compared to un-shifted aged matched controls (Davidson et. al, 2006).

In other studies of the impacts of clock gene mutations on immune parameters, loss of IFN-γ circadian expression is seen in mutant *Per2* animals (Arjona and Sarkar, 2006a, b), along with endotoxic shock resistance following LPS treatment, as well as alterations in NK cell function and decreases in production of IL-1β (Liu et al., 2006), indicating a role for this clock gene in mediating the LPS induced immune response. Circadian rhythmicity is seen to be suppressed in various immune related genes and in circulating leukocytes following *Clock* mutation (Oishi et al., 2003, 2006). Further, in mouse embryonic fibroblasts derived from *Clock* mutant mice possessing a defective clock system, increased levels of the NF-κB component, RelB, are observed as well as altered responsiveness to LPS treatment *in vitro*, with significantly reduced LPS induced levels of IL-1β, Cxcl1 (Chemokine (C-X-C motif) ligand 1) and IL-6 (Bellet et al., 2012). More recently, Logan and colleagues (2013) have described significantly altered rhythms of cytokine and cytolytic factors in splenic NK cells and perturbations in the expression of *Per2* and *Bmal1* in *Per1* deficient mice, indicating that *Per1* acts via NK cellular clocks to modulate immune pathways.

1.12.4 The immune system impacts upon the circadian system

Many studies have highlighted the ability of immune mediators themselves to impact upon circadian timekeeping processes, acting on the expression of clock genes in the SCN and in the periphery and in cell lines (Okada et al., 2008; Cavadini

et al., 2007; Murphy et al., 2007; Tsuchiya et al., 2005; Ohdo et al., 2001; Koyanagi and Ohdo, 2002), as well as phase-shifting SCN rhythmicity resulting in phase advances or delays of circadian locomotor behaviour depending on circadian phase of administration (Marpegan et al., 2005; Sadki et al., 2007; Boggio et al., 2003). Cytokine expression and that of their corresponding receptors is seen to oscillate in the brain in a circadian manner, however their role in timekeeping processes remains unclear. IL-1β shows diurnal variation in expression in the hypothalamus, similar to TNF-α in this region which is also seen to oscillate in the brain stem and forebrain (Taishi et al., 1997; Logan and Sarkar, 2012). IL-1β and TNF-α are found to be expressed within the site of the master oscillator itself, and in the areas surrounding the SCN where circadian oscillations of their receptors are also seen (Beynon and Coogan, 2010; Sadki et al., 2007; Lechan et al., 1990).

1.12.5 Inflammatory mediators cause functional alterations in the SCN

The SCN has been shown to be functionally activated following an immune challenge. Protein synthesis of the immediate early gene *c-fos* is rapidly induced in neurons in response to metabolic activation (Morgan and Curran, 1991; Sagar et al., 1988) and is used as a marker of neuronal metabolic activation (Hoffman et al., 1993). Acutely, following low dose peripheral LPS administration, c-Fos expression is induced in the SCN shell at CT15 (Marpegan et. al., 2005), while a septic LPS treatment has been shown in the acute stages following administration to induce significant expression of c-Fos in the SCN, along with expression of the p65 subunit of the NF-κB pathway (Beynon and Coogan, 2010). Photically induced c-Fos was impaired in the SCN of mice following chronic treatment with 1mg/kg peripheral LPS (Palomba and Bentivoglio, 2008). Trypanosomiasis has been shown to affect SCN functional activation, altering photic induced c-Fos (Peng et al., 1994) and basal c-Fos expression patterns in the SCN (Bentivoglio et al., 1994). Central administration of TNF-α/IFN-γ has been shown to induce c-Fos expression in the SCN shell in the subjective night, and in the SCN core in the subjective day, while in aged mice at both times in the circadian cycle the treatment resulted in c-Fos induction in the dorsomedial shell (Sadki et al., 2007). The SCN projects to the PVN which is involved in regulation of autonomic and endocrine outputs, including production of glucocorticoids (Kalsbeek et al., 2006) and in this way the circadian

system may be involved in the modulation of the immune system (Coogan and Wyse, 2008). The PVN may be directly stimulated by and respond to an immune challenge (Leone et al., 2012), since administration of cytokines or LPS, causes c-Fos activation in the PVN (Marpegan et al., 2005; Sadki et al., 2007; Beynon and Coogan, 2010). Treatment with 50 μg/kg LPS injection in subjective night has been shown to induce PER1 expression in this hypothalamic region at CT15 (Paladino et al., 2010) and at ZT 22 (Takahashi et al., 2001). The LPS receptor TLR-4 is expressed in the PVN and circumventricular organs (Laflamme and Rivest, 2001) and AVP secretion in the rat SCN is increased following LPS slice treatment *in vitro*, indicating that LPS can directly modulate neuroendocrine signalling (Nava et al., 2000). These studies highlight that the circadian system is directly responsive to inflammatory mediators.

1.12.6 Neuroinflammation and the Circadian System

Modulations of the circadian system are seen in many conditions with a neuroinflammatory component. Neuroinflammation can play a harmful role within the CNS due to the activation or over activation of cells involved in the inflammatory response, subsequently causing impairments in neuroprotection and repair and increased neurodegeneration (Taupin, 2008) and there is evidence that inflammation within the brain contributes to both acute and chronic degenerative disorders and may contribute to psychiatric diseases (Lucas et al., 2006). Modulation of circadian timekeeping processes and constituents of the clock have been shown in Alzheimer's Disease (AD) and may be due to underlying neuroinflammation (Hatfield et al., 2004; Ancoli-Israel et al., 1989; Prinz et al., 1982; Okawa et al., 1991; Satlin et al., 1991; Witting et al., 1990; Satlin et al., 1995; Swaab et al., 1985; Zhou et al., 1995). A chronic low grade neuroinflammation is seen to occur in the normal, healthy aging brain including in the SCN (Godbout and Johnson, 2009; Deng et al., 2010) as well as circadian timkeeping impairments (Vitiello et al., 1986; Weitzman et al., 1982; Weinert et al., 2000; Hofman et al., 2006; Kolker et al., 2003; Rosenberg et al., 1991; Zee et al., 1992; Sellix et al., 2012). Altered circadian function is seen in diseases characterized by chronic infection and neuroinflammation, such as Simian immunodeficiency virus (Huitron-

Resendiz et al., 2007), and African trypansomiasis (Lundkvist et al., 2002; Lundkvist et al., 2010).

1.13 Sepsis

1.13.1 Sepsis and Septic Encephalopathy

Sepsis is a profound pathophysiological state elicited by overwhelming infection and successive massive systemic inflammation associated with multisystem organ failure and high mortality rates (Mann et al., 2012). Sepsis is the leading cause of death in critically ill patients, with a mortality rate of severe sepsis up to 70% (Doi et al., 2009). Classically, gastrointestinal, cardiovascular, pulmonary and renal dysfunction comprise the sepsis induced multiple organ failure (Bone et al., 1997; Wheeler and Bernard, 1999). Human sepsis is characterized by an imbalance between the anti-inflammatory and pro-inflammatory responses to a pathogen (Hotchkiss and Karl, 2003), and is seen to involve an inflammatory response stage consisting of a proinflammatory response and an immunosuppressed stage. During the immunosuppressed stage the immune response is depressed and can result in nosocomial infection and death due to decreased lymphocyte proliferation and responsiveness, increased lymphocyte apoptosis and altered antigen presentation (AP) (Hotchkiss and Karl, 2003; Riedemann et al., 2003). A circadian component in sepsis progression and severity exists, with septic patients displaying an increased mortality risk between 2 and 6 a.m. (Hrushesky and Wood, 1997).

Septic encephalopathy is a neurological complication due to sepsis and systemic inflammatory response syndrome (SIRS) evident in up to 70% of patients with severe septic illness and is the most common form of encephalopathy seen in patients in intensive care units (Wilson and Young, 2003; Jacob et al., 2010). The manifestations of SE range from confusion and agitation to delirium, stupor or in life threatening cases, coma (Ebersoldt et al., 2007). Up to 50% of sepsis survivors are seen to suffer from post-septic encephalopathy which is characterized by long-lasting cognitive impairment including attenuations in concentration, attention and memory and/or a global loss of cognitive function (Streck et al., 2008). Neuroinflammation has been implicated in the pathogenesis of various disorders associated with cognitive impairment (Nelson et al., 2002; Katsuse et al., 2003; Perry et al., 2003), including Septic Encephalopathy (SE) (Jacob et al., 2010) and post-septic

encephalopathy is characterized by long-lasting cognitive impairment (Streck et al., 2008).

1.13.2 The CNS is accessed by peripheral inflammatory mediators during sepsis.

SE is suggested to be caused by the immune mediators of the sepsis response, as opposed to in response to a particular infectious agent, since encephalopathy occurs in patients in situations where infection is due to both gram negative and gram positive bacteria, when no causative agent is identifiable and also in response to fungal infections (Jacob et al., 2010). Multiple factors are thought to contribute to this condition including disruption of the blood brain barrier, reduced cerebral blood flow (Davies, 2002), oedema (Gardenfors et al., 2002; Bartynski et al., 2006), increases in proinflammatory mediators (Boos et al., 2005) and inflammation. SE is probably due to the effects of inflammatory mediators on the brain or as a result of brain cells cytotoxic response to these mediators, since reducing inflammation that occurs during the septic state has been shown to alleviate the severity of sepsis (Jacob et al., 2010). The inflammatory process may disrupt the BBB through its effects on cerebrovascular endothelium and astroglial function (Sharshar et al., 2005; Esen et al., 2005), resulting in abnormal neurotransmitter composition and attenuations of neuronal function (Shimizu et al., 1999; Stocchetti, 2005; Semmler et al., 2008). Studies have suggested that inflammation, increased permeability of the BBB, leukocyte recruitment and neuronal apoptosis are contributed to by cytokines and the complement cascasde (Ward, 2008a; Ward, 2008b; Annane, 2009; Bengtson and Heideman, 1988; Guo et al., 2004; Huber-Lang et al., 2002; Jacob et al., 2007; Jacob et al., 2010). Activated leukocytes enter the CNS by adhering to intracellular adhesion molecules (ICAMs) whose expression is increased on the microvessels on the BBB during sepsis (Hofer et al., 2008; Bohatschek et al., 2001). Proteins of the complement cascade and inflammatory mediators such as angiopoietin 1, IL-1β and TNF attract leukocytes to the sites of injury (Smedegard et al., 1989; Boos et al., 2005) and activated leukocytes themselves secrete cytokines (Merrill and Benveniste, 1996). Compromises in the integrity of the BBB during sepsis allows entry of cytokines to the CNS which would normally be prevented due to their size and hydrophilicity (Pan et al., 1997; Gimenez et al., 2004) and further, these can increase BBB permeability themselves and stimulate their own production at

vascular sites (Jacob et al., 2011). Cytokines such as TNF-α stimulate glia to express iNOS in models of SE (Jacob et al., 2007), leading to an increase in NO levels which may play a role in neuronal dysfunction. Excess NO during SE has been shown to be involved in alteration of the regulation of cerebral blood flow, and synaptic transmission, leading to neuroendocrine, behavioural activity and memory formation perturbations (Jacob et al., 2011).

Complement proteins are involved in crosstalk with cytokines and may enhance the inflammatory response by modulating the production of cytokines and chemokines leading to apoptosis, cell necrosis and edema (Jacob et al., 2011). The complement system is implicated in the pathogenesis of sepsis and the complement proteins C3 and C5, and their by-products the anaphylatoxins, C3a and C5a that are generated following complement activation mediate the effects of this (Jacob et al., 2011). Following complement activation, C3 is seen to be up-regulated leading to breakdown of the BBB, increased water content, increased glial activation, and an increase in the expression of TLR4 followed by perturbations in aquaporin 4, TNF and iNOS expression (Ducruet et al., 2009; Komotar et al., 2009; Mocco et al., 2006). In patients with septic shock C3a was seen to be increased (Stove et al., 1996). C5a is increased following administration of endotoxin initially within the cerebral endothelium, then within neighbouring microglial cells and finally deeper within the brain parenchyma, and C5a inhibition is seen to prevent BBB damage. The peripherally induced sepsis pituitary response is prevented, along with a reduction in sepsis induced PVN and amygdala neuronal activation following inhibition of the complement cascade (Flierl et al., 2009; Jacob et al., 2011).

Humoral signalling following sepsis induction exerts immunomodulatory effects. The sympathetic nervous system is stimulated to release epinephrine, which binds to receptors on immune cells modulating the immune response (Padgett and Glaser, 2003). The afferent vagus nerve regulates the release of these hormones by recognizing the proinflammatory cytokines and stimulating components of the central stress response system (Elenkov et al., 2000), leading to the secretion of cortisol via HPA axis activation (Webster et al., 2002), and epinephrine via sympathetic nervous system stimulation (Padgett and Glaser, 2003), both of which play immunoregulatory roles. Crosstalk between the neuroendocrine pathways, cytokines and the autonomic nervous system has been suggested to play important roles during sepsis in the modulation of inflammation (Elenkov et al., 2008). The

HPA axis through the production and release of glucocorticoids inhibits pro-inflammatory cytokine expression (Webster et al., 2002). The cholinergic anti-inflammatory pathway also plays roles in the modulation of the inflammatory response, whereby the parasympathetic nervous system is stimulated to release acetylcholine which regulates the immune response (Pavlov et al., 2003).

Altered neurotransmission may impact upon the CNS during SE. Neurotransmitters of the sympathetic nervous system modulate inflammation during sepsis, and epinephrine action through its activation of adrenergic receptors and increased levels of intracellular cAMP is seen to have a net anti-inflammatory effect (Van der Poll et al., 1996; Van der Poll, 2000). Glutamatergic neurotransmission is thought to play roles in SE, since inhibition of its synaptic release in a rat model has been shown to reduce sepsis induced neurological effects and improve survival (Toklu et al., 2009). Further, increased levels of tryptophan and indolamine have been shown in the brain of rats during sepsis (Freund et al., 1985), highlighting further that sepsis causes alterations in neurotransmitters.

1.13.3 Experimental Sepsis Models

Experimental sepsis in animal models can be induced by a variety of mechanisms, the most commonly used methods being cecal ligation and puncture (CLP) or peripheral administration of endotoxin, a high dose of LPS (Dejager et al., 2011).

LPS administration is a widely used experimental sepsis model attempting to mimic the pathophysiological changes seen in septic patients. LPS, also known as endotoxin, is a toxic, soluble component of the gram negative bacterial cell wall (Marpegan et. al., 2005). LPS effects are dose dependant and comprise fever, acute phase responses and septic shock in animals (Rivest et. al., 2000). At low doses, LPS induces fever and a brain mediated acute phase reaction termed "sicknesss behaviour" which is mediated by temporarily expressed proinflammatory cytokines in the brain (Dantzer, 2004) and characterised by lethargy, piloerection, grooming, decreased social interaction and anhedonia. After LPS administration in mice, the onset of systemic clinical signs that compromise sickness behaviour are seen to occur rapidly (Remick et al., 2000). High-dose LPS administration quickly results in a hypodynamic cardiovascular state, which is characterized by decreased cardiac

output and increased peripheral vascular resistance (Brackett et al., 1985; D'Orio et al., 1987). A decrease in total white blood cell count is seen with reduced levels of neutrophils and lymphocytes (Remick et al., 2000). High-dose LPS treatment induces a rapid increase in systemic cytokine levels which has been shown to be transient (Buras et al., 2005). Peripheral LPS treatment has been shown to induce the expression of various inflammatory mediators including TNF-α, IL-6, IL-1α and IL-1β both in the brain and the periphery (Zetterstrom et al., 1998), and TNFα, IL6 and IL-1β have been shown to be essential in the LPS induced acute behavioural alterations known as sickness behaviour (Dantzer, 2001). Cytokine production exhibits a bell-shaped curve over an 8-h period after injection, with levels reaching a peak between 1.5 to 4.5 h and then declining (Remick et al., 2000).

The use of endotoxin administration as a sepsis model has various advantages. Endotoxin models are reproducible, and the use of LPS can be easily standardized in experimental settings, and LPS is a relatively pure compound that is reliably measured (Fink et al., 1990). The value of endotoxin administration as a sepsis model is supported by studies in healthy human volunteers, where low-dose endotoxin injection results in pathophysiologic perturbations similar to those reported in sepsis patients (Fink et al., 1990). A single endotoxin administration offers the advantage of avoiding the need for a surgical procedure, and therefore presents fewer concerns than surgical models with respect to animal welfare (Nemzek et al., 2008), in general, however, these experimental animal models show a more rapid disease progression than is observed in human sepsis patients (Buras et al., 2005; Deitch, 1998; Hollenberg, 2005). Further, experimental animal models of LPS may not accurately replicate some features of human sepsis such as the initial hyperdynamic cardiovascular state and the magnitude and rate of induction of the cytokine response state (Buras et al., 2005; Deitch, 1998; Fink et al., 1990).

In the CLP model, perforation of the bowel results in leakage of faecal contents into the peritoneum, which establishes an infection with mixed bacterial flora and provides an inflammatory source of necrotic tissue (Wichterman et al., 1980; Ayala et al., 2000). The severity of CLP induced sepsis can be adjusted by increasing the size of the needle and subsequently the puncture size or the number of punctures (Wichterman et al., 1980). Sickeness behaviour symptoms are observed within a few hours of CLP in experimental animal models (Ebong et al., 1999a; Ebong et al., 1999b; Nemzek et al., 2004). The CLP model of sepsis is thought to

very closely mimic the clinical situation, in relation to the onset and progression of the sepsis (Buras et al., 2005; Hubbard et al., 2005), reproducing the cardiovascular function changes seen in humans sepsis patients, and is seen to induce a progressive release of proinflammatory mediators, in line with what is observed in the clinical situation (Chaudry et al., 1979; Ebong et al., 1999a; Ebong et al., 1999b). The haemodynamic and metabolic phases of human sepsis are recreated by the CLP model (Wichterman et al., 1980), as well as cell specific apoptosis and immune responses (Hotchkiss et al., 2003; Ayala and Chaudry, 1996). Following CLP in rats, a mixed population of enteric bacteria is seen, including *Proteus mirabilis, E. coli, Bacteriodes fragilis* and *Enterococcus*, and several bacterial species may be present in blood cultures (Wichterman et al., 1980).

A limitation of CLP as a sepsis model is that surgical intervention is required, raising various issues in relation to animal welfare. Additionally, the state induced by CLP can be affected post-surgery, anesthesia and postoperative hypothermia can modulate outcome, as can the use of anesthetic drugs which can impact upon the inflammatory response (Gallos et al., 2004; Hansbrough et al., 1985), and post-surgical treatments such as antibiotic administration can affect bacteria dissemination, as well as sepsis severity depending on the antibiotic used (Enoh et al., 2006; Newcomb et al., 1998; Nemzek et al., 2008). Further, the CLP model is not standardized within the published sepsis literature and care must be taken when making comparisons between studies, due to potential differences in surgical technique and postoperative care (Nemzek et al., 2008). During CLP, the amount of caecum ligated can induce variability into the technique, since increased mortality is associated with larger amounts ligated (Singleton and Wischmeyer, 2003). Additionally, it is difficult to control for the leaked amount of faecal material between studies, which could account for variability in disease severity.

1.13.4 The acute systemic immune response to a septic LPS treatment

The body recognizes LPS as a PAMP and consequently the innate immune system mounts an immune response (Banks and Robinson, 2010). LPS exerts its effects by binding through TLR4 present on macrophages and monocytes which subsequently triggers intracellular signalling pathways, leading to activation of nuclear transcription factors (Dantzer, 2004). LPS administration induces NF-κB

dependant signalling which results in transcription of proinflammatory cytokines (Rivest et al., 2003). As described, high-dose LPS treatment induces a rapid increase in systemic cytokine levels which has been shown to be transient (Buras et al., 2005), both in the brain and the periphery (Zetterstrom et al., 1998). In plasma, TNF levels increased following LPS treatment, peaking at 1.5hrs then quickly declining, while IL-6 levels peaked approximately 4hrs post LPS, and rapid induction of the chemokines MIP-2 and CXCL1 was seen in plasma (Remick et al., 2000). The susceptibility of mice to LPS induced endotoxic shock differs depending on the time of the light dark cycle at which it is administered, and susceptibility to lethal doses has been shown to increase dramatically during the resting period in mice (Halberg et al., 1960). Most inflammatory immune cells and factors show increased expression during the rest phase (Leone et. al., 2007) and mortality following LPS administration in L:D is directly proportional to the induction of inflammatory cytokines and chemokines such as IL-1β, IL-6, MCP1 and MIP1α in serum (Marpegan et. al., 2009).

Peripheral administration of LPS in humans and animals has been shown to activate HPA axis activity, increasing circulating levels of corticosterone and ACTH (Linthorst and Reul, 1998). The HPA axis through the production and release of glucocorticoids inhibits pro-inflammatory cytokine expression (Webster et al., 2002). 1 mg/kg LPS i.p. treatment causes increased levels of corticosterone and blood vessel inflammation 24hrs following treatment (Chung et al., 2010). Cytokines can impair the expression of glucocorticoid receptors (GR) and reduce their function (Pace et al., 2007). It has been shown that activation of NF-κB, STAT5 and p38 MAPK by cytokines disrupts the translocation of glucocorticoid receptors to the nucleus from the cytoplasm and nuclear protein-protein interactions which inhibit GR-DNA binding, reducing the function of glucocorticoids (Pace et al., 2007). Further, it has been suggested that cytokine mediated alterations of the function of the HPA axis and glucocorticoid receptor function may further modulate inflammation since glucocorticoids play an essential role in regulation of inflammatory processes (Capuron and Miller, 2011).

1.13.5 LPS treatment impacts upon the CNS.

LPS may affect the brain by a number of mechanisms including crossing the BBB and directly activating cells within the CNS that respond to LPS including microglia (Marzolo et al., 2000), astrocytes (Chakravarty & Herkenham, 2005) and brain endothelial cells (Reyes et al., 1999; Verma et al., 2006) and those that express TLR4 (Chakravarty & Herkenham, 2005). Indeed, TLR4 and the monocyte CD14, both known to recognize LPS, are expressed in the choroid plexus, leptomininges and circumventricular organs, that is in those regions close to the BBB (Rivest et al., 2003). BBB breakdown leads to alterations in ionic homeostatis and allows inflammatory cells and toxic metabolites to access the brain leading to neuronal damage and inflammation within the CNS (Nishioku et al., 2009). However, other studies have shown that minimal amounts of peripherally administered LPS cross the BBB and do not appear sufficient to induce the neuroimmune reactions seen following peripheral LPS treatment, except possibly at the highest experimentally used doses and for the most sensitive CNS functions, and therefore suggest that these neuroimmune reactions in response to peripherally administered LPS are likely mediated through LPS receptors located outside the BBB (Banks and Robinson, 2010).

Outside of the brain, LPS may also indirectly impact upon the brain through interactions with the afferent and vagal nerves (Romeo et al., 2001; Goehler et al., 1999), acting at circumventricular organs (Blatteis et al., 1983), causing the release of inflammatory mediators in the periphery able to cross the BBB (Qin et al., 2007) or by altering the permeability of the BBB (Xaio et al., 2001) and the response of cells comprising the BBB to LPS and the subsequent release of substances such as cytokines at these sites (Quan et al., 2003; Verma et al., 2006). Studies have shown the long-lasting effects of LPS on the brain to be mediated by TNF-α, since studies in TNF-α receptor knockout mice have shown that TNF-α receptors are necessary for a systemic LPS treatment to produce TNF-α mRNA production and activation of microglia in the brain (Qin et. al 2007). IL-1β has also been suggested to be involved in the transmission of the peripheral inflammatory response following LPS treatment to the brain via the cerebral vasculature (Turrin et al., 2001) subsequently leading to upregulation of proinflammatory cytokine synthesis in the brain (Turrin et al., 2001; Shaw et al., 2001; Terrazzino et al., 2002).

1.13.6 The effects of LPS treatment on the brain and behaviour

Central and peripheral LPS administrations induce similar neuroimmune responses (Gottschall et al., 1992; Banks and Robinson, 2010). Peripheral LPS treatment induces central upregulation of expression of a variety of substances including nitric oxide, prostaglandins and interleukins (Singh and Jiang, 2004; Larson & Dunn, 2001; Sugita et al., 2002) including proinflammatory cytokines such as IL-1, IL-6, IL-8 and also TNF-α (Qin et al,. 2007, Herber et al., 2006), and the chemokine MCP-1 (Thompson et al., 2008; Qin et al., 2008) acutely in the brain following LPS treatment and these temporarily expressed proinflammatory cytokines in the brain mediate sickness behaviour (Dantzer, 2004). Systemic LPS impacts on sleep architecture and sleep-wake behaviour (Krueger and Majde, 1994). Sickness behaviour can also be induced by central or peripheral treatment with TNF-α (Porter et al., 1998) or IL-1β (Bluthe et al., 1995; Swiergiel et al., 1997). Behavioural output is altered during sickness behaviour, and hypoactivity is exhibited (Franklin et al., 2007). Alterations in cytokine levels in the CNS and the neuroinflammatory response may lead to neurobehavioral impairments and delirium (Lemstra et al., 2007).

Microglial activation and cytokine release in the CNS has previously been shown in response to LPS induced sepsis (Godbout et al., 2005; Van Dam et al., 1992; Buttini et al., 1996). Further, peripheral LPS treatment and subsequent microglial activation and cytokine release have been associated with behavioural deficits (Bluthe et al., 1992; Godbout et al., 2005; Combrinck et al., 2002). Activated microglia have been shown in the brains of human sepsis patients, irrespective of age (Lemstra et al., 2007). Neuronal and glial cells (microglia and astrocytes) form a cytokine network within the CNS, capable themselves of releasing cytokines, and containing cytokine receptors, responding to cytokine information and in turn modulating this (Rothwell et al., 1996; Haas & Schauenstein, 1997). Microglia and astrocytes also synthesize complement proteins, and activation of the complement cascade has been shown to play roles in the brain following LPS treatment, leading to upregulation of CD45, TNF-α and TLR4, along with an upregulation in activated microglia and iNOS (Jacob et al., 2007). Activated microglia release inflammatory mediators which are shown to be neurotoxic *in vitro* (Ekdahl et al., 2003) such as IL-1β, IL-6, TNF-α, nitric oxide, and reactive oxygen

species (Pocock and Liddle, 2001; Hanisch, 2002; Gebicke-Haerter, 2001; Vallieres et al., 2002). Studies have shown an upregulation in glia in various regions of the CNS both acutely (Chung et al., 2010; Jacob et al., 2007; Semmler et al., 2005) and chronically (Hauss-Wegrzyniak et al., 2000; Weberpals et al., 2009) following LPS treatment. In SE models, increases in levels of the immune signaller nitric oxide are seen, due to elevated iNOS (NOS2) expression as a result of cytokines such as TNF-α stimulating glia to express iNOS (Jacob et al., 2007). 24hrs following 10mg/kg LPS treatment in rats an upregulation of iNOS is seen in the striatum, cerebellum, hippocampus and midbrain (Semmler et al., 2005).

It has been suggested that inflammatory cytokines and nitric oxide generation may cause perturbations in neuronal function and cause neurodegeneration during septic encephalopathy (Semmler et al., 2008). Sustained nitric oxide generation impacts upon neuronal survival (Boje et al., 1992; Leist et al., 1997; Heneka et al., 1998), and NO also plays roles in neuronal activity (Mori et al., 2001; Wang et al., 2004). Inflammatory cytokines TNF-α and IL-1β also influence neuronal viability (de Bock et al., 1998; Venters et al., 2000) and the function of neurons (Pickering et al., 2005; Tancredi et al., 1992; Kelly et al.,2001). Acutely, following sepsis induction, Semmler et al. (2008) have also shown neuronal loss in the hippocampus and cortex, along with up-regulated microglial activation in the cortex associated with an increase of the immunomediators iNOS, MCP-1, Il-1β, TNFα and TGF-β. Further, a single septic i.p. LPS treatment has been shown to irreversibly damage dopaminergic neurons which are subsequently seen to degenerate between 7 and 10months post-treatment (Qin et al., 2007). Three months following 10mg/kg LPS treatment, neuronal loss in the prefrontal cortex and hippocampal subregions, and reduced cholinergic innervation of the parietal cortex was evidenced in rats (Semmler et al., 2007).

Together, these studies highlight that sepsis is a profound pathophysiological state and highlight the impact of sepsis on the CNS both acutely and in the long term.

1.14 Aims of Study:

A bidirectional communication is suggested to exist between the immune system and the circadian system (Coogan and Wyse, 2008), and while there is much

evidence regarding the regulation of immune function by the circadian system (reviewed in Scheiermann et al., 2013), there is less information regarding the impact of immune processes on the timekeeping system. Various studies have assessed the acute impact of immune mediators on the circadian timekeeping system, however there is a gap in the literature as to the chronic effects of an immune challenge on the circadian timekeeping system. The current set of studies sought to address this gap, and examine the long term impact of sepsis, a substantial immune challenge, on circadian timekeeping processes, utilizing a septic endotoxin model known to chronically impact on the innate immune system in the CNS (Weberpals et al., 2009; Qin et al., 2007; Bossu et al., 2012). These studies will enhance our knowledge of the impact of immune processes on timekeeping processes, and describe a previously unknown impact of the chronic effects of experimental sepsis on the circadian timekeeping system.

The specific aims of this study were:

- To examine post-septic locomotor behavioural rhythmicity as an output measure of the functional activity of the post-septic endogenous oscillator, assessing core circadian locomotor behaviour parameters and circadian resetting behaviour in the long term following the induction of sepsis (Chapter Two).
- To examine the neurochemistry of the SCN both in the acute phase and in the long term following a septic LPS treatment, and examine the possibility of a chronic neuroinflammation within the SCN that could play a role long term in circadian timekeeping processes (Chapter Three).
- To examine the long-lasting impact of LPS induced sepsis on the rhythmic expression of clock gene and IEG protein product expression patterns in the SCN, and to assess post-septic SCN responsiveness and function (Chapter Four).
- To assess the response of the post-septic circadian system to further immune challenge, assessing a priming effect of previous CNS insult following LPS induced sepsis (Chapter Five).

Chapter Two

Analysis of the Effects of Sepsis Induction on Circadian Locomotor Behaviour

2.1. Introduction

The LPS induced sepsis model used in the current set of studies is one that is known to induce a long-lasting neuroinflammation (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012), and modulation of circadian timekeeping processes, including locomotor behaviour, has been shown in response to immune mediators and in conditions of chronic neuroinflammation. It is therefore possible that endotoxin induced sepsis and the post-septic CNS state could chronically attenuate circadian timekeeping processes.

2.1.1 The direct effect of immunomediators on the circadian system

SCN function is modulated by immune factors, which can impact upon the rhythmic expression of clock genes and subsequently affect circadian locomotor behaviour. Treatment with endotoxin has been shown to alter clock gene expression both in the SCN and in the periphery (Okada et al., 2008; Cadivini et al., 2007; Murphy et al., 2007), and further, other inflammatory mediators have been shown to impact upon molecular clock gene oscillations both in the site of the master pacemaker and in peripheral tissues and in cell lines (Tsuchiya et al., 2005; Cavadini et al., 2007; Ohdo et al., 2001; Koyanagi and Ohdo, 2002). TNF-α is seen to interfere with *Dbp* in the SCN, as well as cause an increase in the number of rest episodes during the subjective night in mice, and a reduction in total locomotor activity under a 12:12 LD cycle, but no effect on the FRP under constant conditions (Cavadini et al., 2007). Low dose peripheral LPS has been shown to acutely impact upon circadian behaviour, transiently suppressing locomotor wheel running behaviour (Marpegan et al., 2005). A single treatment with IFN-α directly decreases locomotor behaviour in mice (Crnic and Segall, 1992), while chronic administration

of the cytokine is seen to significantly alter locomotor rhythmicity and cause a decrease in total activity levels (Koyanagi and Ohdo, 2002).

Additionally, direct administration of immune factors is capable of phase-shifting SCN rhythmicity resulting in phase advances or delays depending on the circadian phase at which they are administered, and inflammatory mediators have been suggested to play roles in entrainment of the circadian timekeeping system (Leone et al., 2012). In hamsters, i.c.v. administration of IFN-γ leads to phase advances during the subjective day, but not during the subjective night (Boggio et al., 2003), while centrally administered IL-1β and TNF-α in the early subjective night induce phase delays of locomotor behaviour (Leone et al., 2012).

LPS treatment also induces phase resetting responses. Low dose peripheral treatment administered in the early subjective night, at CT15, but not at other phases, has been shown to induce photic like phase delays that were non additive to the effects of light (Marpegan et. al., 2005; Leone et al., 2012). IL-1β has been shown not to be involved in mediating phase shifts of behaviour following an endotoxin induced immune challenge, however TNF-α plays an essential role in this response, since its inhibition blocks LPS induced phase delays (Leone et al., 2012). Further, TLR4 is suggested to play a role in these LPS induced circadian behavioural responses, since in TLR4 deficient mice, the low dose LPS induced phase delay at CT15, along with the induction of PER1 and c-Fos in the PVN was seen to be decreased or absent (Paladino et al., 2010). Additionally, the immune related transcription factor NF-κB has been implicated in the LPS effects on the circadian system and its presence and action upon the SCN has been shown during LPS induced and photic induced phase shifts (Marpegan et al., 2004, 2005; Paladino et al., 2010). LPS administration has been shown to cause functional activation of the SCN (Marpegan et. al., 2005; Beynon and Coogan, 2010), and to impact upon SCN responsiveness (Palomba and Bentivoglio, 2008). No alterations in period or phase of rhythmic core body temperature or locomotor behaviour are seen in response to a lethal dose of LPS (Marpegan et. al., 2009).

2.1.2. Circadian rhythm perturbations in chronic inflammatory states

As described previously, dysfunction of timekeeping activities has been shown to perturb immune function, as has been shown by a wide variety of chronic jet lag studies (Filipski et al., 2003; Filipski et al., 2006; Fu and Lee, 2003; Miyazaki et al., 2010; Sukumaran et al., 2010; Castanon-Cervantes et al., 2010), and inflammation is a key risk factor in the development of many of the pathologies associated with chronic circadian misalignment. The LPS induced sepsis model used here induces a long-lasting neuroinflammation (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012), and modulation of circadian timekeeping processes is seen in many conditions with a neuroinflammatory component. Neurological diseases and disorders such as Alzheimer's Disease, are well documented as having underlying neuroinflammation. Alzheimer's patients show alterations in the circadian system (Hatfield et al., 2004), including sleep-wake cycle disturbances (Ancoli-Israel et al., 1989; Prinz et al., 1982), perturbations in the phase and amplitude of the sleep rhythm (Okawa et al., 1991; Satlin et al., 1991; Witting et al., 1990; Satlin et al., 1995) and reductions in the neuropeptides vasopressin (Swaab et al., 1985) and vasoactive intestinal polypeptide in the SCN (Zhou et al., 1995), highlighting modulations of the circadian system in AD patients, possibly due to underlying neuroinflammation.

A chronic low grade neuroinflammation is seen to occur in the normal, healthy aging brain including in the SCN (Godbout and Johnson, 2009; Deng et al., 2010), along with an increase in microglial activity (Jurgens and Johnson, 2010). In healthy aging, circadian impairments are seen, including alterations in core body temperature (CBT) rhythms, (Vitiello et al., 1986; Weitzman et al., 1982), damped circadian amplitude (Weinert et al., 2000) and functional alterations of peripheral oscillators (Sellix et al., 2012) and decreases in the neuropeptides AVP and VIP in the SCN (Hofman et al., 2006). Furthermore, attenuations are found in the senescent SCN in photic responsiveness (Kolker et al., 2003), photic induced resetting (Rosenberg et al., 1991) and synchronization (Zee et al., 1992; Sellix et al., 2012).

Altered circadian function is seen in diseases characterized by chronic infection and neuroinflammation, such as Simian immunodeficiency virus, which shows alterations in clock controlled rhythms of CBT and locomotor activity (Huitron-Resendiz et al., 2007), and African trypansomiasis (Lundkvist et al., 2002;

Lundkvist et al., 2010). Trypanosomiasis has been shown to affect SCN functional activation, altering photic induced c-Fos (Peng et al., 1994) and spontaneous c-Fos expression in the SCN (Bentivoglio et al., 1994). Human African trypanosomiasis (HAT) consists of a hemolymphatic stage followed by a meningoencephalitic stage whereby the parasite *Trypanosoma brucei* (*Tb.*) and inflammatory cytokines cross the BBB, invading the CNS (Etet et al., 2012). *T.b. brucei* infected rodent models show alterations in some, but not all circadian rhythms (Kristensson et al., 2010) including perturbations in CBT (Berge et al., 2005; Chevrier et al., 2005; Etet et al., 2012) and in ultradian and circadian organization of rhythmic locomotor behaviour (Grassi-Zucconi et al., 1995). Trypanosomes secrete prostaglandins which interact with the host's immune response causing the release of proinflammatory cytokines and neuroinflammatory signalling has been suggested to be involved in the attenuations in circadian rhythms and in sleep patterns that characterize the disease (Kennedy, 2009; Kristensson et al., 2010). Indeed studies in an experimental rat model of African trypanosomiasis utilizing the *T.b. brucei* parasite, show that it is at the same time that CNS parenchyma invasion by the parasite and inflammatory mediators occurs that alterations in circadian rhythms become more marked, including perturbations in the oscillations of CBT, rest-activity, total sleep time and Slow Wave Activity (SWA) become significant and more severe (Etet et al., 2012), supporting the role of neuroinflammation in the circadian alterations.

Given the bidirectional communication between the immune system and the circadian system, the impact of centrally acting inflammatory mediators on circadian timekeeping, and the fact that peripheral LPS induced sepsis is characterized by significant increases in proinflammatory cytokines (Qin et al,. 2007, Herber et al., 2006) and activation of the HPA axis (Linthorst and Reul, 1998), dysfunction of the innate neuroimmune system following sepsis and SE could perturb the circadian timekeeper and circadian output. Inflammatory mediators might impact upon the SCN, modulating SCN output and locomotor activity rhythms, affecting synchronization and entrainment of the post-septic circadian timekeeping system, or core circadian locomotor behavioural parameters. The primary aim of this portion of the study was to examine locomotor behavioural rhythmicity as an output measure of the functional activity of the post-septic endogenous oscillator, examining the long-term effects of a substantial immune challenge on circadian organization and

the SCN's ability to coordinate timekeeping processes. For the purposes of these experiments, we have studied the core circadian parameters as well as the entrainment and resetting properties of circadian locomotor behaviour in the long term post-sepsis. These studies are the first to document the long-lasting effects of sepsis on the circadian pattern of locomotor activity.

2.2. Materials & Methods

2.2.1 Ethical Approval

All protocols were approved by the Research Ethics Committee at the National University of Ireland, Maynooth and were licensed by the Department of Health and Children, Ireland. Animals were treated in accordance with the Cruelty to Animals Act, 1876 and the SI No.17 – European Communities (Amendment of Cruelty to Animals Act, 1876) regulations, 1994 (European Directive 86/609/EC). All efforts were made to minimize the number of animals used and any potential suffering. Where experimental procedures allowed, all animals were treated with a single intra-peritoneal (i.p.) drug administration. To ensure minimisation of animal usage, animals used for behavioural monitoring were also used for Immunohistochemical analysis. Brain sections not relevant for the purposes of these experiments were supplied for Immunohistochemical analysis for use in another related project.

2.2.2 Animals and housing

The animal model used in this study is the C57BL/6 strain of mouse (*Mus musculus*) which is a naturally melatonin-deficient inbred strain. C57/BL6 mice are well characterised from a chronobiological aspect with extensive characterization of their circadian parameters having previously been performed (Schwartz and Zimmerman, 1990). For the purposes of these experiments, adult C57BL/6 male mice (24-30g) were obtained from Charles River, UK or Harlan, UK, at 6 weeks old. Animals were group housed on arrival. At the beginning of each experiment these animals were between 8 and 10 weeks old and were used for the duration of these experiments up until the age of 4-5 months.

For the purpose of behavioural monitoring, animals were individually housed in polypropylene cages (33cm long x 15cm wide x 13cm high) equipped with steel

running wheels (11.5cm diameter) with food and water available *ad libitum*. The lighting conditions were adjusted via a timer on the outside of the cabinet therefore the cabinet was not unnecessarily opened. The light source was standard fluorescent light bulbs with an average 150 lux luminance level in each individual cage. Group housed animals were housed in groups of 10 in polypropylene cages (44.5cm long x 28.5cm wide x 10.2cm high) equipped with appropriate environmental enrichment with food and water available *ad libitum*. Animals were maintained on a 12:12 light dark (LD) photoperiod for at least 2 weeks prior to any experimentation. When animals were housed in constant darkness, daily checks were carried out with the aid of a red safety light (<1 lux). Temperature and humidity were kept constant with an average temperature of 21 $\pm 1^{o}$C and humidity of 50$\pm$10%. Cages were ventilated via axial fans to prevent the build-up of pheromones and these fans produced white noise at the level of 50dB.

2.2.3 Treatments

2.2.3.1. Sepsis induction

For the purposes of these experiments, animals were treated with the endotoxin Lipopolysaccaride from Gram Negative Bacteria, *Escherichia coli.* (serotype 0111.B4; Sigma, Ireland) to induce sepsis. The introduction of bacterial components into the abdominal cavity (e.g. LPS administration) is a commonly used experimental animal model of sepsis (Nemzek et al., 2008). A single i.p. injection was utilized, LPS 5mg/kg dissolved in 0.9% sterile saline (NaCl). This is a recently described model whereby a single treatment i.p. LPS (5mg/kg) in mice induces septic shock followed by a long-lasting neuroinflammation and central upregulation of TNF-α and components of the IL-1β system (Qin et al, 2007; Weberpals et al, 2009), thereby inducing a chronic effect as a result of an acute treatment. Control animals were treated in parallel with an equal volume of 0.9% NaCl. The model avoids the stress of repeated i.p. injections to the animals. All treatments were at Zeitgeber Time (ZT) 6-8, where ZT0 = lights on. LPS treatment elicited the full spectrum of sickness behaviour evident 1hr after treatment. ~10% of LPS 5mg/kg treated animals displayed a significant moribundity requiring euthanasia or mortality in the first 72hrs following treatment. Following LPS treatment, animals were

allowed to recover for at least two weeks prior to any experimentation or manipulation of the photoperiod.

2.2.3.2. Non-photic phase-shifting treatment.

8-hydroxy-2-dipropylaminotetralin hydrobromide (8-OH-DPAT) a selective 5-HT_{1A} agonist (Tocris Bioscience, Bristol, UK) is known to induce non-photic phase shifts of the mouse circadian system when applied 6hrs prior to activity onset (Horikawa and Shibata, 2004; Gardani and Biello, 2008). A single i.p. injection of the serotoninergic agonist was therefore utilized at CT6 at a dose of 5mg/kg which has previously been shown in mice to induce maximal non-photic phase shifts (Horikawa and Shibata, 2004).

2.2.3.3. NF-κB inhibition

Animals were treated with ammonium pyrrolidinedithiocarbamate (PDTC) ($C_5H_{12}N_2S_2$), a potent antioxidant and pharmacological inhibitor of the transcription factor Nuclear Factor κB (NF-κB) (Zhu et al., 2002), 10 minutes prior to LPS 5mg/kg i.p. treatment. Animals received a single 200mg/kg i.p. injection of PDTC dissolved in 100mM DMSO, a dose previously shown in rats to maximally inhibit NF-κB activation and the expression of the proinflammatory genes TNF-α, COX-2, cytokine-induced neutrophil chemoattractant, and ICAM-1 and their products when administered prior to LPS treatment (Liu et al., 1999). Control animals were treated in parallel with an equal volume of sterile Saline (0.9% NaCl) to DMSO i.p.

2.2.4 Sepsis scoring

A sepsis score was calculated for control and LPS treated animals and PDTC and LPS treated animals immediately after and 1hr post-treatment and subsequently at regular intervals for 48 hrs post-treatment. Scores were assigned on a five point scale assessing alterations in the parameters of behaviour, appearance, dehydration and respiration. Each parameter was graded on a scale from 0-4 as follows: 0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe. These scores were seen to revert back to zero approximately 48hrs following LPS treatment. At this time any animals that continued to display a high sepsis score or significant moribundity were humanely culled.

2.2.5 Circadian Behavioural Analysis

Animals were singly housed in cages equipped with steel running wheels for the purposes of behavioural monitoring. The cages were fitted with micro-switches connected to a data acquisition system computer using the Chronobiology Kit by Stanford System (Santa Cruz, California) for recording of daily rhythms of locomotor activity. Each wheel revolution generated a switch closure measured by the data acquisition system and collected every 5 minutes which then produced actograms or actigraphs of activity rhythms. Onset of activity was defined as ZT12 (where ZT0 is the time at lights on under a 12:12 LD cycle). Using the actogram data for each animal, phase shifts were calculated by fitting a line-of-best-fit through activity onsets for one week prior to treatment and for one week post-treatment (Fig. 2.1). The difference between the two lines was calculated by two to three researchers and the results averaged to exclude experimenter bias.

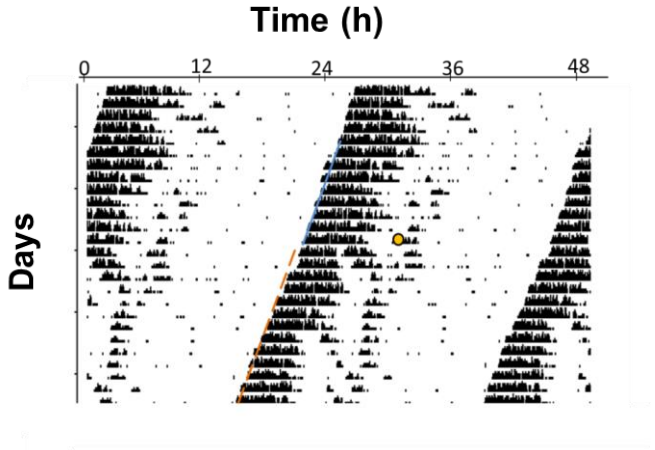


Figure 2.1: Line of Best Fit Analysis. *Sample double plotted actogram illustrating the line of best fit method utilized to assess locomotor activity onsets following application of a phase-shifting stimulus to a subject free running in DD. The application of the stimulus is indicated by the yellow circle. The lines illustrate the lines of best-fit through activity onsets. The blue line highlights the line of best fit through activity onsets for the 7 cycles prior to the stimulus. The red line indicates the line of best fit for the 10 days post the phase resetting stimulus. Note the exclusion of transient days from analysis of the locomotor phase resetting response.*

The Chronobiology Kit Chi Squared periodogram is a mathematical time-series analysis which calculates the free running period and rhythm amplitude from the actogram data. (Figure 2.2).

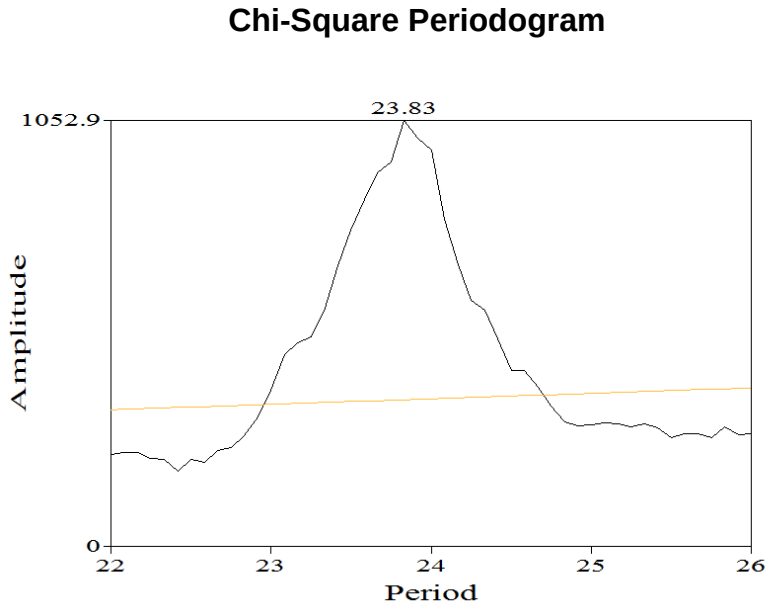


Figure 2.2: Chi-Square Periodogram. *Sample chi-square periodogram calculated by the Chronobiology Kit showing the free running period and amplitude values of the activity rhythm.*

2.2.6. Jetlag Experiments

For the purposes of these experiments, adult male mice were housed in individual cages equipped with running wheels and were allowed to habituate to a 12:12 LD cycle (150 lux, lights on 0500h) for two weeks prior to being treated with either LPS 5mg/kg i.p (n=11) or saline (n=12).

Actogram data and circadian behavioural parameters were examined the week beginning 7 days following septic LPS treatment (n=5-8 per group). The average FRP, rhythm amplitude and total wheel running average in the week beginning 7 days post-treatment were calculated for each animal by two independent researchers blind to the experimental procedure and differences assessed between groups by independent t-test. Actogram data and the phase angles of entrainment were examined for each animal in both treatment groups under the LD photoperiod in the 7 days beginning one week post-treatment. The time of activity onset for each daily cycle was calculated for each animal in both treatment groups and compared to the known dark cycle onset time for the photoperiod. This was done for each cycle over a 7 day period starting 7 days following LPS treatment or saline vehicle.

Activity onset prior to onset of the dark cycle was defined as a positive phase angle of entrainment, and a negative phase angle if locomotor activity began after lights off. Differences between the onsets were assessed for each daily cycle and a weekly average was then calculated. The weekly average phase angle of entrainment was quantified by two independent researchers blind to the experimental procedure and analysed for each group by means of independent samples t-test.

The animals were allowed to recover for two weeks following the immune challenge prior to experimental manipulation of the photoperiod. Two different "jetlag" protocols were adhered to, a 6hr jetlag manipulation was applied to one group of experimental animals (12 saline, 11 LPS), consisting of a 6hr phase advance of the L:D cycle followed two weeks later by a 6hr phase delay of the L:D cycle. The second group of experimental animals (8 saline, 8 LPS), were subject to a 10hr jetlag protocol consisting of a 10hr phase advance of the L:D cycle followed by a 10hr delay of the photoperiod 14days later. For the purpose of a phase advance of the photoperiod, the dark phase of the light dark cycle is shortened so that lights on occurs earlier, resulting in a "short day". For the first cycle following the 6hr phase advance of the LD cycle, the dark phase of the cycle consists of 6 hrs of in total and consists of 2hrs in total following a 10hr advance of the LD cycle. The animals were maintained under these 12:12 LD photoperiods for a further two weeks, following which the photoperiod was delayed, whereby lights off occurred later resulting in a "long day". For the first cycle following the 6hr phase delay of the photoperiod the dark phase of the cycle was lengthened by 6hours and so the animals, dark phase of the cycle was 18hrs in total. Following a 10hr delay of the LD cycle the dark phase of the cycle was lengthened by 10hrs resulting in a dark phase of 22hrs in total. This 12:12 LD schedule was maintained for a further 14 days.

Following each phase shift paradigm, circadian locomotor activity rhythms were analysed. The animals' rates of re-entrainment to these 'jetlag' protocols were monitored. The first day of the shifted cycle was defined as the day of the change in light onset. The time taken to re-entrain to a shift of the light dark photoperiod was determined by calculating the total duration of the activity rhythm and subsequently, calculating the rhythm midpoint for each animal prior to and following the phase shift. The effects of masking can impact upon activity onset while activity offset is an unmasked index of behaviour and so to assess re-entrainment to a phase shift of the light:dark cycle it is more accurate to utilize the midpoint between activity onset

and offset. When activity midpoint following the phase shift was seen to show a difference of 6 or 10hrs to the activity midpoint prior to the phase shift, depending on protocol, the animal was determined to have a resynchronized rhythm. The number of days taken for this difference to be seen was calculated for each animal by two researchers blind to the experimental procedure and analysed by independent samples t-tests. Further, in order to assess the rate of change in activity onset, the time of activity onset for each daily cycle was assessed for each animal in both treatment groups and was analysed in the 7 days following manipulation of the photoperiod by mixed between-within ANOVA, with the time of activity onset as the dependant variable for 8 days following the shift of the light/dark cycle. Following each phase shift of the light dark cycle, the circadian parameters of free running period and rhythm amplitude were calculated for each animal using the Chronobiology Kit Chi Squared procedure and analysed for each group by independent samples t-test. In order to assess locomotor activity levels, total wheel revolutions were calculated for each animal for each day during the course of the experiments using the Chronobiology Kit and analysed for each group by independent samples t-test.

2.2.7. Photic Phase Response Curve

To facilitate construction of the photic phase response curve, 30 adult male mice were singly housed in cages equipped with running wheels and housed under a 12:12 LD cycle (150 lux, lights on 0500h) for 2 weeks to allow for habituation. Animals were then treated with either LPS 5mg/kg i.p (n=14) or Saline (n=16). Following recovery, animals were transferred into constant darkness (0 lux) and allowed to free-run for a duration of 20 weeks. During this time the animals were subjected to an Aschoff type 1 protocol (Aschoff, 1965), receiving a 30 minute light pulse (~150 lux) every 14 days at random times across the circadian cycle. Each animal received 6 light pulses (LP) in total. The animals' locomotor activity was examined under DD in the 14 days following each light pulse prior to administration of the next light pulse. Actograms and phase shift magnitudes were assessed and quantified by three independent researchers blind to the CT at which each light pulse was applied. The photic phase shift magnitudes were calculated for each animal utilizing the line of best fit method, fitting the line through activity onsets 7 days

prior to the light pulse and the onsets for 7 cycles starting 4 days after the light pulse to minimise interference from transients. Following quantification of the phase shifts, the time of the circadian cycle at which the light pulses were administered were calculated and grouped in 3hr CT bins and a phase response curve was constructed plotting phase shift magnitude against circadian time of the light pulse. Phase shift magnitudes were examined between groups by two-way factorial ANOVA and independent t-tests with Bonferroni corrections were used for analysis of phase shift magnitude at the individual circadian time bins. The free running period and rhythm amplitude were assessed for each animal 10 days before the light pulse and 10 days after the light pulse using the Chronobiology Kit Chi Squared procedure and analysed for each group by independent samples t-test.

2.2.8. Non-photic Phase Shifts

In order to examine the effects of a non-photic stimulus on circadian locomotor activity following the induction of sepsis, animals were singly housed in running wheel cages under a 12:12 light dark cycle (150 lux, lights on 0500h) and allowed to habituate for 2 weeks prior to being treated with either LPS 5mg/kg i.p (n=7) or saline (n=7). The animals were allowed to recover for two weeks following the immune challenge prior to being transferred into constant darkness (0 lux). Animals were maintained under constant dark conditions for 14 days and then received a 5mg/kg i.p. injection of the serotoninergic agonist 8-OH-DPAT at CT6 (where CT12 is the onset of activity). Animals were left to free run for a further two weeks in DD, during which time their locomotor activity rhythms were monitored and the phase-shifting effects of 8-OH-DPAT treatment assessed. Using the actogram data for each animal, the magnitudes of the non-photic phase shifts following administration of 8-OH-DPAT at CT6 were quantified for each animal for each treatment group. The line of best fit method was used to assess the non-photic phase shift magnitudes, fitting the line through activity onsets 7 days before the light pulse and the onsets for 7 cycles starting 4 days after the light pulse to minimise interference from transients. The actograms and differences between the lines were rated by two independent researchers blind to the experimental procedure. The animals were then transferred to a 12:12 light dark cycle and maintained under these lighting conditions for two weeks prior to treatment with a 5mg/kg i.p. 8-OH-DPAT

injection at CT6, 1hr after which they were culled. Phase shift magnitudes were assessed between groups by independent samples t-test.

2.2.9. Skeleton Photoperiods

In order to verify that rhythmicity exhibited by the animals under L:D cycles is in fact truly entrained circadian behaviour and not owing to masking by the light dark cycle, 16 male mice were individually housed in cages equipped with running wheels and habituated to a 12:12 LD cycle for 2 weeks (150 lux, lights on 0500h). The animals were then treated with either LPS 5mg/kg i.p injection (n=7) or saline (n=9) and allowed to recover for 14 days prior to manipulation of the photoperiod. The light dark cycle was initially shortened to a skeleton photoperiod whereby light occurred during only the first and last hour of the previous light cycle followed by the original dark phase (1L:10D:1L:12D). The animals were maintained under these conditions for 21 days and their locomotor activity patterns monitored. Following 3 weeks under the skeleton photoperiod, the light:dark cycle was then made a half skeleton photoperiod where the first light phase was removed so that the only light phase occurred during the final hour of the original light cycle (IL:23D). Actogram data and the phase angles of entrainment were examined and calculated as described previously for each animal in both treatment groups under both photoperiods for each cycle over the 42 day period. Differences between the onsets were assessed for each daily cycle and a weekly average was then calculated for each animal for each week under the two skeleton photoperiods. The weekly average phase angle of entrainment was quantified by two independent researchers blind to the experimental procedure and analysed for each group by means of independent samples t-test.

2.2.10. The effects of sepsis on circadian parameters exhibited under constant conditions

In order to assess free-running circadian parameters, animals previously treated with either LPS 5mg/kg i.p. or saline upon recovery were housed in individual running wheel cages under a 12:12 LD cycle (150 lux, lights on 0500h) for two weeks to allow for habituation prior to experimentation. Animals were then transferred into either constant dark (8 saline, 7 LPS) or constant light (8 saline, 7 LPS) with light intensity levels of either 0 lux or 150 lux (LL) and were maintained

under these conditions for a period of 14 days during which time their locomotor activity patterns were monitored. Using the actogram data, the circadian parameters of free running period and rhythm amplitude were quantified for each animal in each treatment group under both lighting conditions by two independent researchers blind to the experimental procedure using the Chronobiology Kit Chi square periodogram and analysed for each group by independent samples t-test.

2.2.11. Behavioural responses following treatment with PDTC prior to LPS treatment

The involvement of NF-κB in the LPS mediated effects on circadian rhythmicity was examined by administration of PDTC prior to LPS treatment and monitoring of locomotor activity rhythms. Animals were housed in individual cages equipped with running wheels under a 12:12 LD (150 lux, lights on 0500h) cycle for two weeks prior to treatment to allow for habituation. PDTC 200mg/kg i.p. injection or saline vehicle was administered 10 minutes prior to LPS 5mg/kg (i.p.) or control. Three experimental groups consisting of PDTC and LPS (n=5), PDTC and control (n=6) and control and LPS (n=5) were examined. Mixed between within ANOVA was used to assess statistically significant differences between sepsis scores following treatment with a control injection followed by a septic LPS treatment and the sepsis score reached when PDTC was administered prior to a septic LPS treatment. The animals were allowed to recover for two weeks following the immune challenge and re-entrainment to a 6hr phase advance of the photoperiod was assessed as outlined above (Section 2.2.6).

The animals were then transferred into constant darkness (0 lux) and allowed to free-run in order to assess whether treatment with PDTC prior to LPS 5mg/kg would attenuate the enhanced late subjective night photic induced phase shifts. The animals were held under these conditions for 14 days prior to receiving a light pulse for 30 mins at CT22, the advance portion of the phase response curve at which post-septic animals have previously exhibited larger phase shift magnitudes in a response to a 30 min light pulse compared to controls. Animals were maintained for a further two weeks in DD following application of the light pulse and their locomotor activity rhythms monitored. The magnitudes of the resultant phase shifts were quantified for the three treatment groups. The line of best fit method was used to

assess the photic phase shift magnitudes, fitting the line through activity onsets 7 days before the LP and the onsets for 7 cycles starting 4 days after the LP to minimise interference from transients. The actograms and differences between the lines were rated by two independent researchers blind to the experimental procedure. Phase shift magnitudes were compared between groups using one way ANOVA.

2.3. Results

2.3.1. The acute effects of LPS 5mg/kg treatment on circadian locomotor activity

Animals exhibited the classic symptoms of sickness behaviour following i.p. LPS 5mg/kg treatment, including lethargy, piloerection, grooming and decreased social interaction, and these behaviours were scored for each animal and contributed part of the sepsis score quantified for each animal across the following 48hrs. Further, LPS treated animals did not display wheel running activity for a number of cycles following treatment, which can be clearly observed from the representative actogram for LPS treated animals Figure 2.4. When locomotor activity resumed, it did so as would be expected following a period of entrainment, with the onset of locomotor activity continuing in phase with the onset of running wheel activity displayed on the last cycle prior to LPS 5mg/kg i.p. treatment, i.e. no alterations in the phase relationship between the activity onset and the onset of the dark phase of the entraining L:D cycle (Figure 2.3). Saline control animals displayed entrained circadian locomotor activity with no interruption in wheel running following treatment.

Independent t-tests showed that there were no significant differences between the phase angles of entrainment in the week beginning 7 days post-treatment between the treatment groups (LPS, 4.25 $\pm$ 3.82 min. vs. saline controls, 5.09 $\pm$ 4.01 min, $P>0.05$). Independent t-test analysis showed that there were no significant differences between the mean free running period values in the week beginning 7 days post-treatment between the treatment groups (LPS, 23.8 $\pm$ 0.00 hrs. vs. saline, 23.9 $\pm$ 0.17 hrs, $P>0.05$), nor were there any differences observed at this time in the mean rhythm amplitude between groups (LPS, 1365.2 $\pm$ 116.6 vs. saline, 768.9 $\pm$ 224.9, $P>0.05$). Independent t-test analysis was also used to examine whether there

were statistically significant differences between the average total wheel running values in the week 7 days post-treatment between the treatment groups (LPS, 9472.1 ± 3333.5 vs. saline, 5090.9 ± 4599.9 revolutions, P>0.05). (Fig.2.3).

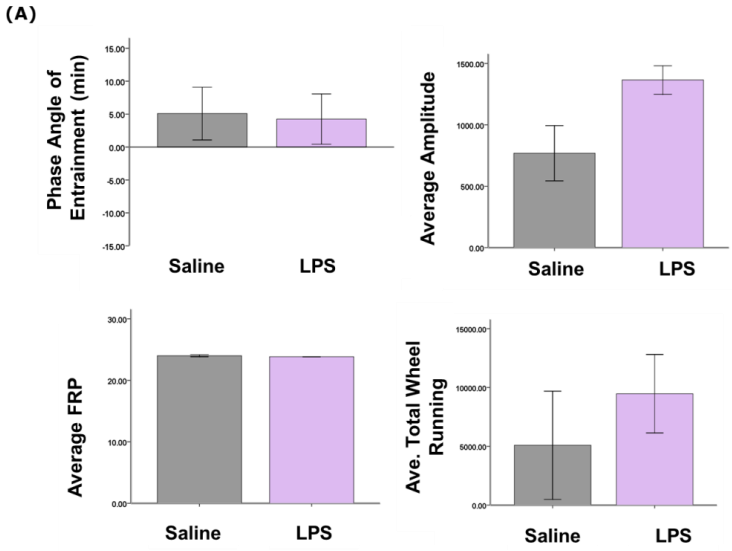


Figure 2.3: Examination of behavioural parameters in the week beginning 7 days following septic LPS treatment*. (A) Bar graphs showing the average phase angle of entrainment, FRP, amplitude and total wheel running average in the week beginning 7 days post-treatment (n=5-8 per group).*

2.3.2. Jetlag Experiments

2.3.2.1. Locomotor Activity following implementation of a 6hr Jetlag paradigm

When exposed to a 6 hour advance of the L:D cycle, post-septic animals re-entrainment to the new photocycle was faster than that seen in control animals. Mixed between within ANOVA was utilized to analyse the time of onset of wheel running activity for each individual animal in both treatment groups for the 7 days following the phase advance shift and revealed a significant time x treatment interaction ($F_{6,126}$=2.53, P<0.01; Figure 2.4). The time to entrain to the new photoperiod was 5.09±0.48 days for post-septic animals vs. 7.08±0.46 days for controls (P<0.01; Fig. 2.4 C). When these animals were then exposed to a 6 hour delay of the L:D photoperiod, mixed factorial ANOVA analysis of the time of onset of wheel running activity for each individual animal in both treatment groups for the 7 days following the shift revealed no time x treatment interaction ($F_{6,78}$=0.37, P>0.05) (Fig. 2.4). Independent t-test found the mean time to re-entrain was 3.1±0.2 days for post-septic animals vs. 3.2 ± 0.3 days for controls (P>0.05; Fig. 2.4

D). Visual inspection of actogram data showed saline controls exhibited transient days as expected following a phase advance of the photoperiod with locomotor activity showing gradual advances in locomotor behaviour onset until entrainment was achieved, however this is not seen in some of the post-septic animals (Fig. 2.4).

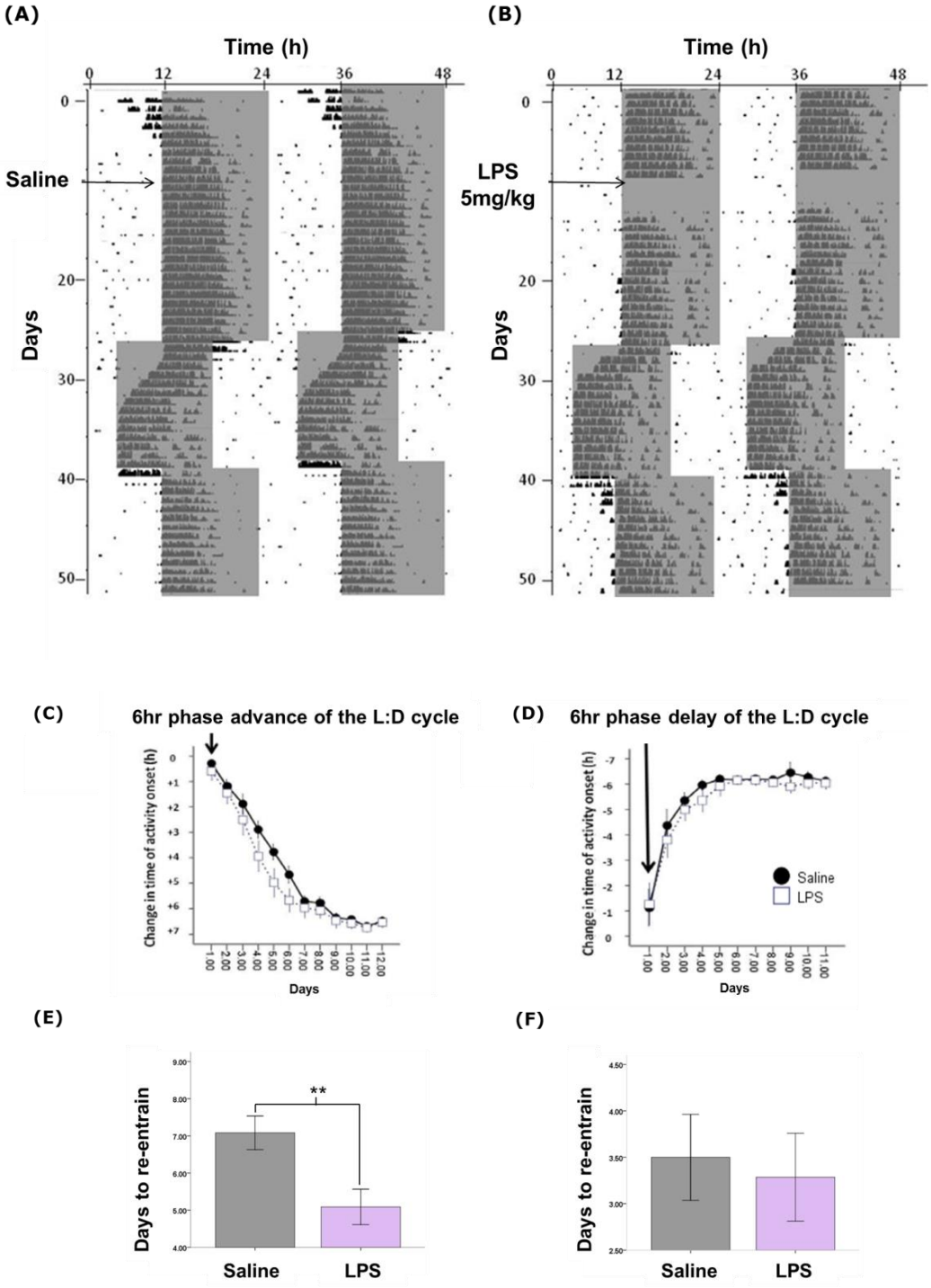


Figure 2.4: Altered rates of re-entrainment to 6hr phase shifts of the light:dark cycle in post-septic animals. *(A) and (B) Sample double plotted actograms from*

*saline and LPS treated animals who then underwent a 6hr advance of the L:D cycle, followed by a 6hr delay of the L:D cycle. The shaded area of the graphs is the dark phase of the L:D cycle. Note suppression of wheel running activity for a number of cycles following LPS treatment, but resumption of activity at the expected phase. (C) and (D) illustrate the activity onsets for the periods following the shift of the light:dark cycle. The post-septic animals show accelerated re-entrainment to the advanced schedule. Note the more rapid adjustment of the activity onsets in the LPS group vs. controls following the advance (C) but not the delay (D). (E) and (F) Bars graphs showing the average number of days to re-entrain (+/-SEM) to the shift of the L:D cycle for control and post-septic animals. (**=P<0.01).*

2.3.2.2. Re-entrainment following 10hr phase shifts of the photoperiod

When exposed to a 10 hour advance of the L:D cycle, post-septic animals showed more rapid re-entrainment to the new photoperiod than controls. Independent t-test analysis found the mean time to re-entrain was 8.9 $\pm$ 0.6 days for control animals and 7.1 $\pm$ 0.3 days for post-septic animals ($P<0.05$). When these animals were then exposed to a 10 hour delay of the L:D photoperiod, t-test assessment found there to be no significant difference in the rate of re-entrainment displayed between the treatment groups, with average time taken to entrain to the new photoperiod of 4.88 $\pm$ 0.23 days for post-septic animals and 4.71 $\pm$ 0.29 days for controls ($P>0.05$). (Fig. 2.5).

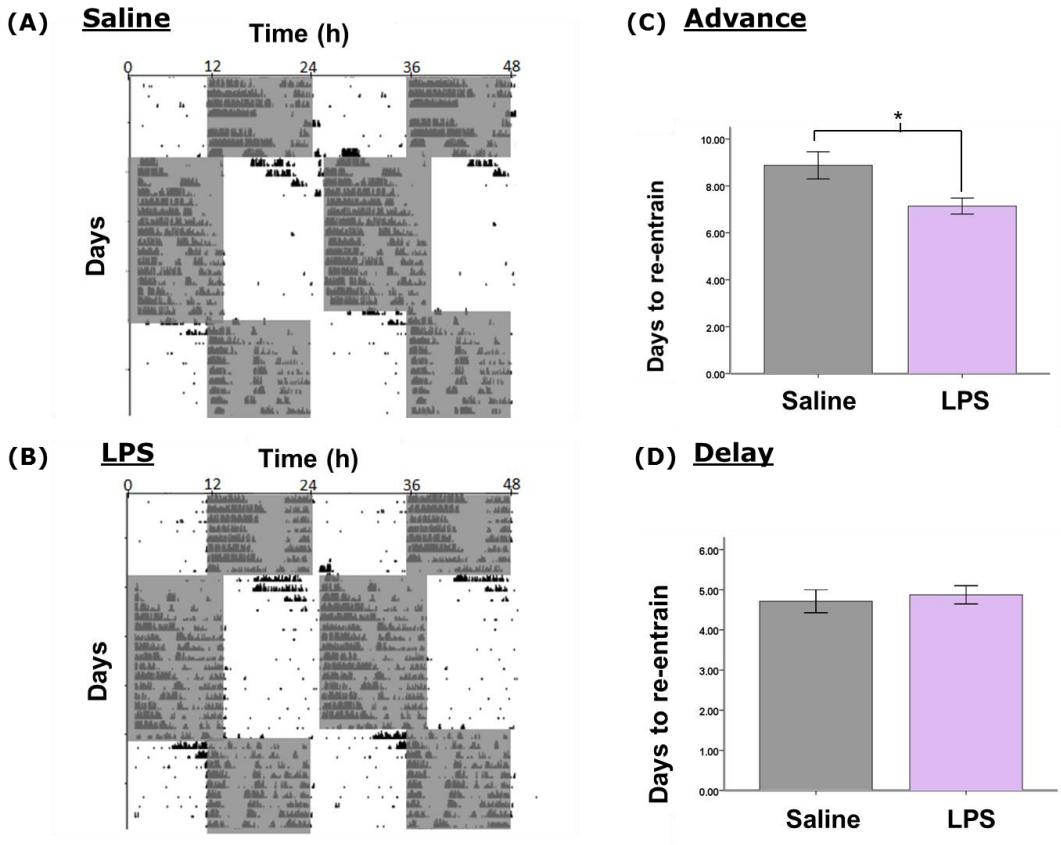


***Figure 2.5**: **Altered rates of re-entrainment to 10hr phase shifts of the light:dark cycle in post-septic animals.** **(A)** and **(B)**are sample double plotted actograms from saline and LPS treated animals who then underwent a 10hr advance of the L:D cycle, followed by a 10hr delay of the L:D cycle. The shaded area of the graphs is the dark phase of the L:D cycle. **(C)** and **(D)** Bars graphs showing the average number of days to re-entrain to the phase shifts of the L:D cycle. (*=P<0.05).*

2.3.3. The effects of sepsis on the basic parameters of the circadian locomotor activity rhythm.

Upon termination of circadian locomotor activity monitoring under experimental jetlag paradigms, the basic circadian parameters of free running period and rhythm amplitude were examined under the different lighting conditions. When released into both LL and DD conditions, monitoring of actogram data revealed no differences between the treatment groups in their transition to the different conditions, with locomotor activity continuing in phase with that of the previous L:D cycle, showing the appropriate advance (under DD) or delay (under LL) from activity onset on the last cycle of the L:D photoperiod to the onset of activity on the initial cycle under the new housing conditions. The free running periods and rhythm

amplitudes were examined for 14 days under constant light and constant dark conditions by Chi^2 periodogram analysis and subsequent assessment by t-tests for both treatment groups. Under LL, there were no significant differences between post-septic animals and saline controls with mean free running periods of 24.99 $\pm$ 0.19 hrs and 25.13 $\pm$ 0.10 hrs respectively (P>0.05). Independent t-test analysis of rhythm amplitude revealed that there were no significant differences between that of LPS treated (708.24 $\pm$ 76.83) and saline controls (748.83 $\pm$ 85.53, P>0.05). (Figure 2.6).

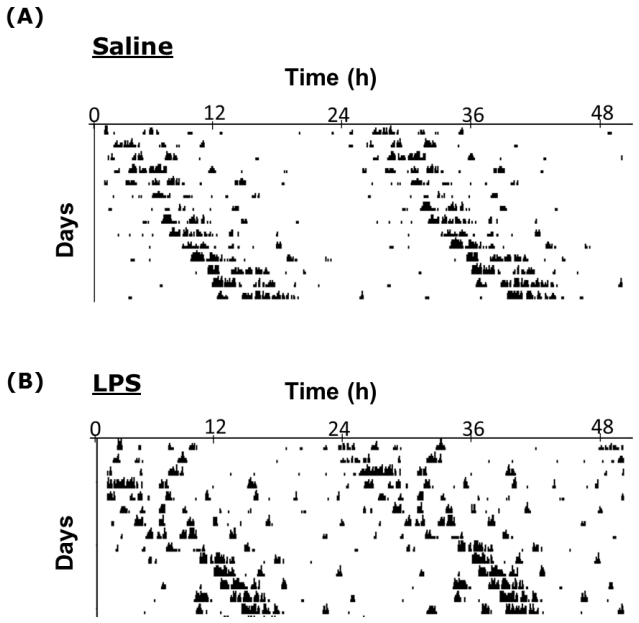


Figure 2.6. Locomotor activity Rhythms under LL. (A) *and* ***(B)*** *are sample double plotted actograms from a control and an LPS-treated animal respectively, showing locomotor behaviour under LL conditions.*

Comparison of mean free running periods in DD between post-septic treated animals and saline controls by independent t-test found no significant differences between the treatment groups, with free running periods of 23.6 $\pm$ 0.11 hrs and 23.73 $\pm$ 0.14 hrs respectively (P>0.05). Under housing in DD conditions, rhythm amplitude was not found to be significantly different between post-septic animals (1382.7 $\pm$ 227.7) and saline controls (1357.48 $\pm$ 232.7, P>0.05, independent t-test). (Figure 2.7).

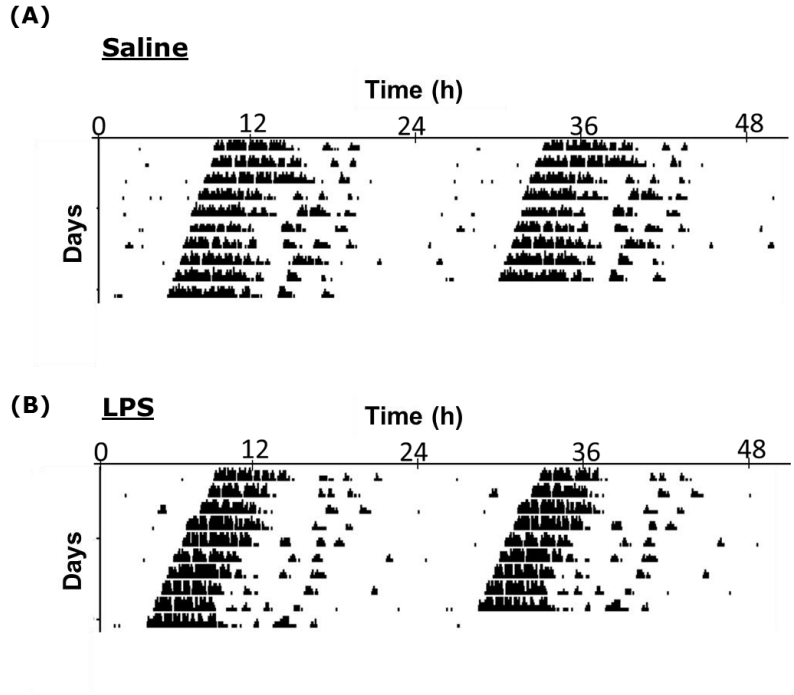


Figure 2.7. Locomotor activity Rhythms under DD. (A) *and* ***(B)*** *are sample double plotted actograms from a control and an LPS-treated animal respectively, showing locomotor behaviour under DD conditions.*

Independent t-test was utilized to analyse the mean wheel revolutions per hour for a period of 14 days while maintained under a 12:12 LD cycle for both treatment groups as a measure of overall activity levels. There were no differences in the amount of wheel running between the post-septic animals and saline controls, with mean revolutions in LPS-treated animals of 7676.48 $\pm$ 2095.7 vs. 9055 $\pm$3641.73 in controls ($P>0.05$). Analysis of rhythm amplitude under a 12:12 L:D cycle revealed that there were no significant differences between the mean rhythm amplitude of LPS treated animals (885.67 $\pm$ 38.2) and saline controls (865.93 $\pm$ 42.3, $P>0.05$, independent t-test). (Table 2).

Table 2: Assessment of core circadian parameters under various lighting conditions. *Table illustrating the average free running period values, rhythm amplitude and total wheel-running activity for saline and post-septic animals under different lighting conditions. (n.s.) denotes not significant.*

	Control	LPS
Rhythm Amplitude in LD	865.93 +/- 42.3	885.67 +/- 38.2 (n.s.)
Free running period in LL (h)	25.13 +/- 0.10	24.99 +/- 0.19 (n.s.)
Rhythm Amplitude in LL	748.83 +/- 85.53	708.24 +/- 76.83 (n.s.)
Free running period in DD (h)	23.73 +/- 0.14	23.6 +/- 0.11 (n.s.)
Rhythm Amplitude in DD	1357.48 +/- 232.7	1382.7 +/- 227.7 (n.s.)
Wheel revolutions per day	9055 +/- 3641.73	7676.48 +/- 2095.7 (n.s.)

2.3.4. Locomotor activity rhythms under Skeleton Photoperiods

2.3.4.1. Assessment of the phase angle of entrainment under skeleton and half skeleton photoperiods

An apparently normal pattern of circadian locomotor activity is exhibited by both post-septic animals and saline controls under a 12:12 L:D cycle. In order to examine whether this pattern of activity reflects appropriate circadian entrainment, or whether underlying circadian abnormalities were being masked by the L:D cycle, we assessed the entrainment of a group of animals to skeleton photoperiods.

The phase angle of entrainment was firstly assessed under a 12:12 LD cycle and independent t-test analysis showed no significant differences between the phase angle of entrainment for LPS treated animals (2.10 $\pm$ 5.69 min) vs. controls (-0.54 $\pm$ 1.47 min, $P>0.05$). Upon transfer from the 12:12 LD photocycle to the skeleton photoperiod (1L:10D:1L:12D), all animals maintained their entrained activity profiles, no alterations were seen in the relationship between locomotor activity onset on the first cycle of the new photoperiod and the onset of running wheel behaviour on the last cycle under the 12:12 LD cycle. Both saline and LPS animals began activity after the second light phase in the evening despite being exposed to

10hrs of darkness following the morning light phase. Comparison of mean phase angle relationship values during the first week under the skeleton photoperiod in post-septic animals and saline controls by independent t-test revealed no significant differences between the treatment groups, with mean phase angles of 18.01 $\pm$ 5.31min and 10.17 $\pm$ 1.90 min respectively ($P>0.05$). Independent t-tests showed that there were no significant differences between the phase angles of entrainment during the second (LPS, 21.11 $\pm$ 4.13 min. vs. saline controls, 15.29 $\pm$ 2.47 min, $P>0.05$) or third weeks (LPS, 26.08 $\pm$ 2.16 min. vs. saline controls, 18.25 $\pm$ 4.38 min, $P>0.05$) of housing under the skeleton photoperiod seen between the groups. (Fig. 2.8).

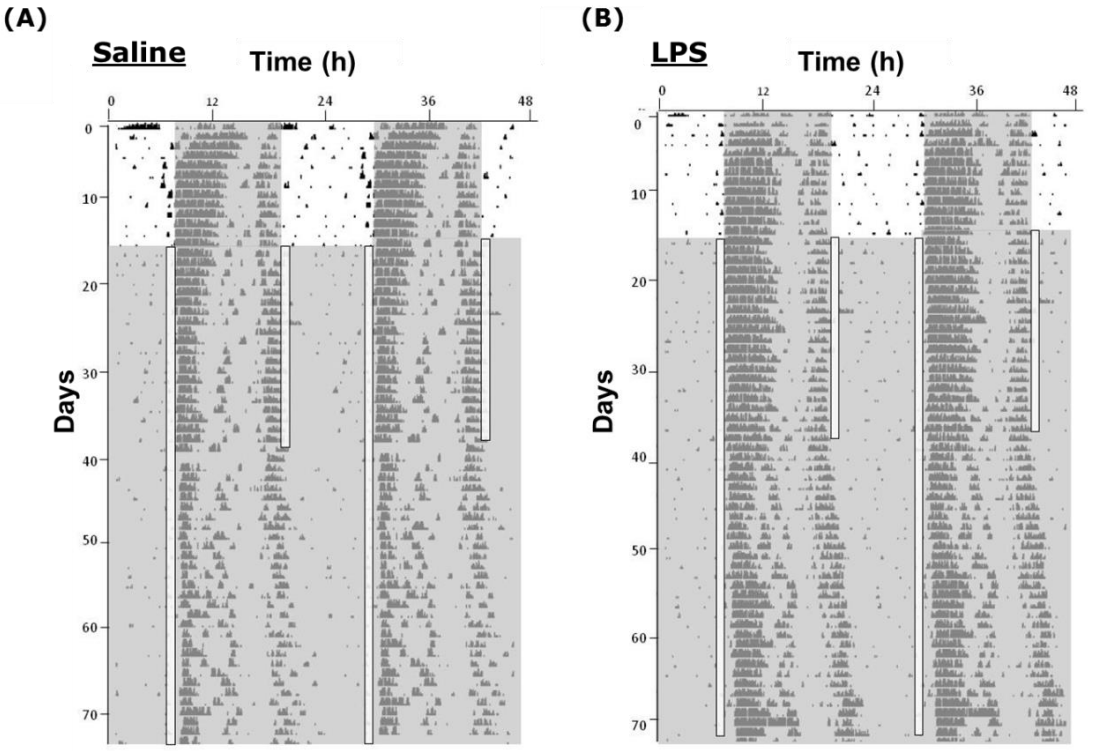
When the morning light pulse was removed under the half skeleton photoperiod (1L:23D), control and LPS animals remained entrained, with the onset of locomotor activity on the first cycle of the new photoperiod continuing in phase with the onset of running wheel behaviour on the last cycle under the skeleton photoperiod. Assessment of the phase angle relationship values during the first week under the skeleton photoperiod by independent t-test in post-septic animals and controls showed there to be no significant differences between the groups, with mean phase angle values of 39.11 $\pm$ 5.43 min and 28.63 $\pm$ 3.95 min respectively ($P>0.05$). Independent t-tests assessed that there were no significant differences between the phase angles of entrainment during the second (LPS, 46.76 $\pm$ 11.89 min. vs. saline controls, 42.77 $\pm$ 5.19 min, $P>0.05$) or third (LPS, 67.77 $\pm$ 12.33 min vs. saline controls, 51.39 $\pm$ 6.55 min, $P>0.05$) weeks of housing under the skeleton photoperiod, seen between the groups (Fig. 2.8). The similarities in circadian behaviour in post-septic animals under skeleton and half skeleton photoperiods when compared to controls at any point during this experiment indicate that normal entrainment is exhibited by post-septic animals.

2.3.4.2. The Free Running Period and Rhythm Amplitude in DD following maintenance under skeleton photoperiods

Upon termination of analysis of locomotor behaviour under skeleton and half skeleton photoperiods, the animals transfer into DD conditions was assessed and the free running periods and rhythm amplitudes were examined for 14 days under these conditions by Chi^2 periodogram analysis and subsequent assessment by t-tests.

Visual monitoring of actogram data revealed no differences between the treatment groups in their transition to constant conditions, with locomotor activity continuing in phase with the previous photocycle.

Comparison of mean free running periods in DD following prolonged housing under skeleton and half skeleton photoperiods by Chi^2 analysis and independent t-test found no significant differences between post-septic animals and saline controls, with mean free running period values of 23.98 $\pm$ 0.04 hrs. and 23.93 $\pm$ 0.03 hrs. respectively ($P>0.05$). Rhythm amplitude was not found to be significantly different between post-septic animals (1513.08 $\pm$ 206.75) and saline controls (1234.28 $\pm$ 234.38, $P>0.05$, independent t-test) maintained under DD.



(C)

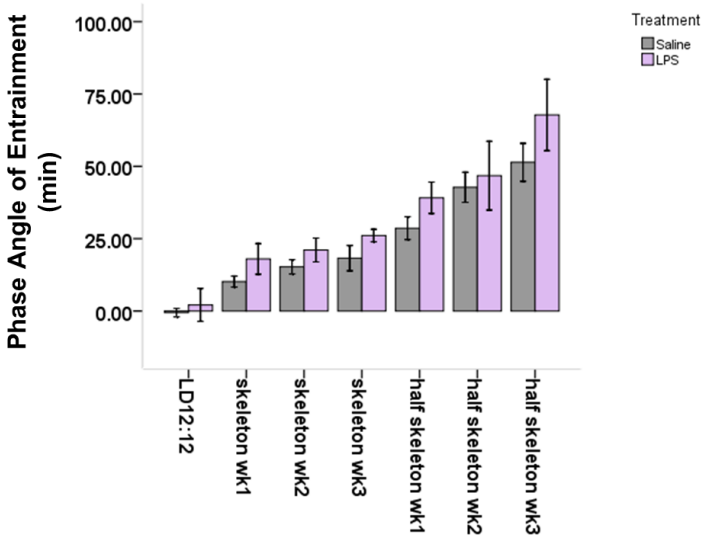


Figure 2.8: Entrainment to skeleton photoperiods. (A) *and* ***(B)*** *are sample double plotted actograms from a control and an LPS-treated animal respectively showing entrainment to a 12:12 L:D cycle, then a 1:10:1:12 skeleton photoperiod and subsequently a 1:11:12 half skeleton photoperiod. White bars in figures represent light phase.* ***(C)*** *Graph illustrating the average phase-angles of entrainment for each week under the experimental photoperiods for the control and post-septic animals.*

2.3.5. Locomotor Activity rhythms in response to a non-photic stimulus applied in the subjective day.

We examined the phase shifts elicited by a non-photic stimulus following application of the 5-HT1a/7 agonist 8-OH-DPAT at CT6. Comparison of 8-OH-DPAT induced phase shifts following administration in the early subjective day by independent t-test found that phase shifts of similar magnitudes were induced between the treatment groups, with a modest mean phase advance of 0.62 $\pm$ 0.23 hrs exhibited by saline controls which was shown not to differ significantly from those induced in post-septic animals, with a mean phase shift value of 0.77 $\pm$ 0.13 hrs ($P>0.05$, independent t-test; Fig. 2.9).

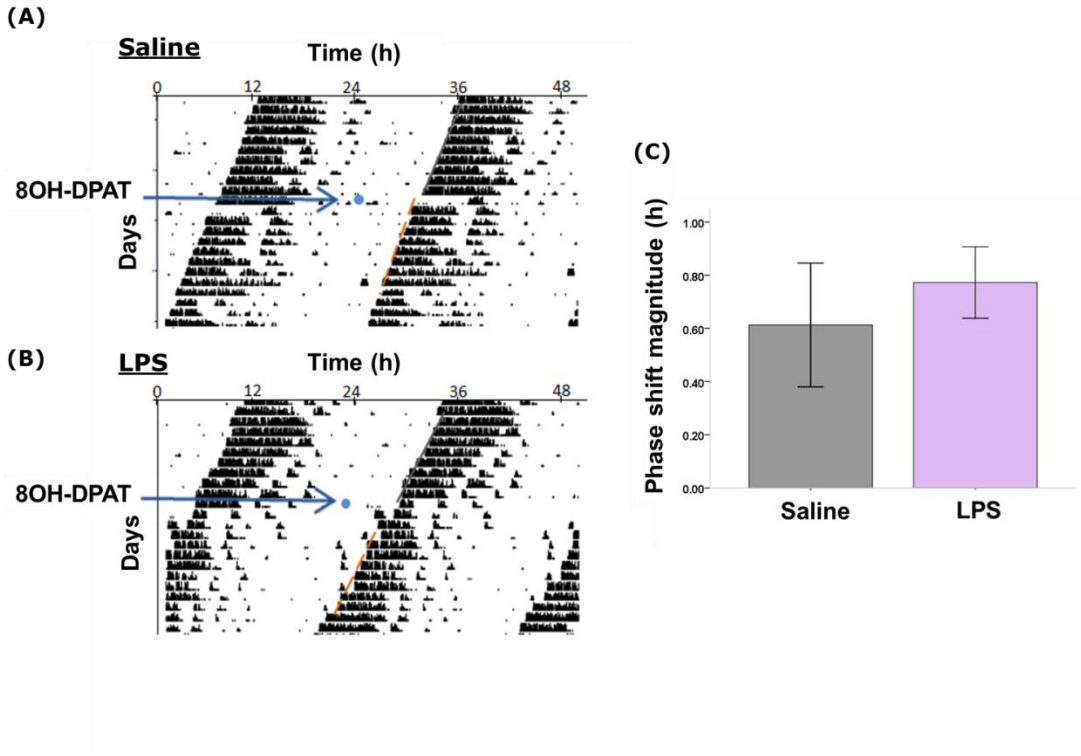


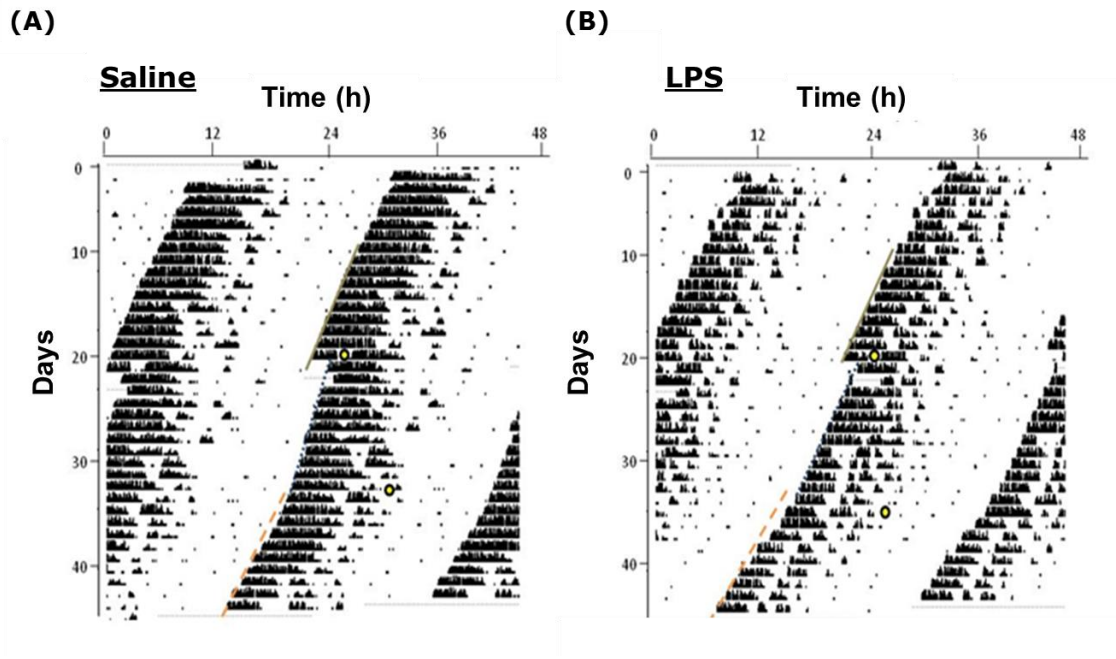
Figure 2.9: Phase resetting following application of a non-photic phase-shifting stimulus. (A) *and* ***(B)*** *are sample double plotted actograms of a control animal and a post-septic animal free running in DD and treated i.p. with 8-OH-DPAT at CT6 (CT6 treatment indicated by blue circle, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days after non-photic stimulus. Note exclusion of transient days from analysis).* ***(C)*** *Bar graph illustrating the modest phase advance induced following 8-OH-DPAT treatment in saline controls and post-septic animals.*

2.3.6. Construction of the Photic Phase Response Curve in post-septic animals

Two Way Factorial ANOVA with independent t-test and Bonferroni corrections were used for analysis of phase shift magnitude at the individual circadian time bins for each individual animal in both treatment groups, and found a significant effect of time ($F_{7,94}$=106.4, P<0.05), no main effect of treatment ($F_{1,94}$=0.31, P>0.05), but a significant interaction effect between CT of photic stimulation and treatment interaction term ($F_{7,94}$=2.4, P<0.05). Subsequent analysis of phase shift magnitudes exhibited by each treatment group at each individual CT bin found a statistically significant increase in phase shift magnitude in post-septic animals compared to controls at one time bin only, CT22-CT24. At CT22-CT24,

there was a statistically significant difference seen in the magnitude of photic phase advances elicited between post-septic animals and controls with average phase advance magnitudes of 1.45 $\pm$ 0.11 hrs and 0.87 $\pm$ 0.06 hrs respectively ($P<0.001$, independent t-test, Fig. 2.10). There were no differences in photic induced phase shift magnitude observed at any other circadian time bin of the PRC between the treatment groups ($P>0.05$).

At other circadian time bins during the subjective night when photic stimulation produces phase shifts of circadian behaviour, there were no alterations seen between treatment groups in phase shift magnitude elicited. At CT12-CT14, photic phase delay magnitudes were found to be similar between post-septic animals and controls with average phase delays of 1.97 $\pm$ 0.34 hrs and 1.55 $\pm$ 0.12 hrs respectively ($P>0.05$, independent t-test). There were no differences seen in the magnitude of photic phase delays between post-septic animals and controls at CT15-CT17 with average phase delays of 2.03 $\pm$ 0.10 hrs and 2.04 $\pm$ 0.22 hrs respectively ($P>0.05$, independent t-test, Fig. 2.10). At CT18-CT20, the mean magnitudes of photic phase shifts for post-septic animals and controls were 0.54 $\pm$ 0.33 hrs and 1.23 $\pm$ 0.30 hrs respectively ($P>0.05$, independent t-test). (Figure 2.10).



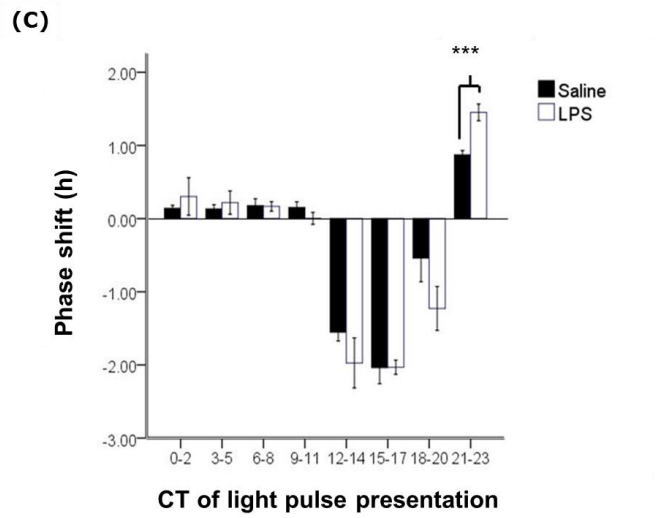


Figure 2.10**: **Altered photic phase-shifting in post-septic animals.** (**A**) and (**B**) are sample double plotted actograms illustrating the locomotor responses to photic stimulation in a saline and a post-septic animal respectively, free running in DD and subjected to light pulses (light pulse indicated by yellow circles, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days after the light pulse. Note the exclusion of transient days from analysis of the locomotor response). (**C**) Shows the photic phase response curve for saline and post-septic animals. Responses were grouped into 3 hour time bins according to LP time. (=P<0.001).*

2.3.7. Behavioural responses following treatment with PDTC prior to LPS treatment

2.3.7.1. Re-entrainment a 6hr phase shift of the photoperiod

Sepsis was scored in control and LPS treated animals (n=6) and in PDTC and LPS treated animals (n=6) and mixed between within ANOVA was used to assess statistically significant differences in mean sepsis scores between groups at regular intervals for 48hrs post-treament. ANOVA revealed a main effect of time for sepsis score post-treatment ($F_{8,80}$=25.43, P<0.001), as well a main effect of treatment ($F_{1,10}$=8.12, P<0.05), and a significant time treatment interaction ($F_{8,80}$=6.55, P<0.01). (Figure 2.11).

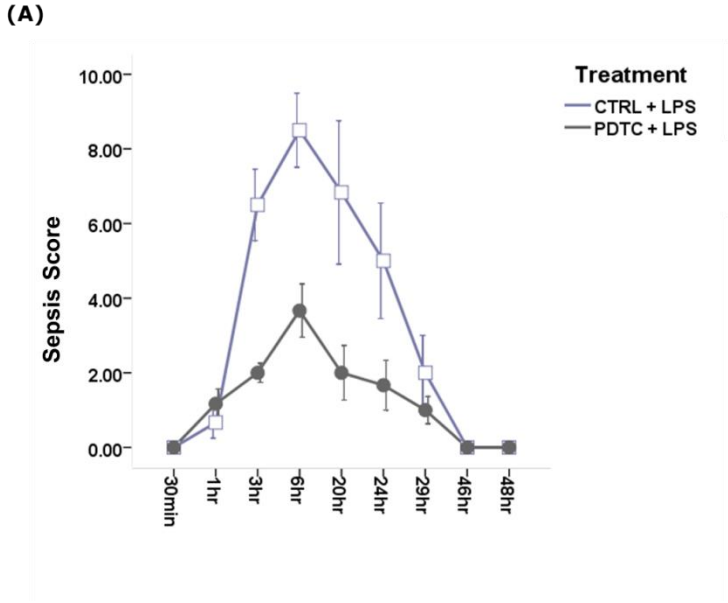


Figure 2.11: Sepsis scoring across 48hrs following treatment. *(A) Graph illustrating the sepsis scores for both control + LPS treated animals (n=6) and PDTC + LPS treated animals (n=6) quantified at regular intervals over 48hr following treatment. Note the lower sepsis scores for the PDTC + LPS treatment group.*

When exposed to a 6 hour advance of the L:D cycle, vehicle and LPS 5mg/kg treated animals re-entrainment to the new cycle was faster than was seen in animals treated with either PDTC 200mg/kg prior to LPS 5mg/kg, or control and 200mg/kg PDTC, or 200mg/kg PDTC prior to a control i.p. injection. One Way ANOVA with the Tukey post-hoc test was utilized to assess statistically significant differences between groups in the mean time taken for each group to re-entrain to the new photoperiod, finding a significant between groups effect ($F_{2,13}$=5.11, P<0.05). Post Hoc analysis (Tukey) revealed that the mean time taken to entrain to the new photoperiod of 5.4 $\pm$ 0.51 days for control and LPS treated animals differed significantly to the rate of re-entrainment seen for PDTC and LPS treated animals 7.0 $\pm$ 0.32 days (P<0.05). Control and LPS treated animals re-entrainment was also shown to be significantly different to the time taken for PDTC and control treated animals which took 7.0 $\pm$ 0.37 days synchronize to the new photoperiod (P<0.05). Comparison of the rates of re-entrainment between PDTC and LPS treated animals and PDTC and control treated animals revealed no significant differences between the groups (P>0.05). (Fig. 2.12)

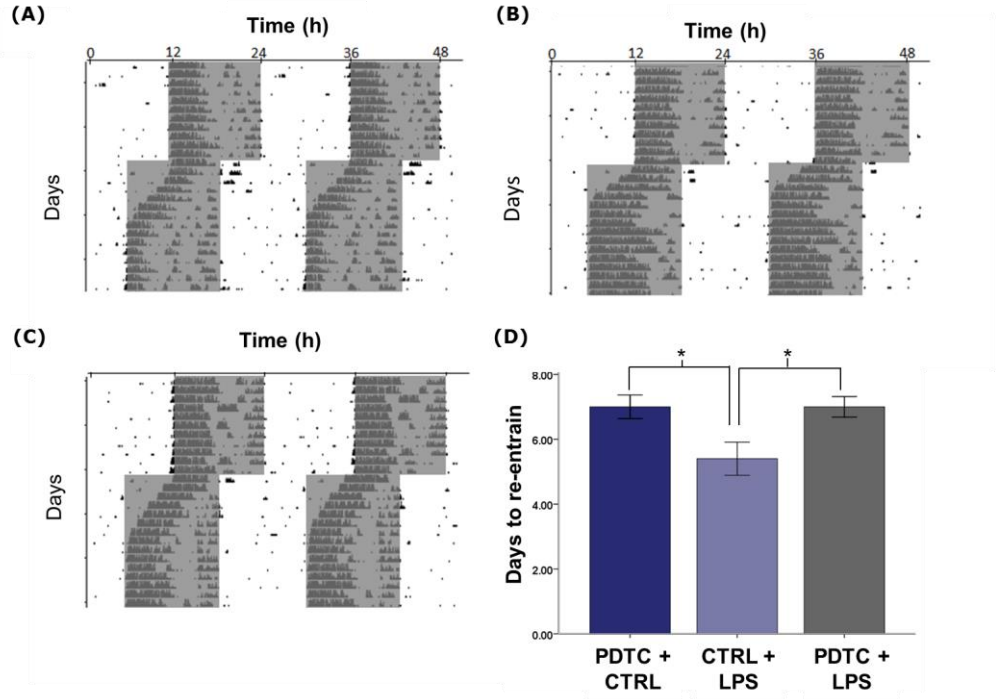


Figure 2.12: Re-entrainment to a 6hr phase advance of the light:dark cycle in PDTC + CTRL, CTRL + LPS and PDTC + LPS treated animals. (A), (B) and (C) *are sample double plotted actograms showing a PDTC + CTRL treated animal, a CTRL + LPS treated animal and a PDTC + LPS treated animal respectively.* ***(D)*** *Bar chart illustrating the average rate of re-entrainment of each group following a 6hr advance of the L:D cycle. (*=P<0.05).*

2.3.7.2. Phase advance resetting following PDTC treatment prior to LPS.

One Way ANOVA with Tukey post hoc test was used to assess whether there were statistically significant differences between groups in the mean phase advance magnitude, finding a significant between groups effect ($F_{2,16}$=22.79, P<0.001). Post Hoc analysis (Tukey) revealed that the mean phase advance magnitude of 1.3 $\pm$ 0.18 hrs revealed for control and LPS treated animals differed significantly from the mean phase shift magnitude seen for PDTC and LPS treated animals 0.92 $\pm$ 0.4 hrs (P<0.001). Control and LPS treated animals mean phase advance magnitude was also shown to be significantly different to that for PDTC and vehicle treated animals (0.89 $\pm$ 0.04 hrs; P<0.001). Comparison of the mean phase advance magnitude between PDTC and LPS treated animals and PDTC and control treated animals were not found to differ significantly between the groups (P>0.05). (Figure 2.13)

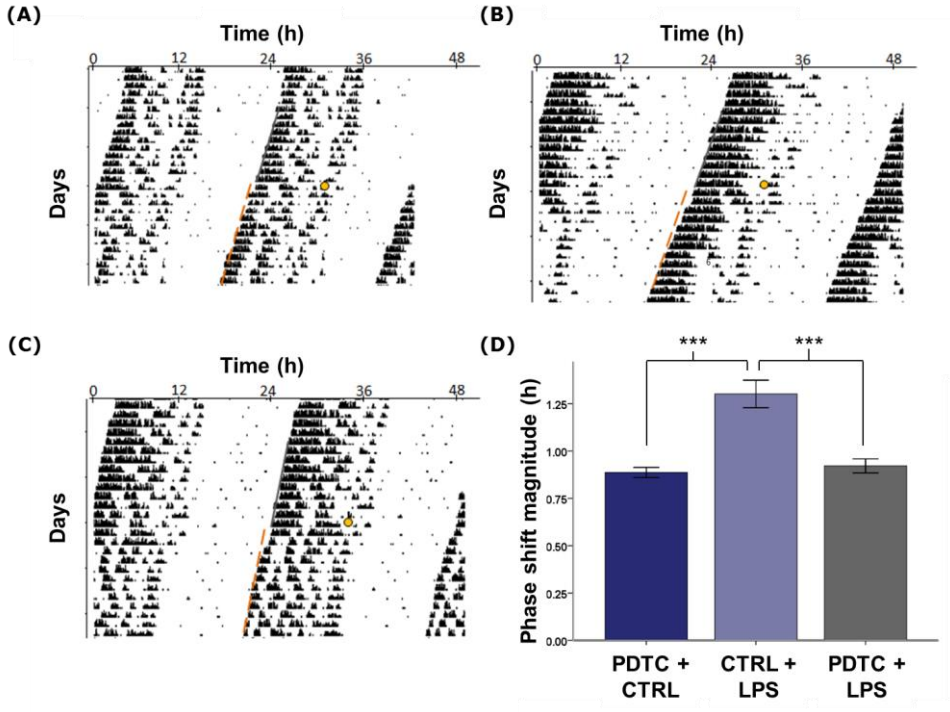


Figure 2.13: Phase resetting to photic stimulation at CT22 in PDTC + CTRL, CTRL + LPS and PDTC + LPS treated animals. (A), (B) and (C) *are sample double plotted actograms showing a PDTC + CTRL treated animal, a CTRL + LPS treated animal and a PDTC + LPS treated animal respectively, free running in DD and subjected to light pulses at CT22 (light pulse indicated by yellow circles, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days after the light pulse. Note the exclusion of transient days from analysis).* ***(D)*** *Bar chart illustrating the average phase shift magnitude of each group following a light pulse at CT22. (***=P<0.001).*

2.4. Discussion:

These experiments performed in post-septic animals reveal long-lasting perturbations in circadian behaviour up to three months following a single septic treatment with LPS. Post-septic animals exhibit alterations in photic phase-shifting, with exaggerated phase shift magnitudes in response to a phase advancing light pulse, and accelerated re-entrainment rates to phase advances of the photoperiod.

Acutely, visible sickness behaviour ensued following peripheral administration of LPS 5mg/kg and sepsis scoring allowed us to confirm successful induction of sepsis in LPS treated animals compared to vehicle treated controls. The induction of sickness behaviour following a septic LPS treatment inhibited rhythmic

wheel running behaviour in LPS treated animals for 2-3 cycles. Once recovered from the initial stages of the septic treatment, locomotor wheel running behaviour resumed in phase with that of the previous activity pattern and in line with that of the entraining light dark cycle. The assessment of total wheel running activity, phase angle of entrainment, FRP and rhythm amplitude in the week starting 7 days post-treatment found these post-septic behavioural parameters to be similar to controls and therefore there were no perturbations in the short term and no alterations prior to experimental manipulation of the photoperiod. However there was some variability in the average total wheel running values obtained within the groups, and the sample sizes may need to be increased and this parameter reassessed in order to confirm that there were no differences between groups.

For assessment of wheel running activity under experimental jetlag paradigms, we sought to characterize locomotor behaviour in animals having experienced septic shock in response to rapid 6hr and 10hr phase shifts of the light dark cycle, mimicking eastward and westward trans meridian travel. The rates of resetting in post-septic animals in response to phase advances of the photoperiod were found to be significantly more rapid than in controls, however there were no alterations found in resetting rates between post-septic animals or saline controls in response to either 6hr or 10hr phase delays of the light dark cycle. It must be pointed out that re-entrainment to a phase delay of the photoperiod has been shown to occur much faster than resetting to a phase advance, 3 days vs. 7 days respectively following a single one hour phase shift (Reddy et al., 2002) and it may be that synchronisation to the new light dark cycle following a phase delay simply takes place too quickly for any alterations in resetting to be observed between the groups. With respect to a 6hr phase delay of the photoperiod, given the short period of time that is required to reset activity to a phase delay of the light dark cycle, it is indeed possible that re-entrainment to this phase delay might occur too rapidly to observe any between groups differences. We would anticipate however, re-entrainment to an abrupt 10hr phase delay of the photoperiod which would require a longer time for resetting of locomotor behaviour to be completed, would highlight any differences in delay resetting between the groups, however no variations in resetting rates were observed.

The resetting rates to the 6hr phase delay shown by both treatment groups and the re-entrainment to a 6hr phase advance shown by our control animals, but not

post-septic animals, are in line with previously published results showing that re-entrainment to an abrupt phase advance of the photoperiod takes approximately 7 cycles, while resetting to a phase delay takes approximately 3 cycles (Reddy et al., 2002). Photic stimulation resulting in a phase advance causes transient shifts of locomotor activity onset over several cycles but an immediate shift in offset of activity, while the opposite is seen for onsets and offsets following a phase delay (Meijer and Schwartz, 2003). Large phase advances of the photoperiod cause gradual daily advances in locomotor behaviour of approximately 1-2 hours until re-entrainment to the new photoperiod has been achieved (LeGates et al., 2009). While this gradual advancing is seen in control animals, some post-septic animals appear to exhibit unstable advances in behaviour in the cycles following the 6hr advance shift (see Fig. 2.4).

Alterations in the basic circadian parameters and entrainment properties could account for the significantly more rapid re-entrainment rates of locomotor activity to shifts of the light dark photoperiod. The more rapid resetting visible in our post-septic animals and the unstable daily advances could be due to alterations in the free running period, since proportionately larger daily phase advances would be required of an animal with a shorter endogenous free running period (LeGates et al., 2009). However there are no alterations observed in the period between the groups under either LD, LL or DD conditions and therefore altered resetting cannot be attributed to perturbations in this parameter. Additionally, there were no alterations in total wheel running activities between the groups under any lighting condition. A damped or weak oscillator resulting in a decrease in pacemaker amplitude could result in decreased resistance of the SCN to external perturbing stimuli and a more robust response (Pulivarthy et al., 2007; Abraham et al., 2010) and could underlie enhanced resetting. Our experimental analysis of locomotor rhythmicity in LL would indicate that there is no decrease in pacemaker amplitude of post-septic animals, since post-septic animals display rhythmic, robust locomotor behaviour throughout the 14 days they were maintained under LL, and no alterations were observed in the FRP or rhythm amplitude between groups, and were there an underlying weakened pacemaker arrhythmicity would be expected to be observed. Further, no alterations were observed in the FRP or rhythm amplitude under DD, and additionally, the examination of actogram data of post-septic animals while free running in DD indicated that the post-septic behaviour was not being masked by the

presence of light. Were there an effect of masking of locomotor activity due to the presence of an entraining stimulus, upon removal of that stimulus, animals locomotor activity would exhibit an alteration in the phase relationship with perturbations in the phase of activity onset on the first day of constant conditions to that of the previous activity onset on the last cycle when under the influence of a Zeitgeber. Upon transfer to DD constant conditions there were no alterations in the phase angle relationship in either post-septic animals or controls. Furthermore, assessment of locomotor behaviour under skeleton and half skeleton photoperiods indicates that the behavioural rhythms of LPS treated animals are in fact entrained circadian patterns of locomotor activity and not merely due to the effects of negative masking. There were no perturbations in entrainment properties in post-septic animals and no differences between the treatment groups in the phase angle relationship under any of the lighting schedules, along with appropriate timing of activity, with no unexpected bouts of wheel running during the subjective day. Increases were observed in the difference between activity onset and the time of application of the dark phase upon increasing time spent under half skeleton photoperiods in both post-septic animals and controls, which is standard locomotor behaviour under these conditions, since the phase angle of entrainment is known to be more stable under a two pulse skeleton photoperiod than under a one light pulse half skeleton photoperiod.

That there were no alterations seen in entrainment properties under constant conditions or under housing in skeleton photoperiods was surprising, however, it is clear that the attenuations in resetting shown by post-septic animals cannot be contributed to alterations of the core circadian parameters examined in these studies.

Given the phase dependency of locomotor activity attenuations in post-septic animals, mice were exposed to 30 minute light pulses at all CT's while free running in DD i.e. an Aschoff type 1 protocol (Aschoff, 1965) to fully characterize the photic responsiveness of these subjects and construct a Phase Response Curve. Construction and assessment of the photic phase response curve of the post-septic animals was required, since it seemed a reasonable explanation that alterations could be present within its shape, accounting for the altered resetting and impaired SCN functional activation. By and large, the overall shape of the PRC in post-septic animals remained unaffected, with no variations in the proportions of the phase

advance, phase delay, or unresponsive regions. Upon construction of the PRC, following a phase advancing light pulse in the CT22-24 portion of the late subjective night, we observed a significant increase in the magnitude of the phase advance response in post-septic animals compared to controls, but no alterations in phase shift magnitude at any other phase of the circadian cycle including no attenuations in phase delay responses at any point between the groups. The notably larger phase shifts exhibited by post-septic animals in response to photic stimulation at CT22-24, the time at which a phase advancing light pulse produces maximal responses, points to a role of altered light processing in post-septic animals or a decrease in the amplitude of the endogenous pacemaker and the increased capacity for a perturbing stimulus to impact upon the SCN and subsequent output from it.

The significantly more rapid resetting to a phase advance of the light dark cycle and the enhanced phase shift magnitude to a phase advancing light pulse seen in post-septic animals was shown to be attenuated by administration of 200mg/kg PDTC, a potent inhibitor of the NF-κB pathway, prior to LPS 5mg.kg i.p. treatment, and values were seen to be in line with control animals. NF-κB has been implicated in the LPS-induced effects on the circadian system and the presence and action of this immune related transcription factur upon the SCN has been shown during LPS induced and photic induced phase shifts (Marpegan et al., 2004, 2005, Paladino et al., 2010). In hamsters, treatment with PDTC blocks light induced phase advances of locomotor behaviour (Marpegan et al., 2004), while application of sulfasalazine, an NF-κB inhibitor, has previously been shown to block the phase-shifting effects of peripheral LPS treatment in the early subjective night in mice (Marpegan et al., 2005). PDTC treatment was also shown to prevent the high sepsis scores elicited in LPS treated animals, with a significant decrease in sepsis scores between animals treated with a control injection and LPS, and those treated with PDTC prior to LPS. This is probably due to the inhibition of the release of proinflammatory cytokines following LPS treatment by PDTC administration and indicates that the induction of sepsis does indeed precede the alterations in photic resetting in LPS treated animals.

Enhanced sensitivity to light in post-septic animals could be responsible for either the accelerated resetting to a single day shift of the photocycle or the exaggerated phase shift magnitude following a phase advancing light pulse, however there are no alterations observed in phase delay reentrainment or reentrainment in response to a phase delaying light pulse, and no alterations in entrainment under

skeleton or half skeleton photoperiods, which might be expected to be attenuated were there perturbations in photic sensitivity. Further, it's possible that post-septic animals are not accurately perceiving or processing light information and the integrity of the retinohypothalamic tract or transmission from it may be compromised, however alterations in RHT transmission impact upon the phase angle of entrainment, and the current study observes no differences in this between post-septic animals and controls. The assessment of photic IEG induction in Chapter Three will shed further light on the photic sensitivity of post-septic animals.

The question as to what is underlying this altered resetting then arises, since we know that it is not due to attenuations in core circadian parameters. The resetting responses of the circadian system can involve various different components of the timekeeping system. Coupling and intercellular signalling in the SCN plays an important role in photic responsiveness and circadian entrainment. Alterations in the expression of neuropeptides that play roles in input to (VIP) and output from (AVP) the SCN could result in a decrease in pacemaker amplitude which could underlie post-septic circadian behavioural changes, since perturbations in the expression of these neuropeptides have previously been shown to affect the amplitude of rhythmicity in the SCN and the amplitude of output rhythms (Aton et al., 2006; Brown et al., 2007; Brown and Nunez, 1989; Mihai et al., 1994). The assessment of these neuropeptides was carried out and results from these data presented in Chapter Three. At the electrophysiological level, intercellular synchronization can influence phase-shifting responses (VanderLeest et al., 2009) and SCN electrical activity has shown to be impaired following a cocktail of IFN-γ and TNF-α (Lundkvist et al., 2002). Alterations in the amplitude or in the functional output of the SCN could account for the changes seen in post-septic animals behaviourally. Enhanced resetting in mice has previously been suggested to be due either to reduced pacemaker amplitude allowing increased efficacy of perturbing stimulants in phase-shifting the oscillator or an increase in the strength of phase-shifting input to the pacemaker (Winfree, 2001; Vitaterna et al., 2006). This enhanced resetting is a situation analogous to what we find in these studies and reduced pacemaker amplitude could therefore account for the exaggerated phase shift magnitudes in response to light and accelerated re-entrainment, since the circadian system appears to be more easily perturbed in post-septic animals. However, pacemaker amplitude is not always responsible for the enhanced phase shift responses and while many of

our results may point to a reduction in the amplitude of the pacemaker, we have shown there to be no alterations in the free running period or rhythm amplitude under any lighting conditions, no effects of negative masking on locomotor activity, and no alterations in phase upon transfer to constant conditions, all of which could be affected from a reduction in pacemaker amplitude. Furthermore, application of a non-photic stimulus, treatment with serotoninergic agonist 8-OH-DPAT, was found not to produce alterations in the magnitude of phase shifts elicited between groups, indicating that the circadian system of post-septic animals is not simply perturbed by any type of stimulus and ruling against a reduction in amplitude of the pacemaker. Any perturbations found in rhythmicity of post-septic animals were specific to phase advancing stimuli and all involve interactions between light and the timekeeping system.

It is clear that alterations in behavioural rhythm resetting are only seen during the advance portion of the phase response curve or in response to phase advancing stimuli, with these animals showing accelerated re-entrainment to advances of the L:D cycle, and showing larger magnitude phase shifts in response to light pulses delivered in the late subjective night (but no changes at other phases). The mechanisms by which the alterations in advance resetting occur in post-septic animals remain unclear. Studies have suggested that the difference in direction of phase shift responses could be due to changes in the expression of clock genes or their protein products (Reddy et al., 2002; Yan and Silver, 2002; Yan and Silver, 2004) and so alterations in these might cause alterations in behavioural responses. The circadian expression of clock gene protein products will be assessed in Chapter 4 to examine whether alterations in these could be involved in the attenuation of rhythmic behaviour seen in post-septic animals.

The intracellular signalling pathways underlying shifts of locomotor activity vary depending on the direction of the phase shift. In the late subjective night, the portion of the phase response curve at which photic stimulation induces phase advances of locomotor behaviour, the activation of a cGMP-dependent pathway that involves the activation of guanylyl cyclase and of a cGMP dependent protein kinase (PKG) is involved in the phase shift of behaviour (Tischkau et al., 2003; Agostino et al., 2004; Golombek et al., 2004). It's possible that alterations in the cGMP-PKG

pathway, associated with phase advances but not delays, could underpin the attenuations seen in advance resetting in post-septic animals.

Glucocorticoid rhythms have been demonstrated to influence the speed of re-entrainment to shifts in phase (Davidson et al., 2009), with faster resetting kinetics in rhythmic behavioural locomotor activity seen in response to manipulation of the photoperiod (Sage et al., 2004; Mohawk et al., 2005; Kiessling et al., 2010; Pezuk et al., 2012). Further, impairment of the HPA axis is seen in humans and in experimental sepsis studies in rodents, both in the acute and post-acute phase (Carlson et al., 2006; Polito et al., 2011), and inflammatory mediators have been shown to alter corticosterone rhythms (Shinohara et al., 2008). Examination of rhythmic corticosterone following the induction of the sepsis and during the time course at which manipulations of the light dark cycle resulted in altered resetting would be interesting, however would require further extensive studies.

It may be that similiarties exist between results we present in these studies, and those reported from studies on the effects of healthy ageing on circadian rhythmicity. In hamsters, it has previously been shown that ageing is associated with larger magnitude phase shifts following a phase advancing light pulse (Rosenberg et al., 1991) along with more rapid resetting to phase advances (Zee et al., 1992). These results however are not consistent, since other studies of aging have found slower rates of resetting or phase shifts of smaller magnitude along with a dampened circadian amplitude (Kiessling et al., 2010) which is in contrast with what we present in these studies. Ageing is associated with a low-grade central neuroinflammation (Godbout and Johnson, 2009), including the presence of neuroinflammation in the SCN (Deng et al., 2010) and so assessment of SCN neurochemistry in post-septic animals will assess whether there are parallels between the post-septic and ageing SCN (Chapter 3). Moreover, phase shifts in locomotor behavioural rhythmicity have been induced following response of the SCN to central proinflammatory cytokines (Leone et al., 2012), so assessment of the SCN post-sepsis is important to discern whether any of the changes we observed in behavioural locomotor activity might be due to actions of immune mediators on the SCN. Examination of the effects of sepsis induction on the acute and long term neurochemical composition of the SCN was performed and is presented in Chapter 3, the data from which further describes the effects of sepsis on circadian timekeeping.

Acute sepsis has been shown to affect cerebral glucose utilization, and analysis of glucose uptake has shown a decrease in cerebral metabolism associated with a decrease in cerebral blood flow and alpha activity in EEG (Semmler et al., 2008). Further studies have noted a consistent tendency for LPS induced sepsis to decrease cerebral glucose utilization in the long term following sepsis induction in the hippocampus, frontal cortex and striatum (Weberpals et al., 2009). Alterations in neuronal metabolism could play a role in behavioural impairments following LPS treatment. Attenuations in synaptic plasticity in the post-septic brain could play a role in the attenuations in circadian behaviour, and studies have shown that two months following a single septic LPS treatment, synaptic alterations are evidenced in the post-septic brain (Weberpals et al., 2009). Additionally, it is possible that the induction of sepsis might cause neuronal loss in the SCN which could account for attenuations in circadian rhythmicity. TUNEL analysis has revealed a significant increase in apoptotic cells in the brain 24hrs post LPS treatment in the hippocampus, cerebellum, midbrain and cortex, however this was at a higher dose (10mg/kg) than that used in the current set of experiments (Semmler et al., 2005). Further, experimental work using peripheral LPS 5mg/kg treatment has shown delayed neurodegeneration of dopaminergic neurons in the substantia nigra (Qin et al., 2007). Assessment of apoptotic markers in the SCN following LPS induced sepsis in Chapter 3 further clarifies this. Attenuations in neurotransmission in the post-septic brain could cause alterations within the CNS as a result of LPS induced cytokine modulations, since studies of the CNS in sepsis have observed evidence for altered neurotransmission (Van der Poll et al., 1996, Van der Poll, 2000; Toklu et al., 2009; Freund et al., 1985)

To date there have been no comprehensive studies examining circadian parameters as output measures of post-septic encephalopathy. The results from the assessment of circadian activity patterns in post-septic animals show that the core circadian parameters do not differ significantly in the circadian systems of post-septic mice and controls, but that an underlying mechanism is influencing resetting behaviour.

Chapter Three

The Effects of a Septic LPS treatment on SCN Neurochemistry.

3.1 Introduction

3.1.1. Septic doses of LPS treatment induces a long-lasting neuroinflammation

A single septic LPS treatment in the periphery has been shown to have a long-lasting impact on the innate immune system in the CNS (Weberpals et al., 2009; Qin et al., 2007; Bossu et al., 2012). In response to septic LPS treatment, the proinflammatory cytokine TNF-α is seen to rise in the periphery in the acute phase following treatment and to subside in the periphery approximately one week following LPS 5mg/kg treatment, but to stay elevated in the brain (Qin et al., 2007; Bossu et al., 2012). Elevated levels of cytokines are observed in the CNS 8 weeks post LPS 5mg/kg treatment, including upregulation of TNF-α in the prefrontal cortex and cerebellum, and an increase in IL-1β in the prefrontal cortex (Weberpals et al., 2009). Additionally, 5mg/kg LPS treatment results in an increase in NOS2 expression in the hippocampus and frontal cortex, as well as a sustained microglial activation in the frontal cortex and synaptic alterations up to 8 weeks following the immune challenge, and nitric oxide appears to play a role in these long-lasting LPS consequences (Weberpals et al., 2009). Acute sepsis has been shown to affect cerebral glucose utilization, and analysis of glucose uptake has shown a decrease in cerebral metabolism associated with a decrease in cerebral blood flow and alpha activity in EEG (Semmler et al., 2008). Further studies have assessed cerebral glucose uptake two months following sepsis induction, and there was a consistent tendency for LPS induced sepsis to decrease cerebral glucose utilization in the hippocampus, frontal cortex and striatum however this didn't reach significance in the sample in these studies (Weberpals et al., 2009). Alterations in neuronal metabolism could play a role in behavioural impairments following LPS treatment.

Other studies using a model of chronic i.c.v. infusion of LPS to induce neuroinflammation in rats observes an increase in the level of GFAP positive astrocytes and reactive microglia throughout the brain, in particular in the

hippocampus (Hauss-Wegrzyniak et al., 1998), and a long-lasting upregulation of microglia has been shown 37 days following the final LPS administration (Hauss-Wegrzyniak et al., 2000). Additionally, this chronic neuroinflammation model causes the induction of TNF-α, IL-1β and β-amyloid precursor protein mRNA levels within the basal forebrain region and hippocampus, as well as degeneration of CA3 hippocampal pyramidal neurons (Hauss-Wegrzyniak et al., 1998). Further, chronic LPS infusion has been shown to cause loss of NMDA receptors in the hippocampus and entrohinal cortex (EC) (Rosi et al., 2004) and a time-dependent, but not dose-dependent, degeneration of nucleus basalis magnocellularis (NBM) cholinergic cells (Willard et al., 1999). Additionally, following 10mg/kg LPS treatment in rats, TUNEL analysis revealed a significant increase in apoptotic cells in the brain 24hrs post-treatment, in the hippocampus, cerebellum, midbrain and cortex (Semmler et al., 2005).

LPS 5mg/kg i.p treatment has been shown to cause elevated TNF-α levels in the brain up to 10 months following the immune challenge, along with irreversible damage to dopaminergic neurons in the substantia nigra (SN) which are seen to degenerate between 7 and 10months post-treatment (Qin et al., 2007). Studies by Bossu and colleagues (2012) assessing the impact of a septic LPS treatment on the CNS have shown chronic region specific cytokine alterations in the CNS 10 months following treatment, and have shown both TNF-α and IL-18 to be upregulated in the hippocampus, cerebellum and frontal cortex.

At these timepoints, in the long-term following LPS treatment, when circulating levels of proinflammatory cytokines have returned to baseline levels, the elevation of inflammatory mediators in the brain is therefore independent of the initial systemic reaction, and is being propelled in the CNS itself, leading to the view of a self-propelling neuroinflammation (Qin et al., 2007; Bossu et al., 2012). The precise mechanisms underlying the chronic neuroinflammatory state following a septic LPS treatment have not been fully elucidated, but have been suggested to involve the action of TNF-α in the periphery in the early stages, leading to microglial activation in the CNS and subsequently a self-propelling neuroinflammation by the further induction of cytokines in the CNS such as TNF-α, IL-1β (Qin et al., 2007) and IL-18 (Bossu et al., 2012), leading to a loss of neurons in the later stages following peripheral inflammation (Qin et al., 2007; Semmler et al., 2007) and lasting behavioural alterations (Bossu et al., 2012).

3.1.2. LPS induced neuroinflammation and subsequent cognitive alterations

Peripheral LPS treatment and subsequent microglial activation and cytokine release is associated with behavioural deficits (Bluthe et al., 1992; Godbout et al., 2005; Combrinck et al., 2002). Alterations in cytokine levels in the CNS and the neuroinflammatory response may lead to neurobehavioral impairments and delirium (Lemstra et al., 2007). Neuroinflammation and microglial activation are implicated in the pathogenesis of various disorders associated with cognitive impairment such as AD, Lewy body dementia and AIDS dementia (Nelson et al., 2002; Katsuse et al., 2003; Perry et al., 2003). LPS treatment causes alterations in cognitive function. The influences of cytokines on behaviour are mediated by cytokine action on the neuroendocrine pathway, neural plasticity, effects on neurocircuitry and function of neurotransmitters (Capuron and Miller, 2011).

Peripheral LPS has been shown to exert effects on sleep architecture and sleep-wake behaviour (Krueger and Majde, 1994). Promptly following LPS treatment, sickness behaviour is seen to develop and peaks between 2 and 6 hours, and is seen to slowly decline after 6hrs, while at 24hrs post LPS treatment depressive like behaviour is expressed (Frenois et al., 2007; Dantzer et al., 2008). Sickness behaviour is mediated by temporarily expressed proinflammatory cytokines in the brain (Dantzer, 2004) and can be blocked by administration of the anti-inflammatory IL10 or the IL-1 receptor antagonist, IL-1RA, supporting the role that proinflamamtory cytokines are outlined to play in the acute phase reaction (Kent et al., 1992; Bluthe et al., 1995; Avitsur et al., 1997; Dantzer et al., 2008).

Alterations in learning and memory are seen both acutely and chronically following LPS administration. Administration of low dose endotoxin to healthy volunteers results in an increase in levels of systemic proinflammatory cytokines, as well as behaviour and memory perturbations (Reichenberg et al., 2001; Krabbe et al., 2005). Acutely in mice, when administered hours before assessment of cognitive function, LPS treatment causes learning and memory impairments (Arai et al., 2001; Sparkman et al., 2005). Single LPS treatment has been shown to cause spatial memory deficits up to 11 days following the immune challenge (Shaw et al., 2001). *In vivo* and *in vitro*, LPS has been shown to reduce hippocampal long-term potentiation (LTP) (Cunningham et al., 1996; Commins et al., 2001; Jo et al., 2001) which is an indicator of synaptic efficiency in the formation of learning and memory

(Malenka and Nicoll, 1999; Malenka and Bear, 2004). In similar studies, the inflammatory suppression of LTP was ameliorated by the inhibition of NOS2 (Mori et al., 2001; Togashi et al., 2001; Wang et al., 2004). Further, in a model of cerebral ischemia, inhibition of NOS2 by aminoguanidine was seen to attenuate learning and memory deficits (Mori et al., 2001).

In the long term, LPS treated animals also show impaired spatial memory performance 2 months post-treatment, and this was protected in the absence of NOS2 derived nitric oxide (Weberpals et al., 2009), highlighting the role of immune mediators in LPS induced cognitive dysfunction. Further, chronic LPS infusion has been shown to cause significant impairment in spatial memory (Hauss-Wegrzyniak et al., 1998), while three months following 10mg/kg LPS treatment, memory deficits have been evidenced in studies in rats (Semmler et al., 2007). More recently, it has been shown that ten months following a septic peripheral immune challenge with endotoxin, LPS induced cognitive impairments are evident, and these have been suggested to be possibly as a result of LPS induced delayed loss of neurons (Bossu et al., 2012).

3.1.3. LPS treatment and its effects on the SCN and the circadian system

As discussed previously, various proinflammatory cytokines are known to impact upon circadian behaviour including TNF-α (Cavadini et al., 2007; Nygard et al., 2009), IL-6 (Motzkus et al., 2002), IL-1β (Cavadini et al., 2007), IFN-α (Ohdo et al., 2001; Koyanagi and Ohdo, 2002) and IFN-γ (Lundkvist et al., 2002; Kwak et al., 2008). The SCN expresses cytokine receptors and regulatory molecules (Lundkvist et. al., 1999; Sadki et. al., 2007) and responds in the acute phase to a substantial peripheral immune challenge, showing functional changes and alterations in clock gene expression following LPS administration (Beynon and Coogan, 2010; Marpegan et al., 2005; Okada et al., 2008).

Septic doses of LPS have been shown to induce a substantial increase in the expression of the IEG c-Fos in the SCN acutely, as well as activation of the p65 subunit of the NF-κB pathway (Beynon and Coogan, 2010), while low LPS doses are also seen to cause functional activation of the SCN as evidenced through c-Fos expression (Marpegan et. al., 2005). Mice treated with LPS i.p. for 60 days exhibit altered SCN function as illustrated through a decrease in photically-induced c-Fos

expression, shown to be reversible over time (Palomba and Bentivoglio, 2008). The first day following LPS treatment, the clock gene *Per2* is shown to be suppressed in the SCN as is the clock controlled gene *Dbp,* with normal expression restored from 48hrs post-treatment. *Per1, Per2* and numerous clock controlled genes expression was inhibited in the periphery in the liver 24hrs following LPS with restoration of rhythmicity 2days post the immune challenge (Okada et al., 2008). The neurotransmitter AVP is increased in the SCN following *in vitro* treatment of SCN slices with LPS (Nava et al., 2000).

The PVN has also been shown to be affected following LPS treatment. In rats following 2.5mg/kg LPS treatment, *Cox-2* mRNA levels have been shown in blood vessels in the PVN, peaking 2 h post-treatment (Quan et al., 1998). Further, following either i.v. or i.p. LPS treatment, levels of TNF-α and IL-6 are significantly increased in the PVN in the acute phase (Kakizaki et al., 1999). Additionally, the circadian clock gene *Per1* has been shown to be induced in the PVN of the hypothalamus following LPS administration (Takahashi et al., 2001).

Given the long-lasting neuroinflammation induced by LPS treatment, the influence of inflammatory mediators on the CNS and on behaviour and the consequences systemic LPS exerts on the SCN and circadian behaviour, we examined the possibility that LPS induced circadian attenuations may be due to alterations in the expression of immune mediators in the SCN. The specific aims of this portion of the study were, to examine the neurochemistry of the SCN both in the acute phase following a peripheral septic LPS treatment, and in the long term, when attenuations in circadian locomotor behaviour are observed in post-septic animals.

3.2. Materials & Methods:

3.2.1. Animals and Housing:

Adult C57BL/6 male mice (8-10wks old) used to assess SCN neurochemistry in the acute phase, 24hrs following LPS 5mg/kg treatment, were group housed as outlined in section 2.2.2. In order to examine SCN neurochemistry in the long term following LPS 5mg/kg treatment, adult C57BL/6 male mice (4-5mths old) housed

individually in polypropylene cages equipped with steel running wheels (described in section 2.2.2) and used for the assessment of circadian behavioural rhythmicity following a septic LPS treatment, were used upon termination of behavioural monitoring (approximately 3mths post-treatment) were terminally anaesthetized and subject to transcardiac perfusion and assessment of the SCN by immunohistochemistry.

3.2.2. Treatment:

LPS 5mg/kg treatment and PDTC administration prior to LPS treatment were performed as outlined previously (section 2.2.3.3 and section 2.2.3.1 respectively).

3.2.3. Transcardiac perfusion and tissue preparation:

All perfusions were carried out in the middle of the lights on phase (ZT4-8), unless otherwise noted. Animals were terminally anesthetised with an i.p. injection of sodium pentobarbital (Euthathal, Merial Animal Health, UK), the chest cavity opened and animals perfused transcardially with approximately 30mls of 0.9% NaCl at 4˚C, followed by perfusion with approximately 50mls of 4% paraformaldehyde (PFA, Sigma) in 0.1M Phosphate Buffer (PB) at 4˚C. Brains were carefully removed, post-fixed overnight in 4% PFA for 24hrs at 4˚C and were then cryoprotected in 30% Sucrose (Sigma) in 0.1M PB for a further 24hrs at 4˚C. The brain was removed from the 30% sucrose, and cut caudally removing the cerebellum and rostrally to remove the preoptic region with a single edged razor blade. Brains were mounted on a freezing stage microtome (Leica), utilizing the minimal amount of mounting medium, with the caudal cut surface attached to the stage and the ventral aspect facing the blade. Brains were quick frozen with dry ice and 30µm thick serial coronal sections were cut throughout the rostrocaudal extent of the SCN. All sections through the SCN were collected and divided in 4 series, obtaining approximately 5 or 6 sections through the SCN in each series. The sections were stored in 0.1M PB, pH 7.4 (Sigma) with 0.1% sodium azide at 4°C to inhibit microbial growth prior to being processed for immunohistochemistry.

3.2.4. Immunohistochemistry Protocol:

For immunohistochemistry purposes the avidin-biotin-peroxidase complex (ABC)/Nickel DAB colourometric protocol method (Beynon and Coogan, 2010,) was used following this protocol:

Free floating sections in 12 well tissue culture plates were washed at room temperature for 10min in 0.1 M PB (pH 7.4) twice. They were then washed for 10 min in PBX consisting of 0.1 M PB and 0.03% Triton X-100 (Sigma). PBX perforates the cell membrane. Sections were incubated with 0.1M PB and 1.5% Hydrogen peroxide (H_2O_2) for 20 minutes. H_2O_2 inactivates endogenous peroxidases and reduces non-specific background staining. Sections were then subjected to another set of 10 minute washes as before, 2 x PB and 1x PBX. A non-specific antibody blocking step was then carried out in 0.1M PBX with either 5% Normal Goat Serum (NGS, for rabbit and rat polyclonals) or Normal Horse Serum (NHS, for goat polyclonals) for 60min at room temperature. Sections were incubated with primary antisera raised in either rabbit, rat or goat diluted in 0.1M PBX and 2% NGS or NHS (blocking solution) for 24-48hrs at 4°C.

Table 3: Primary Antisera used for Immunohistochemical analysis

Antibody	Dilution	Raised in	Supplier	Product Code
c-Fos (4)	1:2000	Rabbit	Santa Cruz Biotechnology	sc-52
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
GFAP	1:1000	Rabbit	Sigma, St. Louis, MO, U.S.A	G4546
IBA-1	1:1000	Rabbit	Wako, Denmark	019-1974
Cleaved Caspase-3 (Asp175)	1:200	Rabbit	Cell Signaling Technology, Danvers, MA, U.S.A.	#9661
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
NF-κB p65 (C-20)	1:200	Rabbit	Santa Cruz Biotechnology	sc-372
p-IKK α/β (Ser 180/ Ser 181)-R	1:200	Rabbit	Santa Cruz Biotechnology	sc-23470-R
p-IκB- α (Ser32/36)	1:200	Rabbit	Santa Cruz Biotechnology	sc-101713
AVP	1:5000	Rabbit	Millipore, Temecula, CA, U.S.A.	AB1565
VIP	1:1000	Rabbit	Millipore, Temecula, CA, U.S.A.	AB982

Following incubation with the primary antisera, sections were put through a series of washes as before, two PB and one PBX wash, and incubated with the appropriate biotinylated secondary antibody (1:400 biotinylated goat anti-rabbit Jackson Immuno research Labs, 1:400 biotinylated anti-goat or 1:400 anti-rat) diluted in 0.1M PBX and 2% NGS or NHS (blocking solution) for 70 min at room temperature. Due to the photosensitive nature of the biotinylated secondary antibody, sections were shielded from light for the remainder of the experiment. Sections were then treated using the avidin-biotin method (0.4%) in 0.1M PBX with a Vectastain Elite Universal Kit (Vector Laboratories) for 90min at room temperature. Sections were washed twice for 10min in 0.1M PB and then in 0.1M sodium acetate (pH6, Sigma) for 10min. Immunoreactivity was visualised with light sensitive nickel-enhanced diaminobenzidine, (3, 3'-diaminobenzidene with ammonium nickel chloride, NiDAB), (pH6), as chromogen, 1000ul NiDAB and 60ul catalyst, glucose oxidase (5mg/ml) per well. Once sufficient staining was obtained, sections were washed in 0.1M sodium acetate to stop the reaction and then underwent three final washes in 0.1M PB. Tissue sections were finally stored in 0.1M PB at 4˚C, then mounted onto gelatine coated slides(1% gelatine and 0.05% chromium potassium sulphate) and allowed to dry before being put through dehydrating and delipifying steps. They were dehydrated through a graded series of 3min ethanol washes, 70%, 90% and 100%, and were subsequently cleared in a series of 3 min delipifying steps in 2% Histoclear (National Diagnostics, UK) washes. Slides were left to air-dry overnight before being coverslipped using mounting media (Eukitt, Fluka Analytical).

All sections were treated in exactly the same manner at all stages of tissue processing to minimise any interassay variability. As much as possible, tissue sections from different experimental groups were reacted in parallel when processed for the same antibody, otherwise standardised immunostaining procedures were ensured between assays, including immunoreactivity times when incubated in NiDAB.

In parallel with IHC staining for an antibody of interest, immunizing peptide blocking experiments were performed where an immunizing blocking peptide for the antibody was available, in order to examine whether non-specific binding of the antibody to proteins other than the antigen might be taking place (see Section 3.5, Figure S1).

3.2.5. Quantitative Analysis:

Photomicrographs of the mid-rostrocaudal levels of the SCN were taken using a digital camera connected to an Olympus BX-51 light microscope equipped with an image analysis digital system (ImageJ 1.43, NIH, USA). All images were taken using the same camera and magnification settings. For analysis, brain sections were examined under either the 10X or 40X objective lens. Between 3 and 6 images were evaluated for each individual animal and region depending on the rostro-caudal extent of the area being examined and a mean value obtained for each animal. Immunoreactive cells in each region of interest were quantified using either image analysis software or by quantification of immunoreactive (ir) cell number by an observer by manual counting for the purpose of analysing immunoreactive nuclear staining. Brain sections were counted according to anatomical location throughout the rostro-caudal extent of the SCN. The Suprachiasmatic nuclei were measured at the mid rostro-caudal level, including the core and shell regions of these structures. The observer was blinded to the experimental procedure during optical density measurements or quantification of immunoreactive cells per SCN. The difference in integrated optical density (IOD) of immonostaining in the SCN or the number of immunoreactive cells that displayed clear nuclear staining in the SCN of all animal groups was evaluated for quantitative analysis. The light intensity was kept constant while all measurements were taken to standardize IOD measurements for analysis. A previously described method whereby the image was binarised for analysis was

used for integrated optical density measurements (Vilaplana and Lavialle, 1999). Results are given as mean values (IOD or cell number) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

3.2.6. Assessment of Acute effects of LPS on the SCN

In order to examine whether a septic dose of LPS would cause the expression of immune factors in the SCN or would induce signs of apoptosis, adult male mice were group housed in colonies of ten in cages equipped with appropriate environmental enrichment under a 12:12 LD cycle (150 lux, lights on 0700) for one month, allowing for habituation. Animals were treated at ZT6 with either an LPS 5mg/kg i.p. injection (n=3-5) or saline vehicle i.p. (n=3-5) and, while still in the acute phase following sepsis induction, 24 hours later (ZT6), were terminally anaesthetized and perfused transcardially as previously outlined in section 3.2.3. The immunohistochemistry protocol outlined in section 3.2.4 was adhered to for staining purposes. Brains were then processed by immunohistochemistry for the microglial marker F4/80, the proinflammatory cytokines TNF-α and IL-6, the immediate early genes protein products EGR-1 and ARC and for NOS2, a signaller known to be induced by neuroimmune processes (all outlined in Table 3).

For each individual animal and each antibody, 3-6 SCN images were examined by means of manual quantification of the number of immunoreactive nuclei in the SCN or assessing the difference in IOD of antibody immunosignal in the SCN as previously outlined in section 3.2.5., and a mean value obtained for each animal for each antibody for all SCN regions of interest. These values were calculated for each animal by two researchers. The observers were blinded to the experimental procedure during IOD or quantification of immunoreactive cells per SCN. The means calculated for each group were compared and analysed by independent samples t-test. Results are given as mean values (IOD or cell number) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

3.2.7. Assessment of apoptosis 24hrs following induction of sepsis

TUNEL (Terminal deoxynucleotidyl transferase-mediated d-UTP Nick End Labeling) Analysis (DeadEnd Fluorometric TUNEL Staining kit, Promega, U.K.) was carried out for the detection and quantification of apoptotic cells in the SCN or

throughout the brain 24hrs following LPS 5mg/kg i.p. (n=4) injection or saline vehicle (n=4). Nuclear DNA fragmentation occurs during apoptosis as a result of the activation of endonucleases that cleave chromosomal DNA, and DNA strand breaks are therefore an important apoptotic hallmark (Gavrieli et al 1992, Darzynkiewicz et al., 2008). The TUNEL method (Gavrieli et al 1992) was utilized on 30 µm thick sections cut through the rostro-caudal extent of the SCN, obtained as previously described (section 3.2.3). Sections were mounted on poly-L-Lysine-coated microscope slides. A 1:10 dilution of Poly-L-Lysine (Sigma) was pipetted onto pre-cleaned glass slides which were then allowed to air dry. Once the slides were dry they were rinsed in deionized water and allowed to air-dry again for 60min. Poly-L-Lysine coated slides were stored at 4°C and used within 7 days. Brain sections containing the optimum mid SCN were floated on very dilute 0.1M PB before placement on Poly-L-Lysine coated slides. The slides with mounted SCN sections were allowed to air-dry overnight at room temperature prior to TUNEL analysis. The TUNEL kit, was used according to manufacturer's instructions. All solutions and reagents outlined below were supplied as part of the TUNEL analysis kit unless otherwise stated.

Slides were immersed in Phosphate Buffered Saline (PBS) for 5 min at room temperature followed by fixation of the cells in 4% PFA in PBS for 5mins. Formaldehyde serves to crosslink low molecular weight DNA fragments to other cellular constituents, thereby preventing their extraction during rinses. Slides were immersed 2X in PBS for 5 min. Permeabilization of the cells was achieved by treatment of the sections with cell permeabilization solution, 100µl of 20µg/ml Proteinase K solution (100mM Tris-HCl, pH8.0; 50mM EDTA) was added to each slide to cover the tissue sections and incubated for 15min at room temperature. The slides were tapped to remove excess liquid and incubated for 10min at room temperature in 100µl of Equilibration Buffer (200mM potassium cacodylate, pH 6.6 at 25°C; 25mM Tris-HCl, pH 6.6 at 25C; 0.2mM DTT, 0.25mg/ml BSA and 2.5mM cobalt chloride) in order to equilibrate the tissue sections. Slides were then put through 2X 5min PBS rinses before being incubated with 100µl ice cold Terminal Deoxynucleotidyl Transferase Recombinant (rTdT) reaction mix (98µl Equilibration buffer, 1µl Biotinylated Nucleotide Mix, 1µl rTdT Enzyme). TdT catalytically incorporates biotinylated nucleotide to the 3'-OH DNA ends. Sections were covered with plastic coverslips to ensure even distribution of the reagent and placed for 1hr in

a humidified chamber (Memmert, Schwaback) at 37°C to facilitate the end-labelling reaction. The slides were taken from the incubator, plastic coverslips removed and in order to stop the reaction, slides were immersed for 15min in 2X SSC made by diluting 20X SSC (87.7g NaCl, 44.1g sodium citrate in 400ml deionized H_2O, adjusted to pH 7.2 with 10N NaOH and brought to a final volume of 500ml) 1:10 in deionized H_2O. Slides were put through 3X 5min PBS washes to remove unincorporated biotinylated nucleotides and endogenous peroxidases blocked by 5min incubation of the slides in 0.3% H_2O_2 at room temperature. Slides were washed 2X for 5min in PBS and 100µl Horseradish peroxidase-labelled streptavidin (Streptavidin HRP) diluted 1:500 in PBS was then added to the slides, which were incubated for 30 min at room temperature. Streptavidin HRP binds to the biotinylated nucleotides. Slides were put through 3X 5min PBS washes and the DAB substrate kit utilized in order to observe TUNEL staining (Promega, U.K.). The Streptavidin HRP bound biotinylated nucleotides were then detected using peroxidase substrate, hydrogen peroxide and the stable chromogen diaminobenzidine (DAB). 100µl DAB solution (50µl DAB substrate 20X Buffer in 950µl deionized water, 50µl DAB 20X Chromogen and 50µl Hydrogen Peroxide 20X) was added to each slide and the slides incubated for approximately 10min until a light brown background stain was achieved. Apoptotic stained nuclei appeared as dark brown. Slides were rinsed several times in deionized water before being coverslipped with permanent mounting medium (Eukitt).

Quantitative analysis of the number of TUNEL-positive cells was carried out for each individual animal by an observer using an Olympus BX-51 light microscope under 10X objective lens by means of manual counting with the aid of a hand held counter. Three SCN images were examined per animal in order to assess the number of TUNEL positive stained cells in the SCN. The Suprachiasmatic nuclei were measured at the mid rostro-caudal level, including the core and shell regions of these structures. The observer was blinded to the experimental procedure for quantification of TUNEL positive cells per SCN. The difference in the number of TUNEL stained cells that displayed clear staining in the SCN of all animal groups was evaluated for quantitative analysis. These values were calculated for each animal by two researchers blind to the experimental procedure and the means for each group were compared and analysed by independent samples t-test. Results are given as

mean values (cell number) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

In order to further assess whether apoptosis had taken place following LPS 5mg/kg treatment, the immunohistochemistry protocol (outlined in section 3.2.4) was used for staining for cleaved caspase-3. Animals were perfused transcardially, brains were removed and then processed by immunohistochemistry as described in section 3.2.3. For each individual animal, 3-6 SCN images were examined by means of assessment of the difference in IOD of antibody immunosignal in the SCN as previously outlined in section 3.2.5, and a mean value obtained for each animal for all SCN regions of interest. These values were calculated for each animal for each antibody in both groups by two researchers blinded to the experimental procedure during IOD measurements of the SCN and analysed by independent samples t-test. Results are given as mean values (IOD) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

3.2.8. The long-lasting effects of sepsis induction on SCN neurochemistry

In order to assess whether or not the induction of sepsis causes long-lasting effects on the neurochemistry of the SCN, both LPS and saline treated animals used in experiments analysing circadian locomotor activity rhythms previously outlined in Chapter Two, were transferred into a 12:12 LD cycle (150 lux, lights on 0700) upon termination of behavioural monitoring. Animals were maintained under this schedule for 14 days and then approximately 3months following the initial induction of sepsis were terminally anaesthetized and perfused transcardially as outlined in section 3.2.3. Brains were carefully removed at ZT6 and processed for immunohistochemistry as outlined in section 3.2.4. Brains were then processed by immunohistochemistry for markers of glia, proinflammatory cytokines, immediate early genes, SCN neuropeptides and for signallers known to be induced by Neuroimmune processes (primary antisera outlined in Table 3).

For each individual animal and each antibody, 3-6 SCN images were examined by means of manual quantification of the number of immunoreactive nuclei in the SCN or assessing the difference in integrated optical density of antibody immunosignal in the SCN as previously outlined in section 3.2.5, and a mean value obtained for each animal for each antibody for all SCN regions of

interest. These values were calculated for each animal by two researchers blind to the experimental procedure and the means for each group were compared and analysed by independent samples t-test. Results are given as mean values (IOD or cell number) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

3.2.9. Assessment of SCN neurochemistry following PDTC administration prior to LPS.

In order to assess the involvement of NF-κB in the LPS mediated effects on SCN neurochemistry, three experimental animal groups treated with either PDTC and LPS (n=10), PDTC and control (n=7) or control and LPS (n=10) used in experiments analysing circadian locomotor activity rhythms previously outlined in section 2.2.11, were transferred into a 12:12 LD cycle (150 lux, lights on 0700) upon termination of behavioural monitoring. Animals were maintained under this schedule for 14 days and then approximately 3 months following the initial treatments were terminally anaesthetized and perfused transcardially as outlined in section 3.2.3. Brains were carefully removed at ZT6 and processed for immunohistochemistry as outlined in section 3.2.4. Brains were then processed by immunohistochemistry for IBA-1 (primary antisera outlined in Table 3).

For each individual animal and each antibody, 3-6 SCN images were examined by means of manual quantification of the number of IBA-1 immunoreactive cells in the SCN as previously outlined in section 3.2.5, and a mean value obtained for each animal for each antibody for all SCN regions of interest. These values were calculated for each animal by two researchers blind to the experimental procedure and the means for each group were compared and analysed by independent samples t-test. Immunoreactivity was compared between groups using One Way ANOVA. Results are given as mean values (immunoreactive cell number) ± standard error of the mean, statistical significance was accepted at $P<0.05$.

3.3. Results:

3.3.1. Examination of immune mediators in the SCN 24hrs post LPS treatment

24 hours following LPS 5mg/kg treatment while animals were still in the acute phase, Immunohistochemical (IHC) analysis was carried out to investigate whether there was any effect of LPS treatment on the expression of immune mediators in the SCN and whether these might be identified as being involved in mediating the effects of LPS on the circadian system.

3.3.1.1. TNF-α expression in the SCN acutely following LPS treatment.

TNF-α expression was assessed in the SCN 24hrs post LPS treatment or saline (Saline 4, LPS 4). Immunohistochemical analysis followed by Integrated optical density measurements of immunoreactive TNF-α expression in the SCN and independent t-test analysis revealed no alterations in the SCN between saline controls (7.22 ± 2.19 IOD/SCN) and post-septic animals (2.56 ± 0.46 IOD/SCN, P>0.05; Figure 3.1), including no significant differences in expression of the proinflammatory mediator in the core region of the SCN (saline, 3.69 ± 1.15 vs. LPS 2.46 ± 0.57 IOD/SCN, P>0.05) or in the dorsomedial shell sub region (saline, 8.50 ± 2.64 vs. LPS, 2.75 ± 0.48 IOD/SCN, P>0.05).

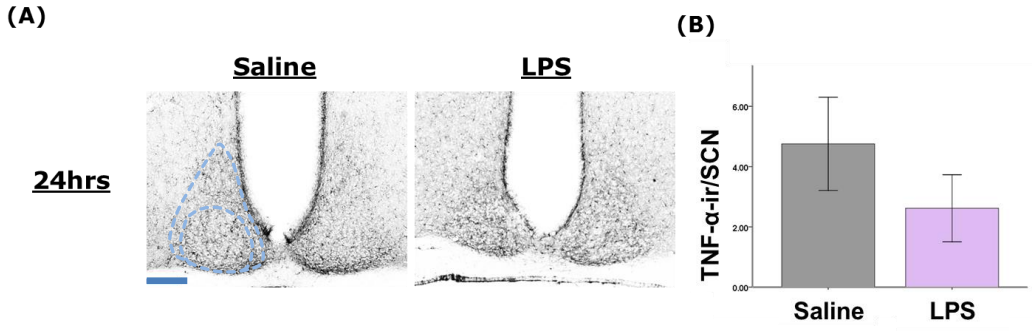


Figure 3.1: TNF-α expression is not altered in the SCN 24hrs post LPS treatment. *(a) Representative photomicrographs of TNF-α expression in the SCN of animals treated 24hrs previously with LPS (scale bar approx. 100 µm). Dashed line outlines the SCN and delineates the shell and core subdivisions. (B) Bar graphs illustrating quantification of levels of TNF-α immunostained cells (n = 4 for each group) in the SCN of the control and LPS treated groups at 24hrs post LPS treatment.*

3.3.1.2. LPS 5mg/kg treatment causes an increase in SCN NOS2 expression.

The SCN was examined for the acute induction of inducible nitric oxide synthase (iNOS or NOS2) in the SCN 24hrs following peripheral LPS (n=4) or saline (n=4). Integrated Optical Density analysis and assessment by independent t-test found there to be a significant difference in the levels of NOS2 expression in the SCN between groups, with a significant upregulation of NOS2 immunosignal in the SCN of post-septic animals (29.67 $\pm$ 3.19 IOD/SCN) compared to controls (12.67 $\pm$ 3.54 IOD/SCN, $P<0.05$; Figure 3.2) 24hrs post-treatment. Immunostaining revealed no difference in ventrolateral core NOS2 expression between controls (22.24 $\pm$ 10.29 IOD/SCN) and LPS treated animals (60.28 $\pm$ 16.56 IOD/SCN, $P>0.05$, independent t-test). Integrated Optical Density measurements of immunoreactive NOS2 found the difference in NOS2 immunosignal to be statistically significant in the SCN shell between the groups, with significantly higher NOS2 immunoreactivity in LPS treated animals (23.09 $\pm$ 4.07 IOD/SCN) and control animals (10.83 $\pm$ 2.61 IOD/SCN, $P<0.05$, independent t-test; Figure 3.2).

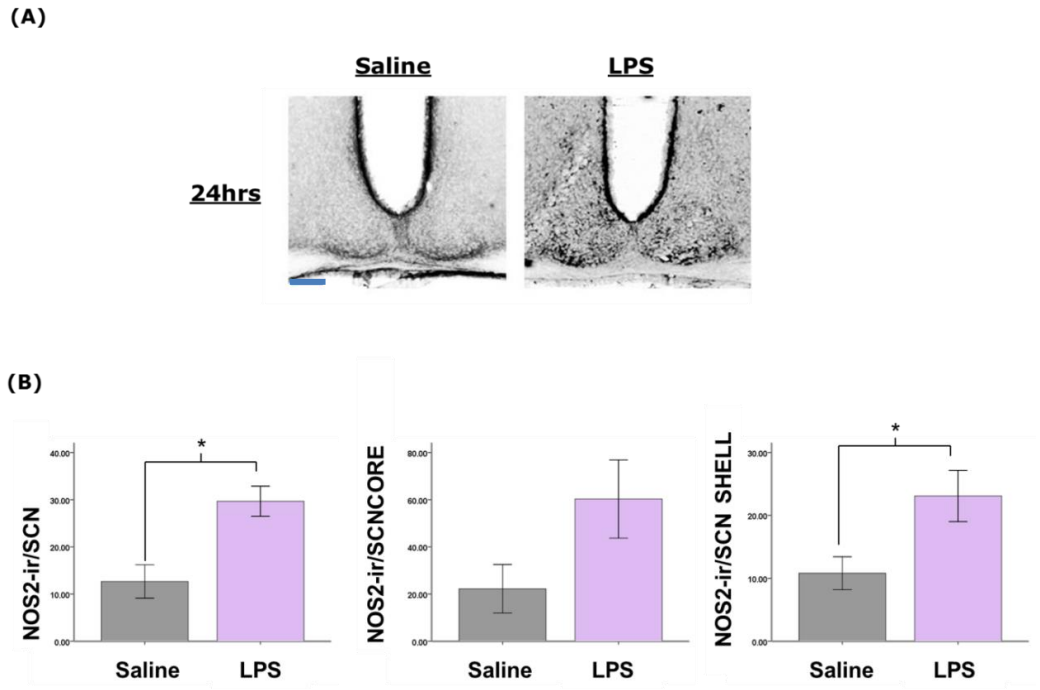


Figure 3.2: NOS2 expression is altered in the SCN 24hrs following LPS treatment. *(A) Representative photomicrographs of NOS2 expression in the SCN of animals treated 24hrs previously with LPS (scale bar approx. 100 µm). (B) Bar graphs illustrating quantification of levels of NOS2 immunostained cells (n = 4 for each group) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals at 24hrs post LPS treatment. (* P<0.05).*

3.3.1.3. LPS 5mg/kg treatment causes an increase in SCN IL-6 expression.

24hrs post LPS 5mg/kg treatment, the expression of the proinflammatory cytokine interleukin-6 (IL-6) was seen to be altered in the SCN. IOD measurements showed a significant difference in immunoreactive IL-6 expression in the SCN between control (1.47 $\pm$ 0.28 IOD/SCN) and post-septic animals (3.58 $\pm$ 0.52 IOD/SCN, $P<0.01$; Figure 3.3) with the LPS treated animals showing significant upregulation of IL-6 immunoreactivity. Upon SCN regional examination, this upregulation in expression was found to be statistically significant in the SCN core region of LPS treated animals (3.37 $\pm$ 0.77 IOD/SCN) compared to saline controls (0.79 + 0.16 IOD/SCN, $P<0.05$, independent t-test), while expression in the SCN shell region was also seen to be statistically different between saline (1.74 $\pm$ 0.35 IOD/SCN) and LPS animals (3.79 $\pm$ 0.61 IOD/SCN, $P<0.05$, independent t-test).

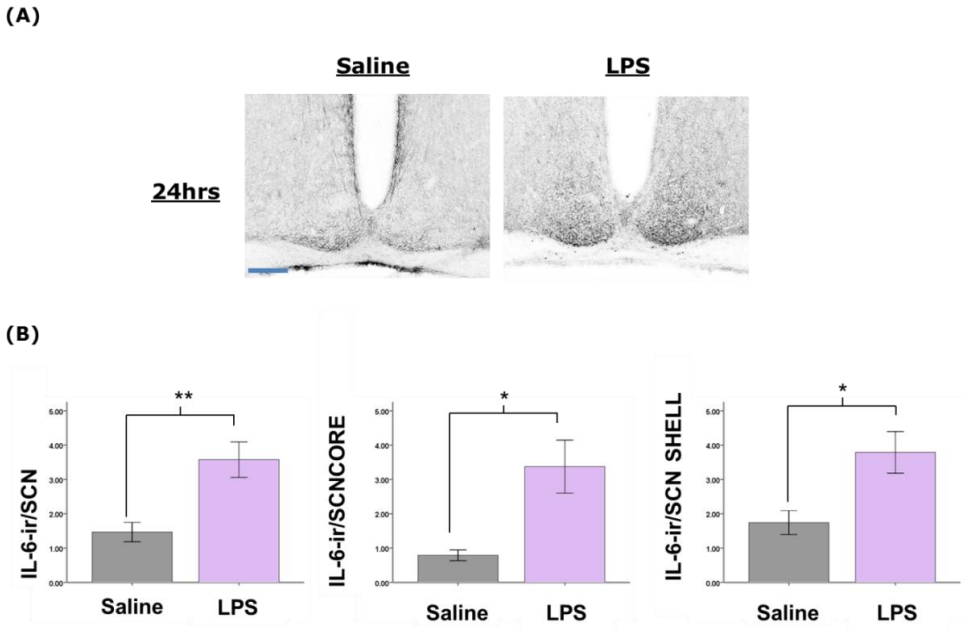


Figure 3.3: IL-6 expression is altered in the SCN 24hrs following LPS treatment. *(A) Representative photomicrographs of IL-6 expression in the SCN of animals treated 24hrs previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of IL-6 immunostained cells (n =5 for each group) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals at 24hrs post LPS treatment (** P<0.01, * P<0.05)*

3.3.2. Immediate Early Gene Expression 24hrs post LPS 5mg/kg treatment

3.3.2.1. The expression of ARC is not altered in the SCN 24hrs following LPS

Comparison of ARC immunosignal in the SCN 24hrs post LPS 5mg/kg treatment by immunohistochemistry combined with manual quantification of immunoreactive nuclei and subsequent independent t-test analysis found levels of expression of ARC immunoreactive nuclei in the SCN of LPS treated animals (n=5) analogous to controls (n=5), with mean values of 0.82 $\pm$ 0.67 and 0.57 + 0.19 ir. cells/SCN respectively (P>0.05; Figure 3.4). Independent t-test analysis of levels of ARC immunoreactive cells revealed no difference in expression in the SCN subdivisions, with similar expression levels in the core (saline, 0.00 $\pm$ 0.00 vs. LPS, 0.10 $\pm$ 0.10 ir. cells/SCN, P>0.05) and shell regions of the SCN (controls 0.50 $\pm$ 0.17 vs. LPS 0.72 $\pm$ 0.57 ir. cells/SCN, P>0.05).

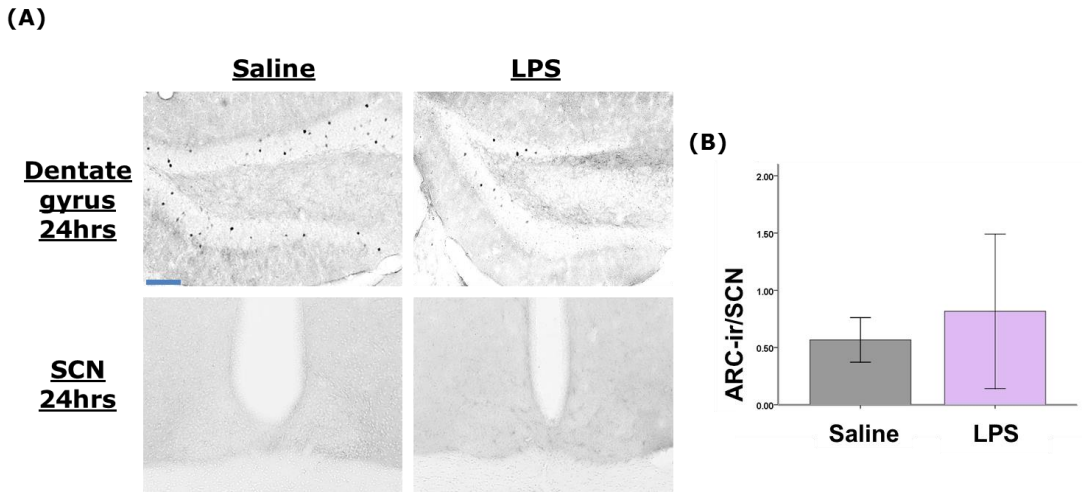


Figure 3.4: ARC expression is not altered in the SCN 24hrs following LPS treatment. *(A) Representative photomicrographs of ARC staining in the hippocampus and SCN of animals treated 24hrs previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of ARC immunostained cells (n = 5 for each group) in the SCN of the control and LPS treated groups at 24hrs post LPS treatment.*

3.3.2.2. SCN EGR-1 immunoreactivity is increased acutely following LPS 5mg/kg treatment.

Immunohistochemical analysis coupled with manual quantification of EGR-1 expression in LPS treated animals (n=5) and vehicle treated controls (n=5) found EGR-1 expression to be significantly increased in the SCN of LPS treated animals (69.93 $\pm$ 23.26, ir. nuclei/SCN) when compared to those treated with saline (2.27, $\pm$ 0.89 ir nuclei/SCN, $P<0.05$, independent t-test; Figure 3.5). When analysing the separate subdivisions of the SCN by independent t-test, the mean number of immunoreactive EGR-1 cells in the ventrolateral core of the SCN was found to be significantly different between the treatment groups, with LPS treated animals displaying an upregulation of EGR-1, with a mean of 20.75 $\pm$ 7.20 ir. cells/SCN and control animals displaying an mean of 0.40 $\pm$ 0.17 ir. cells/SCN ($P<0.05$). The difference in EGR-1 expression levels was also seen to be statistically significant in the dorsomedial shell portion of the SCN, with a significant increase in EGR-1 expression levels in animals treated with LPS (49.18 $\pm$ 16.08 ir. cells/SCN) compared to saline controls (1.91 $\pm$ 0.83 ir. cells/SCN, $P<0.05$, independent t-test; Figure 3.5).

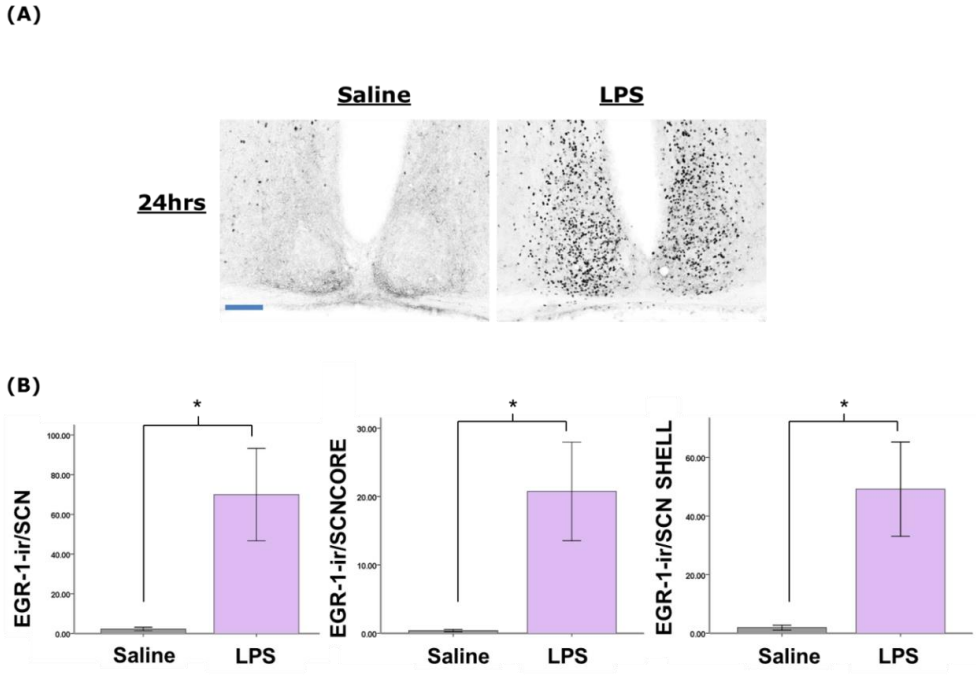


Figure 3.5: The expression of the IEG EGR-1 is altered in the SCN 24hrs following LPS treatment. *(A) Representative photomicrographs of EGR-1 immunoreactive cells in the SCN of animals treated 24hrs previously with LPS (scale bar = 100 mm). (B) Bar graphs illustrating quantification of levels of EGR-1*

*immunoreactivity (n =5 for each group) in the SCN at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals 24hrs post LPS treatment. (*P<0.05).*

3.3.3. Microglial examination in the SCN 24hrs post-septic LPS treatment.

3.3.3.1. F4/80 is upregulated in the SCN 24hrs post LPS treatment

Immunohistochemistry for the microglial marker F4/80 found there to be a significant increase in the expression of the microglial antigen throughout the brain (Figure 3.6) including the SCN in LPS treated animals (n=3) vs. saline controls (n=4). Manual quantification of F4/80 immunoreactive cells in the SCN coupled with independent t-test analysis found a statistically significant difference in F4/80 expression in the SCN between saline (7.13 $\pm$ 1.14 IOD/SCN) and LPS treated animals (13.5 $\pm$ 2.43 IOD/SCN; $P<0.05$). IHC revealed this difference to be significantly significant at the level of the dorsomedial shell of LPS treated animals (10.56 $\pm$ 1.79 IOD/SCN) and saline controls (5.38 $\pm$ 0.97, $P<0.05$), while F4/80 immunoreactivity was not found to be significantly different in the core region between control (1.75 $\pm$ 0.43 IOD/SCN) and LPS treated animals (2.94 $\pm$ 0.64 IOD/SCN, $P>0.05$, independent t-test).

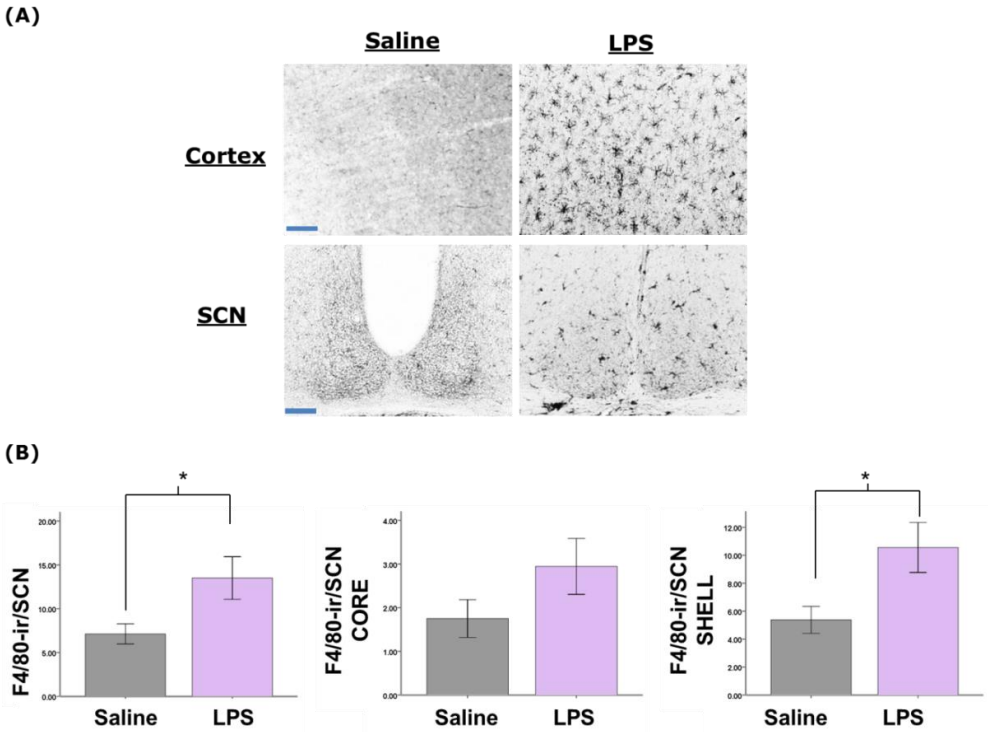


Figure 3.6: 5mg/kg LPS treatment results in upregulation of the microglial

marker F4/80 in the SCN. *(A) Representative photomicrographs of F4/80 expression in the SCN and cortex of animals treated 24hrs previously with either saline or LPS (n = 3-4 for each group). Animals were sampled mid subjective day (ZT5-8) in a 12:12 L:D cycle. Note the significant upregulation of F4/80 in the SCN and cortex of LPS treated animals, and also the activated, de-ramified morphology of the cells (scale bar = 100 µm). (B) Quantification of levels of immunoreactivity for F4/80 (n = 3-4 for both groups) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals 24hrs post LPS treatment. * P<0.05.*

3.3.4. Assessment of apoptosis 24hrs following induction of sepsis

3.3.4.1. Cleaved Caspase-3 expression in the acute phase following LPS treatment.

Cleaved Caspase-3 expression was examined in the SCN 24hrs following LPS 5mg/kg treatment (n=5) or saline (n=5). In the SCN, IOD measurements of cleaved caspase-3 immunoreactive signal and subsequent analysis by independent t-test found levels to be similar between saline controls (0.76 $\pm$ 0.26 IOD/SCN) and LPS animals (1.73 $\pm$ 0.65 IOD/SCN, P>0.05; Figure 3.7). IHC revealed no significant differences in cleaved caspase-3 expression in either the core (saline, 0.38 $\pm$ 0.10 vs. LPS, 1.65 $\pm$ 0.68 IOD/SCN, P>0.05, independent t-test), or in the shell portion of the SCN (saline 0.89 + 0.31 vs. LPS, 1.72 $\pm$ 0.61 IOD/SCN, P>0.05, independent t-test) between treatment groups.

3.3.4.2. TUNEL analysis in the acute phase following LPS treatment.

Following TUNEL analysis, manual quantification of TUNEL positive cells in the SCN of LPS treated animals (n=4) and vehicle treated controls (n=4) coupled with independent t-test analysis found no significant difference in TUNEL positive cells in the SCN of LPS treated animals (0.13 $\pm$ 0.13, TUNEL+cells/SCN) when compared to those treated with saline (0.00 $\pm$ 0.00 TUNEL+cells/SCN, P<0.05; Figure 3.7), and furthermore, examination of the separate subdivisions of the SCN found no difference in TUNEL + cells in either the ventrolateral core or dorsomedial shell regions of the SCN (P>0.05, independent t-test) between the treatment groups.

(A)

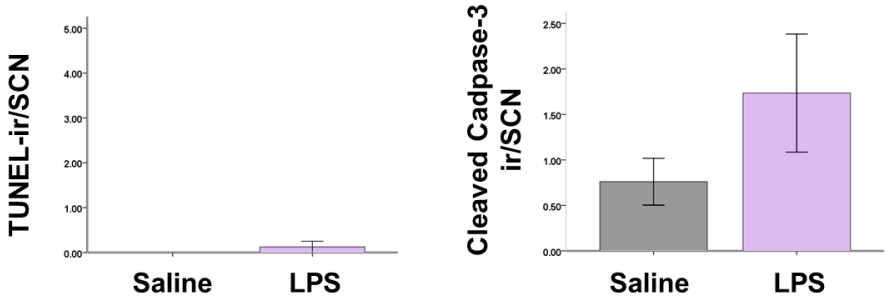


Figure 3.7: Examination of apoptotic markers 24hrs post LPS treatment. *Animals were treated with either saline or LPS and sampled 24 hours later. There were no increases in the markers for apoptosis TUNEL and cleaved caspase-3 (A; n = 4–5 for the saline and acute LPS groups). (A) Bar graphs illustrating quantification of levels of TUNEL and cleaved caspase-3 immunoreactivity in the SCN of control and post-septic animals 24hrs post LPS treatment, with no significant differences in expression of either apoptotic marker in the SCN in LPS treated animals vs. controls.*

3.3.5. Examination of glial markers in the SCN 3mths post LPS treatment

It's possible that immune mediators may be present in the SCN in the long term following sepsis induction, therefore the presence of various immune factors was investigated for three months following LPS 5mg/kg treatment.

Three months following the initial peripheral immune challenge with a septic dose of LPS, the presence of chronic neuroinflammation throughout the brain and in the SCN was examined through Immunohistochemical analysis for the microglial antigens IBA-1, F4/80 and CD-11b and the astroglial marker GFAP.

3.3.5.1. GFAP expression in the post-septic SCN

Immunostaining combined with IOD followed by independent t-test analysis of GFAP immunoreactivity in the SCN (Saline 5, LPS 5) revealed immunosignal for the astroglial marker GFAP in the SCN to be similar between saline (0.88$\pm$ 0.21 IOD/SCN) and post-septic animals (2.38$\pm$ 0.77 IOD/SCN, P>0.05; Figure 3.8). In the SCN subdivisions, there were no statistically significant differences found in GFAP expression in the SCN core region (control, 0.74 $\pm$ 0.19 vs. LPS, 1.38 $\pm$ 0.42

IOD/SCN, P>0.05, independent t-test) or in the shell region (saline, 1.00 ± 0.22 vs. LPS, 2.88 ± 0.91 IOD/SCN, P>0.05, independent t-test) between groups.

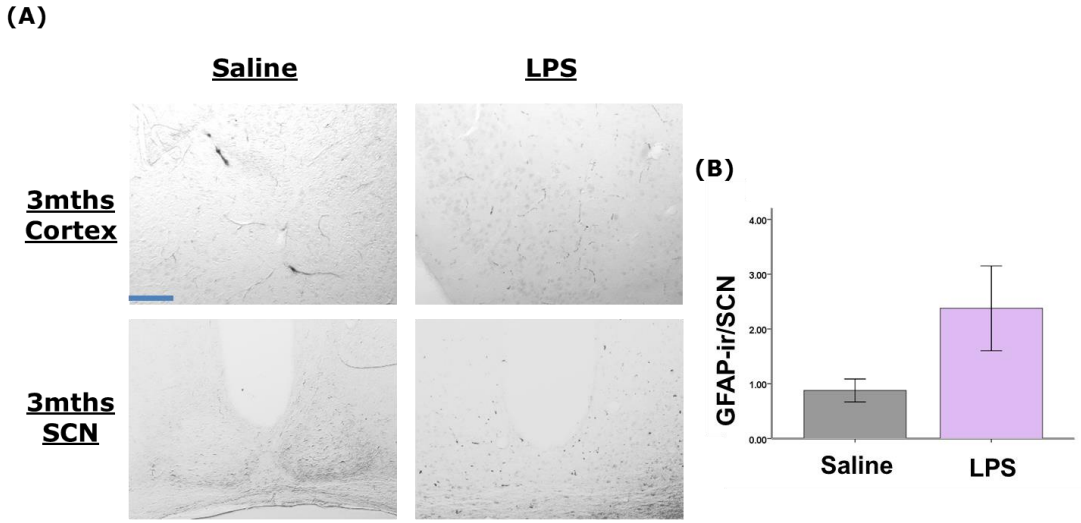


Figure 3.8: GFAP expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of GFAP staining in the cortex and SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of GFAP immunostained cells (n = 5 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.5.2. Long-lasting alterations in CD-11b expression in the SCN.

Immunohistochemical analysis combined with manual quantification of cells expressing the microglial marker CD-11b in the SCN of post-septic animals (n=5) and vehicle treated controls (n=3) followed by independent t-test analysis found there to be a highly significant increase in expression of activated CD-11b in the SCN of LPS treated animals (29.8 ± 2.35, ir. cells/SCN) when compared to those treated with saline (6.77, ± 1.49 ir cells/SCN, P<0.001; Figure 3.9). Further examination of the separate subdivisions of the SCN by IHC, manual quantification and independent t-test of the mean number of immunoreactive CD-11b cells found the expression to be significantly different between the treatment groups at the level of the ventrolateral core of the SCN, with LPS treated animals displaying an upregulation of the microglial marker, with a mean of 10.55 ± 0.44 ir. cells/SCN and control animals displaying an mean of 2.31 ± 0.67 ir. cells/SCN (P<0.001; Figure 3.9). The difference in CD-11b immunoreactivity was also seen to be statistically significant at the levels of the dorsomedial shell of the SCN, with a significant

increase in expression levels in animals treated with LPS (17.8 ± 2.29 ir.cells/SCN) compared to saline controls (4.47 ± 0.83 ir.cells/SCN, P<0.01, independent t-test; Figure 3.9).

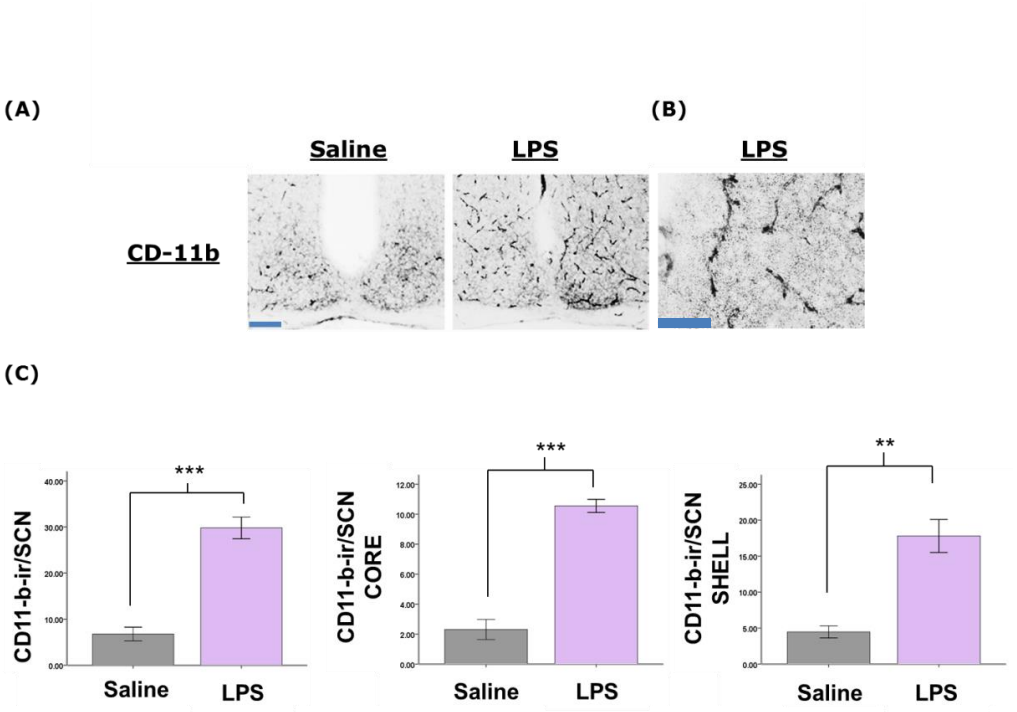


Figure 3.9: Previous sepsis results in a long-lasting upregulation of the microglial marker CD-11b in the SCN. *(A) Representative photomicrographs of CD-11b expression in the SCN of animals treated 3 months previously with either saline or LPS. Animals were sampled mid subjective day (ZT5-8) in a 12:12 L:D cycle. Note the significant upregulation of CD-11b in the SCN, and also the resting, ramified morphology of the cells in the post-septic SCN (scale bar = 100 µm). These are shown in higher magnification in (B) in SCNs from LPS treated animals (scale bar approx. 50 µm). (C) Quantification of levels of immunoreactivity for CD-11b (n =3-5 for both groups) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals at 3mths post LPS treatment. ** P<0.01; *** P<0.001.*

3.3.5.3. F4/80 expression is chronically altered in the SCN following a septic LPS treatment.

Immunohistochemistry for the microglial marker F4/80 and comparison of F4/80 immunoreactive cells in the SCN found there to be a statistically significant increase in expression of the antigen in the SCN of post-septic animals (n=3) vs. controls (n=4). Independent t-test showed a highly significant increase in the levels of F4/80 immunoreactivity in the SCN of post-septic animals (28.8 ± 3.49

ir.cells/SCN) compared to control animals (8.3 ± 1.57 ir.cells/SCN, P<0.01; Figure 3.10). IHC and subsequent analysis by independent t-test revealed significant upregulation in expression of the microglial marker in the core of LPS treated animals (11.5 ± 1.5 ir.cells/SCN) vs. controls (2.6 ± 0.82 ir.cells/SCN, P<0.01; Figure 3.10). This difference in F4/80 immunoreactivity was also found to be significantly different in the dorsomedial shell region with significant alterations in expression between control (5.8 ± 0.78 ir.cells/SCN) and post-septic animals (17.3 ± 2.73 ir.cells/SCN, P<0.01, independent t-test; Figure 3.10).

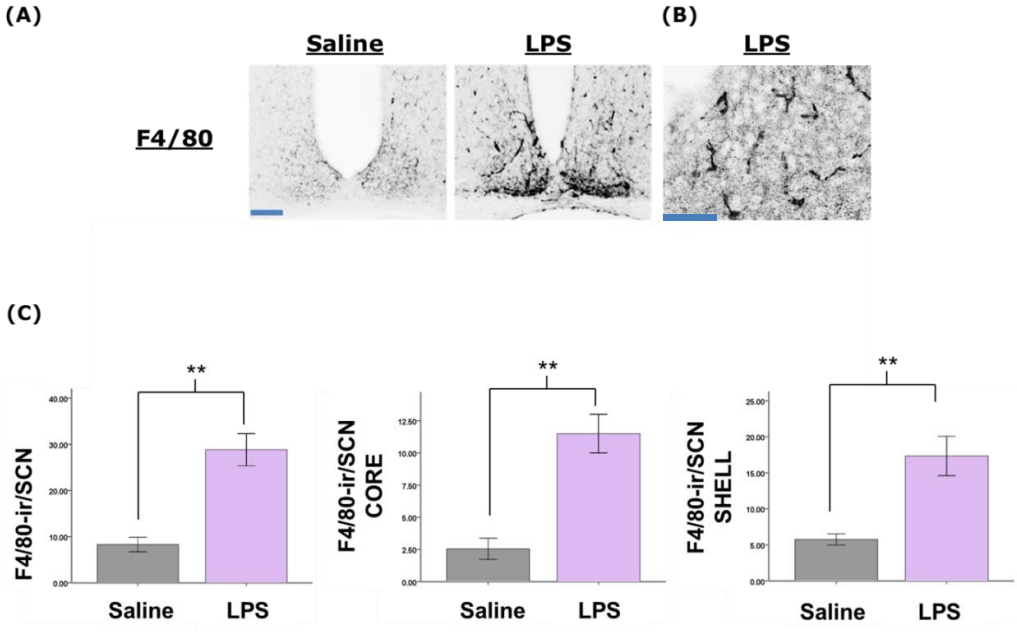


Figure 3.10: Previous sepsis results in a long-lasting upregulation of the microglial marker F4/80 in the SCN. *(A) Representative photomicrographs of F4/80 expression in the SCN of animals treated 3 months previously with either saline or LPS. Animals were sampled mid subjective day (ZT5-8) in a 12:12 L:D cycle. Note the significant upregulation of F4/80 in the SCN, and also the resting, ramified morphology of the cells in the post-septic SCN (scale bar = 100 µm). These are shown in higher magnification in (B) in SCNs from LPS treated animals (scale bar approx. 50 µm). (C) Quantification of levels of immunoreactivity for F4/80 (n =3-4 for both groups) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals at 3mths post LPS treatment. ** P<0.01.*

3.3.5.4. IBA-1 immunoreactivity in the post-septic SCN.

Comparison of IBA-1 expression 3 months post-treatment (Saline 3, LPS 4) by immunostaining combined with manual quantification of IBA-1 immunoreactive cells and subsequent analysis by independent t-test revealed immunostaining for the microglial marker IBA-1 in the SCN to be similar between saline (4.78 $\pm$ 2.63 ir.cells/SCN) and post-septic animals (8.52 $\pm$ 1.60 ir.cells/SCN, $P>0.05$). Examination of the SCN ventrolateral core, found there to be a statistically significant difference in microglial immunoreactivity in post-septic animals, with significantly higher mean values of 1.81 $\pm$ 0.28 ir. cells/SCN in post-septic animals compared to controls 0.39 $\pm$ 0.20 ir.cells/SCN ($P<0.01$, independent t-test). There was no significant difference in IBA-1 immunosignal seen between groups in the SCN shell region (saline, 4.39 $\pm$ 2.56 ir.cells/SCN vs. LPS, 6.71 $\pm$ 1.76 ir.cells/SCN, $P>0.05$, independent t-test).

Further assessment of the morphology of IBA-1 immunostained cells 3mths post-treatment in the SCN under high power followed by manual quantification found there to be a significant increase in the expression of hypertrophic glia in the SCN in post-septic animals (2.21 $\pm$ 0.31 ir.cells/SCN) vs. vehicle treated controls (0.44 $\pm$ 0.22 ir.cells/SCN; $P<0.01$, independent t-test; Figure 3.11). Examination of the separate subdivisions of the SCN by independent t-test revealed this statistically significant difference not to be different in the different subregions between the groups, but to be statistically different in the SCN as a whole when taking the core and shell values together. The mean number of IBA-1 immunoreactive hypertrophic microglia in the ventrolateral core of the SCN was found to be similar between the treatment groups, with LPS treated animals displaying an upregulation of activated microglia, with a mean of 0.38 $\pm$ 0.16 ir. cells/SCN and control animals displaying a mean of 0.00 $\pm$ 0.00 ir. cells/SCN ($P>0.05$; Figure 3.11). While the difference in hypertrophic microglia was seen to approach significance in the dorsomedial shell of the SCN between groups, it was not seen to be statistically significant, with expression levels in post-septic animals of 1.65 $\pm$ 0.45 ir.cells/SCN and saline controls 0.33 $\pm$ 0.19 ir.cells/SCN ($P>0.05$, independent t-test; Figure 3.11).

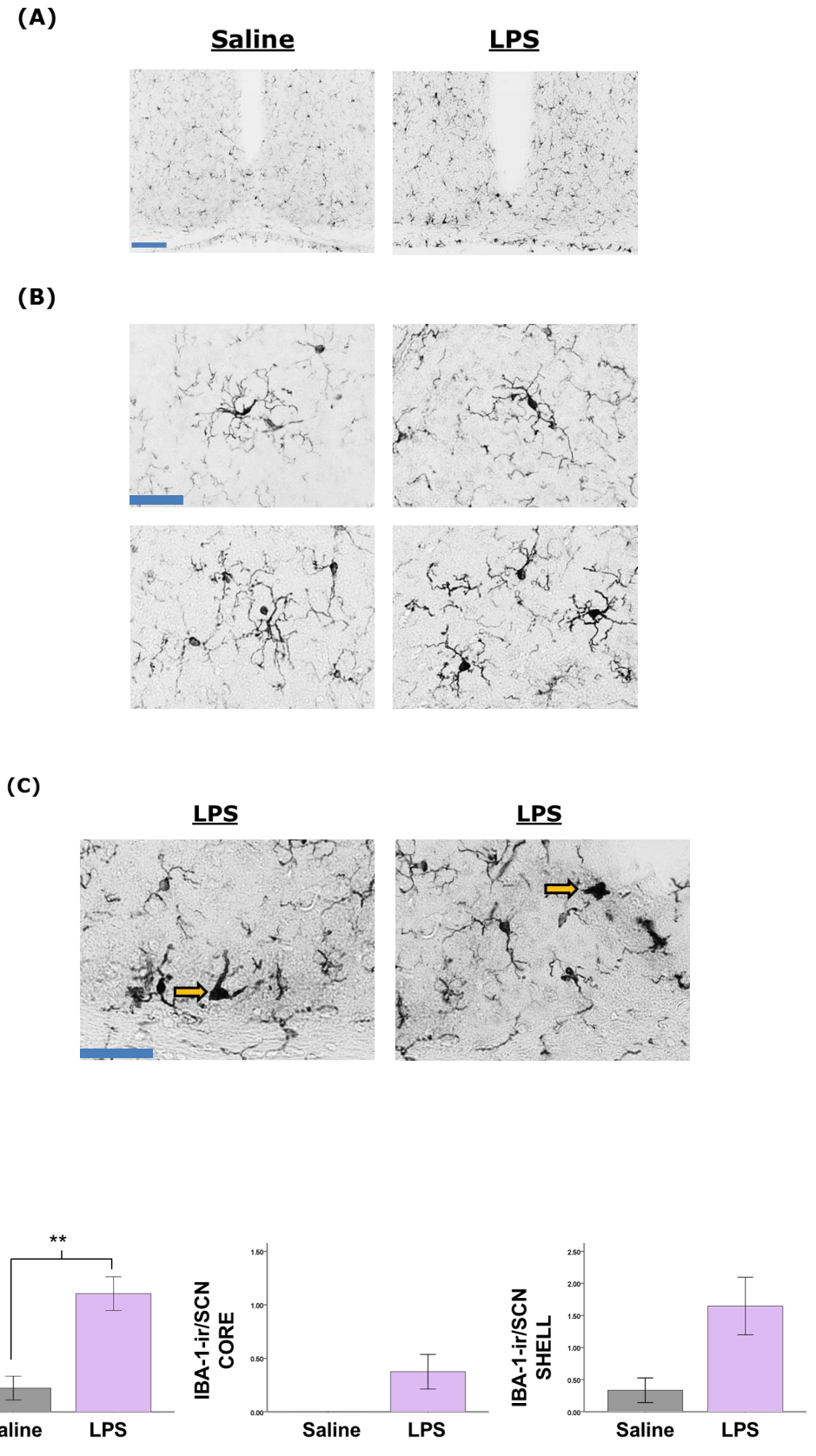


Figure 3.11: Previous sepsis results in a long-lasting upregulation of IBA-1 immunostained cells in the SCN. *(A) Representative photomicrographs of IBA-1 expression in the SCN of animals treated 3 months previously with either saline or*

*LPS (scale bar approx. 100 µm). Animals were sampled mid subjective day (ZT5-8) in a 12:12 L:D cycle. These IBA-1 immunostained microglia are shown in higher magnification in (B) in SCNs from LPS treated animals (scale bar approx. 50 µm). (C) Pictomicrographs of densely stained, activated microglia in the post-septic SCN (scale bar approx. 50 µm). Note the very dense IBA-1 immunostained spherical cells without processes in the SCN of LPS treated animals, which were not evident in the SCN of the saline treated animals. (D) Bar graphs illustrating quantification of levels of very dense IBA-1 microglial immunoreactive cells (n =3-4 for both groups) at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals. ** P<0.01.*

3.3.5.5. Examination of IBA-1 immunoreactivity in the SCN 3mths post-treatment following administration of PDTC prior to LPS treatment.

Comparison of IBA-1 expression by immunostaining combined with manual quantification of IBA-1 immunoreactive cells and subsequent analysis by One Way ANOVA revealed immunostaining for the microglial marker IBA-1 in the SCN to be similar between groups ($F_{2,24}$=3.07, P>0.05). However, further assessment of the morphology of IBA-1 immunostained cells 3mths post-treatment in the SCN under high power followed by manual quantification coupled with One Way ANOVA with the Tukey post-hoc test assessed statistically significant differences between groups in IBA-1 immunoreactive hypertrophic cells, and found a significant between groups effect ($F_{2,24}$=6.05, P<0.01). One Way ANOVA assessed statistically significant differences between groups in IBA-1 immunoreactive hypertrophic cells in the core and shell of the SCN, and found a significant between groups effect in the dorsomedial shell region of the SCN ($F_{2,24}$=6.2, P<0.01), but not in the ventrolateral core ($F_{2,24}$=0.82, P>0.05). Post Hoc analysis (Tukey) revealed that the expression levels of IBA-1 immunoreactive hypertrophic glia of 0.68 $\pm$ 0.17 ir. cells/SCN for control and LPS treated animals differed significantly to the levels of IBA-1 immunoreactive hypertrophic glia seen for PDTC and LPS treated animals 0.14 $\pm$ 0.05 ir. cells/SCN (P<0.01). Comparison of the IBA-1 hypertrophic glia immunoreactivity between control and LPS treated animals and those treated with 200mg/kg PDTC prior to a control i.p. injection which displayed an average expression of hypertrophic glia of 0.27 $\pm$ 0.08 ir. cells/SCN, revealed no significant differences between the groups (P>0.05). Further, IBA-1 immunoreactive hypertrophic glia expression levels were not found to be statistically different

between PDTC and LPS treated animals and PDTC and control treated animals (P>0.05). (Fig. 3.12).

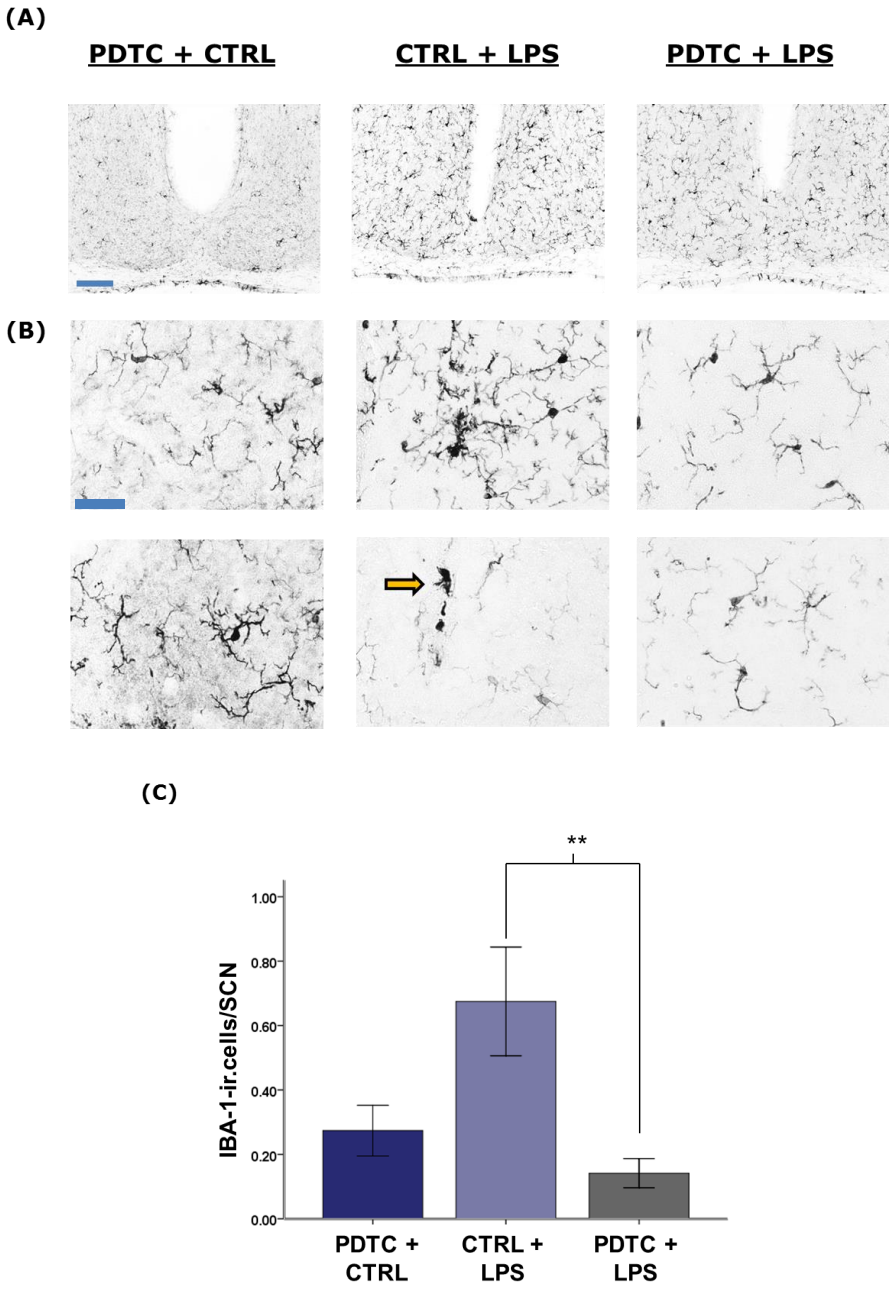


Figure 3.12: PDTC administration prior to LPS 5mg/kg decreases the long-lasting upregulation of IBA-1 immunostained cells in the SCN. *(A) Representative photomicrographs of IBA-1 expression in the SCN of animals treated 3 months previously with either PDTC + control, control + LPS or PDTC + LPS (scale bar = 100 µm). Animals were sampled mid subjective day (ZT5-8) in a 12:12 L:D cycle. These IBA-1 immunostained microglia are shown in higher magnification in (B) in SCNs from each treatment group (scale bar approx. 50 µm). Note the very dense IBA-1 immunostained spherical cells without processes in the SCN of control + LPS treated animals which are not evident in the SCN of the PDTC + LPS treated*

*animals. (C) Bar graph illustrating quantification of levels of very dense IBA-1 microglial immunoreactive cells (n = 7-10 for each group) in the SCN. ** P<0.01.*

3.3.6. Examination of immune factors in the post-septic SCN

3.3.6.1. TNF-α expression in the post-septic SCN.

Three months following the initial treatment, Integrated Optical Density (OD) measurements and independent t-test analysis assessed the expression of TNF-α in the SCN (Saline 7, LPS 6), finding no significant difference in TNF-**α** expression in the SCN between saline controls (4.76 ± 1.55 IOD/SCN) and post-septic animals (2.62 + 1.11 IOD/SCN, P>0.05; Figure 3.13), with no alterations in expression seen in the separate SCN subdivisions, with similar immunoreactivity levels between groups in the ventrolateral core SCN region (control, 3.99 + 1.47 IOD/SCN vs. post-septic animals, 2.17 + 0.97 IOD/SCN, P>0.05) and the dorsomedial shell region (controls, 5.29 + 1.70 IOD/SCN vs. LPS, 2.92 + 1.22 IOD/SCN, P>0.05, independent t-test).

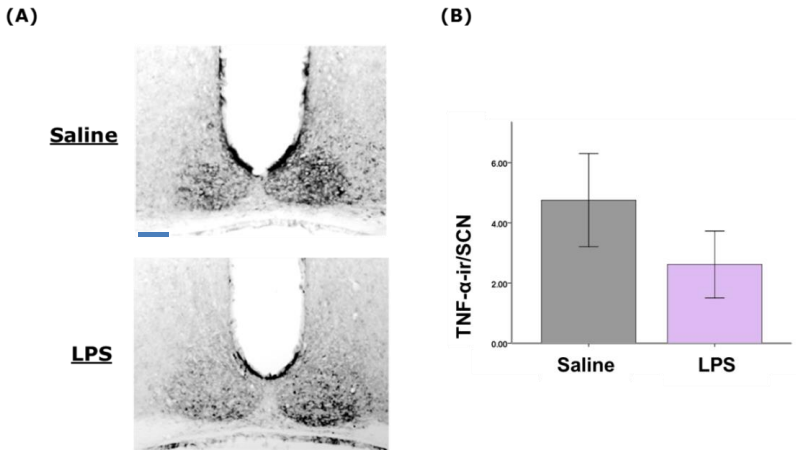


***Figure 3.13: TNF-α expression is not altered in the SCN 3mths following LPS treatment**. (a) Representative photomicrographs of TNF-α expression in the SCN of animals treated 3mths previously with LPS (scale bar approx. 100 µm). (B) Bar graphs illustrating quantification of levels of TNF-α immunostained cells (n =6-7 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.6.2. NOS2 expression in the post-septic SCN.

Immunohistochemistry coupled with IOD measurements of NOS2 expression in the SCN of control (n=7) and post-septic animals (n=7) and subsequent analysis by independent t-test found similar levels of NOS2 immunosignal in the SCN of post-septic animals (3.07 $\pm$ 0.61 IOD/SCN) and saline controls (3.91 $\pm$ 0.69 IOD/SCN, P>0.05; Figure 3.14). Furthermore, there were no significant differences in NOS2 expression in the core SCN region of saline animals (2.98 $\pm$ 0.64 IOD/SCN) vs. LPS (2.86 $\pm$ 0.90, P>0.05), or in the dorsomedial shell SCN subregion between post-septic animals (3.40 $\pm$ 0.72 IOD/SCN) and controls (4.36 + 0.83 IOD/SCN, P>0.05, independent t-test).

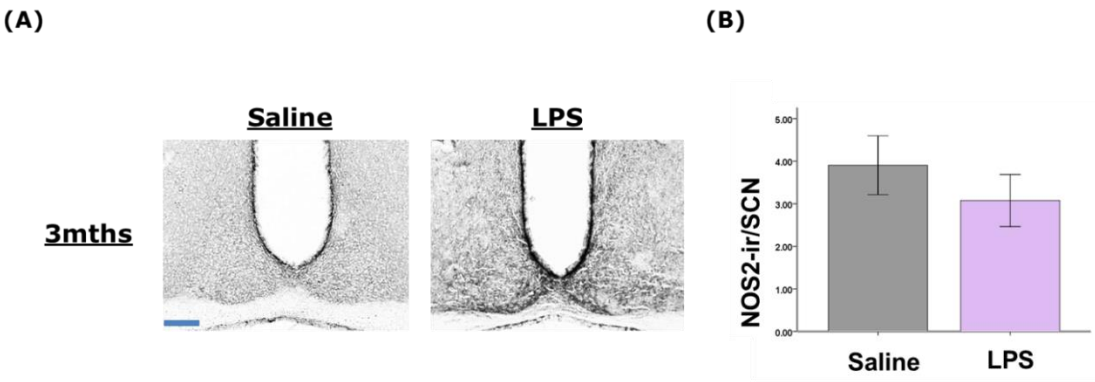


Figure 3.14: NOS2 expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of NOS2 expression in the SCN of animals treated 3mths previously with LPS (scale bar approx. 100 µm). (B) Bar graphs illustrating quantification of levels of NOS2 immunostained cells (n = 7 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.6.3. IL-1β expression in the post-septic SCN.

IHC coupled with IOD measurements assessed the immunoreactivity of IL-1β in the SCN 3mths following treatment with either saline (n=4) or LPS (n=4), finding no statistically significant difference in IL-1β expression in the SCN between control (3.06 + 1.21 IOD/SCN) and post-septic animals (4.65 + 0.33 IOD/SCN, P>0.05, independent t-test; Figure 3.15). IOD followed by independent t-test analysis found no significant differences in the expression of the antigen in the dorsomedial shell SCN region of LPS treated animals (5.65 + 0.32 IOD/SCN) vs.

controls (3.15 + 1.39 IOD/SCN, P>0.05), nor were there any statistically significant differences in IL-1β expression in the ventrolateral core subdivision between post-septic animals (2.75 + 0.29 IOD/SCN) and controls (2.62 + 0.48 IOD/SCN, P>0.05).

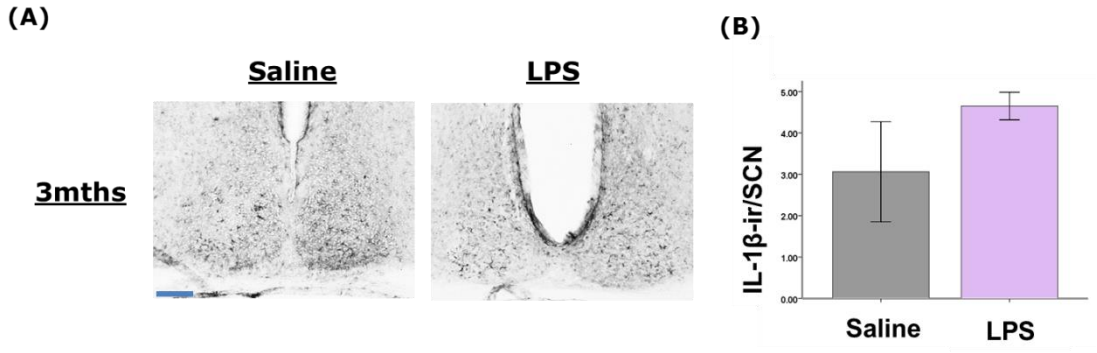


Figure 3.15: IL-1β expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of IL-1β expression in the SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of IL-1β immunostained cells (n = 4 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.6.4. IL-6 expression in the post-septic SCN.

Immunohistochemistry followed by IOD measurements and independent t-test analysis examined for statistically significant differences in IL-6 expression 3mths post-treatment in the SCN (Saline 5, LPS 5), finding no significant differences in expression between post-septic animals (1.60 + 1.13 IOD/SCN) and controls (3.05 + 1.48 IOD/SCN, P>0.05; Figure 3.16). Additional assessment of IL-6 immunosignal in the SCN regional subdivisions found no significant differences in expression in the core of LPS animals (0.71 $\pm$ 0.59 IOD/SCN) when compared to controls (1.81 $\pm$ 0.96 IOD/SCN, P>0.05, independent t-test). Furthermore, no alterations were seen in IL-6 expression between LPS and saline treated animals in the dorsomedial shell, with mean immunoreactivity levels of 1.92 $\pm$ 1.37 IOD/SCN and 3.74 $\pm$ 1.79 IOD/SCN respectively (P>0.05, independent t-test).

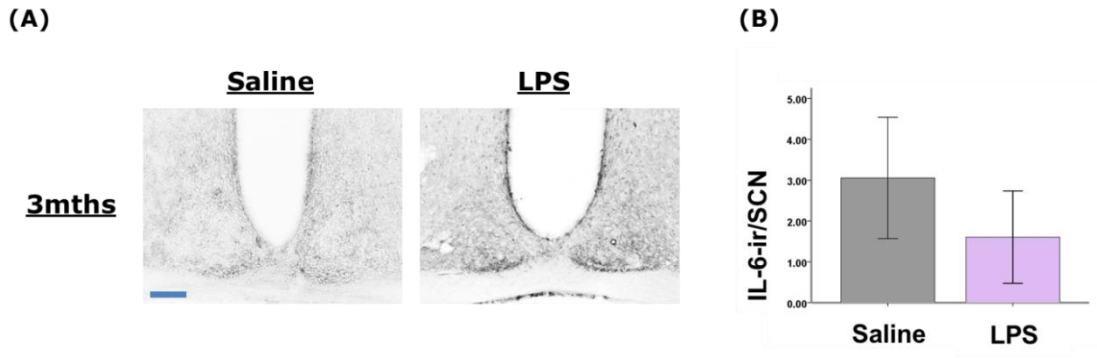


Figure 3.16: IL-6 expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of IL-6 expression in the SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of IL-6 immunostained cells (n = 5 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.7. Examination of components of the NF-κB pathway in the SCN 3mths following LPS treatment

3.3.7.1. Phosphorylated (p)-IκK expression in the post-septic SCN.

Three months following the initial immune challenge, IHC followed by Integrated Optical Density measurements and independent t-test analysis assessed the expression of p-IκK in the SCN (Saline 5, LPS 5), finding no statistically significant alterations in the p-IκK immunosignal throughout the SCN as a whole between post-septic (6.04 $\pm$ 1.97 IOD/SCN) and saline animals (11.18 $\pm$ 2.47 IOD/SCN, P>0.05; Figure 3.20).

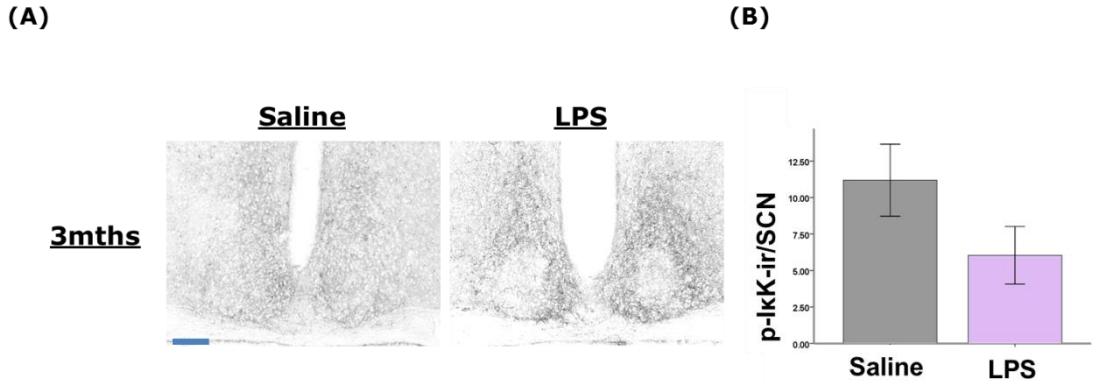


Figure 3.20: p-IκK expression is not upregulated in the SCN 3mths following LPS

treatment. *(a) Representative photomicrographs of p-IκK expression in the SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of p-IκK immunostained cells (n = 5 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.7.2. p-IκB expression in the post-septic SCN.

Similarly, 3mths post LPS treatment, immunostaining and Integrated Optical Density measurements followed by independent t-test analysis for p-IκB immunoreactivity in the SCN (Saline 5, LPS 5) found no significant differences in p-IκB expression in the SCN sections overall between post-septic animals (6.45 ± 1.13 IOD/SCN) and saline animals (7.25 ± 0.97 IOD/SCN, P>0.05, independent t-test; Figure 3.21).

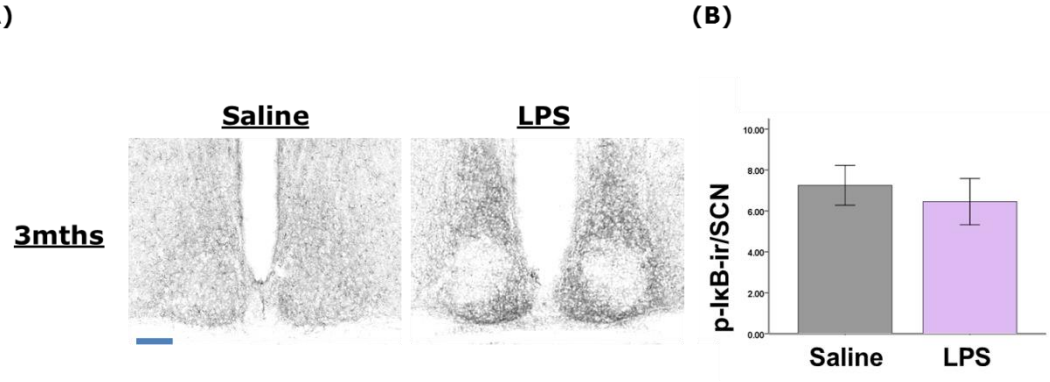


Figure 3.21: p-IκB expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of p-IκB expression in the SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of p-IκB immunostained cells (n = 5 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.7.3. p65 NF-κB expression in the post-septic SCN.

Immunostaining combined with IOD and subsequent assessment by independent t-test of p65 NF-κB immunoreactivity in the SCN three months following initial treatment (Saline 4, LPS 4) revealed the p65 NF-κB immunosignal to be similar in the SCN of saline controls (1.48 ± 0.39 IOD/SCN) and post-septic

animals (1.13 ± 0.46 IOD/SCN, P>0.05; Figure 3.22). Analysis of the different SCN subdivisions found there to be no difference in p65 NF-κB immunoreactivity in the ventrolateral core region (control, 1.32 ± 0.28 IOD/SCN vs. LPS, 1.11 ± 0.54 IOD/SCN, P>0.05, independent t-test) or in the dorsomedial shell region (saline, 1.51 ± 0.42 IOD/SCN vs. LPS, 1.19 ± 0.51 IOD/SCN, P>0.05, independent t-test) between groups.

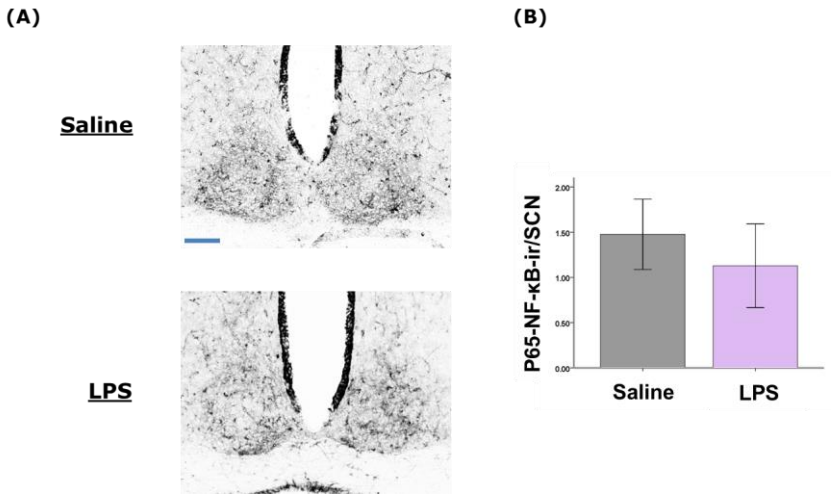


Figure 3.22: p65 NF-κB expression is not altered in the SCN 3mths post LPS. *(a) Representative photomicrographs of p65 NF-κB expression in the SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of p65 NF-κB immunostained cells (n = 4 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.8. The long-lasting effects of sepsis induction on SCN neuropeptide expression

The effects of chronic neuroinflammation on the circadian patterns of expression of the neuropeptides arginine vasopressin and vasoactive intestinal polypeptide were investigated. These are recognised as playing vital roles in SCN function. Perturbation of their expression by neuroinflammation could have significant consequences for circadian processes.

3.3.8.1. VIP expression in the post-septic SCN.

Immunostaining for VIP (Saline 6, LPS 6) combined with manual quantification of immunoreactive nuclei and subsequent analysis by independent t-test revealed VIP expression levels to be similar in the SCN between saline (67.33 $\pm$ 11.31 ir.cells/SCN) and post-septic animals (48.16 $\pm$ 12.63 ir.cells/SCN, $P>0.05$; Figure 3.23). Further examination of the levels of VIP ir. nuclei in the independent SCN subregions followed by independent t-test analysis revealed no difference in expression in the SCN subdivisions, with similar expression levels in the core (saline, 60.16 $\pm$ 21.33 vs. LPS, 47.19 $\pm$ 14.01 ir.cells/SCN, $P>0.05$) and shell regions of the SCN (controls, 55.97 $\pm$ 8.42 vs. LPS, 47.89 $\pm$ 12.02, $P>0.05$).

3.3.8.2. The expression of AVP in the post-septic SCN.

Comparison of AVP immunosignal in the SCN 3 months post LPS 5mg/kg treatment (n=6) or vehicle (n=5) by immunohistochemistry combined with integrated optical density measurements of ir. nuclei found levels of expression of AVP immunoreactive nuclei in the SCN of post-septic animals analogous to controls, with mean values of 30.23 + 12.05 and 32.37 $\pm$ 12.59 ir. cells/SCN respectively ($P>0.05$, independent t-test; Figure 3.23). Independent t-test analysis of mean expression values of AVP immunostained sections revealed no difference in expression in the SCN subdivisions, with similar expression levels in the core (saline, 12.65 $\pm$ 5.38 ir.cells/SCN vs. LPS, 8.43 $\pm$ 2.97 ir.cells/SCN, $P>0.05$) and shell regions of the SCN (controls 41.94 $\pm$ 16.56 ir.cells/SCN vs. LPS 37.72 $\pm$ 13.23 ir.cells/SCN, $P>0.05$).

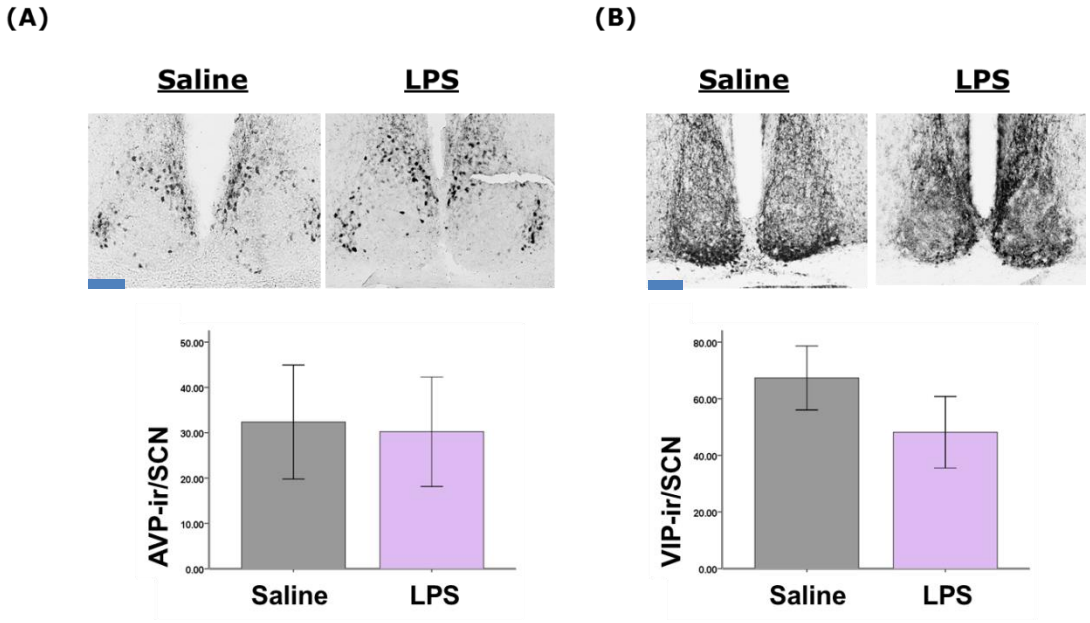


Figure 3.23: SCN neuropeptide expression in the post-septic SCN. *(A) Representative photomicrographs of AVP expression in the SCN of animals treated 3 months previously with either saline or LPS (scale bar = 100 µm) and bar graph illustrating quantification of levels of AVP immunostained cells (n = 5-6 for each group) in the SCN of the control and LPS treated groups, with no significant differences in the expression of AVP in the SCN between the treatment groups. (B) Representative photomicrographs of VIP 3 months following LPS or saline treatment (scale bar = 100 µm) and bar graph illustrating quantification of levels of VIP immunostained cells (n = 6 for each group) in the SCN of the control and LPS treated groups.*

3.3.9. Immediate Early Gene expression in the post-septic SCN.

3.3.9.1. ARC expression in the SCN 3mths post sepsis.

Assessment of spontaneous expression of ARC in the SCN 3mths post LPS 5mg/kg (n=5) or saline treatment (n=4) by immunohistochemistry combined with manual quantification of ir. nuclei found there to be no alterations in the levels of expression of ARC immunoreactive nuclei in the SCN of LPS compared to controls, with mean values of 2.98 $\pm$ 2.18 and 2.33 $\pm$ 0.70 ir. cells/SCN respectively ($P>0.05$, independent t-test; Figure 3.24). Independent t-test analysis of measurements of ARC immunostained sections revealed no difference in expression in the SCN subdivisions, with similar expression levels in the core (saline, 0.79 $\pm$ 0.43

ir.cells/SCN vs. LPS, 1.05 ± 0.76 ir.cells/SCN, P>0.05) and shell regions of the SCN (controls, 1.54 ± 0.66 ir.cells/SCN vs. LPS, 1.80 ± 1.44 ir.cells/SCN, P>0.05).

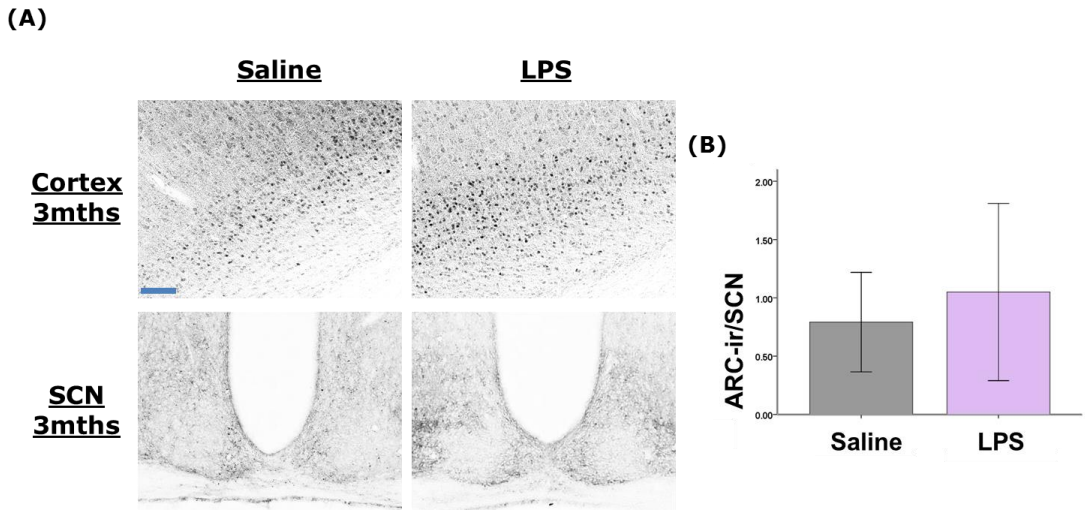


Figure 3.24: ARC expression is not altered in the SCN 3mths following LPS treatment*. (a) Representative photomicrographs of ARC staining in the cortex and SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of ARC immunostained cells (n = 4-5 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.3.9.2. EGR-1 expression in the SCN 3mths post sepsis.

Comparison of spontaneously expressed EGR-1 in the SCN 3mths post LPS 5mg/kg (n=4) or vehicle (n=4) treatment by immunohistochemistry combined with manual quantification of ir. cells and independent t-test analysis found levels of expression of EGR-1 ir. nuclei in the SCN of post-septic animals analogous to controls, with mean values of 0.54 ± 0.21 and 1.17 ± 0.73 ir. cells/SCN respectively (P>0.05; Figure 3.25). Independent t-test analysis of measurements of EGR-1 immunostained sections revealed no difference in expression in the SCN subdivisions, with similar expression levels in the core (saline, 0.25 ± 0.25 vs. LPS, 0.21 ± 0.13 ir.cells/SCN, P>0.05) and shell regions of the SCN (controls, 0.92 ± 0.53 vs. LPS, 0.33 ± 0.19 ir.cells/SCN, P>0.05).

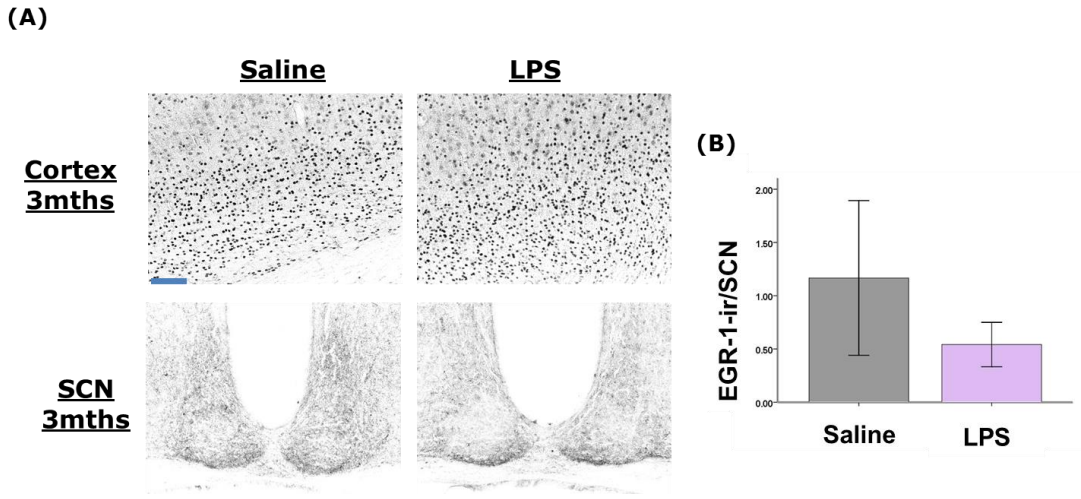


Figure 3.25: EGR-1 expression is not altered in the SCN 3mths following LPS treatment. *(a) Representative photomicrographs of EGR-1 expression in the cortex and SCN of animals treated 3mths previously with LPS (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of EGR-1 immunostained cells (n = 4 for each group) in the SCN of the control and LPS treated groups at 3mths post LPS treatment.*

3.4. Discussion:

The examination of SCN neurochemistry in the acute and chronic phase following LPS treatment in the current set of experiments show that the SCN is acutely responsive following LPS treatment, showing an increase in the expression of inflammatory mediators, as well as displaying a long-lasting upregulation of microglial markers in the post-septic SCN.

3.4.1. Examination of SCN Neurochemistry 24hrs post LPS treatment.

These studies assessing changes in the SCN 24hrs following LPS treatment, show that the SCN responds in the acute phase to a substantial peripheral immune challenge. The current study reveals that LPS treatment induces a significant upregulation of immunoreactive EGR-1 in the SCN of LPS treated animals in comparison to controls 24hrs following treatment, highlighting the effect of the peripheral immune challenge on the SCN. Various different studies have shown that acute LPS treatment can alter circadian/SCN function, and the SCN has been shown

to respond to LPS treatment (Beynon and Coogan, 2010; Nava et al., 2000; Marpegan et al., 2005). The SCN expresses cytokine receptors and regulatory molecules (Lundkvist et. al., 1999; Sadki et. al., 2007) and in this way, inflammatory mediators induced by peripheral LPS may impact upon the SCN causing functional changes and alterations in clock gene expression following LPS administration. Peripheral administration of low dose LPS in the early subjective night can result in phase shifts, along with an increase in c-Fos expression in the SCN (Marpegan et al., 2005), and these LPS-induced effects appear to be dependent on TLR4 signalling (Paladino et al., 2010). AVP production is altered following direct treatment of SCN slice cultures with LPS, indicating that the SCN may express TLR4 and be capable of responding directly to LPS (Nava et al., 2000). Further, peripheral septic LPS treatment has been shown to induce a substantial increase in the expression of the IEG c-Fos in the SCN acutely, as well as activation of the p65 subunit of the NF-κB pathway (Beynon and Coogan, 2010). We also examined the effector IEG ARC in the SCN, since long-lasting neuroinflammation induced by chronic i.c.v. infusion of LPS has previously been associated with an upregulation of ARC in the dentate gyrus (Rosi et al., 2005), however, we observed no evidence of ARC upregulation in the SCN either in the acute phase following LPS treatment or 3 months post sepsis induction.

It does not appear that the dose of LPS used in this study is inducing cell loss in the SCN, since no changes were observed in apoptotic markers, as assessed by TUNEL analysis or cleaved caspase-3 immunostaining 24 hours following LPS treatment. It must be noted that we assessed the SCN alone for apoptosis, and that there may be alterations in other brain regions in TUNEL positive cells and cleaved caspase-3 immunoreactivity and this would require further investigation. Additionally, it is important to note that neither TUNEL anlaysis nor immunohistochemical analysis for cleaved caspase-3 expression would detect cell loss in the SCN as a result of necrosis and since we have not assessed neuronal numbers in the SCN in this study, this remains a possible consequence of septic LPS treatment.

Studies assessing cell loss in the brain following LPS treatment have highlighted that apoptosis and neuronal loss following sepsis induction via endotoxin treatment is dose dependent, and further, appears to be region specific. Following

10mg/kg LPS treatment in rats, TUNEL analysis revealed a significant increase in apoptotic cells in the brain 24hrs post-treatment in the hippocampus, cerebellum, midbrain and cortex (Semmler et al., 2005). At 4hrs or 14days following repeated treatment with LPS (20µg/100µl), there was no observation of TUNEL-positive neurons in the thalamus, cortex or hippocampus (Terrazzino et al. 2002). Further, treatment of mice with the same endotoxin dose as used here and subsequent assessment of neuronal loss in the cerebral cortex or in the hippocampus 8 weeks following sepsis induction, observed no evidence of cell loss at this chronic timepoint (Weberpals et al., 2009). Other experimental work utilizing LPS 5mg/kg peripheral treatment has shown that neurodegeneration is only seen to occur in the CNS 7 months after LPS challenge, and that this loss appeared to be of the dopaminergic neurons in the substantia nigra alone (Qin et al., 2007).

In line with the finding that there was no significant cell loss in the SCN 24hrs post LPS treatment, assessment of SCN neuropeptides three months following the immune challenge did not find any changes in the expression of AVP or VIP in the long term in the post-septic SCN. Alterations in the expression of neuropeptides that play roles in input to (VIP) and output from (AVP) the SCN or communication between the SCN subdivisions could result in a decrease in the amplitude of the central pacemaker and could underlie post-septic circadian behavioural changes. Levels of SCN neuropeptide have previously been shown to be diminished in the SCN in ageing (Hofman et al., 2006), and therefore in relation to neuropeptide expression at this time, the post-septic SCN differs from that of the aged SCN. Other studies however, have shown the neurotransmitter AVP to be increased in the SCN following *in vitro* treatment of SCN slices with LPS (Nava et al., 2000). We observe neither an increase nor a decrease in the post-septic SCN in neuropeptide expression and so the alterations exhibited in post-septic circadian behaviour cannot be attributed to this.

These studies reveal a significant upregulation of F4/80 expressing cells in the SCN 24hrs post LPS treatment showing a de-ramified, activated microglial morphology. These results are in line with findings from other studies where microglial activation and cytokine release in the CNS have previously been shown in response to LPS induced sepsis (Godbout et al., 2005; Van Dam et al., 1992). Further, in the acute phase following LPS treatment, significant upregulation of microglia and astrocytes have been shown to be evident in the cortex 8hrs following

treatment (Jacob et al., 2007), while others have also observed a significant upregulation of activated microglia following LPS treatment, along with an upregulation of NOS2 24hrs post-treatment in the striatum, cerebellum, hippocampus and midbrain, as well as a significant increase in GFAP positive astrocytes in the cortex, striatum and hippocampus (Semmler et al., 2005). We have not assessed astrocytic activation in the acute phase in the SCN post LPS treatment and therefore further work would be required to assess whether reactive astrogliosis takes place in the SCN at this time. The activation of microglia in the acute phase may exert effects on the SCN, since activated microglia are known to release a variety of neuroinflammatory mediators. Microglia and astrocytes are known to synthesize complement proteins, and activation of the complement cascade has been shown to play roles in the brain following LPS treatment, leading to upregulation of CD45, TNF-α and TLR4, as well as an upregulation in activated microglia and NOS2 (Jacob et al., 2007). Similarly, we find that LPS acutely alters NOS2 expression in the SCN of LPS treated animals, with a significant upregulation of NOS2 in the SCN 24hours following peripheral LPS treatment. NOS2 is the inducible isoform of nitric oxide synthase and is a source of nitric oxide during inflammatory processes and appears to play an important role in the pathogenesis of sepsis. Similar to the significant upregulation of NOS2 in the SCN observed here 24hours following a septic LPS treatment, NOS2 is shown to be up-regulated following the induction of sepsis by other groups (Polito et al., 2011). Additionally, in mice lacking NOS2, septic shock and organ failure have been shown to be attenuated (Wei et al., 1995), highlighting the important role that NOS2 plays in sepsis.

Nitric oxide has been shown to be involved in the long-lasting activation of the innate immune system following a peripheral immune insult (Weberpals et al., 2009) and appears to play important roles in the development of neuroinflammation and cognitive alterations induced by peripheral inflammation. An increase in nitric oxide production has been associated with the activation of microglia (Zielasek and Hartung, 1996). Indeed, we observe both an upregulation of NOS2 and microglial activation in the SCN 24hrs post-treatment. However, the upregulation of NOS2 observed in the SCN in the acute phase following septic LPS treatment in these studies is seen to have returned to control levels 3mths after the peripheral immune challenge, while an upregulation in microglial markers remains evident within the

SCN at this time. Studies performed by Weberpals and colleagues show that 8 weeks post LPS treatment, there was a significant upregulation of activated microglia in the CNS, which was seen to be absent in NOS2-/- deficient LPS treated mice, indicating that nitric oxide derived from NOS2 plays a role in the sustained microglial changes and inflammatory state in the brain following LPS 5mg/kg treatment (Weberpals et al., 2009). It is possible that NOS2 may mediate the effects of LPS on the SCN initially, leading to an upregulation in microglia, however the long-lasting microglial changes in the post-septic SCN must then be being mediated further by another mechanism.

There are various mechanisms by which NOS2 might impact upon the circadian system. Cytokines such as TNF-α stimulate glia to express NOS2 in models of SE (Jacob et al., 2007), leading to an increase in NO levels, which may play a role in neuronal dysfunction. Excess NO can disturb the coupling between blood flow and metabolism by altering regulation of cerebral blood flow, and can alter synaptic transmission, leading to neuroendocrine, behavioural activity and memory formation perturbations (Jacob et al., 2011). In rat models of SE, NOS2 plays roles in systemic hemodynamic derangements (Kadoi and Goto, 2004).

NOS2 induction may play roles in LPS induced apoptosis, and a variety of studies have highlighted that NOS2 causes apoptosis as well as delayed loss of neurons (Czapski et al., 2007; Arimoto and Bing, 2003). However, in the current study, we do not observe any evidence for neuronal loss in the SCN 24hrs following LPS treatment and therefore do not attribute any alterations in circadian function to NO induced neuronal loss in the SCN. Additionally, NOS2 derived nitric oxide is suggested to be involved in long term LPS induced cognitive alterations (Weberpals et al., 2009), as well as the inflammatory suppression of LTP (Mori et al., 2001; Togashi et al., 2001; Wang et al., 2004).

NOS2 synthesis has been suggested as a possible factor in impairment of the HPA axis that is seen in studies of septic shock in rats and in humans, whereby a reduction in ACTH synthesis is found (Polito et al., 2011), and NOS2 increase during septic shock could impact upon rhythmic corticosterone and feedback to the SCN. Further, increases in NO levels have been shown to impact upon circadian resetting (Melo et al., 1997). The specific inhibition of nitric oxide prior to treatment with LPS would be interesting along with long term assessment of circadian

resetting given the important role attributed to NOS2 by other studies in the long-lasting activation of the innate immune system following a peripheral immune insult.

3.4.2. Long-lasting increase in microglial markers in the post-septic SCN

We observe long-lasting changes in the expression of microglial markers in post-septic animals in various regions of the brain, including in the SCN, with a significant upregulation of F4/80, CD-11b and IBA-1 in the SCN. Importantly, in comparison to the expression of activated microglia we observe in the SCN acutely following LPS treatment, the morphology of the cells expressing F4/80 and CD-11b does not appear to be that of activated microglia at this time, 3mths post-treatment, but rather these cells exhibit a resting phenotype, expressing a ramified morphology. The current results are in line with recent studies by Puntener and colleagues (Puntener et al., 2012), who's Immunohistochemical analysis utilizing the antigens CD11c, MHC11 and MHC1 indicates that following peripheral infection, microglia within the CNS are activated transiently, appearing to return to their resting morphology 3 weeks following the peripheral immune challenge. Studies have suggested that numbers of activated microglia can decrease upon resolution of the activating stimulus by apoptosis and/or these may lose their activation markers, subsequently returning to the resting state (Jones et al., 1997; Streit et al., 1999). It is therefore possible that the previously activated microglia might change into a ramified form and this could account for the upregulation of F4/80 and CD-11b immunoreactive microglia exhibiting ramified morphology at 3mths post-treatment in the post-septic SCN. The use of another microglial marker was therefore required to further assess the state of the up-regulated microglia, and therefore, we further examined the morphology of the immunostained glia when assessing the levels of activated microglia in the SCN 3months post sepsis, further quantifying those appearing to be hypertrophic, as carried out in other studies assessing microglial activation (Lemstra et al., 2007), through the utilization of the ionized calcium binding adapter molecule 1 (Iba-1) immunoreactive microglial marker. The activation of microglia sees the cells progress through a series of morphological phenotypes, the end stage of which they appear hypertrophic and are seen to resemble phagocytic cells (Kreutzberg, 1996). Compared to resting microglia, activated microglia exhibit a larger cell body with processes that are shorter and

denser than those seen to project from resting microglia (Dheen et al. 2007; Kaur and Ling 1991; Kaur et al. 1985). Reactive microglia are generally evidenced as small, spherical cells with no processes, however an ameboid-like or rod-shaped morphology may also be observed (Davis et al. 1994). Overall, there was no significant difference in the levels of IBA-1 microglial staining in the SCN 3mths following LPS 5mg/kg treatment. However, both active and resting microglia express IBA-1 (Lalancette-Herbert et al., 2007), and this may account for why we do not observe a significant difference between saline treated and post-septic animals in IBA-1 immunoreactivity in the SCN. Further examination of these sections under high power allowed inspection of the morphology of the IBA-1 immunostained cells, and it became clear that at 3 months post LPS treatment, these animals displayed small, spherical glia without processes, a morphology that has previously been described as the characteristic reactive microglial morphology (Davis et al., 1994), and the increased expression of these glia was found to be significant in the SCN at this 3 month time point in the LPS treated animals, but not in the SCN of saline treated controls. Further, when IBA-1 immunoreactivity was assessed in the SCN of animals treated with PDTC prior to LPS compared to those treated with a control injection and LPS, it was found that this upregulation of reactive microglia was diminished following the administration of PDTC prior to LPS treatment, while the upregulation of the densely stained hypertrophic glia remained significant in the SCN of control and LPS treated animals. In line with these results, studies have shown that LPS treatment results in a sustained microglial activation in the frontal cortex 8 weeks following the peripheral immune challenge (Weberpals et al., 2009), while other studies have shown that chronic i.c.v. infusion of LPS in rats has been shown to cause a long-lasting upregulation of microglia 37 days following the last LPS administration (Hauss-Wegrzyniak et al., 2000). Further, activated microglia have been shown in the brains of human sepsis patients, irrespective of age (Lemstra et al., 2007). The current study finds no evidence of astrogliosis in the post-septic SCN, evidenced by no changes in expression of the astrocytic marker, GFAP, keeping our findings in line with those experiments undertaken by Weberpals and colleagues (Weberpals et al., 2009), who also do not observe any evidence for astroglial activation in the hippocampus, frontal cortex or cerebellum 8wks post-septic LPS treatment (Weberpals et al., 2009).

Microglial activation in the CNS parenchyma seems to be vital for communication between the immune system and the brain (Konsman et al., 2002; Combrinck et al., 2002), playing either harmful or beneficial roles. Following CNS insult, microglia activation may play a beneficial role by the release of neurotrophic molecules, promoting aspects of regeneration (Streit et al., 1999; Schwartz, 2003; Ekdahl et al., 2003). In the current study, those immunomediators seen to be up-regulated in the SCN 24hrs post sepsis, namely NOS2 and IL-6 are known to play important roles in the activation of microglia (Zielasek and Hartung, 1996; Kreutzberg, 1996), and NOS2 derived NO production or increased IL-6 expression may play a role in microglial activation in the SCN. Additionally, activated microglia release inflammatory mediators which are shown to be neurotoxic *in vitro* (Ekdahl et al., 2003) such as IL-1β, IL-6, TNF-α, nitric oxide, and reactive oxygen species (Pocock and Liddle, 2001; Hanisch, 2002; Gebicke-Haerter, 2001; Vallieres et al., 2002) as well as induce the activation of the complement cascade, which has been shown to play roles in the brain following LPS treatment, leading to upregulation of CD45, TNF-α and TLR4, activated microglia and NOS2 (Jacob et al., 2007).

Alterations in cognitive function are seen following the activation of microglia. Peripheral LPS treatment and subsequent microglial activation and cytokine release is associated with behavioural deficits (Bluthe et al., 1992; Godbout et al., 2005; Combrinck et al., 2002). Administration of low dose endotoxin to healthy volunteers results in an increase in levels of systemic proinflammatory cytokines, along with alterations in behaviour and memory perturbations (Reichenberg et al., 2001; Krabbe et al., 2005). Further, neuroinflammation and microglial activation have been implicated in the pathogenesis of various disorders associated with cognitive impairment such as AD, Lewy body dementia, and AIDS dementia (Nelson et al., 2002; Katsuse et al., 2003; Perry et al., 2003).

Assessment of the state these up-regulated microglia exist in warrants further investigation. In situations of pre-existing pathology, such as in chronic neurodegenerative disease or in ageing whereby priming of microglia is observed, an enhanced consequence of neuroinflammation is seen (Godbout et al., 2005; Perry, 2004; Cunningham et al., 2005). Evidence points to systemic inflammation elicited by infection or injury in the periphery, in the activation of already primed microglia in the normal healthy ageing brain or in the brains of AD patients, and this can

subsequently impact upon the cognitive decline seen in these patients and further, can result in delerium (Perry et al., 2003). Studies have also shown an enhanced activation of microglia upon central administration of cytokines in old rats compared to young and middle aged rats (Deng et al., 2006). Given the chronic alterations in the CNS in microglial markers following the induction of sepsis, it's possible that microglia in the post-septic SCN may show enhanced activation to further immune challenges. Further, some of the alterations we observe in circadian locomotor behaviour are similar to those exhibited in normal ageing, which exhibits a low grade neuroinflammation and priming of microglia. Certain aspects of this will addressed in Chapter 5.

It is not possible for us to confirm from the current study the role that the long-lasting activated microglial expression played in the alterations in circadian behaviour, or whether in fact it played any role at all. Examination of additional inflammatory mediators that are known to be released by activated glia such as MCP-1 or reactive oxygen species is warranted in the post-septic SCN at this time. Further, assessment of phagocytic or fully activated microglia utilizing the ED-1 antibody which is known not to stain ramified microglia may be beneficial (McClain et al., 2011). It would be interesting to assess whether the LPS induced long-lasting upregulation of microglial markers in the SCN resolves itself over time, and if so, the exact period length of this increase in up-regulated microglial markers in the SCN, and whether normal circadian behavioural resetting might be restored upon resolution of the immunomodulation. Further, it would be interesting to investigate whether administration of a specific inhibitor of activated microglia such as minocycline would ameliorate the long-lasting upregulation of microglial markers observed in the SCN and the long-lasting behavioural alterations, and would shed further light as to whether the chronic microglial activation played a role in the post sepsis circadian behavioural alterations, or whether these effects were being mediated by another mechanism.

3.4.3. Proinflammatory cytokine expression in the SCN in the acute phase following LPS treatment and long term, in the post-septic SCN

Examination of the SCN in the current study revealed there to be no changes in the expression of the proinflammatory cytokine, TNF-α either 24hrs following

LPS 5mg/kg treatment, or 3 months following the initial immune challenge. TNF-α has previously been outlined to play a principal role in the mediation of the effects of LPS on the brain post sepsis, and has been proposed as a potent mediator of the chronic neuroinflammatory state (Qin et al., 2007; Bossu et al., 2012). Previous studies utilizing LPS i.p. treatment have shown a long-lasting upregulation of TNF-α in brain homogenates for many months following the peripheral immune challenge, while levels of TNF-α in the periphery were shown to return to baseline levels within 7 days following LPS treatment (Qin et al., 2007). TNF-α was also shown to be upregulated in the brain in the hippocampus and frontal cortex 7 days following LPS treatment and this significant upregulation was seen to remain up to ten months following LPS (Bossu et al., 2012). Studies by Weberpals and colleagues (Weberpals et al., 2009), utilizing the same endotoxin dose used in this study, have shown a long-lasting upregulation of proinflammatory cytokines in specific regions in the CNS post sepsis, including a significant upregulation of TNF-α mRNA in the cerebellum, cortex and hippocampus 2 months post-treatment (Weberpals et al., 2009). Furthermore an increase in IL-1β is evident in the frontal cortex two months following septic LPS treatment (Weberpals et al., 2009). Examination of the proinflammatory cytokine IL-1β in the current study in the post-septic SCN revealed that there were no alterations in expression of this cytokine 3 months post-treatment. The expression of TNF-α and IL-1β in the SCN have not been previously examined in the long term following sepsis induction, and so it may be that TNF-α and IL-1β are only up-regulated in specific CNS regions following sepsis and that the SCN is a region that does not show alterations in IL-1β or TNF-α expression following LPS treatment. That TNF-α is not found to be up-regulated in the SCN itself does not mean that it does not play a role in the LPS induced effects on circadian locomotor behaviour, acting outside of the SCN to induce changes in the early stages which then induce long-lasting effects. TNF-α and IL-1 are known to activate the NF-κB signalling pathway (Beg et al., 1993), and the p65 NF-κB subunit has been shown to be acutely up-regulated in the SCN following peripheral LPS treatment (Beynon and Coogan, 2010) and the role of TNF-α in mediating the effects of a septic LPS treatment has been documented, and suggested that the cytokine plays a role in the early stages following a septic LPS treatment activating microglia within the CNS, which then results in a self-propelling neuroinflammation (Qin et al., 2007).

The current study observes a significant upregulation of IL-6 in the SCN in the acute phase following a peripheral septic LPS treatment, however 3mths following the initial immune challenge, IL-6 expression levels are seen to be in line with those of controls. Similar to these findings, other studies have shown that in the acute phase, 4hrs following the induction of sepsis by peripheral LPS, levels of IL-6 expression are significantly up-regulated in cortex, cerebellum and hippocampus (Beurel and Jope, 2009), as well as being up-regulated following central injection in the CNS (Beurel and Jope, 2009). IL-6 is suggested to play a dual role within the CNS, with both detrimental and beneficial effects (Gruol and Nelson, 1997), probably determined by its level of expression and the duration of up-regulation (Beurel and Jope, 2009). IL-6 carries out various functions including playing a role in the immune response, being involved in the regulation of inflammation, haematopoesis and cell differentiation, along with being considered a signal in the activation process of microglia (Kreutzberg, 1996). Indeed, mice which overproduce human IL-6 in neurons known as NSEhIL6^{+} mice, spontaneously show increased microglial reactivity as well as spontaneous development of severe astrogliosis (Fattori et al, 1995). Further, glia play a role in mediating the effects of IL-6 (Beurel and Jope, 2009). The significant upregulation of IL-6 and activated microglia observed in the SCN 24hrs following LPS treatment in our experiments are in line with these studies. Since IL-6 is known to play roles in the activation of microglia (Kreutzberg, 1996), this may play a role in the expression of activated microglia in the SCN following LPS treatment. An increase in IL-6 has been associated with severe cognitive impairment (Campbell et al., 1993; Heyser et al., 1997; Dantzer et al., 2008) and additionally, in the CNS, IL-6 is suggested to be involved in the long-lasting effects of sepsis on the brain, including cognitive and behavioural alterations and neuronal loss (Semmler et al., 2007). It may be that the significant upregulation of IL-6 in the acute phase following LPS treatment activates glia within the SCN, leading to further release of other proinflammatory cytokines, that may impact upon circadian timekeeping processes and behaviour, however since IL-6 is not upregulated in the long term the increased microglial expression at this time cannot be attributed to this.

While we ourselves have not assessed the levels of proinflammatory cytokines in the periphery following LPS treatment, other studies using the same endotoxin dose as that used in these experiments have shown that proinflammatory

cytokines are seen to rise in the periphery and in the brain early following LPS treatment, but these levels are seen to subside in the periphery approximately one week following LPS treatment, after the acute response, but to stay elevated in the brain (Qin et al., 2007; Bossu et al., 2012). Studies by Bossu and colleagues (2012) have shown TNF-α to be upregulated 7 days following a septic LPS treatment in the hippocampus and frontal cortex, but not in the hypothalamus. These studies further highlight the region specificity of cytokine alterations following septic LPS treatment in the long term, and have shown both TNF-α and IL-18 to be upregulated in the hippocampus, cerebellum and frontal cortex 10 months following treatment, however examination of these inflammatory mediators in the hypothalamus again at this time point does not reveal any significant upregulation. At these timepoints, in the long-term following LPS treatment, when circulating levels of proinflammatory cytokines have returned to baseline levels, the elevation of inflammatory mediators in the brain is therefore independent of the initial systemic reaction, and is being propelled in the CNS itself, leading to the view of a self-propelling neuroinflammation (Qin et al., 2007; Bossu et al., 2012). We can therefore assume that any alterations observed in SCN neurochemistry or circadian locomotor behaviour, which are assessed following recovery from the acute phase of septic LPS treatment are not due to alterations in circulating cytokine levels. The molecular mechanisms underlying the potentiation of the chronic neuroniflammatory state have not been fully elucidated, but mechanisms have been put forward by other groups and suggest the involvement of TNF-α at the early stages in response to LPS treatment, activating microglia in the brain and leading to a self-propelling neuroinflammation by the further induction of cytokines in the CNS such as TNF-α, IL-1β (Qin et al., 2007) and IL-18 (Bossu et al., 2012), leading to a loss of neurons in the later stages following peripheral inflammation (Qin et al., 2007; Semmler et al., 2007) and lasting behavioural alterations (Bossu et al., 2012).

In support of a self-propelling neuroinflammation, there may be alterations in inflammatory mediators in the long term following LPS treatment that are not seen in the early stages following treatment. For example, IL-18 has recently been implicated in the long-lasting inflammatory response following a single LPS treatment, and is found to be significantly increased in the cerebellum, hippocampus and frontal cortex 10 months after peripheral LPS treatment, with no alterations in its expression 1 week following treatment (Bossu et al., 2012). Furthermore, IL-18 has

been shown to be involved in delayed neuroinflammation in experimental models of trauma and brain ischemia (Jander et al., 2002; Yatsiv et al., 2002) and has also been found to be expressed following LPS treatment in microglia and astrocytes (Conti et al., 1999; Wheeler et al., 1999). Assessment of IL-18 and other inflammatory cytokines in the SCN not undertaken in the current set of experiments would be interesting. There may be alterations in regions of the brain that the SCN projects to such as the PVN, both acutely or 3mths following septic LPS treatment and these could mediate the chronic effects of LPS induced sepsis on locomotor behaviour. Indeed, in the PVN following both i.v. or i.p. LPS treatment, levels of TNF-α and IL-6 are significantly increased in the acute phase following treatment, following an ACTH increase (Kakizaki et al., 1999). The chemokine MCP-1 which acts as a chemoattractant for T cells, basophils, monocytes/macrophages, and NK cells (Jacob et al., 2011) is shown to be up-regulated in the brain in the acute phase following LPS treatment (Thompson et al., 2008; Qin et al., 2008). MCP-1 may play important roles in the activation of the neuroinflammatory response following a peripheral immune challenge, since in MCP-1 KO mice treated with 5mg/kg LPS an exaggerated peripheral response has been shown, with an upregulation in cytokines, but a decrease in microglial activation, CNS levels of chemokines and the proinflammatory cytokines IL-1β and TNF-α, along with a reduction in serum corticosterone levels are also observed (Thompson et al., 2008).

Attenuations in central cytokine expression may also impact upon the HPA axis and activation of the HPA axis is one of the key events in the inflammatory response (Mathias et al., 2000; Takebe et al., 1966). Indeed, inhibition of brain cytokine expression following a septic peripheral endotoxin treatment in MCP-1 KO mice has been shown to reduce serum corticosterone levels (Thompson et al., 2008). Further, in adrenalectomized mice there is an increase in plasma IL-1β and TNF-α levels after LPS treatment along with an increase in mortality which has been shown to be reversed by treatment with glucocorticoids (Beishuizen and Thijs, 2003) and glucocorticoids have been shown to inhibit the induction of NOS2 (Kleinert et al., 1996), which is known to play roles in the long-lasting effects of sepsis (Weberpals et al., 2009), by decreasing cytokine-induced activity of NF-κB (Kleinert et al., 1996), highlighting the importance of HPA axis activation in response to LPS treatment. Alterations in cytokines and signallers such as NOS2 in the SCN may therefore impact upon the HPA axis, leading to attenuations in glucocorticoid

expression and neuroendocrine feedback to the SCN which could underlie the long-lasting changes in the circadian system following the induction of sepsis.

3.4.4. The involvement of the NF-κB pathway in the circadian system post-sepsis

Although evidence suggests that the NF-κB signalling pathway is involved in the SCN in the acute phase following sepsis (Beynon and Coogan et al., 2010; Leone et al., 2006) we do not observe a significant upregulation in components of the NF-κB pathway in the post-septic SCN. The translocation of NF-κB to the nucleus from the cytosol is prevented by tight association with inhibitors known as the I-KappaB's (IκBs) (Baeuerle and Baltimore 1988). In response to signals that activate NF-κB, the enzyme IkappaB kinase (IκK) becomes activated and subsequently phosphorylates IκB which dissociates from NF-κB and becomes degraded, allowing NF-κB to translocate to the nucleus and subsequent NF-κB DNA binding and the induction of the transcription of different components of the inflammatory response including cytokines such as IL-6, NOS, immunoglobulins, Rel family proteins, IκB and neurotransmitters, along with various other genes (Beg et al., 1993; Verma, 1995; Nomura, 2001; Marpegan et al., 2004). Cytokine activation of the NF-κB complex might cause an upregulation in components of the NF-κB pathway in the SCN, however, in the long term following a septic LPS treatment, our assessment of three components of this pathway including the p65 NF-κB subunit, p-IκK and p-IκB found no significant upregulation of these in the post-septic SCN. It does appear that the distribution of expression of both p-IκK and p-IκB may be altered in the post-septic SCN, however this requires further investigation.

NF-κB has been implicated in the LPS effects on the circadian system (Marpegan et al., 2004, 2005, Paladino et al., 2010) and application of sulfasalazine, an NF-κB inhibitor, has previously been shown to block peripheral LPS induced phase shifts in the early subjective night in mice (Marpegan et al., 2005), highlighting the role of NF-κB in mediating the effects of LPS on the SCN. While the SCN itself does not show upregulation of NF-κB pathway components in the long term following LPS treatment, the NF-κB signalling pathway does appear to play a role in mediating the effects of LPS in the acute phase following LPS treatment, since its inhibition by administration of 200mg/kg PDTC, a potent inhibitor of the NF-κB pathway, prior to LPS 5mg.kg i.p. treatment ameliorates the

impact of the septic LPS treatment on the SCN and on circadian behaviour in the long term. In line with this, reducing the inflammation that occurs during sepsis has been shown to alleviate the pathogenesis of sepsis (Jacob et al., 2010). Administration of PDTC, could prevent the inflammation associated with septic encephalopathy, thereby preventing post-septic neuroinflammation and inhibiting the alterations observed in the circadian pattern of locomotor activity and long-lasting microglial activation following the induction of sepsis. As mentioned previously, PDTC treatment was also shown to prevent the high sepsis scores elicited in LPS treated animals, with a significant decrease in sepsis scores between animals treated with PDTC prior to LPS compared to those treated with a control injection prior to LPS, and this was probably due to a decrease in the induction of proinflammatory cytokines. Additionally, the upregulation in hypertrophic microglial cells is shown to be attenuated by administration of PDTC prior to LPS treatment, and values were seen to be in line with control animals, along with the attenuations in circadian resetting exhibited by post-septic animals. The possible decrease in the activation of the inflammatory mediators in the SCN may underlie the decrease in microglial activation in the SCN of animals treated with PDTC prior to LPS. Indeed, NF-κB is known to regulate IL-6 expression (Steinbrecher et al., 2005) and the induction of NOS2 following LPS treatment (Xie et al., 1994), therefore, inhibition of the NF-κB signalling pathway by PDTC administration may inhibit these in the SCN in the acute phase following sepsis. A decrease in inflammatory mediators in the SCN might also account for the unaltered circadian resetting in PDTC + LPS treated animals. Further, NF-κB has been implicated in disruption of glucocorticoid function following its activation by cytokines (Pace et al., 2007), and this could possibly play a role in the LPS induced attenuated behavioural resetting and its inhibition by PDTC may account for why circadian resetting is in line with controls in PDTC and LPS treated animals.

Assessment of NOS2 and IL-6 levels and microglial activation in the SCN in the acute phase following administration of PDTC prior to LPS 5mg/kg treatment would shed further light on this. It is worth noting that NF-κB signalling mediates the transcription of a wide variety of mediators involved in the immune and inflammatory response (Beg et al., 1993; Verma, 1995; Nomura, 2001) and its inhibition may serve to prevent the induction of these, which may play a role in the expression of normal circadian behaviour following PDTC treatment prior to a

peripheral septic LPS dose. Additionally, it would be interesting to assess whether PDTC administration prior to LPS might diminish the increased expression of the effector IEG, EGR-1 in the SCN at 24hrs post-treatment. Additional work must be carried out in order to establish the mechanisms by which NF-κB signalling is involved in the long-lasting alterations in the SCN and the circadian system.

Overall, we observe that the SCN responds to the substantial peripheral immune challenge, however on the whole, there were no major alterations in the SCN between post-septic and LPS treated animals 3months following the initial immune insult. Therefore, the observed alterations in circadian locomotor behaviour seen in post-septic animals at this time indicate that the long-lasting perturbations in circadian behavioural rhythmicity exemplified by these studies are being mediated by other factors.

Attenuations in various other mechanisms may then underlie the perturbations in circadian resetting exemplified by these studies. As previously suggested, cytokine mediated alterations of the function of the HPA axis and glucocorticoid receptor function may play a role, further modulating inflammation (Capuron and Miller, 2011), and this alteration in endocrine function may feedback to the SCN, impacting upon circadian function. Inflammatory mediators induced by peripheral LPS may impact upon the SCN inducing functional changes and alterations in clock gene expression following LPS administration, and the ability of LPS to impact upon clock gene expression has been shown by various different studies (Takahashi et al., 2001; Marpegan et al., 2005; Palomba and Bentivoglio, 2008; Okada et al., 2008). The examination of clock gene expression in Chapter 4 will clarify whether alterations in clock gene expression could underlie the long-lasting changes in behavioural resetting post sepsis. Additionally, in the SCN or regions it projects to such as the PVN, attenuations in the expression of other factors involved in the inflammatory response not examined in these studies may play roles in mediating the effects of LPS following the induction of sepsis. The assessment of cholinergic transmission in the SCN post sepsis would be interesting. The cholinergic anti-inflammatory pathway plays roles in the modulation of inflammation, stimulating the parasympathetic nervous system to release acetylcholine which regulates the immune response (Pavlov et al., 2003). Given that cholinergic input to the SCN is involved in the function of the circadian system (Hut

and Van der Zee, 2011), it's possible that alterations in the cholinergic system such as the loss of cholinergic fibres previously shown in rats following high dose endotoxin (Semmler et al., 2007) could play a role in the behavioural alterations. Attenuations in synaptic plasticity in the post-septic brain could play a role in the attenuations in circadian behaviour. Two months following a single LPS treatment, synaptic alterations are evidenced in the post-septic brain, possibly mediated through nitric oxide production (Weberpals et al., 2009) and given the upregulation we observe in NOS2 expression in the acute phase following LPS treatment, it's possible that synaptic alterations could underlie the alterations in circadian behaviour exhibited post sepsis. Further, microglia induced nitric oxide has been shown *in vitro* to affect the anterograde axonal transport of synaptophysin, which is a synaptic protein involved in synaptic plasticity (Stagi et al., 2005). LPS induced cytokine modulations could also perturb neurotransmitter function or brain circuitry since alterations in neurotransmitter expression have been found in the rat brain during sepsis (Freund et al., 1985), and alterations in these may underlie the perturbations in behaviour in post-septic animals (Capuron and Miller, 2011).

Additional studies assessing these parameters are required in order to fully assess the mechanisms by which LPS exerts its effects on the circadian system. The results from the assessment of the neurochemistry of the post-septic SCN do not show long-lasting expression of proinflammatory cytokines known to impact upon circadian rhythmicity in the SCN of post-septic mice, and assert again, that in the long term following a septic LPS treatment, an underlying mechanism is impacting upon circadian locomotor behaviour. It would be tempting to speculate a mechanism whereby the action of TNF-α in the early stages as shown previously by other studies (Qin et al., 2007) leads to activation of the NF-κB signalling pathway, resulting in the transcription of inflammatory mediators, including the induction of NOS2 and IL-6 expression in the SCN, leading to either microglial activation that induces a long-term self-propelling neuroinflammation as outlined by other groups (Qin et al., 2007; Bossu et al., 2012), or resulting in alterations in neuroendocrine feedback to the SCN through impairment of HPA axis function, or both. Further extensive studies would be required in order to delineate a mechanism by which this LPS induced neuroinflammation impacts upon circadian timekeeping processes.

3.5. Supporting Information

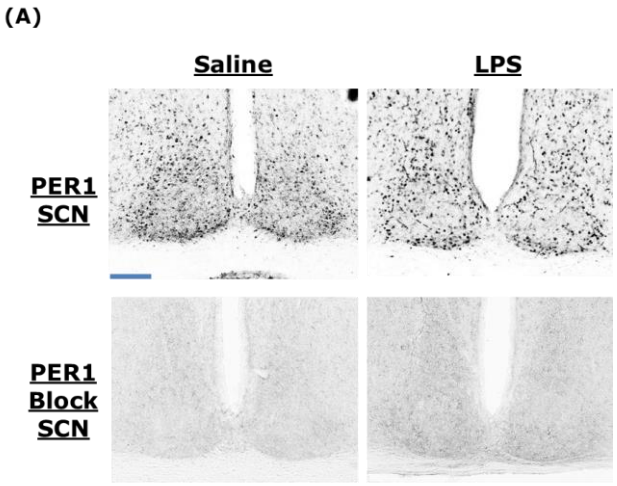


Figure S.1.: Immunizing peptide blocking experiment *(A) Representative SCN photomicrographs of animals treated one month previously with either saline or LPS, illustrating the comparison between normal PER1 IHC stained sections and the staining of sections when the PER1 anibody was neutralized with the PER1 immunizing blocking peptide, confirming that non-specific staining did not occur (scale bar = 100 µm).*

Chapter Four

Analysis of Clock gene and Immediate Early Gene product expression in the post-septic SCN.

4.1. Introduction:

Inflammatory mediators induced by peripheral LPS may impact upon the SCN, inducing functional changes. Treatment with LPS and other inflammatory mediators may attenuate the molecular oscillations of clock gene expression that underpin circadian rhythmicity, both in the master pacemaker itself, and in the periphery, and in this way can impact upon timekeeping processes.

4.1.1. LPS treatment impacts upon central and peripheral clock gene expression

Treatment with endotoxin impacts upon rhythmic clock gene expression both in the SCN and in the periphery, and the ability of LPS to influence molecular oscillations and timekeeping processes has been shown by various different studies (Takahashi et al., 2001; Marpegan et al., 2005; Murphy et al., 2007; Okada et al., 2008). While a low dose LPS treatment is seen not to affect *Per1* or *Per2* expression in the SCN (Takahashi et al., 2001), peripheral 1mg/kg LPS treatment temporarily suppresses both *Per2* and *DBP* mRNA expression levels in the SCN in rats, as well as the expression of *Per1, Per2* mRNA and other genes under circadian control in the periphery, with restoration of expression patterns on the second day after the immune challenge, showing that endotoxin treatment transiently suppresses circadian timekeeping molecular oscillations (Okada et al., 2008). Yamamura and colleagues (2010), have shown that LPS treatment causes a phase dependent reduction in the expression of *Per1* and *Per2* genes in the heart and the liver. Both *Per2* and *Bmal1* have been shown to be significantly upregulated in the periphery in equine blood following LPS treatment (Murphy et al., 2007). Further, these studies have shown LPS induced clock gene alterations to be inhibited by treatment with a cyclooxygenase inhibitor known to reduce PGE2 levels, implicating a role for these in circadian resetting (Murphy et al., 2007). Additionally, human subjects treated with endotoxin *in vivo* show acute alterations in expression levels of various clock

genes in peripheral blood leukocytes (PBL) (Haimovich et al., 2010). Treatment of PER2::luc SCN cultures with either TNF-α or LPS *in vitro* does not affect rhythmic expression patterns (Guenthner et al., 2009). Clock gene expression has also been shown to be altered in other CNS regions in response to LPS treatment. Treatment with 50 μg/kg LPS injection in the subjective night has been shown to induce PER1 expression in the PVN region at CT15 (Paladino et al., 2010) and at ZT22 (Takahashi et al., 2001), and studies have shown stress signals such as LPS to induce upregulation of the *Per1* gene in the PVN CRF neurons, but not in the SCN, in mice (Takahashi et al., 2001).

4.1.2. Inflammatory mediators attenuate clock gene expression in the SCN and in the periphery.

In the SCN, IFN-α is seen to act directly upon SCN neurons, and impact upon clock gene rhythmicity. Mice treated with IFN-α display a time dependent response, with no alterations observed following IFN-α administration at ZT0, while at ZT12, IFN-α treatment causes alterations in the expression of *Bmal1, Per 1, 2* and *3* (Ohdo et al., 2001). Repetitive administration of IFN-α resulted in alterations in photically induced *Per* expression (Ohdo et al., 2001). Further, studies have shown the expression of *Bmal1* and *Clock* to be dampened in the SCN following IFN-α administration, and additionally, this has been seen to cause perturbations in the expression of the clock controlled gene vasopressin which is involved in mediating output from the SCN (Koyanagi and Ohdo, 2002). Administration of TNF-α which is known to be induced following peripheral LPS treatment (Zetterstrom et al., 1998) and is involved in mediating the effects of LPS on the circadian system (Qin et al., 2007) impacts upon clock gene rhythmicity in the SCN, and subcutaneous TNF-α has been shown to alter SCN *Dbp* expression (Cavadini et al., 2007), and it has been suggested that TNF-α which has been shown to be necessary for LPS induced phase delays of locomotor behaviour may elicit these by altering the expression of clock genes (Leone et al., 2012).

A wealth of literature has shown the ability of administration of inflammatory mediators to impact upon clock gene expression in the periphery, both *in vivo* and in cell lines *in vitro*. Continuous IFN-α administration is seen to reduce *Per1* and *Per2* mRNA levels in the liver at ZT14 (Shinohara et al., 2008). Treatment

with IFN-γ at ZT12, but not ZT0, for 6 days was seen to result in alterations in *Per* expression (Ohdo et al., 2001), while in transgenic *Per1-luc* cultures, chronic treatment (4 weeks) with IFN-γ has been shown to significantly reduce rhythm amplitude in individual rat neurons (Kwak et al., 2008). Clock gene expression in mice has been shown to be altered by the proinflammatory prostaglandins, and PGE2 is suggested to act as an *in vivo*-clock resetting stimulus, since peripheral PGE2 treatment is known to phase shift clock genes in the periphery *in vivo*, altering the expression levels of *Per1, Rev-erb-α* and *DBP* (Tsuchiya et al., 2005). *In vitro,* NIH3T3 murine fibroblast cells treated with PGE2 induced acute and transient expression of *Per1* mRNA in a dose dependent manner (Tsuchiya et al., 2005). TNF-α also impacts upon peripheral molecular oscillations. TNF-α interacting with TNFR1 causes the upregulation of *Per1, Per2, Dec* (differentiated embryo chondrocytes)*-1* and *Cry1,* and the downregulation of *Dbp* in fibroblasts *in vitro* (Petrzilka et al., 2009). In fibroblasts *in vitro,* and in the liver of mice *in vivo,* the expression of clock genes that contain E box regulatory elements, i.e. the PAR bZip genes (*Hlf, Tef and Dbp*) and the *Per 1, 2* and *3* genes have been shown to be suppressed by TNF-α (Cavadini et al., 2007). Further highlighting that TNF-α directly affects clock gene expression, treatment of both the human hepatoma HuH7 cell line and human hepatocarcinoma HepG2 cell lines with TNF-α overnight results in dose dependent increase in CRY1 expression (Tong et al., 2012). These authors show that TNF-α upregulates Usp (Ubiquitin Specific Protease) 2a, a deubiquitination enzyme which is responsible for TNF-α induced CRY1 protein stabilization, and suggest that in this way, Usp2a disrupts the circadian clock in response to TNF-α and additionally, suggest that this mechanism may be responsible for chronic TNF-α suppression of the circadian clock. DBP expression was also shown to be decreased following TNF-α treatment, and the *Per2* promoter-driven luciferase reporter activation by BMAL1/CLOCK overexpression was shown to be suppressed in the Huh7 cell line (Tong et al., 2012). More recently, treatment of human fibroblast cells with TNF-α *in vitro* has been shown to significantly increase the expression of *PER3* and *DBP*, while reducing the expression of *NPAS2* and *ARNTL2* (Kouri et al., 2013).

Both IL-1β and IL-6 which are known to be induced in response to LPS both centrally and peripherally (Zetterstrom et al., 1998) impact upon clock gene expression. IL-1β has been found to cause significant alterations in the expression of

Dbp and *Per3* in fibroblasts *in vitro* (Cavadini et al., 2007), and treatment of a human hepatoma cell line (HuH-7) with IL-6 results in significant induction of *Per1* gene expression (Motzkus et al., 2002).

In vivo, the activation of CD40 leads to the production of proinflammatory cytokines, and CD40 stimulation has been shown to lead to alterations in clock gene expression in the liver and is dependent on TNFR1 signalling (Taraborelli et al., 2011). Upon induction of experimental arthritis in mice using a mixture of anti-type II collagen (anti-CII) mAb and LPS, clock gene mRNA expression was disrupted in the spleen, including that of *Bmal1, Per1* and *Per2* and *Dbp*, while PER2 protein expression was disturbed in the nuclei of synovial cells (Hashiramoto et al., 2010). Recent studies by Bellet et al (2012) have suggested there to be communication between the NF-κB pathway and clock gene expression, since RelB-deficient mouse embryonic fibroblasts show alterations in the transcription of clock genes. In *Clock* mutant mouse embryonic fibroblasts that express alterations in the NF-κB pathway component, RelB, attenuations in the transcription of clock genes are shown in response to TNF-α treatment, with lower levels of both *Per2* and *Dbp* mRNAs observed in comparison to TNF-α treated wild type cells, indicating that interplay between the circadian clock and NF-κB pathway components is important in regulating clock gene expression (Bellet et al., 2012).

Together, these studies highlight the impact of immune mediators on molecular clock gene oscillations, highlighting the ability of these factors to impact upon timekeeping processes.

4.1.3. Clock gene alterations are observed in chronic inflammatory conditions

Attenuations in the expression of clock genes have been observed in conditions with an inflammatory component, both in humans and experimental animal models. In male patients with acute Q fever as a result of bacterial infection with the *Coxiella burnetii* (*C. burnetii*), *Per2* has been demonstrated to be up-regulated in the blood (Mehraj et al., 2012), while in female mice infected with *C. burnetii*, the expression of the clock genes *Clock* and *Arntl* was shown to be reduced in the liver, accompanied by an upregulation of *Per2* (Textoris et al., 2010). In both preclinical and clinical AD, the rhythmic expression of clock genes *Per1*, *Cry1* and *Bmal1* is diminished in the pineal, while *Cry1* mRNA was increased in clinical AD

(Wu et al., 2006). Obesity is associated with systemic inflammation (Yaffe et al., 2004), and the expression patterns of *Bmal1* and *Rev-erbα* were seen to be altered in the brainstem of obese mice (Kaneko et al., 2009). More recently, the expression of clock genes has been shown to be disturbed in the synovial membrane of Rheumatoid Arthritis (RA) patients (Kouri et al., 2013), while experimental arthritis is seen to be exacerbated in *Cry* deficient mice, resulting in increased serum levels of matrix metalloproteinase-3, TNF-α, IL-6 and IL-1β, indicating that circadian rhythmicity plays a role in the pathogenesis of arthritis (Hashiramoto et al., 2010). The expression of *Per2* has been shown to be altered in the periphery in the liver of mice with experimental autoimmune encephalomyelitis (EAE) which is characterized by the infiltration of immune cells into the CNS and the presence of inflammatory lesions as well as axonal loss in the brain (Buenafe, 2012). In an experimental rodent model of African trypanosomiasis, *Trypanosoma brucei brucei* (*Tb brucei*) has been shown to impact upon clock gene expression in peripheral oscillators, to induce alterations in *Clock* mRNA in the pineal gland, as well as a reduction in *Bmal1* mRNA in the spleen *in vitro* (Lundkvist et al., 2010). Further, these authors observe a shortened *Per1-luc* period in the pituitary gland, however only 21% of SCN tissues showed flat *Per1-luc* rhythms. In ageing, which is associated with a low-grade central neuroinflammation (Godbout and Johnson, 2009), BMAL1 and CLOCK have been shown to be altered both in the SCN (Asai et al., 2001) and in extra-SCN sites (Wyse and Coogan, 2010). Taken together, these studies highlight the impact of inflammatory conditions and diseases on circadian timekeeping processes.

Given that oscillations of components of the molecular clock in both the SCN and sites of other oscillators are known to drive circadian rhythms of behaviour, and that immune mediators, including LPS, have been shown cause functional alterations in the SCN (Marpegan et al., 2005; Beynon and Coogan, 2010; Palomba and Bentivoglio, 2008; Sadki et al., 2007), and to alter clock gene rhythmicity, and the current study observes perturbations in behavioural circadian rhythms in response to a septic LPS treatment, we examined the oscillations of core molecular clock components in the SCN to determine whether a molecular correlate exists for the behavioural phenotype shown by post-septic animals. These experiments examine the diurnal expression of the clock gene protein products PER1, PER2 and CLOCK

and further, examine c-Fos expression patterns in the SCN, to assess post-septic SCN responsiveness and function. Perturbations in these parameters could be responsible for attenuated behavioural resetting in post-septic animals.

4.2. Materials & Methods

4.2.1. Animals and Housing:

For assessment of IEG and clock gene expression in SCN by immunohistochemical analysis, male C57Bl/6 (obtained at 6-8 weeks old) mice from either Harlan (UK) or Charles River (UK) were used that had been group housed as outlined in section 2.2.2. For the purposes of real-time reporting of circadian gene expression, B6.129S6-*Per2*^{tm1Jt}/J mice were utilized. B6.129S6-*Per2*^{tm1Jt}/J have a firefly luciferase (*luc*) gene inserted in-frame into the 3' end of the endogenous *mPer2* gene between exon 23 and the three prime untranslated region (3' UTR) (Yoo et al., 2004). This allows the observation of the expression of the PERIOD2::LUCIFERASE (or mPER2::LUC) fusion protein in real-time. The mPer2^{Luc} knock-in mice are therefore utilized as a real time reporter of circadian rhythms in mice. Homozygous individuals were then obtained from JAX mice (USA) via Charles River (U.K), and a breeding colony established in NUI Maynooth. Mice homozygous for this mutation show no alterations in the circadian behaviour or in entrainment parameters (Yoo et al., 2004). At the beginning of each experiment these animals were between 8 and 10 weeks old and were used for the duration of these experiments up until the age of approximately 4 months.

4.2.2. Analysis of clock gene expression following induction of sepsis

In order to assess whether previous induction of sepsis has long-lasting effects on the profiles of clock genes expression in the SCN across the 24hr circadian cycle, adult male mice were group housed in colonies of ten in cages equipped with appropriate environmental enrichment under a 12:12 LD (150 lux, lights on 0700h) cycle for two weeks prior to treatment to allow for habituation. Animals were then treated with either LPS 5mg/kg i.p. injection (n=3 or 4 per time point) or saline vehicle (n=3 or 4 per time point). The animals were allowed to recover from the immune challenge for one month, during which time they were maintained under the

12:12 LD cycle. Following this, they were transferred into constant darkness (0 lux) for two cycles.

On the third cycle, the animals were sampled in dim red light (<1 lux) every 4 hours across the twenty four hour circadian cycle. Animals were terminally anaesthetized with 0.1ml i.p. sodium pentobarbital under dim red light (<1 lux) and underwent transcardiac perfusion (outlined in section 3.2.3) at CT2, CT6, CT10, CT14, CT18 and CT22 as outlined previously (Beynon and Coogan, 2010). All brains gathered were subsequently processed for immunohistochemical staining (outlined in section 3.2.4) for the clock gene protein products PER1, PER2, and CLOCK and the immediate early gene c-Fos. Details of all primary antibodies used are detailed in Table 4.

Table 4: Primary Antisera used for Immunohistochemical analysis:

Antibody	Dilution	Raised in	Supplier	Product Code
c-Fos (4)	1:2000	Rabbit	Santa Cruz Biotechnology	sc-52
Per1(N20)	1:500	Goat	Santa Cruz Biotechnology	sc-7724
Per2 (E-20)	1:1000	Rabbit	Alpha Diagnostics, Texas USA.	PER21-A
Clock (H-276)	1:500	Goat	Santa Cruz Biotechnology	sc-25361

For each individual animal and each antibody, 3-6 SCN images were examined by means of visual quantification of the number of immunoreactive nuclei in the SCN, viewed with the light microscope as previously outlined in section 3.2.5, and a mean value obtained for each animal at each time point for each clock gene protein antibody for all SCN regions of interest. These values were calculated for each animal by two researchers. The observers were blinded to the experimental procedure during quantification of immunoreactive cells per SCN. The means calculated for each animal in both groups at each time point were compared and analysed by two way between groups ANOVA, with circadian time and treatment as the between groups factors. Results are given as mean values (immunoreactive cell number per SCN slice) $\pm$ standard error of the mean, statistical significance was accepted at $P<0.05$.

4.2.3. Cosinor Analysis:

In order to analyze the individual 24-h cycle of expression of each clock gene protein products in the SCN and confirm if the rhythm was indeed circadian in nature, a previously described cosinor method was utilized (Nelson et al., 1979). The CircWave 1.4 analyses software (developed by Dr. R.A. Hut, 2007, http://www.euclock.org) employs a forward linear harmonic regression to calculate the profile of the wave with a 24hr period for independent samples utilizing the following function:

$$f(t) = a + \sum_{i=1}^{\infty}\left[p_i \sin i2\pi \frac{t}{\tau} + q_i \cos i2\pi \frac{t}{\tau} \right]$$

Where: a=average; i=1, 2, 3, indicating the fundamental wave, the first harmonic, the second harmonic, …. $(i\text{-}1)^{th}$ harmonic; p_i is the sine coefficient of the $(i\text{-}1)^{th}$ harmonic and q_i the coefficient of the $(i\text{-}1)^{th}$ harmonic; t=timepoint value (modulo τ); f(t) is the calculated function value at time point t. Where: y is the fitted value for time point t; a and b are linear estimates for the sine and cosine contribution to a flat line (when i=0), the fundamental wave (i=1), first harmonic (i=2), second harmonic (i=3), etc. A 24 hour rhythm was confirmed if the null amplitude hypothesis was rejected from an F test producing a significant value $P<0.05$. Values for the amplitude, acrophase and rhythm adjusted mean level during the 24 hour cycle were also provided through the cosinor analysis.

4.2.4. **PER2::LUC** ***Experiments***

In order to assess circadian dynamics in real-time in post-septic animals and saline controls, adult male $mPer2^{Luc}$ knock-in (B6.129S6-*Per2*^{tm1Jt}/J) mice from the NUI Maynooth breeding colony were utilized. Adult male and female $mPer2^{Luc}$ knock-in (B6.129S6-*Per2*^{tm1Jt}/J) mice previously obtained from Charles River, UK., were bred in-house. After weaning, animals were group housed according to sex in colonies of 3 in cages equipped with appropriate environmental enrichment and habituated to a 12:12 LD cycle for 2 weeks (150 lux, lights on 0700h) prior to experimentation. The animals were treated with either LPS 5mg/kg intra peritoneal injection (n=4) or saline (n=5). The animals were allowed to recover

following the induction of sepsis and were maintained for 1 month under a 12:12 LD cycle.

For the purposes of culturing SCN explants, 70% ethanol was used to sterilize any reusable equipment prior to use, these were then placed for 30min under ultraviolet (UV) light in a sterile tissue culture hood to ensure sterilization. All procedures described below were carried out rapidly to ensure the tissue remained living.

One month following the initial LPS 5mg/kg treatment, at ZT4-5, animals were culled by means of cervical dislocation, brains were carefully and rapidly removed and placed on filter paper (Whatman, Sigma, UK) and a few drops of ice cold Hank's Balanced Salt Solution (HBSS, consisting of 100ml Hanks' balanced salt solution 10X, H1641, Sigma, U.S.A.; 4.7ml sodium bicarbonate solution (7.5%, Sigma); 10ml 1M HEPES buffer (Sigma) and 10ml penicillin-streptomycin (10,000 unit/ml-10,000 µg/ml, Gibco, Invitrogen, U.S.A., dissolved in autoclaved Milli-Q water with the total volume adjusted to 1 litre and kept at 4°C prior to use) added immediately. The brain was cut caudally removing the cerebellum and rostrally to remove the preoptic region with a single edged razor blade. The brain was then fixed onto the metal block of a manual advance Vibratome (World Precision Instruments), using the least amount of cyanoacrylate glue necessary (Bostik) with the caudal cut surface attached to the block. The ventral aspect of the brain was positioned to face the vibrating double edged blade, which was angled at 180°. The block was then fixed in place in the vibroslice, and the block and attached tissue submerged in ice cold HBSS for sectioning. 2-3 coronal sections of 300µm thickness containing the SCN were cut through the live brain. The 300µm live brain slice containing the optimum mid SCN region was selected and viewed under a dissecting microscope (Accuscope) and the paired SCN nuclei and retrochiasmatic area identified and isolated from the slice with the aid of two scalpels. Each individual tissue sample was then placed aseptically onto a Millicell culture membrane (Millipore, Ireland) in a 35mm petri dish (Corning, U.S.A.) with 1ml of sterile recording medium consisting of Dulbeccos Modified Eagles Medium (DMEM, with L-glutamine, 1000mg glucose, without phenol red, supplemented with 3.5g of D-glucose powder, Sigma), 4.7ml sodium bicarbonate solution, (7.5%, Sigma), supplemented with 10 mM Hepes buffer (pH 7.2, Sigma), and 2.5ml Penicillin-streptomycin (10,000 unit/ml-10,000 µg/ml, Gibco). All contents were

dissolved in autoclaved Milli-Q water and the final volume adjusted to 1 litre and stored at 4°C prior to use. 0.1 mM beetle luciferin potassium salt (Promega, U.S.A.) was then added just prior to the luciferase activity assay. The medium was used at a temperature of 37°C. Autoclaved vacuum grease (Sigma) was applied around the edge of the culture dish and a 40mm coverslip lid (VWR) placed on top to seal the dish, providing an airtight environment, preventing evaporation of the medium and therefore equipped with the appropriate nutrition, moisture and oxygen supply for maintenance of the slice. A waterproof, light tight, 37°C incubator (Memmert, Schwaback) fitted with Photomultiplier Tube (PMT) detector assemblies (Hamamtsu Photonics. U.K., LTD.) was utilized for continuous monitoring of bioluminescence rhythms from individual SCN culture explants for each animal in both treatment groups for a period of ten days.

The bioluminescence emitted from each individual SCN tissue culture was immediately calculated using Living Image 3.2 software (PMTmonTTL Software by Dr. Shin Yamazaki) and bioluminescence value recorded for one of every two minutes over a period of ten days. An adjacent averaging method with three hour running means was utilized to smooth the data. Data sets were de-trended, whereby the 24hr running average was subtracted from the raw data. A twenty four hour running average was then calculated from the de-trended data, from which a 3hr running average was subsequently calculated. The 3hr running average data was then used for analysis with software developed by Dr. R. Refinetti downloaded from www.circadian.org. The Cosinor software (Cosinor.exe, version 2.03; designed by Dr. Refinetti) utilizes the cosinor method to calculate the mean amplitude and acrophase of the rhythm. The LSP software (Lsp.exe, version 2.07; designed by Dr. Refinetti) utilizes the Lomb-Scargle periodogram procedure to calculate the circadian period of the rhythm. A mean value was therefore obtained for each individual culture for the period, amplitude and acrophase of the bioluminescence rhythm. The means calculated for each culture for each treatment group were compared and analysed by independent samples t-test. Results are given as mean values $\pm$ standard error of the mean, statistical significance was accepted at $P<0.05$.

4.2.5. Photic induction of immediate early gene expression in the SCN.

In order to assess the long-lasting effects of sepsis induction on SCN functional activity, neuronal activation was examined following photic stimulation. In order to examine circadian function in this case, two groups of experimental animals were analysed under different experimental protocols, one where photic stimulation occurred while the animals were held under constant dark conditions, and the other where the experimental animals were maintained under a 12:12 LD cycle. All animals were treated with either LPS 5mg/kg i.p. injection or Saline and were left to recover from the immune challenge prior to experimentation.

For the purposes of the experiments under a 12:12 LD cycle, animals were group housed in colonies of ten with appropriate environmental enrichment and habituated to a 12:12 light dark cycle (150lux, light on 0700) for 14 days prior to treatment. One month after treatment animals received a 30min light pulse (150 lux) at either ZT15 (Saline 7, LPS 5) or ZT22 (Saline 4, LPS 4). Animals were placed back in darkness for 30 min to allow for expression of c-Fos and were culled by means of cervical dislocation one hour after commencement of the light pulse. Brains were carefully removed and, immersion fixed in 4% PFA and processed for immunohistochemistry as outlined in section 3.2.4.

For assessment of photic stimulation under constant dark conditions, 16 adult male mice singly housed in running wheel cages used for construction of the phase response curve were placed into a 12:12 LD cycle and maintained under this photoperiod for 14days. The animals were transferred into constant darkness (0 lux) conditions for two cycles. On the third cycle, animals received a 150 lux, 30 min light pulse at either CT15 (Saline 4, LPS 4) or CT22 (Saline 4, LPS 4). Animals were placed back in darkness for 30 min to allow for expression of the immediate early genes c-Fos, Arc and Egr-1, before being culled by means of cervical dislocation one hour after commencement of the light pulse. Brains were carefully removed, immersion fixed and processed for immunohistochemistry as outlined in sections 3.2.3 and 3.2.4.

For each individual animal, 3-6 SCN images were examined by means of manual quantification of the number of immunoreactive nuclei in the SCN as previously outlined in section 3.2.5, and a mean value obtained for each animal for each antibody for all SCN regions of interest. These values were calculated for each

animal by two researchers blind to the experimental procedure and the means for each groups were compared and analysed by independent samples t-test. Results are given as mean values (cell number) $\pm$ standard error of the mean, statistical significance was accepted at $P<0.05$.

4.3. Results

4.3.1. Analysis of clock gene expression following the induction of sepsis

The molecular correlates of behavioural circadian rhythmicity were examined by assessing the rhythmic expression of clock genes following sepsis induction. Changes in the behavioural rhythm may be mimicked by the cycle of clock gene expression in the SCN. The experiment examined whether the induction of sepsis alters the expression of clock gene protein products PER1, PER2 and CLOCK and the IEG expression patterns in the SCN post-sepsis.

4.3.1.1. PER1 expression across the 24hr circadian cycle

Two Way Repeated Measures ANOVA was utilized to assess statistically significant differences between groups in the mean expression of the clock gene protein product PER1 across the circadian cycle. There was a main effect of time on PER1 expression in the SCN ($F_{5,26}=14.77$, $P<0.001$), but no effect of treatment ($F_{1,26}=2.32$, $P>0.05$) and no significant interaction effect ($F_{5,26}=0.60$, $P>0.05$). Analysis of the separate SCN subdivisions found there to be a significant effect of time on PER1 expression in the SCN core ($F_{5,26}=11.91$, $P<0.001$), but no effect of treatment ($F_{1,26}=0.35$, $P>0.05$), and no interaction effect ($F_{5,26}=0.54$, $P>0.05$). There was a significant effect of time on PER1 expression in the SCN shell ($F_{5,26}=14.47$, $P<0.001$), but no effect of treatment ($F_{1,26}=3.25$, $P>0.05$) and no interaction effect ($F_{5,26}=1.44$, $P>0.05$). (Figure 4.1).

Co-sinor analysis of PER1 expression in the SCN revealed the acrophase of expression to be at CT15.87 in controls and CT 16.27 in post-septic animals. The percentage of variance in PER1 expression accounted for by a 24 hour co-sine wave was found to be 91% in controls and 66% in post-septic animals.

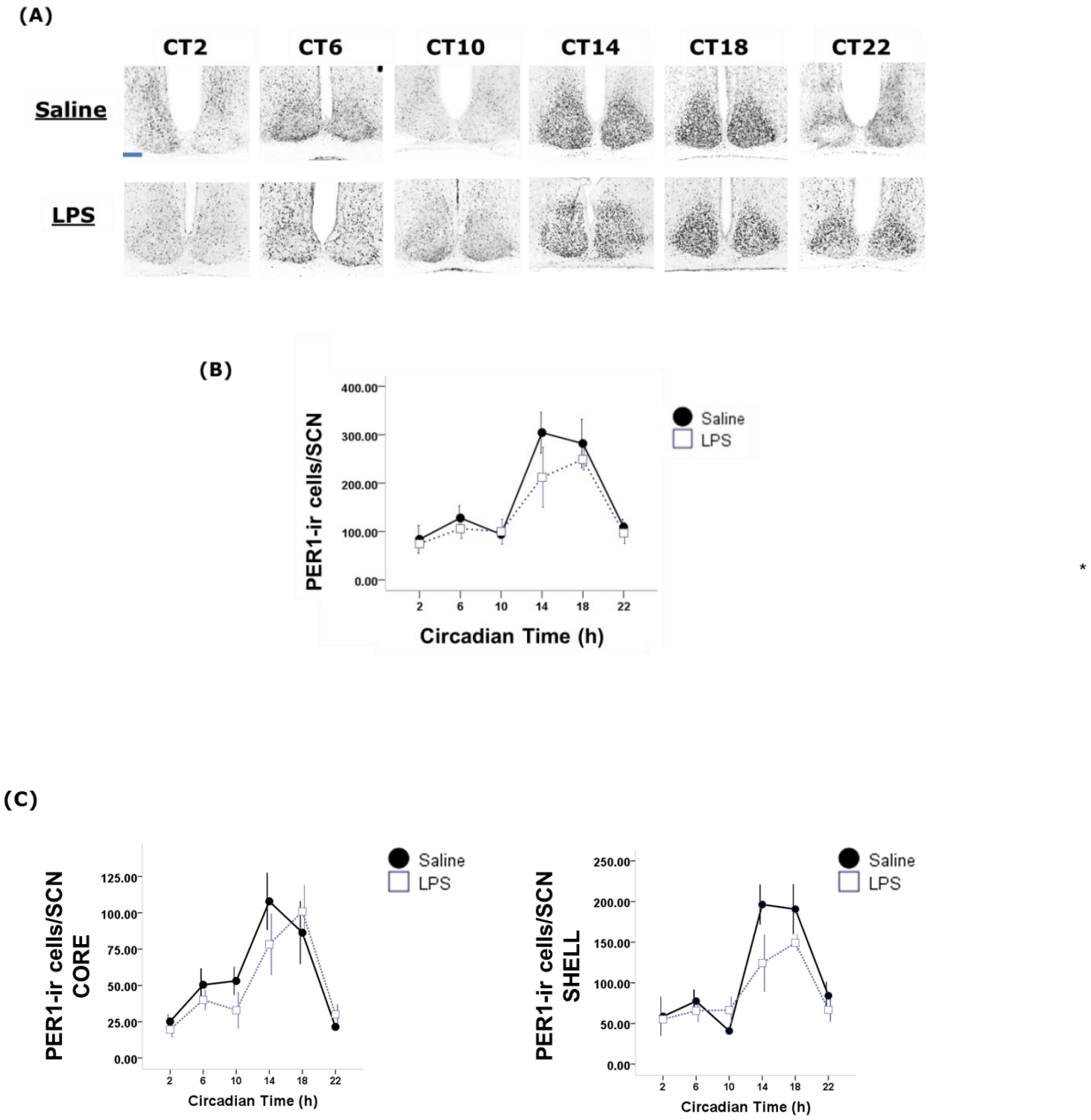
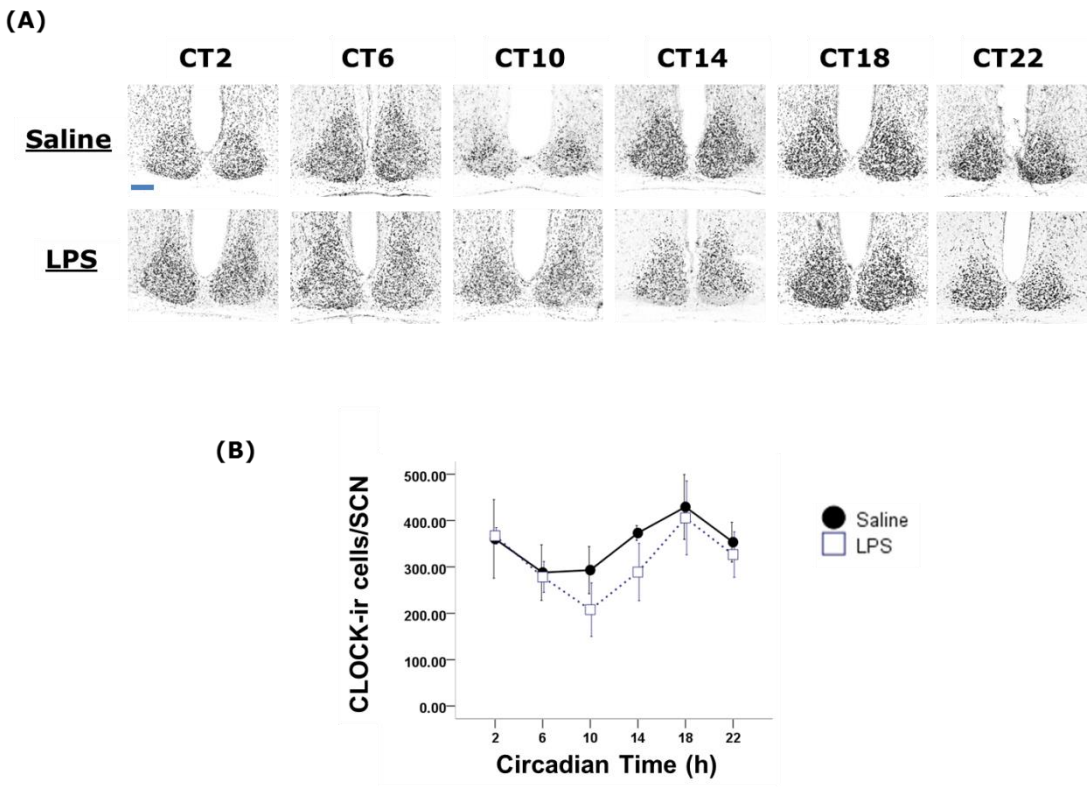


Figure 4.1: Effects of previous sepsis on the expression of the clock gene product, PER1 in the SCN across the 24hr circadian cycle. *Expression patterns of PER1, were examined in the SCN of animals treated one month previous with either saline or LPS and then sampled every 4 hours across the 24 hour cycle in DD. (A) Representative photomicrographs of PER1 expression in the SCN of post-septic animals (n=3-4 per timepoint) and control animals (n=3-4 per timepoint) at CT2, CT6, CT10, CT14, CT18 and CT22 (scale bar = 100 µm). (B) Graph illustrating quantification of levels of PER1 immunoreactive nuclei in the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and illustrating the expression pattern across the circadian cycle. (C) Graphs illustrating quantification of levels of PER1 immunoreactive nuclei in the SCN core and shell subdivisions at CT2, CT6, CT10, CT14, CT18 and CT22, illustrating the expression pattern across the circadian cycle.*

4.3.1.2. CLOCK expression across the 24hr circadian cycle

Two Way Repeated Measures ANOVA was utilized to assess statistically significant differences between groups in the mean expression of the clock gene protein product CLOCK across the circadian cycle. There was no effect of time on CLOCK expression in the SCN ($F_{5,29}$=1.99, P>0.05), no effect of treatment ($F_{1,29}$=1.22, P>0.05) and no interaction effect ($F_{5,29}$=0.23, P>0.05). Analysis of the separate SCN subdivisions found there to be no effect of time on CLOCK expression in the SCN core ($F_{5,29}$=1.20, P>0.05), no effect of treatment ($F_{1,29}$=2.02, P>0.05), and no interaction effect ($F_{5,29}$=0.79, P>0.05). There was no effect of time on CLOCK expression in the SCN shell ($F_{5,29}$=2.33, P>0.05), no effect of treatment ($F_{1,29}$=1.01, P>0.05) and no interaction effect ($F_{5,29}$=0.20, P>0.05). (Figure 4.2).

Assessment of CLOCK expression in the SCN across the circadian cycle by Co-sinor analysis found the percentage of variance in CLOCK expression accounted for by a 24 hour co-sine wave to be similar in control (0%) and in post-septic animals (0%), since CLOCK is not rhythmically expressed in the SCN (Gekakis et al., 1998).



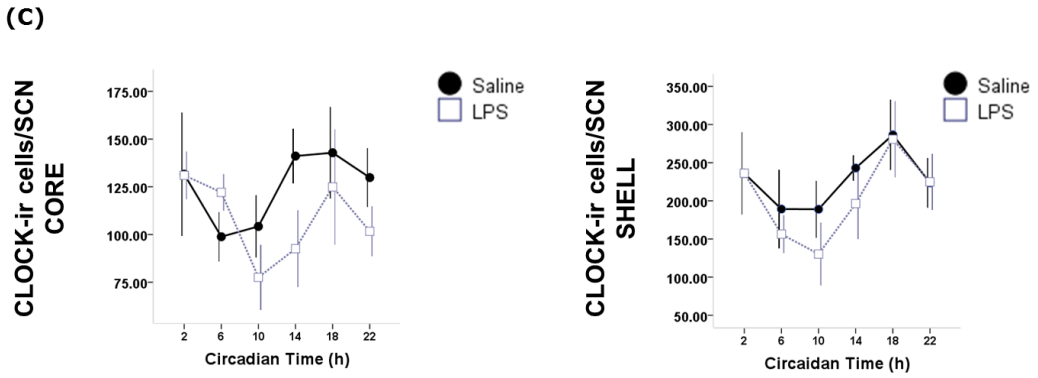


Figure 4.2: Effects of previous sepsis on the expression of the clock gene product CLOCK in the SCN across the 24hr circadian cycle. *Expression patterns of CLOCK, were examined in the SCN of animals treated one month previous with either saline or LPS and then sampled every 4 hours across the 24 hour cycle in DD. (A) Representative photomicrographs of CLOCK expression in the SCN of post-septic animals (n=3-4 per timepoint) and control animals (n=3-4 per timepoint) at CT2, CT6, CT10, CT14, CT18 and CT22 (scale bar = 100 µm). (B) Graph illustrating quantification of levels of CLOCK immunoreactive nuclei in the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and illustrating CLOCK expression across the circadian cycle. (C) Graphs illustrating quantification of levels of CLOCK immunoreactive nuclei in the SCN core and shell subdivisions at CT2, CT6, CT10, CT14, CT18 and CT22, illustrating the expression pattern across the circadian cycle.*

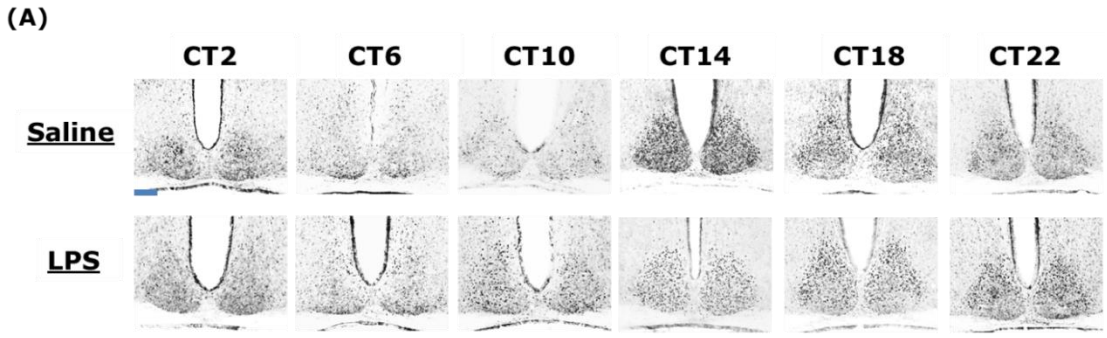
4.3.1.3. PER2 expression across the 24hr circadian cycle

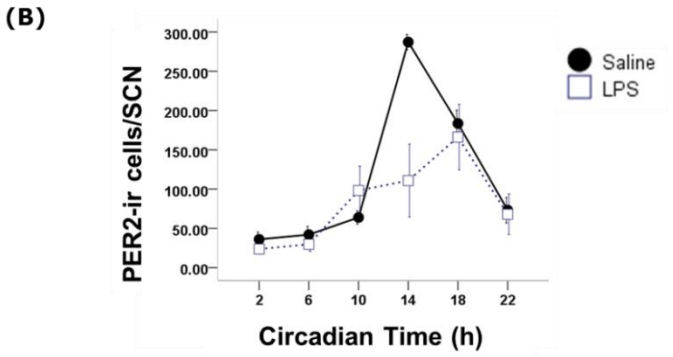
Two Way Repeated Measures ANOVA assessed there to be a statistically significant between groups effect in the mean expression of PER2 in the SCN across the 24hr circadian time period, finding alterations in PER2 expression in the SCN of post-septic animals compared to controls. There was a significant main effect of time on PER2 expression in the SCN ($F_{5,30}$=16.23, P<0.001). A main effect of treatment ($F_{1,30}$=4.72, P<0.05) on PER2 expression in the SCN was found along with a significant time x treatment interaction ($F_{5,30}$=4.26, P<0.01). Analysis of the separate SCN subdivisions found there to be a significant effect of time on PER2 expression in the SCN core ($F_{5,29}$=7.19, P<0.001), but no effect of treatment ($F_{5,29}$=3.05, P>0.05) and no time treatment interaction effect ($F_{5,29}$=1.5, P>0.05). There was a significant effect of time on PER2 expression in the SCN shell

($F_{5,30}$=17.87, P<0.001), but no effect of treatment ($F_{5,30}$=3.95, P>0.05) and no time treatment interaction effect. ($F_{5,30}$=4.61, P>0.05). (Figure 4.3).

Additionally, independent t-test analysis of SCN PER2 expression at CT14 found a statistically significant difference between groups, with significantly lower levels of PER2 immunoreactive cells in the SCN of post-septic animals (110.88 ± 46.5 ir. cells/SCN) vs. controls (287.17 ± 9.73 ir. cells/SCN; P<0.05). When analysing the separate subdivisions of the SCN by independent t-test at CT14, the mean number of immunoreactive PER2 cells was found not to be significantly different in the ventrolateral core of the SCN between the groups (saline, 66.89 ± 9.77 ir. cells/SCN vs. LPS, 32.21 ± 16.08 ir. cells/SCN; P>0.05), but was found to be significantly different at the level of the dorsomedial shell between groups (saline, 212.06 ± 6.9 ir. cells/SCN vs. LPS, 78.5 ± 31.54 ir. cells/SCN; P<0.05).

Examination of PER2 expression in the SCN by co-sinor analysis revealed the acrophase of PER2 expression to be shifted from CT15.9 in control animals to CT17.5 in post-septic animals, and that the percentage of variance in PER2 expression accounted for by a 24 hour co-sine wave is reduced from 91% in controls to 38% in post-septic animals.





(C)

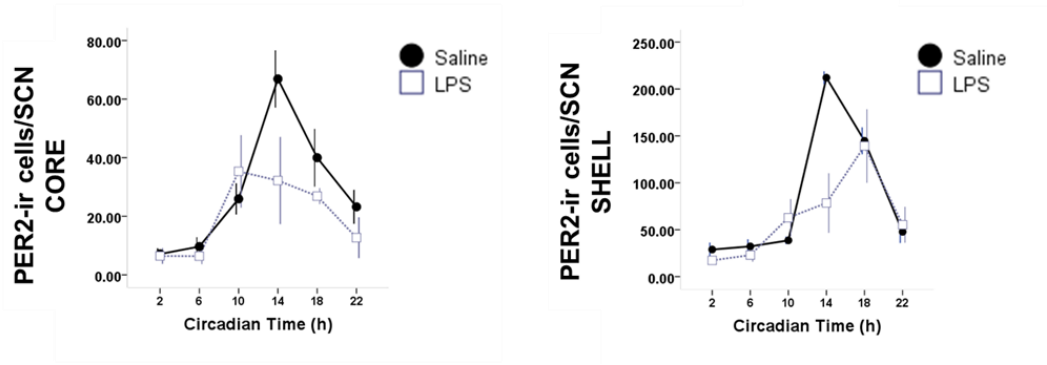


Figure 4.3: Effects of previous sepsis on the expression of the clock gene product, PER2 in the SCN across the 24hr circadian cycle. *Expression patterns of PER2, were examined in the SCN of animals treated one month previous with either saline or LPS and then sampled every 4 hours across the 24 hour cycle in DD. (A) Representative photomicrographs of PER2 expression in the SCN of post-septic animals (n=3-4 per timepoint) and control animals (n=3-4 per timepoint) at CT2, CT6, CT10, CT14, CT18 and CT22 (scale bar = 100 µm). (B) Graph illustrating quantification of levels of PER2 immunoreactive nuclei in the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and illustrating the expression pattern across the circadian cycle. Note the significant change of expression of PER2 in the SCN of LPS treated animals. (C) Graphs illustrating quantification of levels of PER2 immunoreactive nuclei in the SCN core and shell subdivisions at CT2, CT6, CT10, CT14, CT18 and CT22, illustrating the expression pattern across the circadian cycle.*

4.3.1.4. c-Fos expression across the 24hr circadian cycle

Two Way Repeated Measures ANOVA was utilized to assess statistically significant differences between groups in the mean expression of the IEG c-Fos across the 24hr circadian cycle. There was no effect of time on c-Fos expression in the SCN ($F_{5,31}$=0.45, P>0.05), as well as no effect of treatment ($F_{1,31}$=0.21, P>0.05) and no interaction effect ($F_{5,31}$=0.71, P>0.05). Analysis of the separate SCN subdivisions found a main effect of time on c-Fos expression in the SCN core ($F_{5,31}$=0.33, P<0.05), no effect of treatment ($F_{1,31}$=0.14, P>0.05), and no interaction effect ($F_{5,31}$=0.86, P>0.05). There was also a main effect of time on c-Fos expression in the SCN shell ($F_{5,31}$=0.03, P<0.05), no effect of treatment ($F_{1,31}$=0.40, P>0.05) and no interaction effect ($F_{5,31}$=0.80, P>0.05). (Figure 4.4)

Co-sinor analysis of c-Fos expression in the SCN revealed the acrophase of expression to be at CT2.07 in controls and CT9.9 in post-septic animals. The

percentage of variance in c-Fos expression accounted for by a 24 hour co-sine wave was found to be similar in control (0%) and in post-septic animals (0%).

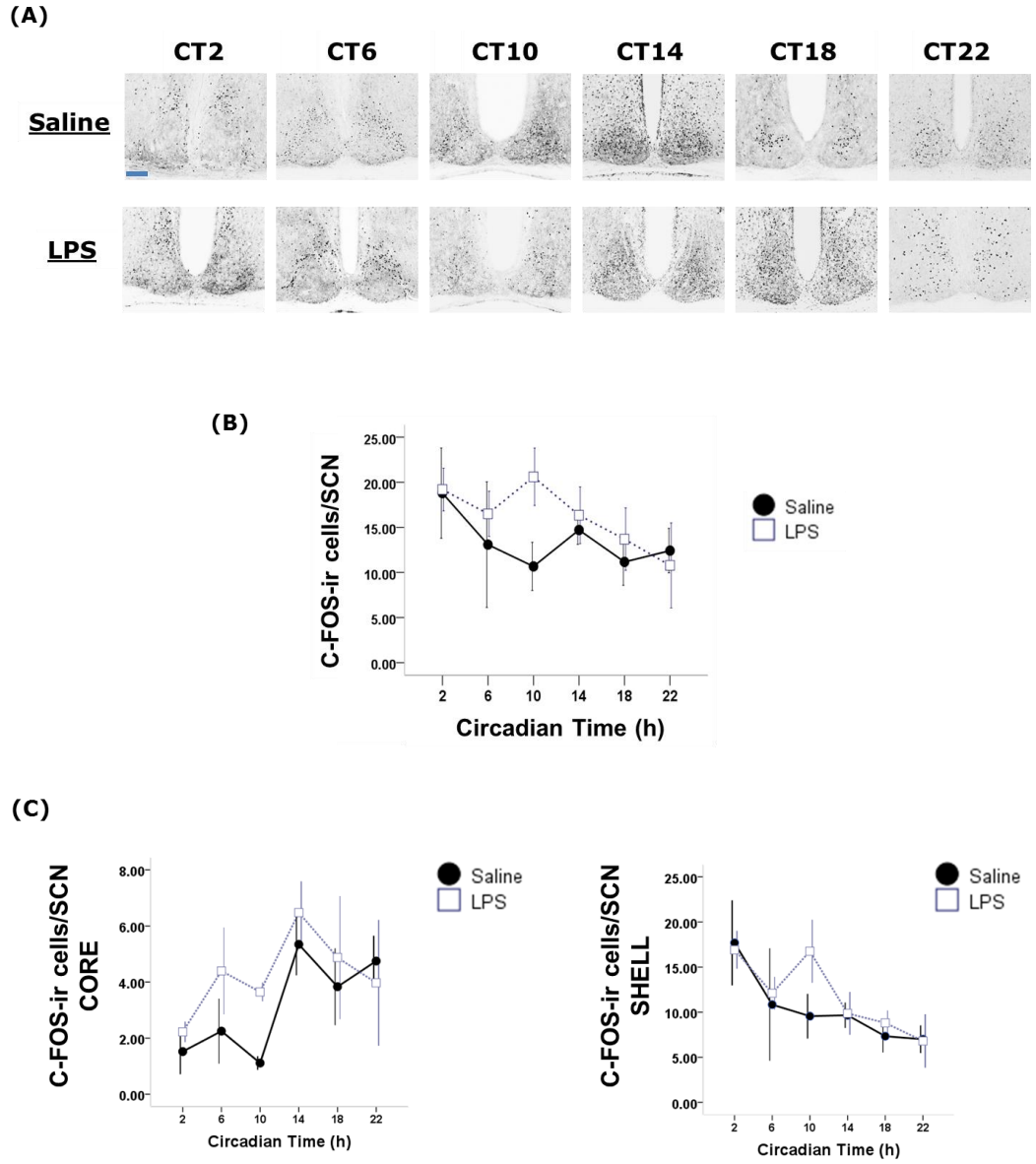


Figure 4.4: Effects of previous sepsis on the expression of the IEG c-Fos in the SCN across the 24hr circadian cycle. *Expression patterns of c-Fos, were examined in the SCN of animals treated one month previous with either saline or LPS and then sampled every 4 hours across the 24 hour cycle in DD. (A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=3-4 per timepoint) and control animals (n=3-4 per timepoint) at CT2, CT6, CT10, CT14, CT18 and CT22 (scale bar = 100 µm). (B) Graph illustrating quantification of levels of c-Fos immunoreactive nuclei in the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and illustrating c-Fos expression across the circadian cycle. (C) Graphs illustrating quantification of levels of c-Fos immunoreactive nuclei in the SCN core and shell subdivisions at CT2, CT6, CT10, CT14, CT18 and CT22, illustrating the expression pattern across the circadian cycle.*

4.3.2. PER2::LUC *Experiments*

The cosinor method was used to calculate the mean period, amplitude and acrophase of the PER2::LUC rhythm for both saline treated controls and post-septic animals, and a mean value was obtained for each individual culture for the period, amplitude and acrophase of the bioluminescence rhythm and subsequently assessed between groups by independent t-test analysis. Rhythm amplitude was not found to be significantly different between post-septic animals (7830.00 $\pm$ 2654.51) and saline controls (9225.00 $\pm$ 2705.05, P>0.05). The acrophase values for the PER2::LUC rhythms were found to be similar between post-septic animals (10.11 + 0.87) and controls (10.98 + 0.68, P>0.05, independent t-test). The Lomb-Scargle periodogram procedure was used to calculate the circadian period of the PER2::LUC rhythm for both saline treated controls and post-septic animals, and a mean value obtained for each individual culture for the period of the bioluminescence rhythm and subsequently assessed between groups by independent t-test analysis. Comparison of mean PER2::LUC period values between post-septic treated animals and saline controls by independent t-test found there to be no significant differences between the treatment groups (saline, 24.27 $\pm$ 0.23 hrs vs. LPS, 24.54 $\pm$ 0.17 hrs , P>0.05). (Figure 4.5).

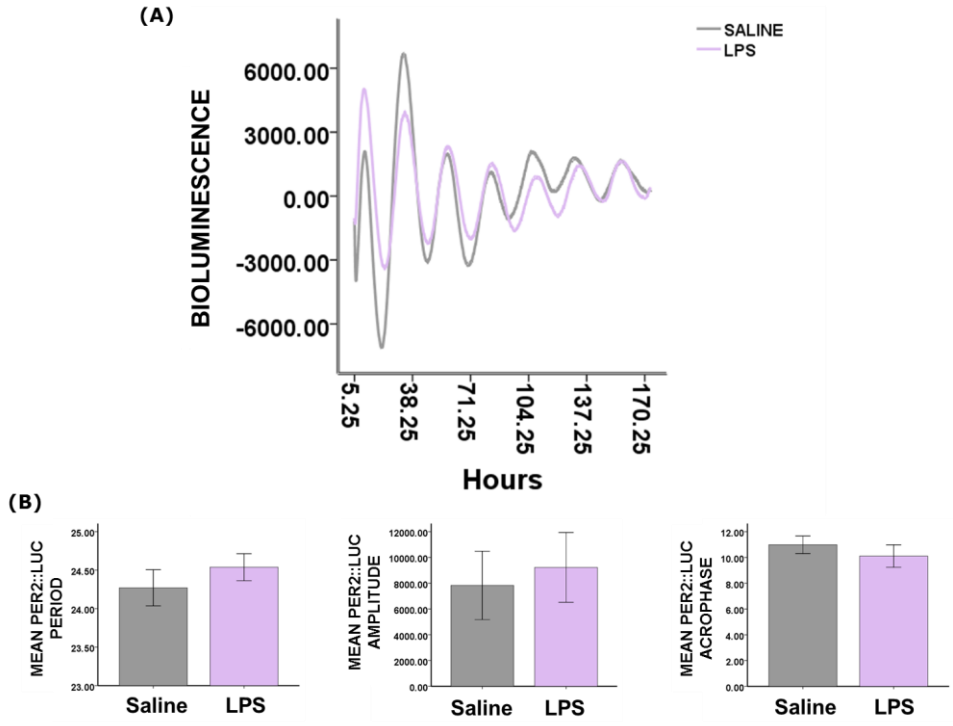


Figure 4.5: Circadian rhythms in PER2::LUC expression in post-septic animals and saline treated controls. *(A) Representative plots of de-trended PER2::LUC*

bioluminescence rhythms from SCN cultures from animals treated one month previous with either saline (n=5) or LPS (n=4) and maintained under 12:12 L:D conditions. Bioluminescence is plotted as time from culture preparation. (B) Bar graphs illustrating PER2::LUC amplitude, acrophase and period values.

4.3.3. Analysis of circadian function through examination of SCN neuronal activation

4.3.3.1. Photically induced c-Fos expression following a 30min light pulse at ZT15

c-Fos expression was assessed in the SCN following a 30min LP at ZT15 by immunohistochemistry, and subsequent analysis of levels of Fos immunoreactivity between the treatment groups by manual quantification found there to be no significant differences in c-Fos immunoreactive nuclei in the SCN between post-septic animals (178.0 ± 15.47 ir.cells/SCN) and vehicle treated controls (197.57 ± 18.86 ir.cells/SCN; P>0.05, independent t-test). Independent t-test analysis of mean expression values of c-Fos in the SCN subdivisions revealed no statistically significant differences in mean expression between groups in the different SCN subregions, with similar expression levels in the core (saline, 47.49 ± 10.37 vs. LPS, 38.60 ± 5.16 ir.cells/SCN, P>0.05) and shell regions of the SCN (controls 160.78 ± 5.95 vs. LPS 144.04 ± 4.90 ir.cells/SCN, P>0.05). (Figure 4.6).

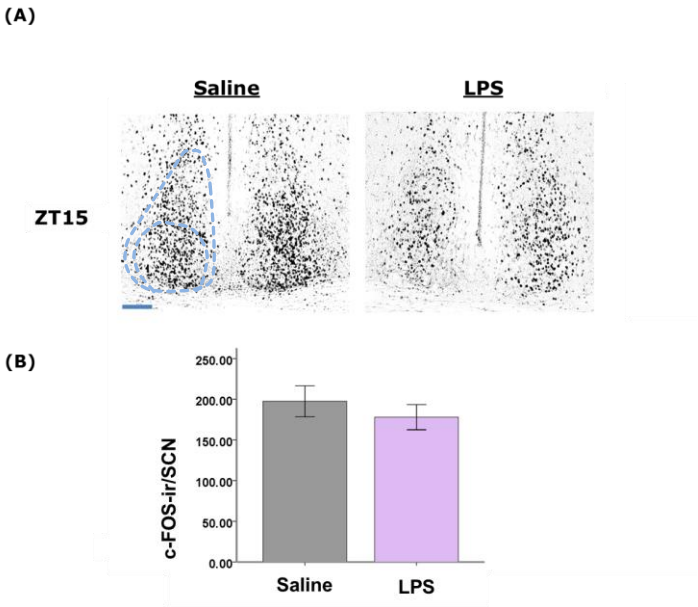


Figure 4.6: c-Fos expression is not altered in the SCN of post-septic animals following photic stimulation at ZT15. *(A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=5) and control animals (n=7)*

following 30 minute light pulses delivered at ZT15 (scale bar = 100 µm). Dashed line outlines the SCN and delineates the shell and core subdivisions. (B) Bar graphs illustrating quantification of levels of c-Fos immunoreactive nuclei in the SCN of control and post-septic animals in response 30min light pulses at ZT15.

4.3.3.2. Assessment of photically induced c-Fos expression at ZT22

In response to 30min photic stimulation at ZT22, immunohistochemistry for c-Fos expression and subsequent analysis of levels of Fos immunoreactivity between the treatment groups, found there to be a significant difference in c-Fos immunoreactive nuclei in the SCN of post-septic animals (43.5 ± 2.40 ir.cells/SCN) when compared to saline treated controls (78.0 + 6.12 ir.cells/SCN; $P<0.01$, independent t-test). A significant difference in c-Fos expression was also found in the SCN subdivisions, with a downregulation in the mean values of photically induced Fos in post-septic animals both in the ventrolateral core of the SCN (15.8 ± 1.16 ir.cells/SCN) compared to saline controls (27.0 ± 1.54 ir.cells/SCN; $P<0.001$, independent t-test) and in the dorsomedial shell, (LPS, 29.67 + 2.44 ir.cells/SCN vs. saline controls, 50.75 + 5.21 ir.cells/SCN; $P<0.01$, independent t-test). (Figure 4.7).

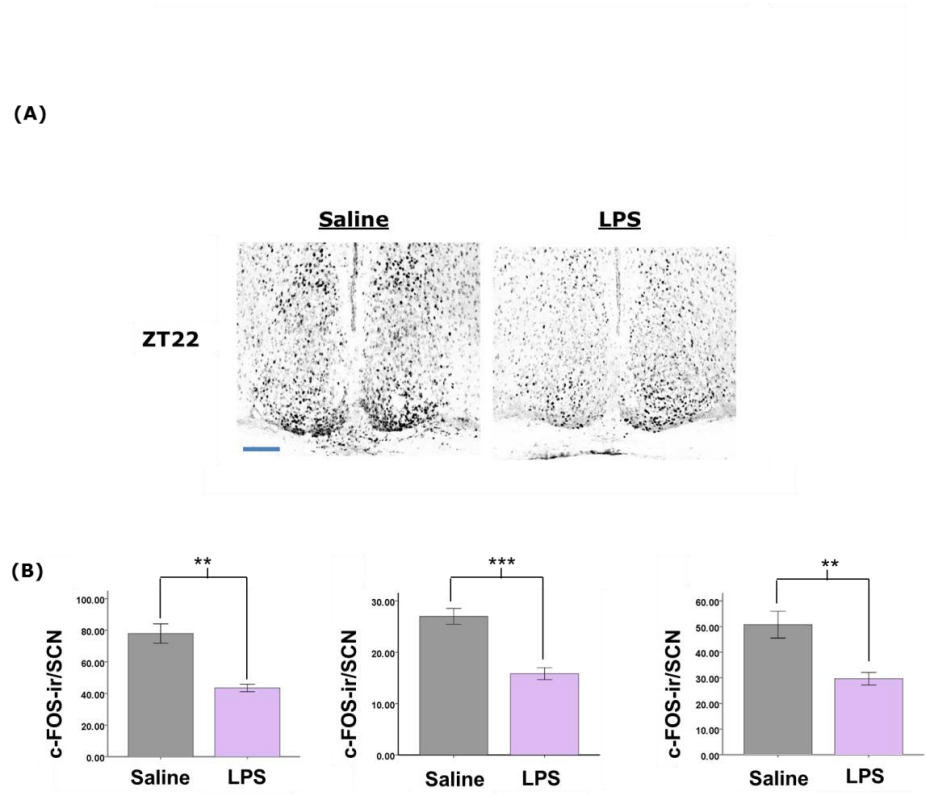


Figure 4.7: Alterations in neuronal activation in the SCN of post-septic animals following photic stimulation at ZT22. *(A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=4) and control animals (n=4) following photic stimulation at ZT22 (scale bar = 100 µm). (B) Bar graphs*

*illustrating quantification of levels of c-Fos immunoreactive nuclei at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals. **P<0.01, ***P<0.001.*

4.3.4. IEG expression following photic stimulation at CT15 and CT22

4.3.4.1. c-Fos expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22.

In response to 30min photic stimulation at CT15, immunohistochemistry for c-Fos expression and subsequent analysis of levels of Fos immunoreactivity between the treatment groups found there to similar levels of c-Fos immunoreactive nuclei in the SCN of post-septic animals (171.08 $\pm$ 7.08 ir.cells/SCN) and saline treated controls (161.86 $\pm$ 11.35 ir.cells/SCN, P>0.05, independent t-test; Figure 4.8). Independent t-test analysis of mean expression values of c-Fos in the SCN subregions assessed there to be no statistically significant differences in mean expression between groups in the different subregions, with similar expression levels in the core (saline, 77.42 $\pm$ 6.67 vs. LPS, 80.25 $\pm$ 4.27 ir.cells/SCN, P>0.05) and shell regions of the SCN (controls, 84.46 $\pm$ 12.43 vs. LPS 90.83 $\pm$ 4.59 ir.cells/SCN, P>0.05).

Comparison of photically induced c-Fos expression at CT22 combined with independent t-test analysis of levels of Fos immunoreactive nuclei levels in the SCN between the treatment groups found there to similar levels of c-Fos immunoreactive nuclei in the SCN of post-septic animals (117.85 $\pm$ 13.07 ir.cells/SCN) and saline treated controls (113.17 $\pm$ 5.57 ir.cells/SCN, P>0.05; Figure 4.8). Independent t-test analysis of mean expression values of c-Fos in the different SCN subregions assessed there to be no statistically significant differences in mean expression between groups, with similar expression levels in the core (saline, 48.96 $\pm$ 2.89 vs. LPS, 52.73 $\pm$ 6.38 ir.cells/SCN, P>0.05) and shell regions of the SCN (controls 64.21 $\pm$ 4.23 vs. LPS 65.13 $\pm$ 7.42 ir.cells/SCN, P>0.05).

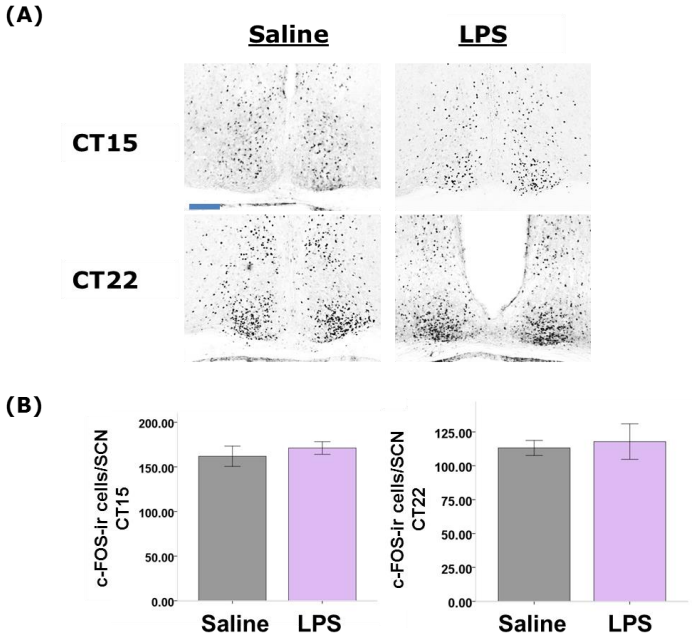


Figure 4.8: c-Fos expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22. *(A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=4 per timepoint) and control animals (n=4 per timepoint) following 30 minute light pulses delivered at either CT15 or CT22 (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of c-Fos immunoreactive nuclei in the SCN of control and post-septic animals in response to 30min light pulses at both CT15 and CT22.*

4.3.4.2. EGR-1 expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22.

In response to a 30min light pulse (LP) at CT15, immunohistochemistry for EGR-1 expression and subsequent analysis of levels of EGR-1 ir nuclei in the SCN by manual quantification coupled with independent t-test analysis found levels of EGR-1 ir nuclei in the SCN of post-septic animals to be analogous to controls, with mean levels of 190.67 $\pm$ 18.35 and 142.44 $\pm$ 23.14 ir. cells/SCN respectively ($P>0.05$; Figure 4.9). Independent t-test analysis of mean expression values of EGR-1 in the different SCN subdivisions found there to be no differences in mean expression levels between groups, with similar expression levels in the retinorecipient core (saline, 80.38 $\pm$ 16.27 vs. LPS, 106.79 $\pm$ 16.33 ir.cells/SCN, $P>0.05$) and in the dorsomedial shell regions of the SCN (controls, 62.06 $\pm$ 14.38 vs. LPS, 83.88 $\pm$ 7.94 ir.cells/SCN, $P>0.05$).

In response to 30min photic stimulation at CT22, EGR-1 immunohistochemical analysis coupled with manual quantification of EGR-1 ir nuclei in the SCN followed by independent t-test analysis found levels of EGR-1 ir nuclei in the SCN of post-septic animals to be similar between control and post-septic animals with mean levels of 60.83 $\pm$ 10.52 and 87.00 $\pm$ 8.39 ir. cells/SCN respectively (P>0.05; Figure 4.9). Furthermore, there were no statistically significant differences in mean expression levels of EGR-1 in the different SCN subdivisions, with similar expression levels in the retinorecipient core (saline, 30.42 $\pm$ 4.36 vs. LPS, 39.96 $\pm$ 4.58 ir.cells/SCN, P>0.05) and in the dorsomedial shell regions of the SCN (controls, 30.42 $\pm$ 6.66 vs. LPS 47.04 $\pm$ 6.03 ir.cells/SCN, P>0.05).

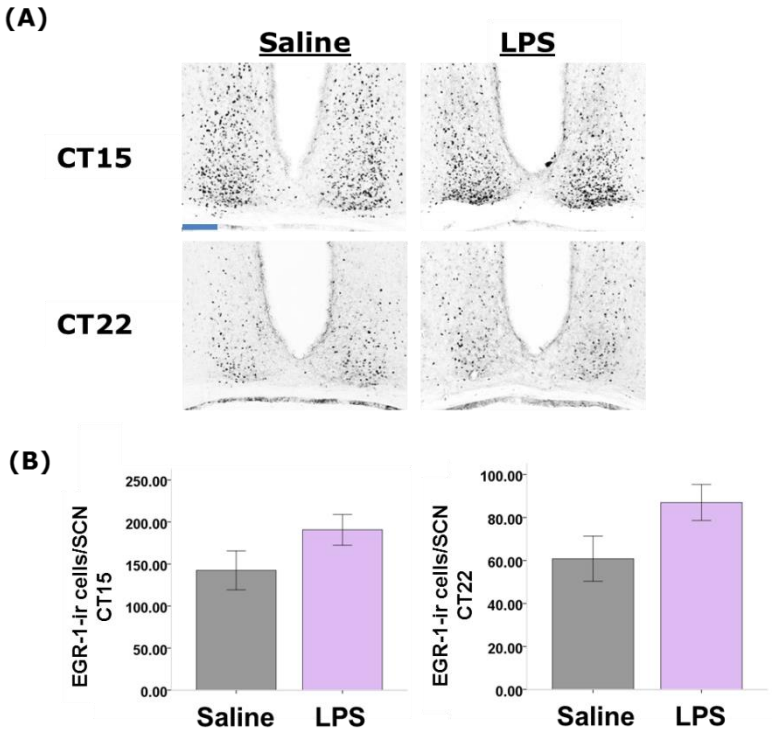


Figure 4.9: EGR-1 expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22. *(A) Representative photomicrographs of EGR-1 expression in the SCN of post-septic animals (n=4 per timepoint) and control animals (n=4 per timepoint) following 30 minute light pulses delivered at either CT15 or CT22 (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of EGR-1 immunoreactive nuclei in the SCN of control and post-septic animals in response to 30min light pulses at both CT15 and CT22.*

4.3.4.3. ARC expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22.

Photic stimulation at CT15 and Immunohistochemical analysis of ARC expression and subsequent analysis of levels of ARC immunoreactive nuclei by manual quantification between the treatment groups, found there to similar levels of ARC ir nuclei levels in the SCN of post-septic animals (23.31 $\pm$ 4.91 ir.cells/SCN) and saline treated controls (22.88 $\pm$ 4.64 ir.cells/SCN, $P>0.05$, independent t-test; Figure 4.10). Independent t-test analysis of ARC ir. nuclei in the SCN subdivisions revealed no statistically significant differences in mean expression values between groups in the independent subdivisions, with similar expression levels in the core (saline, 11.38 $\pm$ 2.34 vs. LPS, 9.54 $\pm$ 2.41 ir.cells/SCN, $P>0.05$) and shell regions of the SCN (controls 11.50 $\pm$ 2.41 vs. LPS, 13.77 $\pm$ 2.57 ir.cells/SCN, $P>0.05$).

Photically induced ARC expression following a 30min light pulse at CT22 was assessed by immunohistochemistry and manual quantification of ir. nuclei between the treatment groups, finding no differences in the levels of ARC ir nuclei in the SCN of post-septic animals (15.88 $\pm$ 5.39 ir.cells/SCN) and saline treated controls (11.17 $\pm$ 3.33 ir.cells/SCN, independent t-test; Figure 4.10). Independent t-test analysis found no statistically significant differences in mean expression values between groups in the different SCN subdivisions, with similar ARC expression levels in the SCN core (saline, 4.91 $\pm$ 2.07 vs. LPS, 7.75 $\pm$ 3.27 ir.cells/SCN, $P>0.05$) and shell regions (controls 6.25 $\pm$ 1.27 vs. LPS 8.13 $\pm$ 2.19 ir.cells/SCN, $P>0.05$).

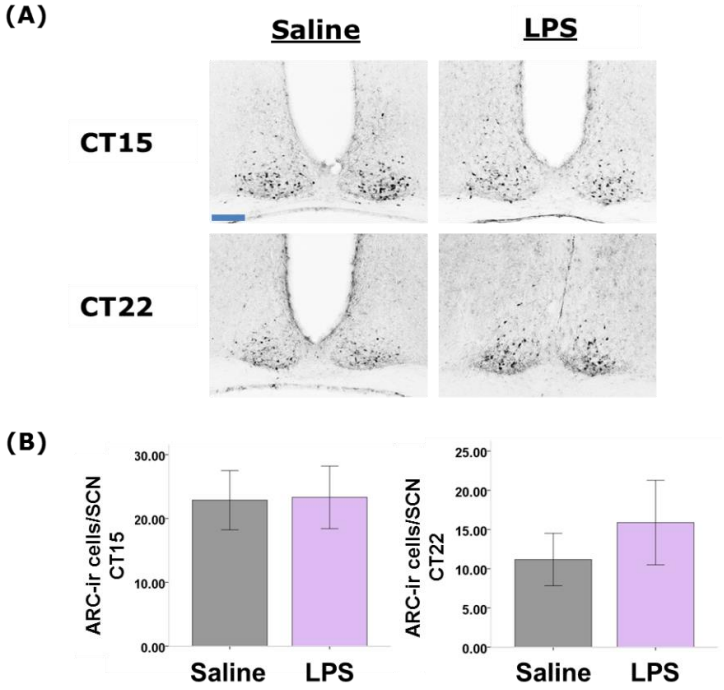


Figure 4.10: ARC expression in the SCN of post-septic animals following photic stimulation at either CT15 or CT22. *(A) Representative photomicrographs of ARC expression in the SCN of post-septic animals (n=4 per timepoint) and control animals (n=4 per timepoint) following 30 minute light pulses delivered at either CT15 or CT22 (scale bar = 100 µm). (B) Bar graphs illustrating quantification of levels of ARC immunoreactive nuclei in the SCN of control and post-septic animals in response to 30min light pulses at both CT15 and CT22.*

4.4. Discussion

Oscillations of components of the molecular clock in both the SCN and the sites of other oscillators are known to drive circadian rhythms of behaviour. Since various studies have shown that proinflammatory mediators induced in response to LPS treatment impact upon the expression of clock genes, we examined the oscillations of core molecular clock components in the SCN to determine whether a molecular correlate existed for the behavioural phenotype exhibited by our LPS treated animals. To the best of our knowledge, the current study is the first to describe long-lasting alterations in the circadian expression of clock genes in the

SCN following a sepsis inducing procedure, as well as perturbations in photically induced IEG expression.

The current set of studies set out to examine the two different patterns of c-Fos expression known to occur in the SCN, that in the SCN core which is observed in response to photic stimulation (Cai et al., 1997; Guido et al., 1999b; Schwartz et al., 1995; Schwartz et al., 2000) and is suggested to be involved in photic entrainment (Aronin and Schwartz, 1991; Colwell and Foster, 1992; Schwartz et al., 2000) and in rhythm maintenance, and that in the dorsomedial shell which is spontaneously expressed across the circadian cycle under constant conditions (Guido et al., 1999b; Schwartz et al., 2000; Sumova et al., 1998), and has been suggested to possibly play a role in the generation of endogenous rhythms (Guido et al., 1999a; Schwartz et al., 2000). Alterations in the pattern of spontaneous c-Fos expression might parallel alterations in behavioural rhythmicity, however the current study observes no alterations in spontaneous c-Fos expression in post-septic animals, and the rhythmic pattern observed in both treatment groups is in line with that described by other studies (Sumova et al., 1998), showing rhythmic expression in the SCN shell (Chambille et al., 1993; Guido et al., 1999b; Schwartz et al., 2000), and peak expression at CT2 (Guido et al., 1999b, Sumova et al., 1998). These findings might reflect the observations from the current study that there are no alterations in any of the core circadian behavioural parameters examined in post-septic animals under constant conditions. The post-septic behavioural attenuations observed all involve interactions with light (Chapter 2), and it is therefore more likely that alterations in photically induced c-Fos would be observed in post-septic animals.

SCN pacemaker function was assessed by examining IEG expression as molecular markers of SCN neuronal activation in response to a light pulse in post-septic animals and saline controls. Photic stimulation during the subjective night causes rapid induction of c-Fos in the SCN within an hour of exposure and is associated with photic induced phase shifts of free running locomotor activity (Kornhauser et al., 1990; Rusak et al., 1990; Ebling et al., 1991; Mead et al., 1992). Given that these studies observe alterations in photic phase advance resetting, it was somewhat surprising that when photically induced c-Fos expression was examined in the SCN in the late subjective night at CT22, paralleling the conditions under which photically induced phase advance resetting attenuations were observed, there were

no alterations in c-Fos immunoreactivity in the SCN of post-septic animals. Similarly, there were no alterations in immunoreactivity of the photically inducible IEG EGR-1 in the SCN in response to photic stimulation at either timepoint in the subjective night in post-septic animals compared to controls. Additionally, we examined the effector IEG product ARC in the SCN, since chronic neuroinflammation induced by centrally delivered LPS has been shown to impact upon the expression of ARC in the hippocampus (Rosi et al., 2005), however ARC was not found to be altered in the SCN. These findings may be in line with those of Palomba and Bentivolgio (Palomba and Bentivoglio, 2008), who have shown photically induced c-Fos in the early subjective night to be attenuated 7 days after the final LPS treatment of a 2 month treatment regime consisting of weekly injections with LPS 1mg/kg, but similar to what is described in these studies, they observed no alterations in photically induced c-Fos 30 days after the last LPS treatment. Studies have suggested that the levels of Fos induced in the SCN correlate with the magnitude of the daily phase shift required for stable entrainment (Beaule and Amir, 1999) and the current study therefore highlights a dissociation in the relationship between phase shift magnitude and c-Fos expression in response to light in the late subjective night under DD conditions, however in line with this, we do not observe any alterations between the treatment groups in c-Fos induction in the SCN in response to a phase delaying light pulse in either DD or in LD in response to photic stimulation in the early subjective night.

Moreover, these studies highlight a dissociation between photically induced c-Fos expression in response to light in the late subjective night under steady state LD conditions and light induced c-Fos expression under housing in constant environmental conditions (DD). In general, photic stimulation at ZT22 results in lower levels of c-Fos induction in the SCN in both post-septic animals and controls compared to levels induced in response to photic stimulation at CT22. However, under steady state LD, photic stimulation at ZT22 caused a statistically significant decrease in levels of photically induced c-Fos activation in the SCN of post-septic animals in response to photic stimulation in the late subjective night (ZT22), but not in the early subjective night (ZT15), highlighting a marked alteration in neuronal activation and hence a functional attenuation in the master pacemaker of these animals under these conditions. Further, this shows alterations in post-septic SCN responsiveness at the portion of the phase response curve during which attenuations

in circadian behaviour are observed, since assessment of immediate early gene expression highlights variations in the responsiveness of neurons in the SCN (Mead et al., 1992). This altered c-Fos activation would tie in with the altered phase advancing light pulse responses shown in post-septic animals and with the attenuations in resynchronization to a phase advancing shift of the photoperiod. However it may be more likely that the photic stimulation during the subjective night while maintained under steady state LD conditions more closely reflects that of the post-septic animals when subjected to phase shifts of the photoperiod.

Any perturbations found in SCN responsiveness and behavioural rhythmicity of post-septic animals were specific to phase advancing stimuli and all involve interactions between light and the timekeeping system. It remains unclear why the current study observes alterations in photically induced c-Fos at ZT22, but no alterations in c-Fos expression when the light pulse was administered at CT22. c-Fos is thought to be involved in entrainment of the circadian system to the environmental LD cycle (Kornhauser et al., 1990) and this might reflect the altered re-entrainment rates observed in response to a phase advance of the photoperiod. Post-sepsis late subjective night photic Fos induction under DD and LD clearly varies, possibly owing to modification of an underlying mechanism that plays a role under conditions of stable entrainment but not under DD, since the alterations in photically induced c-Fos are only seen when maintained under a 12:12 LD cycle. Under DD conditions, the effects of light on the circadian system are only for the duration of the light pulse, whereas under an LD cycle, the animals are subject to a regular entraining photic influence and it is possible that the differences we observe in c-Fos expression at ZT22 could be due to the long-term presence of an entraining stimulus, and it may be that we are observing a prior effect of housing on post-septic SCN photically induced neuronal activation, however what underlies this remains to be investigated.

While c-Fos is associated with circadian function and entrainment (Kornhauser et al., 1990), the precise role of c-Fos in the SCN in the generation of circadian oscillations is not fully understood and has yet to be clearly defined. These studies observe dissociations between light induced Fos expression and the magnitude of phase shifts of behavioural rhythmicity, a situation which has been described by other experimental groups. It has previously been suggested that c-Fos expression levels do not directly determine phase shift amplitudes (Shimomura et al.,

1998) and shown that increased c-Fos expression within the SCN is not necessary for all phase shifts of circadian locomotor activity (Mead et al., 1992), indicating that the relationship between phase-shifting and c-Fos induction is not completely inter-reliant, and that light induced c-Fos may be involved in photic induced phase shifts but is not necessarily essential and therefore may simply contribute to normal entrainment. Studies with mice lacking the PACAP-1 receptor, have shown exaggerated magnitude of phase delays in the early subjective night accompanied by a decrease in c-Fos induction (Hannibal et al., 2001), while in mice lacking the *c-fos* gene, photic responses are still seen and photic entrainment still occurs, however a blunted phase response curve with photic induced phase-shifting responses displaying a decrease in magnitude is observed (Honrado et al., 1996). Additonally, photic c-Fos induction is seen to be maximal in the transition between the delay portion of the phase response curve and the advance portion, however during this time no photic induced phase shifts take place (Sutin and Kilduff, 1992). Transgenic hypertensive TGR (mRen2)27 rats show no induction of c-Fos in response to light but display phase shifts of locomotor activity (Lemmer et al., 2000). Further, *tau* mutant hamsters display significant increases in phase shift amplitude after long periods, 49 cycles in DD, as opposed to the phase shift amplitudes shown when maintained under DD for 2 cycles, while c-Fos induction amplitude is similar irrespective of time spent in DD (Shimomura et al., 1998). Under the same irradiance levels, c-Fos amplitude has also been shown to be unaffected by certain pharmacological agents and anaesthetics, while phase shift amplitude is diminished (Colwell et al., 1993a, b). Further, transgenic mice lacking rods, show Fos induction proportional to photon levels under varying irradiances, similar to that displayed by wildtype animals, while the amplitude of the phase shifts differ between the strains (Lupi et al., 1999). Based on these studies, the photic induction of c-Fos has been suggested to be tightly correlated with direct input of light to the clock, and circadian locomotor activity output responses to be dependent on processes subsequent to this, such as feedback or neural integration from the molecular clockworks (Dkhissi-Benyahya et al., 2000). Indeed, this situation could explain the observations of the current study, where post sepsis, there is either no alteration in c-Fos induction in the SCN at CT22, or a decrease in c-Fos induction at ZT22 paralleling exaggerated phase shift magnitude and circadian resetting. Our SCN locomotor output may be being influenced by mechanisms downstream from the initial SCN response,

resulting in alterations in behavioural rhythmicity. Further, investigation is required to establish whether an input influence might play a role in post-septic SCN responsiveness under stable entrainment conditions.

These findings could also indicate alterations in light processing, masking of the endogenous rhythm, or altered responsiveness of the circadian system across the 24hr cycle. Further, it is clear that all the alterations in behavioural rhythms and IEG expression are seen during the advance portion of the phase response curve, and alterations in the shape of the PRC could account for the changes seen both at a molecular level and behaviourally in the late subjective night. The behavioural analysis and construction of the photic PRC of post-septic animals previously performed however indicate that this is not the case. The PRC of post-septic animals is seen to be similar to that of controls, and therefore we cannot attribute molecular and functional alterations to perturbations in this. Addtionally, the behavioural studies have shown locomotor behaviour to be truly entrained and not seemingly entrained due to the effects of negative masking. It does not appear that the dose of LPS used in this study is inducing cell loss in the SCN, since no changes were observed in apoptotic markers, as assessed by TUNEL analysis or cleaved caspase-3 immunostaining 24 hours following LPS treatment, and therefore we do not believe that this could account for the reduced c-Fos expression in the SCN. Given that IEG responses to photic stimulation are not altered in the SCN following photic stimulation in DD, it also appears as though there are no gross problems in light-sensing or retinohypothalamic tract transmission to the SCN. However, as previously mentioned, it's possible that when maintained under steady state LD conditions, there may be alterations in the photic sensitivity of the post-septic SCN across the circadian cycle, and this requires further investigation.

It is reasonable to suggest that the presence and action of inflammatory mediators on the post-septic SCN may be impacting upon photically induced c-Fos. In trypanosome infected rats, photically induced c-Fos in the subjective night is significantly reduced in the SCN (Peng et al., 1994), indicating alterations in photic entrainment during infection and neuroinflammatory states. Nitric Oxide has been suggested to play a role in photic induced phase shifts of the circadian system and its inhibition has been shown to attenuate SCN c-Fos induction following phase resetting photic stimulation, resulting in a decrease in expression (Amir and Edelstein, 1997). Light induced phase advances at CT18, but not phase delays, and

photic induced c-Fos immunoreactivity at ZT18 have previously been shown to be blocked following administration of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (Plano et al., 2007). While the current study does not observe long-term alterations in nitric oxide expression in the post-septic SCN, alterations are evident in nitric oxide in the SCN in the acute phase following sepsis induction, and it's possible that alterations in nitric oxide signalling may initially impact upon or potentiate another mechanism which may be involved in the long-term alterations in photically induced c-Fos expression in the post-septic SCN that accompanies alterations in photic phase advance resetting.

Various possibilities exist that could underlie this enhanced response in post-septic animals. There may be altered input to the SCN such as alterations in light input or in the threshold response of the SCN to light, variations within the SCN itself such as perturbations in the expression of clock genes, important SCN neuropeptides or intercellular coupling alterations within the nucleus, or modifications in the electrophysiological properties or coupling of SCN output to effector systems, or a combination of these. Factors restricting re-entrainment rates, limiting the photic responsiveness of the master pacemaker may be altered in post-septic animals, however these are factors that remain unknown (LeGates et al., 2009). Further assessment of these parameters is warranted in post-septic animals.

Photic entrainment involves the activation of proteins that participate in the resetting of the transcription-translation feedback loop underpinning circadian oscillations (Meijer and Schwartz, 2003). Alterations in clock gene expression in the post-septic SCN could account for altered behavioural resetting in post-septic animals, since oscillations of components of the molecular clock in both the SCN and the sites of other oscillators are known to drive circadian rhythms of behaviour, and these studies therefore examined the oscillations of core molecular clock components in the SCN, assessing the diurnal expression of the clock gene protein products PER1, PER2 and CLOCK *in vivo*. While normal patterns of CLOCK and PER1 expression were seen in post-septic animals, alterations in the PER2 expression rhythm were found in the post-septic SCN, and the acrophase of PER2 expression was delayed relative to controls. A phase delay of PER2 expression would be expected to result in alterations of the free running period, since alterations in components of the clock should result in alterations of basic clock properties

(Dunlap, 1996) which would then be reflected under constant conditions where the properties of the endogenous oscillator can be observed. Interestingly, however, there were no alterations in free running circadian parameters, despite SCN PER2 expression being altered, and it is therefore possible that as opposed to the PER2 rhythm being phase delayed, that it may be a reduction in peak PER2 expression that is observed in post-septic animals. Somewhat surprisingly, there were no alterations in diurnal expression patterns of PER1 in the SCN between the treatment groups, and while reduction in PER1 expression in post-septic animals trended towards significance at CT14, this was not reached in our sample. It is unclear why PER1 and PER2 expression are not similarly affected in the post-septic SCN. *Per2* is suggested to regulate *Per1 in vivo* (Zheng et al., 1999), and both PER1 and PER2 are involved in photic entrainment (Yan and Silver, 2004), and it might then be expected that alterations in expression would be present in PER1 in post-septic animals, since altered photic resetting responses are observed. Studies assessing clock gene expression following low dose LPS treatment have shown neither *Per1* nor *Per2* expression to be altered in the SCN (Takahashi et al., 2001), while LPS 1mg/kg treatment has been shown to acutely alter *Per2* expression in the SCN, with a return to rhythmic expression levels the day after treatment (Okada et al., 2008), however these effects have not been examined in the long term following LPS administration. A disconnect between *Per1* and *Per2* expression perturbations has been shown in other studies in response to LPS treatment, with *Per2* displaying significant reduction in expression in peripheral tissues while *Per1* is only weakly reduced, and the reasons behind this remain unclear and the mechanisms remain to be established (Yamamura et al., 2010). Modulation through different signalling mechanisms may play a role in this. Although these studies are in the periphery, it is possible that this is a situation which may be analogous to the observations of the current study. Further, other studies assessing the effects of chronic stress on SCN clock gene expression also observe a dampened PER2 expression in the SCN but no alterations in PER1 (Jiang et al., 2011). PER1 and PER2 have differential functions in circadian rhythm regulation (Bae et al., 2001), as well as independent roles in circadian output (Zheng et al., 2001). The differential effects of LPS treatment on clock gene protein product expression may reflect the differential involvement of these proteins in functions of the circadian system that may be attenuated post-sepsis. These clock genes display alternate functions in phase resetting and re-

entrainment, showing differential responses to light, and this differential photic regulation further indicates distinct functions in the SCN (Zylka et al., 1998). While studies have shown PER1 to be photically induced (Yan and Silver, 2004) and antisense oligonucleotides to *Per1* can block resetting (Akiyama et al., 1999), mice lacking PER1 still show entrainment (Bae et al., 2001; Zheng et al., 2001), and studies by Reddy and colleagues (Reddy et al., 2002), have shown that PER1 may not be required for resetting following abrupt photoperiod shifts. PER2 is thought to play a critical role in the maintenance of circadian behavioural rhythmicity in both mice and humans. Targeted disruption of *Per2* involving deletion of 87 residues from the protein dimerization PAS domain ($Per2^{Brdm1}$ mouse strain) has been shown to affect free running rhythms of locomotor behaviour, causing delayed loss of circadian rhythmicity in mice and unstable circadian rhythms (Zheng et al., 1999). Furthermore, PER2 expression in the SCN has previously been shown to have a direct link with behavioural circadian rhythms as evidenced by RNAi mediated knockdown of *Per2* in the SCN, which has been shown to disrupt locomotor activity (Gavrila et al., 2008). Since SCN PER2 plays important roles in rhythmic locomotor output, it is possible that the perturbations in PER2 expression in the current study underlie the altered circadian locomotor behavioural resetting in post-septic animals.

Studies have suggested that attenuations in phase shift responses could be as a result of changes in clock genes or their protein products (Reddy et al., 2002; Yan and Silver, 2002; Yan and Silver, 2004), and it may be that the alterations in PER2 expression in the post-septic SCN underlie the perturbations in circadian resetting. The subjective day is the time when maximal endogenous *Per* gene expression is observed, while peak protein product expression is 6-8hours after this (Reppert and Weaver, 2001), and both PER1 and PER2 proteins show the same endogenous circadian profile, with peak expression levels at the end of the subjective day and in the early subjective night, after which they decline and low levels of expression are observed in the late subjective night (Field et al., 2000). Photic induced phase-shifting of the molecular clock is seen to result in photically-induced *Per1* gene expression in the dorsomedial shell following a phase advancing light pulse, while *Per2* gene expression is seen to be induced in the SCN shell in association with phase delays (Yan and Silver, 2002). The PER proteins have been shown to be differentially increased in the different SCN subregions in response to phase resetting photic stimulation depending on the portion of the PRC at which photic

stimulation occurs, and PER2 induction has been suggested to be important for phase delays, while PER1 induction is important for phase advances (Yan and Silver, 2004). Given that *Per2* induction appears to be involved in photic induced phase delays (Yan and Silver, 2002), it seems likely that alterations in PER2 expression might cause altered phase delays of locomotor behaviour. In contrast to this, we observe no alterations in photic induced delay resetting in post-septic animals, and it is altered photic phase advance resetting that we observe in the current set of experiments. Alterations in endogenous levels of PER expression could impact upon PER induction levels following the application of a phase resetting light pulse, resulting in alterations in photic phase-shifting responses. Previous studies have shown a correlation between phase shift magnitude and light induced *Per* mRNA expression level (Shigeyoshi et al., 1997). A molecular corollary exists between *Per2* induction and the direction of the behavioural response to photic stimulation. *Per2* is differentially induced in response to a phase resetting light pulse, with levels increasing slowly in response to a phase delaying light pulse, peaking at 2hrs following exposure and returning to baseline levels after 6 hours, while in response to a phase advancing light pulse at CT23, rapid induction of *Per2* is observed within 30 minutes of the LP, with levels returning to baseline levels within 2hrs (Zykla et al., 1998). It's possible that there are alterations in *Per2* induction in response to a phase advancing light pulse since perturbations in the induction could underlie why the differences observed in post-septic animals are all in the advance portion of the phase response curve or in response to advance resetting. Additionally, the PER photic induction shows time dependant induction depending on the portion of the phase response curve at which light is applied (Yan and Silver, 2004), and it is possible that alterations in the rate of induction of PER proteins following a light pulse could underlie alterations in photic resetting.

Further work is required to establish whether alterations in induction levels and/or induction rates of PER proteins, in particular PER2, are observed in post-septic animals, and whether this might play a role in altered photic phase resetting. It would be interesting to assess PER2 expression in response to photic stimulation in both the advance and delay portions of the phase response curve in post-septic animals, since this could shed light on the mechanisms underlying altered resetting in post-septic animals.

Studies by Reddy et al., (Reddy et al., 2002) on clock gene expression following abrupt photoperiod shifts suggest that alterations in *Per2*/PER2 may be involved in the regulation of circadian entrainment, since the induction of *Per2* mRNA and PER2-IR has been shown during both advance and delay resetting to phase shifts of the photoperiod (Reddy et al., 2002). These authors suggest that *Per2* is essential for photic entrainment, and the indirect photic regulation of *Cry1* expression in the SCN has previously been suggested to be a possible rate-limiting factor in behavioural re-entrainment during experimental jetlag. Following a photic phase advance, *Per* expression in the SCN resets rapidly and *Cry1* expression synchronizes slowly, paralleling the transient resetting of locomotor activity seen in response to a phase advance, while the expression of *Per* and *Cry* was found to synchronously adjust rapidly in the SCN following a phase delay of the photoperiod, with the rapid readjustment of *Cry* expression suggested to underlie the rapid re-entrainment of locomotor behaviour following a phase delay (Reddy et al., 2002). It's possible that the current studies observe alterations in the readjustment of clock gene expression following a phase advance of the light dark cycle, which could account for the significantly more rapid re-entrainment of post-septic animals to an advance of the photoperiod. Additionally, it has been suggested that the dissociation of clock gene expression and their subsequent readjustment rates might play a role in phase advance resetting following single light pulses in free running DD conditions, however this remains to be examined (Reddy et al., 2002). In the case of abrupt shifts of the photoperiod, these authors have shown that PER2 expression is endogenously high during resetting following a 6hr delay of the photoperiod, and light exposure maintains this expression, and at this time PER2 may impact upon *Cry1* expression, since *Cry1* and *Per2* show synchronous expression. However, PER2 levels are low initially following a 6hr advance, and photic induction of PER2 levels may take time in this instance and impact upon *Cry1* induction. It would be interesting to assess the expression of these clock gene protein products following a phase advance of the photoperiod to examine whether alterations are present in the resetting of these which could play a role in the attenuation of locomotor behaviour seen in post-septic animals. Further, alterations in endogenous levels of expression are seen in the current study and might cause alterations in induction levels and readjustment rates. It is possible that the attenuation in PER2 oscillations revealed by these experiments could lead to altered output signals from the master pacemaker

and underlie the perturbations seen in circadian resetting responses in post-septic animals.

It is important to establish whether the alterations in the post-septic circadian system are due to alterations within SCN properties, or are due to alterations in input to or output from the SCN. Examination of the SCN in isolation would allow us to identify whether alterations in properties within the SCN itself are responsible for altered molecular oscillations and attenuated circadian locomotor rhythmicity in post-septic animals, as opposed to as a result of other influences outside of the SCN. Additionally, it was expected that examination of PER2::LUC post-septic SCN rhythms *in vitro* would clarify whether PER2 expression is truly delayed in post-septic animals, or instead shows dampened expression at the peak of the rhythm. However, our assessment of PER2::LUC rhythmicity *in vitro* found there to be no alterations in PER2 oscillation properties between post-septic animals and saline controls. In comparison to the *in vivo* assessment, *in vitro*, the SCN tissue is in isolation and not subject to the influence of signalling from any peripheral or non-SCN oscillator site. The time point at which PER2::LUC rhythms were examined coincides with time post sepsis induction at which perturbations were observed in circadian resetting, as well as with the attenuations in PER2 expression in the SCN, and photically induced IEG expression *in vivo*. These *in vitro* experiments therefore indicate that when the post-septic alterations in advance resetting take place that these are not due to perturbations manifesting within the SCN itself, but due to outside SCN *in vivo* influences. Therefore, the alterations in PER2 expression in the SCN *in vivo*, may be due to an *in vivo* influence. In the long term post LPS induced sepsis, alterations may manifest in the circadian system at levels downstream from the SCN, by altering molecular oscillations in non-SCN central or peripheral oscillators that are important in the regulation of behaviour and physiological function. Further, it may be that alterations are present at the level of input to the SCN. Influences from oscillators outside of the SCN with post-septic attenuations may be feeding back to the SCN impacting upon the expression of PER2 and post-septic circadian activity rhythms. Therefore, while the changes in clock gene expression within the SCN may underlie attenuated post-septic circadian resetting, it appears that the mechanism responsible for the alterations in PER2 expression rhythms is outside of the SCN itself. These results tie in with the previously

discussed late subjective night photically induced c-Fos observations which were also indicative of an underlying mechanism impacting upon the post-septic SCN, or attenuations in circadian timekeeping processes downstream of the SCN underlying alterations in post-sepsis behavioural resetting.

It is clear that *Per2* plays various roles in physiology, and a variety of studies have shown that *Per2* mutant mice show alterations in physiological parameters. *Per2* mutant mice show arrhythmic circadian locomotor behaviour in DD (Zheng et al., 1999), consume more alcohol (Spanagel et al., 2005), display enhanced sensitivity to cocaine (Abarca et al., 2002) and show altered feeding rhythms (Yang et al., 2009), with food consumption patterns in line with those seen in *Clock* mutant animals (Turek et al., 2005). Further, mutant *Per2* animals show a higher incidence of tumour development following ionizing radiation, displaying an abnormal response to DNA damage, highlighting the importance of *Per2* in tumour suppression (Fu et al., 2002). Additionally, a mutation in *Per2* in humans affecting the phosphorylation of hPER2 by casein kinase 1epsilon is implicated in familial advanced sleep phase syndrome (Toh et al., 2001). Relevant to the current studies, *Per2* is suggested to play a unique role in the regulation of innate immune function (Liu et al., 2006) and *Per2* mutant animals have been shown to be resistant to LPS induced endotoxic shock (Halberg et al., 1960), highlighting involvement of the clock gene in sepsis. Further, *Per2* expression has been shown to be altered in a variety of conditions with an inflammatory component, which could be why the current study observes altered PER2 expression in the post-septic SCN. Treatment with endotoxin has been shown to transiently repress expression of *Per2* and *Dbp* in the SCN, as well as the expression of *Per2* and *Per1* and other genes under circadian control in the periphery, with restoration of expression patterns 2days post the immune challenge (Okada et al., 2008; Cadivini et al., 2007). In the liver of mice with experimental autoimmune encephalomyelitis which is characterized by the infiltration of immune cells into the CNS and the presence of inflammatory lesions as well as axonal loss in the brain, *Per2* expression has been shown to be attenuated (Buenafe, 2012). In male patients with acute Q fever as a result of bacterial infection with the *Coxiella burnetii* (*C. burnetii*), *Per2* has been demonstrated to be up-regulated in the blood (Mehraj et al., 2012), while in female mice infected with *C. burnetii*, the expression of the clock genes *Clock* and *Arntl* were shown to be

reduced in the liver, while *Per2* was shown to be up-regulated (Textoris et al., 2010). Continuous IFN-α administration is seen to reduce *Per2* mRNA levels in the liver at ZT14 (Shinohara et al., 2008), while in fibroblasts *in vitro* and in the liver of mice *in vivo*, *Per2* expression, as well as that of other clock genes, have been shown to be suppressed by TNF-α (Cavadini et al., 2007). More recently, in mouse embryonic fibroblasts deficient in *Clock* and expressing alterations in the NF-κB pathway component, RelB, TNF-α treatment results in lower levels of both *Per2* and *Dbp* in comparison to TNF-α treated wild type cells (Bellet et al., 2012). Further, the proinflammatory cytokines that are known to impact upon clock gene expression have also been shown to impact upon circadian behaviour, including TNF-α (Cavadini et al., 2007; Nygard et al., 2009), IL-6 (Motzkus et al., 2002), IL-1β (Cavadini et al., 2007), IFN-α (Ohdo et al., 2001; Koyanagi and Ohdo, 2002) and IFN-γ (Lundkvist et al., 2002; Kwak et al., 2008). Indeed, alterations in *Per2* expression following LPS treatment have been shown to coincide with alterations in circadian locomotor activity (Okada et al., 2008). Together these studies highlight the impact inflammatory mediators exert on clock gene expression, including that of *Per2*, and it is possible that the long-term alterations we observe in PER2 expression in the post-septic SCN are due to the action of immune mediators on the SCN either directly or indirectly.

While we do not observe any long-lasting alterations in the immunomediators examined in current set of experiments in the post-septic SCN, there may be long-lasting alterations in the expression of other immune mediators in the SCN that could potentially impact upon clock gene expression and underlie the chronic alterations observed in these studies. Further, studies have shown long-lasting upregulation of immune mediators in other regions of the CNS post LPS induced sepsis (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012) which could be impacting upon clock gene expression in extra-SCN sites within the CNS or regions that project to the SCN or are involved in the regulation of behavioural or physiological processes downstream from the SCN. It is possible that cytokines are altering circadian timekeeping mechanisms through their action at extra-SCN sites, since the dorsomedial hypothalamaus, the arcuate nucleus, the habenula and the olfactory bulb are known to be sites of semi-autonomous oscillators (Guilding and Piggins, 2007), and additionally, these CNS regions are known to possess cytokine receptors (reviewed in Coogan and Wyse, 2008). Alterations in sites of other

oscillators might impair the ability of this site to efficiently process timing information communicated to it by the SCN, or impair synchronization to this information, and circadian rhythmicity may therefore be affected downstream from the SCN in regions such as these, thereby impacting upon output processes, modulating certain physiological and behavioural functions. Alterations in these CNS regions could in turn feedback and impact upon clock gene expression in the SCN itself. This would be in line with the notion that it is an *in vivo*, outside of SCN influence impacting upon the post-septic SCN, since it is only *in vivo* that we observe altered PER2 expression, but not *in vitro* when the SCN is in isolation and not influenced by other oscillators in non-SCN central sites or in the periphery.

The PVN is involved in regulation of autonomic and endocrine outputs, including the production of glucocorticoids (Kalsbeek et al., 2006). Marpegan and colleagues (Marpegan et al., 2005) have shown induction of c-Fos in the PVN following a phase resetting LPS treatment, and further, PER1 expression has also been shown to be induced in the PVN following administration of a phase resetting LPS dose (Paladino et al., 2010), indicating that the PVN may play roles in entrainment, however this remains to be examined (Marpegan et al., 2005). Other studies in mice have shown stress signals such as LPS to induce upregulation of the *Per1* gene in the PVN CRF neurons (Takahashi et al., 2001). Given that the PVN has previously been suggested to be involved in mediating output information communicated from the SCN (Kalsbeek et al., 2010), it would be interesting to examine whether changes were evident in clock gene expression or neuronal activation within the PVN in the post-septic animals, since this could play roles in rhythmic outputs. Assessment of these regions and the roles they play, if any, in impacting upon post-septic circadian rhythmicity may be warranted.

Studies have suggested that endocrine signals can impact upon the SCN (Buijs et al., 2006) and it's possible that clock gene expression may be impacted by alterations in endocrine function such as glucocorticoid rhythms which subsequently feed back to the SCN. Inflammatory mediators have been shown to alter corticosterone rhythms (Shinohara et al., 2008), and glucocorticoid treatment or attenuations in GC rhythms alter the expression of clock genes in peripheral tissues (Koyanagi et al., 2006, Yamamoto et al., 2005; Son et al., 2008; Balsalobre et al., 2000a, 2000b; Hayasaka et al., 2007; Pezuk et al., 2012). Further, GC signalling is important for clock gene expression in CNS regions outside of the SCN (Malek et

al., 2007; Lamont et al., 2005; Amir et al., 2004), with a role for adrenal GCs suggested in the regulation of PER2 oscillations in these areas (Lamont et al., 2005). Additionally, attenuations in PER2 rhythms due to GC alterations can be restored by corticosterone replacement (Segall et al., 2006). Glucocorticoids have also been shown to target *Per2* in the periphery (So et al., 2009).

Alterations in GC rhythms may affect circadian behaviour. *Per2* deficient mice do not display a diurnal serum corticosterone rhythm (Yang et al., 2009) and flattened GC rhythms have been shown to be capable of altering circadian behaviour (Koyanagi et al., 2006; Malek et al., 2007), and have been suggested to play a role in behavioural alterations in other studies (Son et al., 2008). In an experimental rodent model of *Tb. brucei* where perturbations in locomotor activity parameters and body temperature rhythms are observed, clock gene alterations are present in peripheral oscillators and in the pineal, as well as attenuations in *Per1-luc* rhythms in the pituitary and in some SCN tissues, and these authors suggest that disturbed endocrine signals as a result of alterations in the pineal and pituitary glands may influence rhythms of locomotor activity and temperature via feedback signalling to the SCN (Lundkvist et al., 2010). Together, these studies highlight a link between clock genes, including *Per2*, and corticosterone rhythms and behaviour. It is possible that attenuations in corticosterone rhythms and clock gene expression in other regions, including regions in the CNS, could feedback onto the SCN, and could be playing a role in post-septic animals in long-term alterations in clock gene expression which could underlie post-septic behavioural perturbations.

Long-lasting alterations in glial function may serve to modulate clock processes, since clock gene oscillations have been shown in SCN derived astrocytes *in vitro* (Prolo et al., 2005). Further, these are known to respond to inflammatory signals (Leone et al., 2006) and it is therefore possible that inflammatory signals might in this way impact upon clock gene expression in these glial cells. Further, TGF-α which has previously been described as a sectretory signal that may be involved in SCN output communication (Kramer et al., 2001) is known to be released by astrocytes (Li et al., 2002), and alterations in clock gene expression in astrocytes in response to inflammatory mediators might in this way impact upon post-septic SCN output.

Clock genes within the SCN generate circadian rhythms in physiology and behaviour by synchronizing peripheral oscillators and their expression of clock genes

via neural and endocrine signalling (Terazono et al., 2003; McNamara et al., 2001; Balsalobre et al., 2000a), as well as entraining non-SCN central oscillators involved in the regulation of behavioural or physiological processes. The molecular clocks at the site of these oscillators then provide circadian modulation of physiological processes, and alterations in the expression of clock genes in the master pacemaker could therefore impact upon molecular oscillations in these extra-SCN sites attenuating physiological output and behaviour.

It is possible that alterations in clock gene protein product expression in the post-septic SCN could lead to alterations in SCN electrical firing rhythms. SCN neuronal firing rhythms are crucial in transmitting circadian timing information to the brain and periphery (Schwartz et. al., 1987), and perturbations in electrical SCN activity as a result of alterations in clock gene expression could lead to altered SCN output. The SCN clockworks most likely regulate downstream events via their regulation of clock controlled genes (ccgs) and their products, and in this way, the core molecular oscillations within the SCN could regulate electrical activity (Reppert and Weaver, 2001). AVP is a clock controlled gene and is suggested to be involved in electrical firing, since during the subjective day, endogenous AVP has been shown to increase the amplitude of SCN neuronal firing (Mihai et al., 1994). The ccg PK2 has been shown to be involved in the regulation of electrical activity of SCN neurons (Ren et al., 2011) and in the generation of circadian locomotor behaviour (Cheng et al., 2002). Individual SCN neurons dissociated in culture show independent firing rhythms (Welsh et al., 1995), and these individual oscillators must be synchronized together, resulting in rhythmic electrical activity and the generation of rhythmic, coherent circadian output rhythms such as behavioural outputs (Schwartz et al., 1987). In *Clock* mutant mice, individual oscillators do not show rhythmic electrical activity, and this is correlated with the arrhythmic locomotor behaviour exhibited by these animals (Herzog et al., 1998). The electrophysiological examination of post-septic SCN neurons might shed light on whether the post-septic SCN functional state is altered as a result of altered clock gene product rhythms.

Further, neural activity and synaptic activity may be required for the generation of circadian oscillations in the core clock genes in the SCN, since various studies have shown that perturbations in these lead to alterations in molecular oscillations. Further the presence of immune mediators may impact upon the electrical activity of the post-septic SCN. Indeed, it has been previously shown that

TNF-α may act through the induction of nitric oxide to modulate the electrical output of the SCN, thereby altering circadian rhythmicity (Nygard et al., 2009). In transgenic mice and Drosophila models where clock cells are hyperpolarized, perturbations have been observed in behavioural and molecular rhythms (Nitabach et al., 2002; Pakhotin et al., 2006). Molecular oscillations have been shown to be attenuated or inhibited by hyperpolarizing Ca^{2+} neurons in culture, or by blocking Ca^{2+} channels (Lundkvist et al., 2005). Other studies by Yamaguchi and colleagues (Yamaguchi et al., 2003) have shown electrical firing rhythms to be involved in the generation of robust rhythmic clock gene expression in individual neurons in SCN cultures. Electrophysiological examination of the post-septic SCN neurons is warranted, since attenuations in this could underlie perturbations in clock gene expression and in post-septic behavioural rhythmicity.

Attenuations in synaptic plasticity in the post-septic brain could play a role in the attenuations in post-septic SCN molecular oscillations. Studies where pharmacological treatments have blocked synaptic transmission in the SCN without affecting membrane depolarization or action potential generation have resulted in attenuations in clock gene expression (Colwell et al., 2011). Assessment of PER2::LUC rhythms *in vitro* revealed perturbations in the expression of clock genes both within individual SCN neurons and across the SCN network, following manipulation of synaptic vesicle recycling, by treatment of SCN slices with either dynasore or botulinum toxin A to inhibit exocytosis and endocytosis (Deery et al., 2009). Further, rhythmic clock gene expression and neural activity were seen to be altered in studies where the secretory vesicle proteins receptor-type tyrosine-protein phosphastase-like N (IA-2) and IA-2β were deleted (Kim et al., 2009). *In vitro*, Stagi and colleagues (Stagi et al., 2005) have shown microglial induced nitric oxide to affect the anterograde axonal transport of synaptophysin, which is a synaptic protein involved in synaptic plasticity, while other studies assessing the long term effects of LPS on the CNS have shown there to be alterations in synaptic proteins involved in synaptic plasticity (Weberpals et al., 2009) and these authors show that these alterations are possibly mediated through nitric oxide production. Given the upregulation we observe in NOS2 expression in the acute phase following LPS treatment, it's possible that synaptic alterations could underlie the alterations in clock gene expression post sepsis. Taken together, these studies highlight the impact of alterations in synaptic properties on clock gene expression and therefore it's possible

that alterations in synaptic properties could impact upon clock gene expression in the post-septic SCN.

It is further highlighted from these experiments, that all the alterations in photic IEG induction and behavioural rhythms are seen during the advance portion of the phase response curve. The results from the current set of studies in post-septic animals again point to an underlying mechanism influencing long-lasting changes in the SCN post sepsis, involved both in rhythmic clock gene expression and in SCN responsiveness. These experiments cannot currently establish a mechanism by which clock gene expression is altered in the post-septic SCN, however they highlight that these alterations are due to an *in vivo* influence. Further work is required to delineate the mechanisms by which alterations in clock gene expression occur *in vivo* and to fully characterise their impact on post-septic circadian behavioural rhythms. To the best of our knowledge, the current study is the first to describe long-lasting alterations in circadian clock gene expression in the SCN following sepsis induction.

Chapter Five

Post-septic circadian responsiveness to a further immune stimulus.

5.1. Introduction

5.1.1. Interactions between pre-existing neuroinflammation and subsequent immune challenge

Interactions have been demonstrated between pre-existing neuroinflammation and a peripheral immune challenge (Combrinck et al., 2002). Exposure to a single acute systemic challenge is not very likely to occur in humans and animals, it is more likely that an infectious pathogen that replicates *in vivo* will be encountered or that they will be exposed to low concentrations of LPS over an extended period of time (Puntener et al., 2012) and systemic infections occur frequently in nature and can exacerbate disease within the CNS (Holmes et al., 2009; Buljevac et al., 2002). The use of a single challenge with LPS, to mimic a single episode Gram-negative bacterial infection has been performed on a background of pre-existing neuroinflammation. A hypersensitive (primed) (Greer and Rietschel, 1978) or hyporesponsive (tolerant) state (Ziegler-Heitbrock, 1995) can ensue following LPS treatment. Cells of the innate immune system of both mice and humans including macrophages, monocytes and fibroblasts are seen to display both endotoxin tolerance and LPS priming (Fu et al., 2012). The function of endotoxin priming and tolerance may be to confer a survival advantage upon organisms, stimulating cells of the innate immune system to react vigorously to further inflammatory challenges in the case of LPS priming, or in the case of tolerance, promoting homeostasis following a substantial immune insult and the accompanying immune response (Fu et al., 2012). However, endotoxin priming and tolerance may play other roles within an organism, since these states have also been associated with the pathogenesis of acute and chronic disease. In septic shock, endotoxin tolerance results in an immunosuppressed state, during which time the occurrence of secondary infections and mortality is seen to increase (West and Heagy, 2002). Endotoxin priming is suggested to be involved in low-grade metabolic endotoxemia, at which time levels

of LPS are elevated in the bloodstream causing an increased frequency of insulin resistance and diabetes, as well as atherosclerosis (Moreno-Navarrete et al., 2010; Wiesner et al., 2010; Kiechl et al., 2001; Slofstra et al., 2006). Both hypo and hyper responsiveness have previously been exemplified in response to LPS treatment in chronic neuroinflammatory conditions.

5.1.2. Hypo-responsiveness following further immune stimuli in conditions of pre-existing CNS immune activation.

Excessive or prolonged activation of the immune system can be detrimental. Endotoxin tolerance is observed in response to secondary immune stimuli, and serves to regulate responses of the innate immune system, preventing over-responsiveness, and is characterized by transient cellular, metabolic and behavioural hypo-responsiveness to recurring or chronic systemic infections (West and Heagy, 2002). *In vivo* and *in vitro*, low LPS doses have been shown to result in a tolerant state where the immune response to further challenge with LPS is altered (Cavaillon and Adib-Conquy, 2006; Biswas and Lopez-Collazo, 2009). A reprogramming of the dose-response relationship between LPS and the induction of cytokines can occur in response to two successive LPS treatments, resulting in a reduction of cytokine production (Fu et al., 2012). Negative regulators of the TLR4 signalling pathway including signalling molecules, transcriptional modulators and soluble factors, as well as epigenetic changes and chromatin modifications of specific genes have been suggested to be involved in endotoxin tolerance (Biswas and Lopez, 2009; Kobayashi et al., 2002; Brint et al., 2004; Sly et al., 2004; Foster et al., 2007; Chang et al., 2009; El Gazzar et al., 2007; Li et al., 2000; Jacinto et al., 2002 Chen et al., 2009a; Chen et al., 2009b). During LPS induced tolerance, sickness behaviours are attenuated following repeated LPS injections as a survival mechanism (Franklin et al., 2007). The stimulation of TLR4 expressing cells has previously been suggested to be required for the induction of endotoxin tolerance (Faggioni et al., 1995). Stimulation of TLR4 which signals to NF-κB results in the transcription of proinflammatory cytokines, while a second LPS stimulation is seen to suppress the induction of these, leading to a decrease in fever, anorexia and lethality (Beutler, 2002; Fraker et al., 1988; Mengozzi and Ghezzi, 1993). Further, LPS tolerant macrophages show a downregulation in surface TLR4 expression (Nomura et al.,

2000). Endotoxin tolerance is characterized by inhibition of TNF-α, attenuations in IL-6 and IL-1, COX2 alterations, perturbations in NF-κB translocation and inhibition of mitogen-activated protein kinase activation (West and Heagy, 2002). However, endotoxin tolerance is not simply a case of down regulated responsiveness, indeed components of the immune response such as the NF-κB p50 subunit, the anti-inflammatory cytokine IL-10 and the TNF receptor type II are in fact increased during tolerance and are seen to be involved in proinflammatory cytokine down regulation (Ziegler-Heitbrock, 1995). Anti-inflammatory cytokines including TGF-β and IL-10 are also thought to play a primary role in the tolerance state (Randow et al., 1995). Additionally, IL-1β has been shown to play a role *in vivo* in LPS tolerance, whereby a down regulation of TLR4 is observed, along with an increase in both glucocorticoid levels and the levels of GC receptors (Alves-Rosa et al., 2002). The HPA axis is involved in endotoxin tolerance (Beishuizen and Thijs, 2003; Cavaillon et al., 2003), and glucocorticoids are thought to play a primary role in the tolerant state induced by endotoxin treatment (Ikeda et al., 2006). Indeed, adrenalectomized mice do not display endotoxin tolerance (Evans and Zuckerman, 1991; Parrillo et al., 1993; Zuckerman and Qureshi, 1992), highlighting the importance of glucocorticoid activity in this process.

Various studies have described tolerance in response to secondary immune stimuli. In rats, a single LPS treatment 3 weeks prior to induction of adjuvant arthritis is seen to cause resistance to inflammation (Harbuz et al., 2002). In whole blood of sepsis patients treated *ex vivo* with LPS a decreased expression of the proinflammatory cytokines TNF-α, IL-6 and IL-1β was observed for up to 10 days (Ertel et al., 1995). Treatment of macrophages with a high dose of LPS *in vitro* sees a reduction in proinflammatory cytokine induction upon subsequent stimulation with a high dose of LPS (Biswas and Lopez-Callazo, 2009). The effects of endotoxin tolerance have been shown to persist up to three weeks *in vivo* (West and Heagy, 2002). *In vitro*, in response to low levels of LPS, both mouse macrophages and human monocytes have been shown to be unable to respond to further challenge with LPS (Dobrovolskaia and Vogel, 2002; del Fresno et al., 2009; Foster et al., 2007), while *in vivo*, endotoxin tolerance has been shown in mice, and monocytes/macrophages were shown to be responsible for this (Cavaillon and Adib-Conquy, 2006). Further, *in vitro*, the epigenetic changes associated with gene activation were not observed in cultured cells previously treated with LPS in

response to an additional LPS challenge (Chan et al., 2005). Additionally, i.c.v. administration of LPS upon four consecutive days has been shown to cause reduced production of systemic TNF-α (Faggioni et al., 1995), while studies in rats show that repeated LPS treatment for 6 consecutive days reduces HPA axis responsiveness (Takemura et al., 1997).

Variations have been shown between the periphery and the CNS during endotoxin tolerance. In the periphery during endotoxin tolerance, peripheral cytokines are no longer seen to be induced, while studies have shown that LPS induced cytokine expression can remain evident in the CNS at this time (Chen et al., 2005; Faggioni et al., 1995). Additionally, Puntener et al. (2012) have recently shown that in response to systemic infection, innate immune cells in the CNS do not become tolerant, but rather become primed. Other studies have shown that in the CNS, the responsiveness of the inflammatory mediators MCP-1, TNF-α, IL-6 and IL-1α can be exacerbated following multiple frequent LPS challenges (Erickson and Banks, 2011), further highlighting the alternative response induced in the CNS during the LPS tolerant state.

5.1.3. Hyper-responsiveness during chronic neuroinflammatory states in response to further stimuli.

The sensitization or priming of the CNS in conditions of neuroinflammation aims to stimulate cells of the innate immune system to react vigorously to further inflammatory challenges (Fu et al., 2012), however systemic infection is known to have a detrimental effect on both humans and animals that suffer from chronic neurodegeneration (Cunningham et al., 2009; Holmes et al., 2009). Further, there is evidence to suggest that priming of the brain occurs in chronic inflammatory conditions (Drake et al., 2011) such as arthritis, diabetes, atherosclerosis and obesity and these conditions are known to be associated with systemic inflammation (Yaffe et al., 2004). The molecular mechanisms underlying endotoxin priming are not currently understood but may involve both intra-cellular and inter-cellular processes (Zhang and Morrison, 1993; Fu et al., 2012). In primed conditions, cells of the innate immune system display altered responsiveness to inflammatory stimuli. Low dose treatment with LPS is seen to result in the priming of macrophages due to reprogramming of their responsiveness, and following a subsequent challenge with a

high dose of LPS, an enhanced proinflammatory cytokine production is seen (Hirohashi and Morrison, 1996; Shnyra et al., 1998; West and Koons, 2008; Zhang and Morrison, 1993; Henricson et al., 1993). Human blood cells primed by pre-exposure to a low dose of LPS show attenuations in the expression of the proinflammatory cytokine IL-6 upon subsequent challenge with a high dose of LPS (Nakamura et al., 2004). It has been suggested that glucocorticoid action within the brain could result in exaggerated inflammatory responses to stimulation of the immune system (de Pablos et al., 2006; Munhoz et al., 2010). Both adrenelectomy and GC receptor function inhibition with RU486 have been shown to inhibit primed microglial responses to LPS *ex vivo* following *in vivo* psychological stress (Frank et al., 2012), implicating a role for glucocorticoids in microglial priming.

Microglia are suggested to play a prominent role in the priming of the CNS during conditions with neuroinflammatory components. On-going pathology within the CNS primes microglia, whereby these are excessively reactive to subsequent immune challenges including those in the periphery (Cunningham et al., 2005; Lunnon et al., 2011). In aging, evidence points to the presence of "primed" microglia, which are seen to be excessively reactive to immune challenges or noxious stimuli (Dilger and Johnson, 2008) including in response to LPS treatment (Godbout et al., 2005). Additionally, obesity which is characterized by chronic inflammation is also seen to exhibit more reactive microglial cells in response to an immune challenge, in line with a "primed" state (Bilbo and Tsang, 2010). In Alzheimer's Disease patients with systemic infection or inflammation, primed microglia have been suggested to play a role in the exaggeration of clinical symptoms (Holmes et al., 2009; Holmes et al., 2011). Further, morbidity is seen in patients with AD following a systemic immune challenge due to an infection in the periphery such as pneumonia, or a urinary tract infection (Burns et al., 1990), and these may subsequently cause an increase in cytokine expression in the CNS (Combrick et al., 2002).

Epigenetic changes have been suggested to play a role in priming of the innate immune system (Puntener et al., 2012). A trained immunity mediated by epigenetic changes in the immune system has been suggested to exist in vertebrates, invertebrates and plants, and in mammals this is due to cells of the innate immune system appearing to display an immunological memory of past immune insults (Netea et al., 2011). Early life infections have been shown to be capable of

attenuating the threshold of IL-1β production in the hippocampus via long-lasting priming of microglia (Williamson et al., 2011). A hyper-responsiveness has been shown in animals maintained in standard animal house conditions compared to those raised and maintained in a specific pathogen free (SPF) environment in response to adenoviral vector, including a stronger inflammatory response and reduced reporter gene synthesis (Ohmoto et al., 1999).

5.1.4. Alterations in behaviour during chronic neuroinflammatory states in response to further stimuli.

Alterations in cognitive function and mood are observed in humans in response to systemic infection or inflammation, along with changes in activity in certain CNS regions, and this is due to communication between the brain and the peripheral immune system (Reichenberg et al., 2001; Krabbe et al., 2005; Bucks et al., 2008). However, in chronic neurodegenerative conditions, systemic infection has been shown to have a detrimental effect on both humans and animals (Cunningham et al., 2009; Holmes et al., 2009). In animal models, acute working memory impairments are observed following a systemic inflammatory stimulus in both animals with pre-existing neurodegenerative disease (Murray et al., 2012) and in aged animals (Chen et al., 2008). Systemic inflammation elicited by infection or injury in the periphery may activate already primed microglia in the normal healthy ageing brain or in the brains of AD patients, and this can impact upon the cognitive decline observed in these patients and additionally, can result in delirium (Perry et al., 2003). Acute cognitive impairments are seen to occur in association with peripheral infection in the elderly population (Wofford et al., 1996; Chiovenda et al., 2002) and in the demented, and often results in delirium, whereby transient perturbations in attention and cognition are evidenced (Elie et al., 1998). Elderly patients often exhibit symptoms consistent with delirium when suffering from pneumonia (Janssens and Krausse, 2004). Further, systemic viral or bacterial infections are seen to significantly contribute to morbidity in the elderly population (Aw et al., 2007). In experimental rodent models, an exaggerated behavioural response is seen in aged rodents in response to viral or bacterial infections (Godbout et al., 2005, Barrientos et al., 2006). Further, in comparison to younger cohorts, aged mice have been shown to exhibit exaggerated deficits in hippocampal-

dependent learning and memory, depression like behaviours and anorexia following peripheral challenge with LPS (Godbout et al., 2005; Chen et al., 2008; Godbout et al., 2008; Dilger and Johnson, 2008). In response to LPS treatment, aged animals have been shown to exhibit primed microglial responses (Godbout et al., 2005) and this may be involved in predisposing this population to enhanced features of depression (Godbout et al., 2008). In the ME7 mouse model of prion disease, A 500µg/kg i.p. LPS challenge 15weeks following disease inoculation was seen to result in acute neurological alterations followed by the earlier development of progressive irreversible neurological impairments that were shown to be more severe in these animals in comparison to wild-type ME7 mice (Cunningham et al., 2009).

5.1.5. Experimental administration of LPS and primed responsiveness

The use of a single challenge with LPS to mimic a Gram-negative bacterial infection has been performed in previous studies in order to assess the impact of a systemic challenge on chronic disease states exhibiting a neuroinflammatory component. APP expression has been shown to be altered following 500µg/kg peripheral LPS treatment (Brugg et al., 1995). Additionally, a 500µg/kg i.p. LPS challenge to the ME7 mouse model of prion disease is seen to increase neuronal apoptosis (Cunningham et al., 2005), while a single LPS 200µg/kg treatment administered to aged mice is seen to cause tyroxine hydroxylase (TH)-positive neuronal death in the SN (L'Episcopo et al., 2011). Further, peripheral 1mg/kg LPS induces long term iNOS, NADPH oxidase labelling and TH-positive neuronal loss in a mouse model of PD (Gao et al., 2011). Discordant cytokine responsiveness is observed in conditions of healthy ageing which exhibits a low-grade neuroinflammation, in response to peripheral LPS treatment (Godbout et al., 2005; Chen et al., 2008), and activated microglia are shown to be increased in the brain in this state (Godbout et al., 2005). Further, studies in aged rats have shown an exaggerated cytokine response following administration of *E. coli* (Barrientos et al., 2006). In aged mice in response to i.c.v. LPS administration an enhanced cytokine response is observed in the brain, highlighting that the response of the innate immune system in the CNS in this state is due to factors within the CNS (Huang et

al., 2008). Together, these studies highlight an exaggerated responsiveness to noxious stimuli in conditions where a long-lasting neuroinflammation is evident.

Given that in conditions of chronic neuroinflammation "priming" is observed in the CNS and subsequent immune stimuli result in exaggerated cytokine responsiveness and exaggerations of cognitive impairment, the current set of experiments examine the possibility that a single septic LPS treatment, known to induce chronic inflammation within the CNS (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012) may result in priming or sensitization of the CNS, and in response to a secondary stimulus such as peripheral infection, an exaggerated or more vigorous response may be elicited. There may be an on-going activity of the innate immune system in the post-septic CNS which would in turn lead to an increased immunological risk upon subsequent exposure to an immune challenge, and given the interactions between the circadian system and inflammatory mediators, an exaggerated response of the master pacemaker may be observed in post-septic animals as well as enhancement of circadian resetting properties. Assessment of the SCN and circadian system responsiveness in further immune challenged post-septic animals would be interesting and may provide insight into whether there are similarities between the post-septic brain and that of other chronic neuroinflammatory states.

In mice, systemic low doses of LPS result in photic-like phase delays of circadian locomotor behaviour when administered at CT15, but it has no significant effect when injected at other circadian times (Marpegan et al., 2005). Given the low does used in these experiments and the alteration in circadian output, we used this LPS induced phase delay as a method for assessing a priming effect of previous CNS insult following a subsequent immune challenge.

5.2. Materials & Methods

5.2.1. Animals and Housing:

For assessment of the SCN in both post-septic and saline treated animals in response to low dose peripheral LPS treatment, adult C57BL/6 male mice (8-10wks old) were group housed as outlined in section 2.2.2. For the purpose of behavioural

monitoring, animals were individually housed in polypropylene cages (33cm long x 15cm wide x 13cm high) equipped with steel running wheels (11.5cm diameter) with food and water available *ad libitum*.

5.2.2. Behavioural responses following low dose LPS treatment after previous induction of sepsis

In order to establish whether an altered behavioural response would be seen in post-septic animals compared to controls following a second immune insult, 22 adult male mice were individually housed in cages equipped with running wheels and habituated to a 12:12 LD cycle for 2 weeks (150 lux, lights on 0500h). The animals were treated with either LPS 5mg/kg i.p injection (n=12) or saline (n=10) and were allowed to recover for two weeks following the induction of sepsis prior to being transferred into constant darkness. Animals were maintained under these constant darkness conditions for 14 days and allowed to free run. Animals then received either i.p. saline or a second (i.p.) injection of LPS at a dose of 100 µg/kg at CT15, the early subjective night, the time at which administration of low dose LPS has previously been shown to induce photic like phase delays of locomotor activity (Marpegan et al., 2005). Following treatment, the animals were maintained in DD for a further 14 days and their actogram data monitored to allow for assessment of phase shifts, before receiving a third treatment of either LPS or saline at CT15. Those that received saline vehicle upon the second treatment occasion were then treated with i.p. LPS 100µg/kg and those having received low dose LPS as second treatment were administered saline control. Following the third treatment, the animals' activity patterns in DD were followed for 14 days and phase shifts of locomotor activity rhythms assessed.

The line of best fit method was used to assess the phase shift magnitudes from the actogram data, fitting the line through activity onsets 7 days before and 10 days after treatment with LPS 100µg/kg or vehicle. The differences between the lines from the actograms were rated by two independent researchers blind to the experimental procedure. Following each CT15 treatment, the circadian parameters of free running period and rhythm amplitude were calculated for each animal using the Chronobiology Kit Chi Squared procedure and analysed by mixed between-

within groups ANOVA and for each group by Bonferroni corrected independent samples t-test.

5.2.3. Assessment of SCN neurochemistry in post-septic animals following an LPS 100µg/kg i.p. treatment

In order to establish whether altered neurochemical responses would be seen in the SCN in post-septic animals compared to controls following a second immune insult, 6 week old male mice were group housed in colonies of three in cages equipped with appropriate environmental enrichment and habituated to a 12:12 LD cycle for 2 weeks (150 lux, lights on 0500h) prior to experimentation. The animals were treated with either LPS 5mg/kg i.p injection (n=4-5) or saline (n=7-9). One month later, both treatment groups received a second treatment, an (i.p.) injection of LPS at a dose of 100 µg/kg at ZT1-2, 4 hours after which (ZT5-6) they were perfused transcardially and processed for immunohistochemistry as outlined previously in sections 3.2.3 and 3.2.4. Immunohistochemistry was carried out for the immediate early genes c-Fos and EGR-1 and the microglial markers CD-11b and F4/80 (details of primary antisera outlined in Table 3).

For each individual animal and each antibody, 3-6 SCN images were examined by means of IOD or manual quantification of the number of immunoreactive nuclei in the SCN, viewed with the light microscope as previously outlined in section 3.2.5, and a mean value obtained for each animal for each antibody for all SCN regions of interest. These values were calculated for each animal by two researchers. The observers were blinded to the experimental procedure during quantification of immunoreactive cells per SCN. The means calculated for each group were compared and analysed by independent samples t-test. Results are given as mean values (IOD or cell number) $\pm$ standard error of the mean, statistical significance was accepted at $P<0.05$.

5.2.4. Assessment of SCN neuronal activity in post-septic animals 2hrs and 9hrs following an LPS 100µg/kg treatment

In order to establish whether post-septic animals would exhibit altered neuronal activity in the SCN following a further low dose LPS treatment compared to controls, 6 week old male mice were group housed in colonies of three in cages

equipped with appropriate environmental enrichment and habituated to a 12:12 LD cycle for 2 weeks (150 lux, lights on 0500h) prior to experimentation. The animals were treated with either LPS 5mg/kg i.p injection (n=3-4) or saline (n=4-5). The animals were allowed to recover following the induction of sepsis and were maintained for 3 months under a 12:12 LD cycle. Both post-septic and saline treated controls then received an i.p. injection of LPS at a smaller dose of 100 µg/kg at ZT1-2 and held under the 12:12 LD cycle. Animals were then terminally anaesthetized and perfused transcardially (as outlined previously in section 3.2.3) in order to examine whether alterations are seen in the SCN at different time points when LPS 100 µg/kg treatment is administered 3 months following LPS5mg/kg or saline. The first group of animals (no sepsis + LPS 4, post-sepsis + LPS 3) were perfused 2 hours after LPS 100 µg/kg treatment (ZT3-4), while the second group of animals (no sepsis + LPS 5, post-sepsis + LPS 4) were perfused 9 hours later (ZT10-11). Immunohistochemistry was carried out as described previously in section 3.2.4 for the IEGs c-Fos and EGR-1 (details of primary antisera outlined in Table 3).

For each individual animal and each antibody, 3-6 SCN images were examined by means of manual quantification of the number of immunoreactive nuclei in the SCN, viewed with the light microscope as previously outlined in section 3. 2.5, and a mean value obtained for each animal for each antibody for all SCN regions of interest. These values were calculated for each animal by two researchers blinded to the experimental procedure during quantification of immunoreactive cells per SCN. The means calculated for each group were compared and analysed by independent samples t-test. Results are given as mean values (cell number) $\pm$ standard error of the mean, statistical significance was accepted at $P<0.05$.

5.3. Results:

5.3.1. Circadian behavioural response following low dose LPS treatment after previous induction of sepsis

We examined the phase shifts elicited by an LPS 100µg/kg i.p. treatment or saline following administration at CT15 in post-septic animals and those originally

treated with saline. Mixed between–within groups ANOVA revealed a main effect for shift magnitude elicited by the different treatments ($F_{1,20}$=6.79, P<0.05) and an interaction effect for prior condition and acute treatment ($F_{1,20}$=8.65, P<0.01). 4 or 6 weeks following the initial treatment with LPS 5mg/kg or saline as control, LPS 100µg/kg i.p. treatment at CT15 induced phase delays with a mean phase delay magnitude of 0.68 $\pm$ 0.14 hrs in animals initially treated with saline prior to a low dose LPS treatment, and this was found to be statistically different to the phase delay magnitude induced in post-septic animals, who displayed a significantly lower average phase delay value of 0.22 $\pm$ 0.08 hrs (P<0.01, independent t-test). Subsequent independent t-test analysis revealed that 4 or 6weeks following the initial treatment with LPS 5mg/kg or saline as control, saline vehicle administered at CT15 did not induce significant differences between the groups in phase shift magnitude elicited, with a mean phase delay magnitude of 0.10 $\pm$ 0.07 hrs in animals initially treated with saline prior to a low dose LPS treatment, and a mean phase delay magnitude of 0.25 $\pm$ 0.13 hrs induced in post-septic animals (P>0.05, independent t-test). Further, paired t-test analysis revealed a significant difference in phase response elicited following low dose peripheral LPS treatment and that following saline control injection in no sepsis animals (P<0.05) but not in post-septic animals (P>0.05). (Figure 5.1).

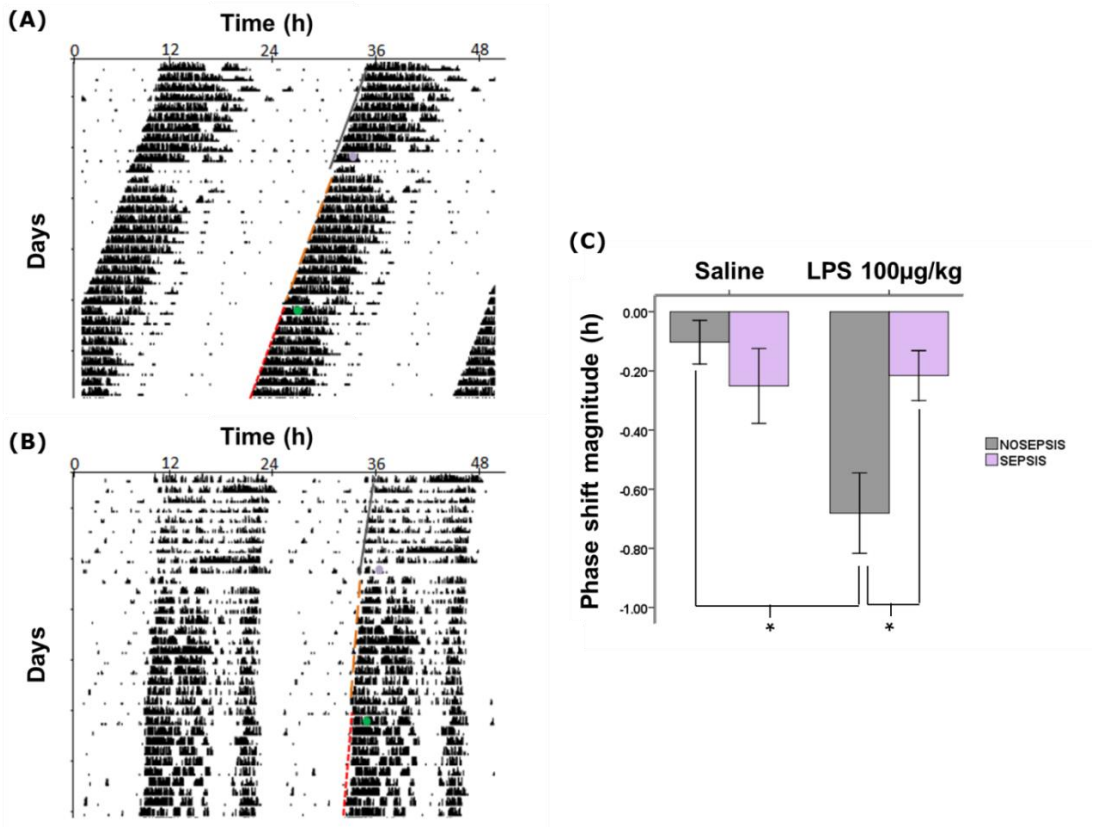


Figure 5.1: Phase resetting is attenuated following low dose LPS in post-septic animals. *(A) and (B) Sample double plotted actograms from saline (n=10) and post-septic animals (n=12) respectively, free running in DD and treated i.p. with either 100µg/kg LPS at CT15 (CT15 LPS treatment indicated by purple circle, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days after LPS treatment) followed 14 days later by saline injection at CT15 (CT15 saline treatment indicated by green circle, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days after saline treatment). Note suppression of locomotor behaviour in the hours following the low dose peripheral LPS treatment. (C) Bar graph illustrating phase shift magnitude in post-septic animals and those treated with saline control originally, not having been subjected to sepsis. (*=P<0.05).*

5.3.2. The Free Running Periods and Rhythm Amplitudes following LPS 100µg/kg i.p. or control treatments in the early subjective night.

The free running periods and rhythm amplitudes were examined for 7 days following each CT15 treatment by Chi^2 periodogram analysis and subsequent assessment by ANOVA for both treatment groups.

Mixed between within ANOVA was used to assess statistically significant differences between groups in the mean rhythm amplitudes in the 7 days prior to treatment and in the 7 days following both the LPS 100µg/kg i.p. treatment and the saline treatment, revealing a main effect of time for rhythm amplitude ($F_{2,19}$=14.15, P<0.001), however finding no between groups effect ($F_{2,19}$=1.02, P>0.05). Mean rhythm amplitude values for saline and LPS 100µg/kg treated animals and post-septic animals treated with LPS 100µg/kg did not differ during the 7 days following the LPS 100µg/kg treatment or following the saline treatment at CT15 (P>0.05, independent t-test). (Table 5).

Mixed between within ANOVA was used to assess statistically significant differences between groups in the mean FRP in the 7 days prior to treatment and in the 7 days following both the LPS 100µg/kg i.p. treatment and the saline treatment, finding neither a main effect of time for FRP ($F_{2,19}$=0.15, P>0.05), nor a between groups effect ($F_{2,19}$=1.84, P>0.05). Mean FRP values for saline and LPS 100µg/kg treated animals and for post-septic animals treated with LPS 100µg/kg did not differ during the 7 days following the LPS 100µg/kg treatment (P>0.05, independent t-test), nor was there any statistically significant difference in mean FRP between the groups following saline treatment at CT15 (P>0.05, independent t-test). (Table 5).

Table 5: Assessment of FRP and rhythm amplitude prior to and following CT15 treatments. *Table illustrating the average free running period and rhythm amplitude values for saline and post-septic animals prior to CT15 treatments and in the 7 days following CT15 saline treatment and CT15 LPS 100µg/kg treatments. (n.s.) denotes not significant.*

	No sepsis	Post-septic animals
Rhythm Amplitude prior to shift	1717.13 +/- 204.54	1470.87 +/- 170.38 (n.s.)
Free running period prior to shift (h)	23.78 +/- 0.06	23.81 +/- 0.03 (n.s.)
Rhythm Amplitude post LPS shift	1005.45 +/- 122.62	1099.78 +/- 95.51 (n.s.)
Free running period post LPS shift (h)	23.77 +/- 0.05	23.79 +/- 0.02 (n.s.)
Rhythm Amplitude post saline vehicle	1161.24 +/- 136.11	1095.15 +/- 121.37 (n.s.)
Free running period post saline vehicle (h)	23.72 +/- 0.05	23.82 +/- 0.03 (n.s.)

5.3.3. Assessment of SCN neurochemistry in post-septic animals following an LPS 100µg/kg i.p. treatment

5.3.3.1. c-Fos expression in response to LPS 100µg/kg i.p. one month following sepsis or no sepsis.

Immunohistochemical analysis coupled with manual quantification of c-Fos expression in post-septic and saline treated controls that received peripheral LPS 100µg/kg, revealed that c-Fos expression was significantly increased in the SCN of post-septic animals (80.72 ± 6.62 ir. nuclei/SCN) when compared to those treated with saline originally (58.04 + 6.30 ir nuclei/SCN, $P<0.05$, independent t-test; Fig. 5.2). When analysing the separate subdivisions of the SCN by independent t-test, the mean number of immunoreactive c-Fos cells in the ventrolateral core of the SCN was not found to be significantly different between the treatment groups, with post-septic animals displaying a mean of 21.88 ± 4.54 ir. cells/SCN and control animals displaying an mean of 15.89 ± 1.93 ir. cells/SCN ($P>0.05$; Fig. 5.2). The difference in c-Fos expression levels was seen to be statistically significant in the dorsomedial shell region of the SCN, with a significant increase in expression levels in post-septic animals (58.83 ± 3.83 ir.cells/SCN) compared to saline controls (41.30 + 4.74 ir.cells/SCN, $P<0.05$, independent t-test; Fig. 5.2).

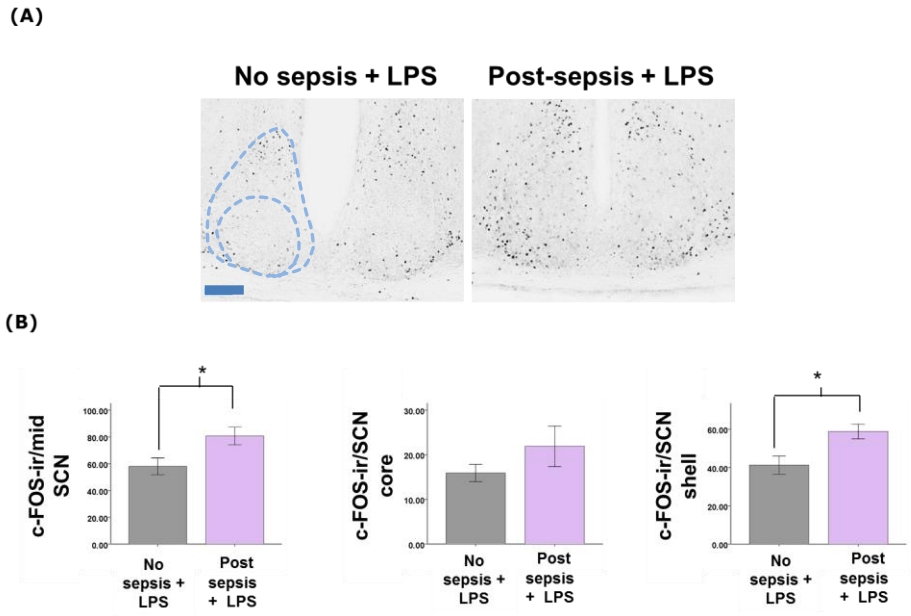


Figure 5.2: Alterations in neuronal activation in the SCN of post-septic animals following low dose peripheral LPS treatment. *(A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=5) and*

animals treated originally with saline (n=9) 4hrs following low dose LPS 100µg/kg treatment at ZT1-2 (scale bar = 100 µm). Dashed line outlines the SCN and delineates the core and shell subdivisions. (B) Bar graphs illustrating quantification of levels of c-Fos immunoreactive nuclei at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and post-septic animals treated with 100µg/kg LPS treatment. (P<0.05).*

5.3.3.2. EGR-1 expression in response to LPS 100µg/kg i.p. one mth following sepsis or no sepsis.

Immunohistochemical analysis of EGR-1 followed by quantification of the immediate early gene protein product's expression in post-septic and saline treated controls that received a second treatment of LPS 100µg/kg, found no significant differences in expression levels in the SCN between post-septic animals (17.90 ± 4.74 ir. nuclei/SCN) and saline controls (19.92 + 4.01 ir nuclei/SCN, P>0.05, independent t-test; Figure 5.3). When analysing the separate subdivisions of the SCN by independent t-test, there were no statistically significant differences found in the mean number of immunoreactive EGR-1 cells in either the ventrolateral core of the SCN (LPS, 4.60 ± 2.52 vs. controls 4.91 ± 1.56 ir.cells/SCN, P>0.05) or in the dorsomedial shell region of the SCN (LPS, 13.30 ± 2.34 vs. 15.01 ± 2.66 ir.cells/SCN, P>0.05, independent t-test).

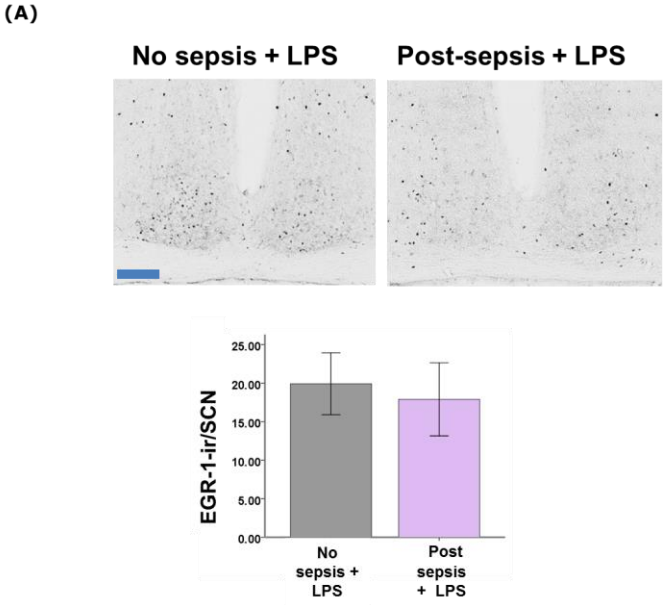


Figure 5.3: EGR-1 expression in the SCN of post-septic and no sepsis animals following low dose peripheral LPS treatment. *(A) Representative photomicrographs of EGR-1 expression in the SCN of post-septic animals (n=5) and animals treated originally with saline (n=8) following low dose LPS 100µg/kg treatment at ZT1-2*

(scale bar = 100 µm). (B) Bar graphs illustrating EGR-1 expression in the SCN of post-septic animals and animals treated originally with saline 4hrs following low dose peripheral LPS 100µg/kg treatment at ZT1-2.

5.3.3.3. CD-11b expression in response to LPS 100µg/kg i.p. one month following sepsis or no sepsis.

Immunohistochemical analysis for the microglial marker CD-11b followed by IOD of the immunostain in the SCN of both post-septic animals and saline treated controls that received peripheral LPS 100µg/kg, found no significant differences in expression levels in the SCN between post-septic animals (2.21 $\pm$ 0.25 ir./SCN) and saline controls (1.95 $\pm$ 0.36 ir./SCN, P>0.05, independent t-test; Figure 5.4). When analysing the separate subdivisions of the SCN by independent t-test, there were no statistically significant differences found in the mean CD-11b immunosignal in either the ventrolateral core of the SCN (LPS, 2.56 $\pm$ 0.27 vs. controls 2.02 $\pm$ 0.40 ir./SCN; P>0.05) or in the dorsomedial shell region of the SCN (LPS, 2.03 $\pm$ 0.36 vs. 1.93 $\pm$ 0.36 ir./SCN, P>0.05, independent t-test).

Quantification of the number of immunostained microglial cells in post-septic and saline treated controls that received a second treatment of LPS 100µg/kg, was also performed, and similarly, found no significant differences in microglial expression in the SCN between post-septic animals (7.96 $\pm$ 3.48 ir.cells/SCN) and saline controls (8.91 $\pm$ 2.83 ir. cells/SCN, P>0.05, independent t-test). Further assessment of the morphology of CD-11b immunostained cells under high power and by manual quantification in post-septic and saline treated controls that received a second treatment of LPS 100µg/kg, found no significant differences in microglial morphology or in the expression of activated glia in the SCN between post-septic animals (0.79 $\pm$ 0.31 ir.cells/SCN) and saline controls (0.49 $\pm$ 0.40 ir.cells/SCN, P>0.05, independent t-test).

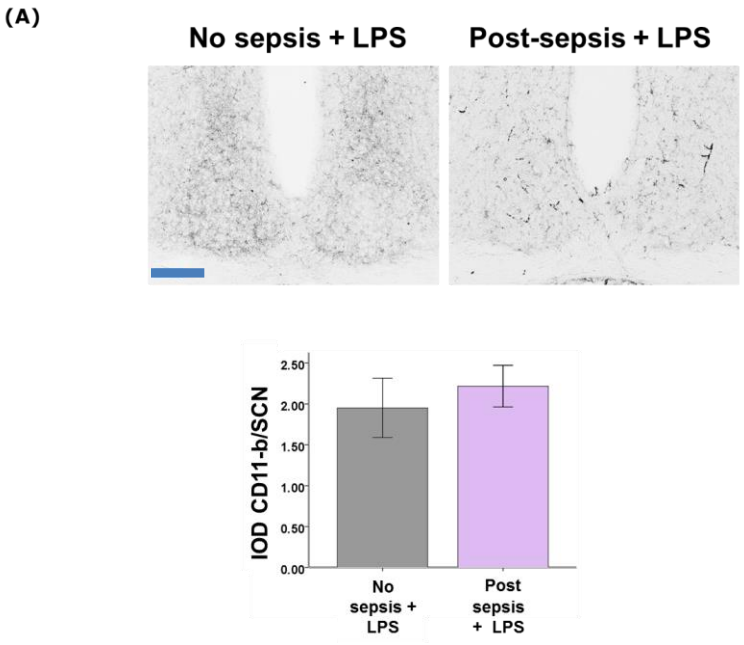


Figure 5.4: CD-11b expression in the SCN of post-septic and no sepsis animals following low dose peripheral LPS treatment. *(A) Representative photomicrographs of CD-11b expression in the SCN of post-septic animals (n=4) and animals treated initially with saline (n=8) following low dose LPS 100µg/kg treatment at ZT1-2 (scale bar approx. 100 µm). (B) Bar graphs illustrating CD-11b expression in the SCN of post-septic animals and no sepsis animals 4hrs following low dose peripheral LPS 100µg/kg treatment at ZT1-2.*

5.3.3.4. F4/80 expression in response to LPS 100µg/kg i.p. one mth post sepsis or no sepsis.

Immunohistochemical analysis for the microglial marker F4/80 followed by IOD of the immunosignal in the SCN of both post-septic animals and saline treated controls that received peripheral LPS 100µg/kg, found no significant difference in microglial expression in the SCN between post-septic animals (2.14 $\pm$ 0.64 IOD/SCN) and saline controls (1.87 $\pm$ 0.26 IOD/SCN, P>0.05, independent t-test; Figure 5.5). When analysing the separate subdivisions of the SCN by independent t-test, there were no statistically significant differences found in the mean F4/80 immunosignal in either the ventrolateral core of the SCN (LPS, 1.80 $\pm$ 0.55 vs. controls 1.94 $\pm$ 0.62 IOD/SCN P>0.05) or in the dorsomedial shell region of the SCN (LPS, 2.23 $\pm$ 0.68 vs. 2.12 $\pm$ 0.31 IOD/SCN, P>0.05, independent t-test).

Manual quantification of immunostained microglial cells in post-septic and saline treated controls that received a second treatment of LPS 100µg/kg, was also performed and similarly found no significant differences in microglial expression in the SCN between post-septic animals (7.5 ± 2.26 ir.cells/SCN) and saline controls (6.12 ± 3.15 ir. cells/SCN, P>0.05, independent t-test). Further assessment of the morphology of F4/80 immunostained cells under high power and by manual quantification in post-septic and saline treated controls that received a second treatment of LPS 100µg/kg, found no significant differences in microglial morphology or in the expression of activated glia in the SCN between post-septic animals (0.60 ± 0.37 ir.cells/SCN) and saline controls (0.21 ± 0.08 ir. cells/SCN, P>0.05, independent t-test).

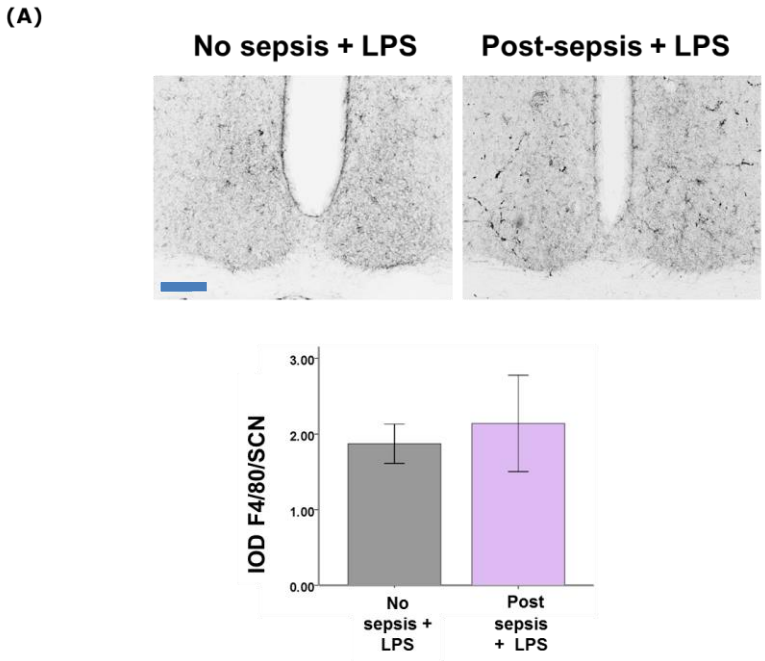


Figure 5.5: F4/80 expression in the SCN of post-septic and no sepsis animals following low dose peripheral LPS treatment. *(A) Representative photomicrographs of F4/80 expression in the SCN of post-septic animals (n=5) and animals treated initially with saline (n=7) following low dose LPS 100µg/kg treatment at ZT1-2 (scale bar = 100 µm). (B) Bar graphs illustrating F4/80 expression in the SCN of post-septic animals and animals treated initially with saline 4hrs following low dose peripheral LPS 100µg/kg treatment at ZT1-2.*

5.3.4. Assessment of SCN neuronal activity in post-septic animals 2hrs and 9hrs following an LPS 100µg/kg treatment 3mths post-sepsis or no sepsis.

Neuronal activation was assessed 2 and 9hrs following an LPS 100µg/kg i.p. treatment 3mths following sepsis or no sepsis. Immunohistochemical analysis of the immediate early genes c-Fos and EGR-1 coupled with the manual quantification of their expression in the SCN was performed at ZT3-4 and ZT10-11, 2hrs and 9hrs post-treatment respectively.

5.3.4.1. c-Fos expression 2hrs and 9hrs following LPS 100µg/kg i.p. 3mths post sepsis or no sepsis.

Mixed between within repeated measures ANOVA was used to assess whether there were alterations in the expression of c-Fos in the SCN following LPS 100µg/kg at different time points and whether there was an effect of treatment. There was no statistically significant effect of time ($F_{1,4}$=2.85, P>0.05) or between groups effect ($F_{1,4}$=0.29, P>0.05) on c-Fos expression levels in the SCN, with c-Fos expression levels at two hours (LPS, 28.00 $\pm$ 4.75 vs. saline, 30.97 $\pm$ 2.39 ir.cells/SCN) and at nine hours (LPS, 49.78 $\pm$ 13.93 vs. saline, 42.22 $\pm$ 7.46 ir.cells/SCN) post-treatment similar between groups (P>0.05). (Figure 5.6).

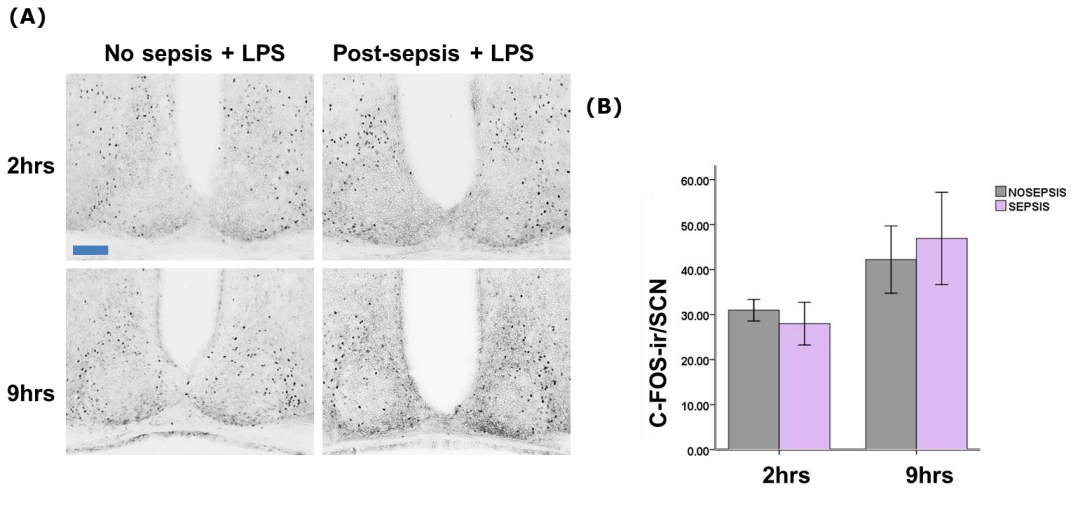


Figure 5.6: c-Fos expression in the SCN of post-septic and no sepsis animals 2 and 9 hrs following low dose peripheral LPS treatment. *(A) Representative photomicrographs of c-Fos expression in the SCN of post-septic animals (n=3-4) and animals treated originally with saline (n=4-5) 2 and 9 hrs following low dose*

LPS 100µg/kg treatment at ZT1-2 (scale bar = 100 µm). (B) Bar graphs illustrating expression levels of the IEG c-Fos in the SCN of post-septic animals and animals treated originally with saline 2 and 9hrs following low dose peripheral LPS 100µg/kg treatment at ZT1-2.

5.3.4.2. EGR-1 expression 2hrs and 9hrs following LPS 100µg/kg i.p. 3mths post sepsis or no sepsis.

The EGR-1 expression following LPS 100µg/kg was assessed between groups across time by Mixed between within repeated measures ANOVA, finding a statistically significant effect of time ($F_{1,4}$=31.81, P<0.01) on EGR-1 expression in the SCN, with EGR-1 levels decreasing from 2hrs post-treatment to 9hrs hours post LPS in both treatment groups, however analysis found no between groups effect ($F_{1,4}$=6.92, P>0.05). (Figure 5.7).

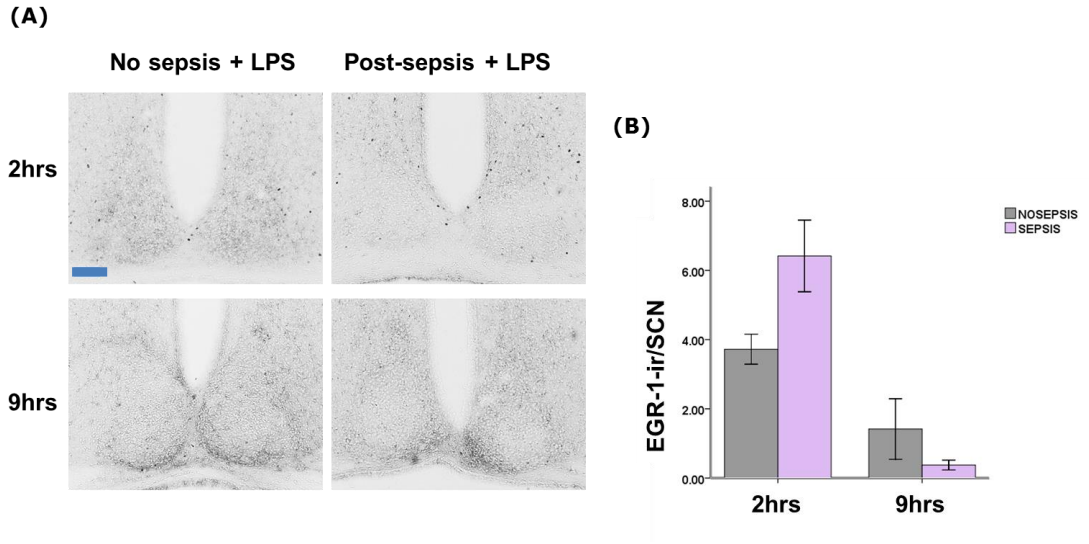


Figure 5.7: EGR-1 expression in the SCN of post-septic and no sepsis animals 2 and 9 hrs following low dose peripheral LPS treatment. *(A) Representative photomicrographs of EGR-1 expression in the SCN of post-septic animals (n=3-4) and animals treated originally with saline (n=4-5) 2 and 9hrs following low dose LPS 100µg/kg treatment at ZT1-2 (scale bar = 100 µm). (B) Bar graphs illustrating expression levels of the IEG EGR-1 in the SCN of post-septic animals and animals treated originally with saline 2 and 9hrs following low dose peripheral LPS 100µg/kg treatment at ZT1-2.*

5.4. Discussion:

In order to fully characterise the state of the post-septic SCN and circadian system and discern whether similarities exist between it and other conditions displaying a chronic neuroinflammatory component in response to further immune stimuli, we examined whether subsequent exposure to a peripheral immune challenge would result in a primed response as has been shown in ageing and neurodegenerative disease states. Additionally, examination of the brain in this manner would shed further light on post-septic circadian timekeeping mechanisms. To the best of our knowledge, these are the first studies assessing the responsiveness of the post-septic circadian system to a further peripheral immune challenge.

Peripheral LPS treatment has been shown to induce photic like phase delays of circadian locomotor behaviour when administered in the early subjective night at CT15 (Marpegan et al., 2005). A phase delaying LPS treatment did not result in a primed responsiveness in the post-septic circadian system, in fact the current study observes a significant reduction in phase delay magnitude in post-septic animals compared to animals initially treated with saline in response to a phase delaying LPS treatment. Animals previously treated with saline and subjected to a peripheral 100µg/kg LPS treatment over four weeks later show a phase delay in line with those shown by Marpegan et al (2005), who show that a 25 µg/kg LPS treatment induces a phase delay of approximately 43 minutes, while 250µg/kg treatment induces larger phase delays in the range of 60 minutes, but there is no significant difference in the delay magnitudes induced by the different doses. There was a decrease in rhythm amplitude following both low dose LPS and control treatments, however this was not due to an effect of prior treatment. Additionally, in the current study, LPS i.p. treatment caused a transient suppression of locomotor wheel running activity that was seen to last for a few hours following the peripheral CT15 treatment, and this suppression is as expected and has previously been observed in response to low dose peripheral LPS treatments shown by other studies (Marpegan et al., 2005; Paladino et al., 2010; Leone et al., 2012).

Various studies have shown the LPS induced phase delay to be decreased or inhibited in response to manipulation of immune signalling mechanisms. Administration of sulfasalazine, an NF-κB inhibitor prior to peripheral 25µg/kg LPS

treatment at CT15 reduced the LPS induced phase delay to approximately 15 minutes (Marpegan et al., 2005), a phase delay magnitude similar to that observed for the animals previously subjected to sepsis in the current study. Following 50µg/kg LPS peripheral treatment at CT15, TLR4-/- mice display significantly reduced LPS induced behavioural phase delays and do not show the LPS induced wheel running inhibition, while c-Fos and PER1 induction in the PVN were found to be significantly lower than in control animals (Paladino et al., 2010). Additionally, inhibition of TNF-α (but not of IL-1β) is shown to prevent the phase delays in wheel-running activity induced by peripheral LPS treatment, highlighting that TNF-α acts at the level of the hypothalamus to mediate the effects of a peripheral immune challenge on circadian behaviour (Leone et al., 2012). While administration of i.c.v TNF-α inhibitor blocked the LPS induced phase delay, the inhibition of locomotor activity was not blocked, indicating that LPS acts via alternate mechanisms to inhibit locomotion and to induce behavioural phase delays (Leone et al., 2012). The inhibition of LPS induced phase delays following administration of an inhibitor of an immune signalling pathway has been suggested to be possibly due to suppression of the release of LPS induced proinflammatory cytokines within the hypothalamus or in the periphery (Marpegan et al., 2005) and further, given the dependence of the LPS induced phase delay on TNF-α, TLR4 and NF-κB signalling mechanisms (Marpegan et al., 2005; Paladino et al., 2010; Leone et al., 2012), it is possible that alterations in the immune response of post-septic animals are indicated by the attenuations of LPS induced phase delays. Indeed the data presented by Leone et al (2012) following inhibition of TNF-α is analogous to the observations of the current study, whereby the LPS induced phase delay was significantly blunted in the post-septic animals, but the transient suppression of locomotor behaviour was still evident. It may be that the current study observes alterations in TNF-α release in the post-septic SCN in response to a further peripheral LPS treatment which could account for the attenuation in the LPS induced phase delay observed in post-septic animals. Further, the electrical activity of SCN neurons is altered following TNF-α treatment, with an increase observed in spontaneous firing rate, and inhibition of nitric oxide was seen to abolish these effects, indicating that TNF-α may act through the induction of nitric oxide to modulate the electrical output of the SCN, thereby altering circadian rhythmicity (Nygard et al., 2009). The assessment of SCN electrical activity in post-septic animals and saline controls following low dose LPS treatment would be

interesting, and may shed further light on post-septic circadian responsiveness. Further, it's possible that in the post-septic SCN alterations may be present in NF-κB signalling. The p65 NF-κB subunit has been shown to be acutely up-regulated in the SCN following peripheral LPS treatment (Beynon and Coogan, 2010) and administration of sulfasalazine, an NF-κB inhibitor, has been shown to block the LPS induced phase delay (Marpegan et al., 2005).

The current study observed an altered neuronal activation in the SCN of post-septic animals, with a significant upregulation of c-Fos activation evidenced in the SCN of post-septic animals following a peripheral low dose LPS treatment, over 4 weeks following the initial septic LPS treatment. This upregulation is indicative of an enhanced activation of neurons within the SCN, showing that the SCN responsiveness of these animals is attenuated, and therefore there are alterations in the circadian timekeeping system of these animals in response to stimulation by the peripheral innate immune system. In contrast to this, at 3mths following the initial LPS treatment, assessment of IEG expression 2 and 9 hrs post low dose peripheral LPS treatment showed there to be no significant differences between groups in IEG expression levels. It may be that this altered SCN neuronal activation dissipates with time and further work is required to establish whether this is the case. However the assessment of neuronal activation at 3mths post the initial treatment was at different times of the circadian cycle to that over 4 weeks following the initial treatment, and this could possibly play a role in SCN responsiveness and neuronal activation, since it has previously been shown that cytokine induction of Fos in the SCN varies depending on the time of the circadian cycle at which cytokine administration takes place (Sadki et al., 2007), and therefore further investigation is warranted to discern whether this is due to the period of time between subsequent LPS treatment or due to CT of assessment.

LPS treatment has been shown to impact upon the SCN and cause alterations in c-Fos expression. Palomba and Bentivolgio (2008) have shown photically induced c-Fos in the early subjective night to be attenuated 7 days after the final LPS treatment of a 2 month treatment regime consisting of weekly injections with LPS 1mg/kg. Additionally, a septic treatment of LPS has been shown in the acute stages following administration to induce significant expression of c-Fos in the SCN, along with expression of the P65 subunit of the NF-κB pathway, although this dose is much higher than that used in the current set of experiments (Beynon and Coogan,

2010). While c-Fos is associated with circadian function (Kornhauser et al., 1990), the precise role of c-Fos in the SCN in the generation of circadian oscillations is not fully understood and has yet to be clearly defined. Assessment of immediate early gene expression highlights variations in the responsiveness of neurons in the SCN (Mead et al., 1992). The dorsomedial shell has been suggested to have a role in synchronization via non-visual stimuli (Moga and Moore, 1997; Moore et al., 2002), and this SCN region has been suggested as a possible site for interaction between endogenous stimuli and the circadian system, and further, studies have suggested the SCN dorsomedial shell region to be the site where immune-circadian interactions take place, since low dose peripheral LPS induces c-Fos expression in the dorsomedial shell of the SCN (Marpegan et al., 2005). The current studies also support the notion that the dorsal portion of the SCN is involved in immune-circadian interactions, given that it is in the shell region of the SCN that c-Fos induction is observed following the peripheral LPS treatment. However, this dorsal SCN activation is altered in post-septic animals, since we observe significantly higher c-Fos expression in the dorsomedial shell of the post-septic SCN in response to a peripheral low dose of LPS, which in this context might indicate alterations in circadian-immune interactions at the level of the SCN. We do not observe any alterations in EGR-1 expression following low dose peripheral LPS treatment between the treatment groups.

A decreased neuronal activation in the post-septic SCN in response to subsequent LPS treatment might be expected since blunted LPS induced phase delays are observed in post-septic animals, and might reflect a decreased circadian responsiveness. However, we have not assessed neuronal activation in the SCN at CT15 in DD, the time at which the phase delaying LPS treatment was administered, and this would be interesting, since it may be that alterations exist at this time in post-septic animals in SCN responsiveness. It is clear however, that the SCN is indeed responding to the LPS treatment and therefore, it does not appear as though the induction of proinflammatory cytokines are suppressed in the post-septic CNS. Studies assessing c-Fos expression in the PVN in response to LPS at CT15 have shown decreased neuronal activation as evidenced by Fos induction in TLR4-/- mice (Paladino et al., 2010), showing that manipulation of immune signalling mechanisms appears to affect c-Fos induction in response to CT15 LPS treatment. It's possible that the enhanced post-septic SCN activation to a low dose LPS treatment could be

due to an exaggeration in cytokine levels within the post-septic SCN, indeed Sadki and colleagues (Sadki et al., 2007) have suggested that c-Fos expression in the SCN in response to cytokine treatment could possibly be dose dependent, and therefore an exaggerated cytokine release within the CNS might impact upon SCN c-Fos induction. However, an exaggerated proinflammatory cytokine release might also result in an exaggerated phase resetting in post-septic animals and in contrast to this, the current study observes a significant reduction in the LPS induced phase delay magnitude. Suppressors of cytokine signalling (SOCS) molecules play roles in regulating the intracellular effects of cytokines (Alexander, 2002) and SOCS 1 and 3 show diurnal variation in expression in the SCN, and have been suggested to be involved in the regulation of the sensitivity of the SCN to immune stimuli, since the time at which their expression is low correlates with both the phase at which cytokine induced c-Fos SCN expression is high (Sadki et al., 2007) and the time at which LPS induces circadian phase delays of locomotor behaviour take place (Marpegan et al., 2005). Examination of SOCS expression in the post-septic SCN might be interesting and may shed light on post-septic SCN responsiveness to further inflammatory stimuli as well as the post-septic innate immune system within the CNS.

It may be that we are observing long term alterations in the SCN or in the circadian timekeeping system specifically in the post-septic animals. Analysis of the PVN might provide further insight into the effects of small dose LPS on the post-septic circadian system. The SCN projects to the PVN which is involved in regulation of autonomic and endocrine outputs, including production of glucocorticoids (Kalsbeek et al., 2006) and it has been suggested that in this way the circadian system may be involved in the modulation of the immune system (Coogan and Wyse, 2008). The LPS receptor TLR4 is expressed in the PVN (Laflamme and Rivest, 2001), indicating that the PVN may be directly stimulated by and respond to an immune challenge with LPS (Leone et al., 2012), and further, administration of cytokines or LPS, causes c-Fos activation in the PVN (Marpegan et al., 2005; Sadki et al., 2007). Marpegan and colleagues (Marpegan et al., 2005) have shown strong induction of c-Fos in the PVN following LPS administration at CT15, and further, 50 μg/kg LPS treatment in the subjective night has been shown to induce PER1 expression in this hypothalamic region at CT15 (Paladino et al., 2010). Other studies have shown stress signals such as LPS to induce upregulation of the *Per1*

gene in the PVN CRF neurons, but not in the SCN (Takahashi et al., 2001). Given that the PVN has previously been suggested to be involved in mediating output information communicated from the SCN (Kalsbeek et al., 2010), it would be interesting to examine whether changes were evident in clock gene expression or neuronal activation within the PVN in the post-septic animals following low dose LPS treatment.

In these studies, a chronic upregulation of the microglial markers F4/80 and CD-11b has been observed in the post-septic SCN, however, in comparison to immunostaining at twenty four hours following LPS treatment, the microglia observed at the 3 month time point post-sepsis are morphologically distinct and do not appear traditionally activated, but rather display a resting phenotype. In response to an immune challenge in the CNS, microglia become activated and are seen to transition from a resting, ramified state to an active ameboid, de-ramified morphology and then through an intermediate "primed" state (Perry, 2004). It has previously been suggested that microglial priming may play roles in the neuroinflammatory state which is characterized by low cytokine levels, an increase in markers for activated microglia and an over-response to inflammatory stimuli (Hains et al., 2010). Certain aspects of this primed condition appear to correlate with the present studies, as well as the long-lasting neuroinflammatory state outlined by other groups using the same model as used here (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012). We therefore sought to examine whether primed microglia existed in the SCN of post-septic animals and would show an enhanced responsiveness to further inflammatory stimuli, since the CNS in these animals had previously shown an innate immune response, as well as altered SCN neurochemistry with a long-lasting upregulation of microglial markers, but no long-lasting alterations in cytokines or inflammatory mediators examined. However, following low dose peripheral LPS treatment, microglial examination through immunohistochemical staining for F4/80 and CD-11b revealed neither a significant change in the expression of microglial markers in the post-septic SCN compared to saline controls, or a difference in microglial morphology.

That we do not observe an alteration in microglial number or morphology in response to further LPS treatment does not necessarily mean that the microglia we observe in the post-septic SCN are not primed. In line with the microglial

observations in these studies, studies in ME7 mice have proposed microglia to be in a primed but not proinflammatory state, since microglia appearing to be activated in this instance do not significantly express the pro-infammatory cytokines IL-6 or IL-1β (Walsh et al., 2001) but do synthesise prostaglandin E2 (Minghetti et al., 2000). The long-lasting upregulation of microglial markers observed in the SCN in the current study is in the absence of an increased expression of proinflammatory cytokines examined at the three month time point, and so while the microglia in the post-septic SCN might not be traditionally activated, they may be in a primed state. Other studies utilizing tomato lectin immunohistochemistry to assess activated microglia in an ME7 mouse model of prion disease following an intracerebral injection of LPS, do not show alterations in microglial morphology in these animals subject to chronic neurodegeneration compared to controls (Cunningham et al., 2005), however while these microglia appear similar, those in the ME7 mice are shown to exhibit IL-1β staining (Cunningham et al., 2005). While CD-11b and F4/80 immunohistochemical analysis in the post-septic SCN does not reveal a morphologically activated phenotype, IBA-1 immunohistochemical staining has previously shown hypertrophic microglia at the three month time point in the post-septic brain. In line with this, morphologically activated microglia have been shown in the aged brain of both humans and rodents, and these show increased expression of CD68 and MHC II (Godbout and Johnson, 2009; Lucin et al., 2009), and it has previously been described that microglia may be primed or reactive and exhibit an increased expression of MHC II, and not necessarily produce inflammatory cytokines (Perry et al., 2003). Additionally, in aged mice, peripheral LPS resulted in overexpression of TLR2 in microglia and subsequently caused exaggerated induction of pro-inflammatory cytokines compared to young adults (Henry et al., 2009). Examination of markers such as MHC II and TLR2, which have shown to be upregulated on the surface of primed microglia is deserved in the post-septic SCN. Further, we have not examined IBA-1 immunostaining in the post-septic SCN following further LPS challenge, and it would be interesting to further assess microglial morphology using this marker. Given the exaggerated induction of proinflammatory cytokines by primed microglia in response to further stimulation, examination of proinflammatory cytokine expression in the post-septic SCN may be warranted following a peripheral low dose LPS treatment, and would help to clarify the state of the post-septic microglia within the SCN.

It is important to point out that we do not observe any alterations in microglial expression between post-septic animals and those treated with saline initially, despite post-septic animals having previously shown chronic upregulation of SCN microglia. It may be that the animals not having undergone septic LPS treatment are showing an increase in microglial expression in response to low dose LPS treatment. Indeed, various studies have shown the ability of low dose LPS treatment to induce a microglial increase in the CNS. Assessment of the neocortex following a single low dose peripheral 0.5mg/kg LPS treatment in mice has shown a significant upregulation in microglia both acutely and chronically, at 2, 7 and 28 days post-treatment (Kondo et al., 2011), while other studies in mice have shown 0.1mg/kg peripheral LPS treatment increases brain mRNA expression levels of MAC-1, a marker for microglial activation, and treatment with minocycline reduces this (Masocha, 2009). Given that we have previously observed significantly increased microglial expression in the post-septic SCN compared to controls, it might be expected that following low dose LPS treatment there would be higher levels of microglia in the post-septic SCN, were the low dose LPS treatment increasing SCN microglial expression, however this is not seen in post-septic animals. It may be then that there are alterations in the post-septic SCN in response to inflammatory mediators and further investigation of the low dose LPS microglial increase in the SCN is warranted.

Studies have shown that in situations of pre-existing pathology, proinflammatory cytokine expression is increased following a peripheral LPS treatment (Cunningham et al., 2005), examination of the expression of proinflammatory cytokines in the post-septic brain following a 100µg/kg LPS treatment would be interesting. In rats, peripheral LPS 100ug/kg has been shown not to cross the blood brain barrier, however to be capable of inducing proinflammatory responses as well as an increase in iNOS, through binding of specific receptors in the cerebral vascular endothelium (Singh and Jiang, 2004) and this could result in an innate immune hyper-responsiveness within a primed CNS, as well as enhanced behavioural responses. It has previously been indicated that the release of cytokines following LPS treatment which go on to act on the SCN is the method by which LPS impacts upon circadian function (Leone et al., 2006) and it is known that direct administration of immune factors affects locomotor behaviour (Crnic and Segall,

1992; Koyanagi and Ohdo, 2002) and is capable of phase-shifting SCN rhythmicity (Marpegan et al., 2005; Sadki et al., 2007; Boggio et al., 2003). Given the enhanced post-septic SCN neuronal activation observed in response to 100µg/kg LPS treatment, it may be that alterations in pro-inflammatory cytokine responses take place in the post-septic CNS and SCN at this time, accounting for the enhanced SCN neuronal activation. As already mentioned, the SCN in mice is seen to express mRNAs for TNF receptors I and II, and TNF-α has been suggested to act through the induction of nitric oxide to modulate the electrical output of the SCN, thereby altering circadian rhythmicity (Nygard et al., 2009), and further, it is TNF-α that has been shown to be required for the LPS induced CT15 phase delay (Leone et al., 2012). Alterations in TNF-α release in the post-septic SCN in response to a subsequent peripheral LPS treatment could possibly account for the attenuation in the LPS induced phase delays observed in post-septic animals. Indeed, various studies in conditions of pre-existing CNS pathology have highlighted altered proinflammatory cytokine responsiveness in response to both central and peripheral LPS treatment. ME7 mice treated with intracerebral LPS show marked microglial iNOS and IL-1β expression, as well as substantial neutrophil infiltration in comparison to controls (Cunningham et al., 2005). iNOS is commonly observed in neuroinflammatory studies, and further, the presence of the inflammatory mediator and NO synthesis hallmarks subsequent activation of primed microglia (Cunningham et al., 2005) and macrophages (Johnson et al., 1983). Increases in the levels of cytokine mRNA expression are seen in the brain following peripheral LPS treatment in both presenilin-1 mutant mice (Lee et al., 2002) and in amyloid precursor protein (APP) overexpressing mice (Sly et al., 2001). Further, ME7 mice treated i.p. with 500 µg/kg LPS resulted in the increased expression of IL-1β in microglia, and the induction of IL-6, TNF-α, PTX3 (pentraxin 3), and iNOS compared to control animals, along with neuronal apoptosis (Cunningham et al., 2005). Additionally, upon administration of a second systemic LPS challenge, microglia are seen to be the primary source of IL-12 that is produced (Stalder et al., 1997).

Together, these studies highlight the extent to which cells within the CNS are primed during conditions of chronic neurodegeneration and inflammation, showing enhanced cytokine responses to subsequent inflammatory challenges. Examination of proinflammatory cytokine expression in the post-septic SCN may prove beneficial following a peripheral low dose LPS treatment, helping to clarify whether the

microglia within the SCN at this time are in a primed state, and further, would help discern whether the alterations observed in the LPS induced phase delays in post-septic animals were due to alterations in the post-septic innate immune responsiveness, or due to alterations at the level of the SCN or circadian timekeeping system. Were there an exaggerated induction of proinflammatory cytokines, either higher levels do not have an effect on the phase delay magnitude at this time, or alterations within the post-septic SCN or output from it might result in the effects of this not being reflected in behavioural resetting.

It has been suggested that glucocorticoid action within the brain could result in exaggerated inflammatory responses to stimulation of the immune system (de Pablos et al., 2006; Munhoz et al., 2010). Further, peripheral administration of LPS activates HPA axis activity, increasing circulating levels of corticosterone and ACTH (Linthorst and Reul, 1998) and the HPA axis through the production and release of glucocorticoids from the adrenal cortex exert potent immunosuppressive and anti-inflammatory effects (Munck and Guyre, 1991; Auphan et al., 1995; Meier, 1996; Barnes, 1998; Sternberg, 2001; Webster et al., 2002). Additionally, the HPA axis and glucocorticoids have been suggested to play a role in endotoxin tolerance (Beishuizen and Thijs, 2003; Cavaillon et al., 2003; Ikeda et al., 2006; Evans and Zuckerman, 1991; Parrillo et al., 1993; Zuckerman and Qureshi, 1992) and in primed microglial responses to LPS (Frank et al., 2012). Together these studies highlight the GC involvement in LPS and CNS immune responses. A long-term increased HPA axis sensitivity is observed following cytokine treatments. IL-1β is induced by LPS treatment, and a single IL-1β treatment induces an increased responsiveness of the HPA axis which is seen to last up to 22 days, but not at 42 days post-treatment and involves the PVN (Schmidt et al., 2001). Further, TNF-α, also known to be involved in LPS responses (Qin et al., 2007) was seen to cause long-lasting HPA sensitization which was associated with increased intra-PVN noradrenergic storage (Hayley et al., 1999). Cytokine mediated alterations of the function of the HPA axis and glucocorticoid receptor function may further modulate inflammation (Capuron and Miller, 2011), and this alteration in endocrine function could feedback to the SCN, impacting upon circadian immune function and SCN responsiveness, and have implications for circadian resetting in response to further immune stimuli, and therefore examination of a possible role for glucocorticoids in the post-septic responses to further LPS treatment would be interesting.

Since prior LPS administration and activation of the innate immune response could result in either a hyper or hypo response within the CNS in response to further immune challenges, and both *in vivo* and *in vitro*, low LPS doses have been shown to result in a tolerant state where the immune response to further challenge with LPS is altered (Biswas and Lopez-Collazo, 2009; Yoza and McCall, 2011), the possibility that endotoxin tolerance may account for the attenuations in the post-septic circadian system in response to a subsequent immune stimulus must be addressed. Indeed, the suppression of the LPS induced phase delay would indicate that the LPS effects upon the circadian system are attenuated, and it's possible that this could be due to a tolerant state within the CNS induced by the previous septic LPS challenge. Further, endotoxin tolerance has been shown to be acutely induced in studies utilizing the same LPS dose as that used in the current study followed by CLP (Wheeler et al., 2008). The diminished LPS induced phase delay might be observed in states of endotoxin tolerance as a result of suppression of the expression of pro-inflammatory mediators known to phase shift the clock (Leone et al., 2012) upon subsequent exposure to LPS. However, we do not believe the results from the current set of studies can be explained by endotoxin tolerance, since the effects of endotoxin tolerance have only been shown to persist *in vivo* up to three weeks (West and Heagy, 2002), and therefore upon further peripheral LPS treatment over four weeks following the initial LPS treatment, it is unlikely that endotoxin tolerance is accountable for the attenuation in LPS induced phase delays of the circadian locomotor rhythm. In line with this assessment, endotoxin tolerance was not observed following two peripheral 50ug/kg LPS treatments at CT15 29 days apart (Leone et al., 2012). Further, were the post-septic CNS in a state of endotoxin tolerance at this time, then it is unlikely that an exaggerated SCN c-Fos responsiveness would be observed following a peripheral immune treatment, given that this highlights that the SCN is responding to the inflammatory challenge and it therefore appears as though the induction of proinflammatory cytokines is not prevented in the post-septic CNS. Moreover, studies have shown that while in the periphery during endotoxin tolerance peripheral cytokines are no longer induced, LPS induced cytokine expression remains evident in the CNS (Chen et al., 2005; Faggioni et al., 1995) and therefore the impact of cytokine induction may still allow a phase delay and the suppression of this could not be explained by endotoxin tolerance.

The alterations observed in clock gene expression in the post-septic SCN could play a role in the effects of a low dose LPS treatment on the post-septic SCN and on circadian behaviour. Inflammatory mediators induced by peripheral LPS may impact upon the SCN inducing functional changes and alterations in clock gene expression following LPS administration, and the ability of LPS to impact upon clock gene expression has been shown by various different studies (Takahashi et al., 2001; Marpegan et al., 2005; Palomba and Bentivoglio, 2008; Okada et al., 2008). Additionally, alterations in clock genes have been shown to affect immune responses. Murine studies using chronic jet lag paradigms or SCN ablation to induce impairment of molecular clock function have shown an increased rate of tumour growth, linked to perturbations in circadian oscillations in the levels of circulating lymphocytes (Filipski et al., 2003, 2006). CJL mice show altered clock gene expression in the periphery, the SCN and in immune cells, as well as enhanced sensitivity to endotoxin administration (Castanon-Cervantes et al., 2010). Loss of IFN-γ circadian expression is seen in mutant *Per2* animals (Arjona and Sarkar, 2006a, b) along with alterations in NK cell function and decreases in proinflammatory cytokine production of IL-1β and endotoxic shock resistance following LPS treatment (Liu et al., 2006), indicating a role for the clock gene in mediating the LPS induced immune response. Since TNF-α has been shown to impact upon clock gene expression (Cavadini et al., 2007; Petrzilka et al., 2009), and to be necessary for the LPS induced phase delays at CT15, it has been suggested to elicit phase shifts by altering the expression of clock genes (Leone et al., 2012), it is therefore possible that TNF-α release following low dose peripheral LPS treatment could further alter clock gene expression in the post-septic SCN, impacting upon the phase shifts induced. Alterations in clock gene expression in the post-septic SCN could account for altered responsiveness in the post-septic animals to further endotoxin treatment, and given that the current study finds alterations in the expression of PER2 in the post-septic SCN *in vivo*, it's possible that these attenuations could play a role in the immune response or the response of the SCN in post-septic animals to phase-shifting immune stimuli.

It seemed a likely consequence that in response to further peripheral immune stimulation, a primed response would be seen within the post-septic brain. Systemic infection has been shown to lead to sustained upregulation of microglial markers

(Weberpals et al., 2009) and prolonged cytokine synthesis within the CNS (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012, Puntener et al., 2012), and more recently, with priming of brain innate immune cells to subsequent focal inflammatory challenge by LPS in the brain parenchyma (Puntener et al., 2012). Further, since ageing is associated with a low-grade central neuroinflammation (Godbout and Johnson, 2009), including in the SCN (Deng et al., 2010) and the current study observes similarities between circadian resetting in post-septic animals, and those in studies assessing the effects of healthy ageing on circadian resetting (Rosenberg et al., 1991; Zee et al., 1992), and the ageing CNS also exhibits primed responsiveness to inflammatory stimuli, it seemed plausible that the post-septic SCN might be primed and that further perturbations in circadian resetting might be observed in the post-septic brain to immune stimuli. Additionally, conditions of chronic neurodegenerative disease known to have an inflammatory component exhibit primed responses within the CNS as well as exaggerated behavioural responses to peripheral immune insults, and LPS administration through both i.p. routes and direct administration to the CNS have been used to model chronic neurodegenerative disease states. Chronic i.c.v. infusion of 0.25µg/h of LPS has been used to model Alzheimer's Disease (Hauss-Wegrzyniak et al., 1998), while 2µg intranigral LPS delivery has been used to model Parkinson's Disease (PD) (Herrera et al., 2000), and the single peripheral septic LPS treatment used in the current set of studies has been used to model pathological features of both AD and PD (Qin et al., 2007). However, it has been suggested that these models do not fully represent the pathogenesis of these neuroinflammatory diseases (Cunningham, 2013). While both the CNS of chronic neuroinflammatory diseases and that following LPS treatment show increased microglial cells, the levels of proinflammatory cytokines induced by LPS are much higher than those in the chronic disease states and may therefore not fully reflect the pathogenesis of these diseases (Cunningham et al., 2013), for example, low expression levels of IL-1β, TNF-α and IL-6 are seen in animal models of PD and AD (Sly et al., 2001; Depino et al., 2003), similar to the CNS of the ME7 prion disease model (Walsh et al., 2001; Cunningham et al., 2002; Betmouni et al., 1996). With this in mind, long-term primed responses may not be exhibited by the cells of the innate immune system within the post-septic CNS, and it is clear that primed behavioural responses are not shown by the post-septic circadian system in response to a phase delaying LPS treatment.

Overall, the current results indicate that either the innate immune response of the post-septic animals is altered in how it responds to a low dose LPS challenge, or that the altered response can be explained by perturbations specific to the SCN or the circadian system itself. Immune factors have been suggested to play roles as entraining factors of the circadian system, given their ability to phase shift circadian locomotor rhythms (Marpegan et al., 2005) and the current study observes an alteration in circadian resetting properties in post-septic animals in response to peripheral LPS treatment. The results of the current study are reminiscent of those where components of the innate immune system are inhibited or immune receptors absent (Marpegan et al., 2005; Paladino et al., 2010; Leone et al., 2012). Additionally, alterations are observed in the induction of c-Fos expression in the SCN in post-septic animals and while this could be indicative of altered input to the SCN, is may also be due to an altered SCN response to immunomodulation. Further, it may be that circadian resetting alterations are due to perturbations within the SCN itself, or output from the SCN, or further, that it is downstream from the SCN that alterations are manifested, such as at the level of the PVN resulting in alterations in output.

As well as the mechanisms previously mentioned, there are multiple routes by which peripheral LPS may affect the CNS (Capuron and Miller, 2011), and therefore, there are a variety of parameters that may be attenuated and could impact upon the way in which the post-septic CNS reacts to additional challenge, or previous attenuations may be further modulated following subsequent exposure to inflammatory stimuli, and these could underlie the post-septic circadian resetting alterations. Peripheral infections may propel the process of degeneration in the CNS (Combrick et al., 2002). In models of chronic neurodegeneration subjected to subsequent immune stimuli, acute neuronal death is observed (Cunningham et al., 2005) and so assessment of apoptosis in the post-septic SCN following a low dose peripheral LPS treatment may be warranted, given that the model used in this study is one which shows delayed neuronal loss in the substantia nigra (Qin et al., 2007), and subsequent inflammatory stimuli may exacerbate this or indeed induce apoptosis in other CNS regions. Additionally, it would be interesting to assess the electrophysiological properties of SCN neurones post-sepsis, and to further examine the neurochemical composition of the SCN in post-septic animals, since changes in the GABAergic network have been shown in the SCN in ageing (Palomba et al.,

2008), and such alterations could explain alterations in SCN function and responsiveness.

Attenuations in neurotransmission in the post-septic brain could cause alterations within the CNS as a result of LPS induced cytokine modulations, since studies of the CNS in sepsis have observed evidence for altered neurotransmission (Van der Poll et al., 1996, Van der Poll, 2000; Toklu et al., 2009; Freund et al., 1985) and alterations in these parameters could underlie the perturbations in phase delays in post-septic animals in response to further LPS treatment. The outflow of the neurotransmitter Acetylcholine (Ach) has been shown to be affected by IL-1β expression (Taepavarapruk and Song, 2010), which is known to be released in response to LPS treatment. Further, the assessment of cholinergic transmission in the SCN post sepsis would be interesting. The cholinergic anti-inflammatory pathway plays roles in the modulation of inflammation, stimulating the parasympathetic nervous system to release acetylcholine which regulates the immune response (Pavlov et al., 2003). Given that cholinergic input to the SCN is involved in the function of the circadian system (Hut and Van der Zee, 2011), it's possible that alterations in the cholinergic system such as the loss of cholinergic fibres previously shown in rats following high dose endotoxin (Semmler et al., 2007) could play a role in the altered responses to LPS and attenuations in LPS induced phase delays in the current experiment. In animals with pre-existing cholinergic neuronal loss, acute working memory impairments are observed following a systemic LPS challenge and this can be inhibited by administration of donepezil, an acetylcholinersterase inhibitor (Field et al., 2012). Together, these studies highlight that alterations in cholinergic pathways and neurotransmission could potentially play a role in attenuated responses following LPS treatment.

The current studies further illustrate that there are long-term perturbations in the post-septic circadian system, however the data from the current study cannot elucidate the mechanism by which this happens. It's possible that these attenuations could be due to alterations within the post-septic innate immune system within the CNS, or indeed may be due to changes in the post-septic SCN or circadian timekeeping mechanisms themselves. Further, given that there are altered responses in the long term post sepsis to peripheral immune stimuli, it may be that there are alterations in circadian-immune cross talk. Further work is required to delineate the mechanisms underlying this altered circadian responsiveness.

Chapter Six

General Discussion

The overall goal of these studies at the outset was to examine the long-term effect of a substantial immune challenge on timekeeping processes. The model used in the current set of studies is one that exerts a long-lasting impact on the innate immune system in the CNS, and this long-lasting neuroinflammation causes alterations within the CNS including delayed neurodegeneration, alterations in learning and memory, alterations at the level of synaptic proteins, as well as changes in cerebral metabolism (Weberpals et al., 2009; Qin et al., 2007; Bossu et al., 2012), however, there are no studies that investigate the chronic alterations at the level of the master pacemaker or the impact upon circadian timekeeping mechanisms. These studies have fulfilled the original objective, and have characterized the long-lasting impact of a profound immune insult on circadian behaviour, on SCN molecular oscillations and SCN responsiveness. The core circadian locomotor behaviour parameters and circadian resetting behaviour were assessed in the long-term post-sepsis, and attenuations in post-septic photic resetting and reentrainment have been described. Further, these studies have highlighted chronic changes in post-septic SCN clock gene protein product expression, attenuations in post-septic SCN functional activation in response to both photic and immune stimulation, as well as alterations in post-septic circadian resetting in response to phase resetting immune stimuli. The current set of studies therefore add to the experimental evidence describing the long-lasting impact of a systemic immune challenge on CNS function, and address a gap in the literture as to the chronic effects of a profound immune challenge on the circadian timekeeping system.

Strong links have been shown in recent years in communication between the immune system and the circadian system. The data presented here highlights important influences between the immune system and the circadian system, and adds to the literature on peripheral immune signals reaching the SCN. The acute impact of immune mediators on the circadian system has been well documented, and immune mediators have been shown to affect various circadian parameters including electrical firing rhythms (Nygard et al., 2009), behavioural phase resetting (Marpegan et al., 2005; Paladino et al., 2010; Leone et al., 2012) and clock gene

expression both centrally and peripherally (Okada et al., 2008; Koyanagi and Ohdo, 2002; Cavadini et al., 2007). Studies assessing the impact of immune mediators on the SCN clock all appear to have shown a decrease in the expression of clock and clock controlled genes, in line with the dampening of clock gene protein product expression observed in the long-term in the current studies (Okada et al., 2008; Koyanagi and Ohdo, 2002; Cavadini et al., 2007; Logan and Sarkar, 2012), therefore the results presented here are in line with, and contribute to, the literature highlighting the repression of clock gene expression following immune activation. Further, the effects of chronic LPS treatment have been shown to impact upon the master pacemaker (Palomba and Bentivoglio, 2008), and the current studies now add to this, outlining the chronic effects of a single septic LPS challenge on the SCN and circadian timekeeping processes, and support the notion that chronic inflammation might underlie circadian timekeeping perturbations, since circadian alterations have been shown in studies of chronic neuroinflammatory states and chronic infection (Hatfield et al., 2004; Rosenberg et al., 2001; Huitron-Resendiz et al., 2007; Lundkvist et al., 2002; Kennedy, 2009; Kristensson et al., 2010). It was thought that a chronic neuroinflammation could possibly occur within the SCN, given the long-lasting neuroinflammation induced in other regions of the CNS by the septic endotoxin model (Qin et al., 2007; Weberpals et al., 2009; Bossu et al., 2012), and that this could play a role long term in circadian timekeeping processes, however, the experimental results obtained cannot attribute the long-lasting perturbations of the post-septic circadian system to the chronic presence and action of the examined proinflammatory mediators at the site of the SCN, but do show long term microglial changes, the significance of which requires further investigation.

Overall, the results from these studies point to an as yet unknown mechanism underpinning the long-lasting changes in the post-septic circadian system. These studies cannot currently elucidate a mechanism by which timekeeping processes are impacted post-sepsis, and raise many new questions which could form the basis of future investigations addressing whether the post-septic perturbations are due to an altered input to the SCN, given the alterations in post-septic SCN responsiveness, due to perturbations in output from the SCN, or whether it may be downstream from the SCN that alterations are manifested. Further extensive studies would be required in order to delineate a mechanism by which LPS induced sepsis chronically impacts upon circadian timekeeping processes.

An interesting future endeavour would be to examine electrical neuronal firing rhythms in the post-septic SCN, since these are crucial in the generation of coherent circadian output and transmitting circadian timing information to the brain and periphery (Schwartz et. al., 1987). Intercellular synchronization can influence phase-shifting responses (VanderLeest et al., 2009), and inflammatory mediators are known to impact upon the SCN at the electrophysiological level (Kwak et al., 2008; Nygard et al., 2009; Lundkvist et al., 2002), and therefore perturbations in post-septic SCN electrical activity could potentially underlie alterations in circadian resetting post-sepsis.

Additionally, it would be insightful to examine SCN functional activation in response to low dose LPS treatment at CT15, as would the examination of what underlies the perturbations in photic SCN functional activation observed under LD conditions but not under DD, and would further add to our knowledge of the post-septic circadian system.

Due to the specificity of photic induced perturbations and SCN photic responsiveness post-sepsis all involving phase advances or occurring during the advance portion of the PRC, future studies should examine parameters whose perturbation could underlie these phase specific attenuations, such as examination of the NO-cGMP-PKG pathway (Plano et al., 2012). Enhanced late subjective night photic induced phase advances of locomotor activity (Plano et al., 2012) and accelerated resetting following a 6h phase advance of the photocycle (Agostino et al., 2007) are seen following prevention of cGMP degradation, and it may be that perturbations in this pathway could underlie the attenuations in post-septic photic phase advance resetting and photoperiod advance reentrainment. The examination of the degree of molecular desynchrony in the SCN that is suggested to underlie gradual phase advance resetting (Reddy et al., 2002) would also be interesting in post-septic animals.

Further, glucocorticoid rhythms have been demonstrated to influence the speed of re-entrainment to shifts in phase (Davidson et al., 2009), and studies have pointed to a role of the corticosterone rhythm in entrainment of behavioural rhythmicity through modulation of light effects on the pacemaker. The SCN responds to glucocorticoid information (Briski et al., 1997) and an SCN-adrenal gland feedback loop has been suggested (Kiessling et al., 2010), whereby the SCN receives photic information and subsequently signals to the adrenal gland through neuronal

connections influencing the expression of clock genes in the adrenal, the adrenal in turn feeds back via the indirect communication to the SCN, possibly via glucocorticoid sensitive serotonergic neurons in the midbrain raphe (Sage et al., 2004), regulating the SCN's control of locomotor rhythmicity (Kiessling et al., 2010).

In situations where corticosterone levels or rhythms are attenuated, faster resetting kinetics in rhythmic behavioural locomotor activity (Kiessling et al., 2010; Sage et al., 2004; Mohawk et al., 2005), as well as more rapid resetting of the phase in the periphery and in brain regions, including the SCN (Pezuk et al., 2012), are seen in response to phase shifts of the photoperiod. Stable entrainment can be seen in cases where underlying glucocorticoid rhythms are out of phase with the natural rhythm and its relationship to the light dark photoperiod (Albers et al., 1985), and experimental manipulation of the glucocorticoid rhythm does not impact upon locomotor behaviour under DD or conditions of stable entrainment, including no effect on locomotor behavioural synchronization or entrainment properties (Sage et al., 2004). Further, restoration of rhythmic corticosterone secretion in phase with the new photocycle following the photoperiod shift re-establishes normal rates of synchronization (Sage et al., 2004).

It has been suggested that circulating glucocorticoids act to stabilise rhythmic locomotor behaviour, by strengthening the resistance of the timekeeping system to photoperiodic manipulations (Sage et al., 2004), or preventing uncoupling following phase shifts of the photoperiod (Pezuk et al., 2012), and that when normal rhythmic glucocorticoid secretion is absent, the animals are dependent on the L:D cycle alone and so reentrain faster to the phase shift (Sage et al., 2004). Similarities exist between the above studies and the data we have obtained on locomotor behaviour in post-septic animals, with no alterations in locomotor entrainment properties or core circadian parameters, but attenuated resetting kinetics. Further, impairment of the HPA axis is seen in humans and in experimental sepsis studies in rodents, both in the acute and post-acute phase (Carlson et al., 2006; Polito et al., 2011), and inflammatory mediators have been shown to alter corticosterone rhythms (Shinohara et al., 2008). Therefore, it's possible that alterations in these rhythms could underlie the changes seen in the behavioural experiments post-sepsis during advance re-entrainment and further studies assessing rhythmic corticosterone following the

induction of the sepsis and during the time course at which manipulations of the light dark cycle resulted in altered resetting would be interesting.

The sepsis inducing endotoxin model used in the current set of studies provides important insights into the long-lasting impact of a profound immune insult on the circadian system in mice, however currently, we can only speculate what effects this might have in nature and in the human population. Care must be taken when interpreting translatability of results between experimental animal models and human sepsis patients, since human sepsis patients are treated with a variety of therapies, while many animal models, such as that used in these studies, show a lack of supportive interventions (Buras et al., 2005). Furthermore, LPS induced sepsis in animal models may not accurately replicate some important features of human sepsis, such as the rate of disease progression, the magnitude and rate of induction of cytokine responses, and the initial hyperdynamic cardiovascular state observed in human sepsis (Buras et al., 2005; Deitch, 1998; Hollenberg, 2005; Fink et al., 1990; Nemzek et al., 2008). Mice have also been shown to be more resistant to endotoxin treatment than humans, and this may limit the extrapolation of results (Copeland et al., 2005), and previous studies assessing sepsis treatments in experimental animal sepsis models did not translate in human clinical trials (Buras et al., 2005; Deitch, 1998). Additionally, these studies are performed in a nocturnal animal model, the results from which may not directly translate to diurnal species and further, the animal model used in these studies is naturally deficient in melatonin, and melatonin rhythms play strong roles in the circadian regulation of the immune response (Logan and Sarkar, 2012), and the attenuation of melatonin rhythms has been shown in the early stages of sepsis in humans (Li et al., 2013). It might be pertinent for extrapolation of results and transferability to examine the long-lasting impact of sepsis on circadian timekeeping processes in a diurnal animal model, and in the presence of rhythmic melatonin.

Immunohistochemistry is a widely used method of protein detection that is fast and is adequately sensitive when utilizing an appropriate detection antibody, however IHC only allows qualitative or semiquantitative analysis of protein expression, and a further limitation of this method lies in the fact that quantification of immunostaining may be somewhat subjective (Guardigli et al., 2005). Protein detection by other methods such as Western blotting and ELISA allows quantitative,

objective analysis. Additionally, real-time PCR is a very sensitive technique allowing the quantification of the mRNA encoding the protein of interest (Bustin, 2000; Guardigli et al., 2005). It might be interesting to utilize these protein detection techniques to further assess the post-septic CNS, however these methods have their own associated limitations (Guardigli et al., 2005). The main advantage of Immunohistochemistry over these other protein detection techniques is the ability of IHC not only to detect the presence of a particular protein, but to demonstrate the location of this within a tissue or cell, and further, antigens can be detected in the context of cellular morphology (Ramos-Vara, 2011; Webster et al., 2009) which was required for, and proved informative in the current set of studies.

Recently, studies in septic patients have shown circadian attenuations in the acute phase following sepsis induction, and have highlighted a potential link between the progression of early stage sepsis and circadian rhythms (Li et al., 2013). The current studies now add to the literature concerning circadian perturbations and sepsis, and illustrate for the first time the long-lasting effects of sepsis on circadian rhythms in mice. Up to 50% of sepsis survivors are seen to suffer from post-septic encephalopathy which is characterized by long-lasting cognitive impairment including attenuations in concentration, attention and memory and/or a global loss of cognitive function (Streck et al., 2008), and neuroinflammation has been implicated in the pathogenesis of various disorders associated with cognitive impairment (Nelson et al., 2002; Katsuse et al., 2003; Perry et al., 2003), including Septic Encephalopathy (Jacob et al., 2010). Further, circadian rhythms have been suggested to play various roles in cognition (Schmidt et al., 2007). In light of the results of the current study which show long-lasting perturbations in circadian timekeeping following utilization of the septic endotoxin model, along with the fact that this model is known to cause a long-lasting neuroinflammation in the CNS, it is possible that circadian attenuations could be involved in the long term cognitive impairment that persist following recovery from the acute phase of septic shock.

These studies have highlighted the long-lasting impact of LPS induced sepsis on the circadian timekeeping system, but it is important to note, that these studies have not shown any major perturbations in the post-septic SCN's ability to

coordinate timekeeping processes under conditions of stable entrainment or under constant conditions, so on a daily basis, post-septic animals do not show obvious behavioural attenuations, and in fact, their behaviour appears similar to controls. It is in response to photic manipulations, specifically phase advancing stimuli and advances of the photoperiod, and phase resetting immune stimulation that post-septic circadian responses are attenuated. Immune factors have been suggested to play roles as entraining factors of the circadian system, given their ability to phase shift circadian locomotor rhythms (Marpegan et al., 2005) and the results from the priming experiments superimposing phase resetting immune stimulation onto a background of attenuated circadian responses and SCN molecular rhythms were interesting, and pose questions regarding immune communication with the circadian system following recovery from the acute phase of sepsis. It may be that there are alterations in circadian-immune cross talk post-sepsis, and is possible that a profound immune challenge such as sepsis results in a disconnect between the circadian system and the immune system, perturbing the bidirectional communication.

Chronic circadian disruption has been shown to significantly perturb immune responses, and circadian coordination of the immune system has been suggested to promote responsiveness to an immune challenge, and disruption of this coordination could impose vulnerability upon organisms (Castanon-Cervantes et al., 2010). Immune mediators have been shown to impact upon the organisation of the clock and timekeeping processes, which could in turn result in further perturbation of the immune system through dysregulated clock processes, and vulnerability of an organism to future challenge (Logan and Sarkar, 2012). Interestingly, experimental evidence indicates that the response to phase-advancing schedules, as opposed to phase delay schedules, appears to be more detrimental for health (Davidson et al., 2006). The responsiveness of the post-septic circadian system may be restricted or modulated in circumstances where perturbations of circadian timing which could impact upon immune function are encountered in a system already attenuated and vulnerable having been subjected to a severe immune challenge. However, it's possible that this might also detrimentally impact upon circadian responsiveness post-sepsis to the external environment, given the necessity of accurate phase-shifting responses of the circadian system to perturbing stimuli for adequate entrainment and adaptation of organisms.

In conclusion, the findings from this project provide further insight into immune circadian communication, however there is still much work to be done assessing the impact of the immune response on circadian timekeeping processes.

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