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Is there a role for NF-κB in the suprachiasmatic circadian clock?

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

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Abstract

Circadian rhythms are recurring patterns in behavioural, physiological, and other parameters that display periods of approximately every twenty four hours. The molecular basis of the circadian timekeeping involves clock genes which act as transcription factors in a serious of feedback loops. Disruption of clock genes in transgenic models leads to dysregulation of the temporal architecture of behaviour and physiology. The master circadian pacemaker is the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. There are circadian oscillators in numerous brain areas and peripheral organs and tissues.

NF-κB is composed of dimers of members of the Rel family, including p65 (RelA), p50, p52, c-Rel and Rel B. NF-κB transcription factors are required for regulating cell survival and differentiation and also are important for inflammatory and immune responses. Dysregulation of this transcription factor has been shown to lead to inflammatory and autoimmune diseases. NF-κB is expressed in neurons and glia throughout the brain.

We hypothesised that the NF-κB pathway may play a role in the generation and perhaps the phase setting of the circadian rhythms in the master mammalian circadian pacemaker. Understanding the role NF-κB system may play in the free running circadian clock, may be of great importance in understanding the effects of ageing, neuroinflammation and neurodegeneration on circadian rhythms. We set out to examine whether there might be a role for the NF-κB system in the master suprachiasmatic clock. We have examined expression of constituents of the pathway across the circadian cycle in the SCN, we examined the effects of pharmacological inhibition of the pathway on circadian processes *in vivo* and *in vitro* and have examined whether there is evidence for circadian regulation of NF-kB-mediated transcription in the SCN. Furthermore, we examined whether the NF-κB pathway played a role in ageing.

Overall, we found no evidence that the NF-kB pathway is involved in the basal functioning of the SCN clock or in photic resetting. We do find some evidence that the NF-kB pathway may have a role in transducing immune events in the SCN. This data suggest that while the NF-kB pathway may be involved in circadian function in peripheral clocks, it does not appear to play a major role in the SCN.

Abbreviations

3v – third ventricle

5HT - serotonin

AD - Alzheimer's disease

ALS - amyotropic lateral sclerosis

ANS - autonomic nervous system

ARC - arcuate nucleus

Arc - activity-regulated cytoskeletal-association protein

AVP - arginine vasopressin polypeptide

BAFF - B-cell activating factor

BBB – blood brain barrier

BMAL1 - Brain and Muscle ARNT-like protein 1

BNST - bed nucleus of the stria terminalis

CALB - calbinin

CK2 - casein kinase 2

CLC - cardiotrophin-like cytokines

CLOCK - Circadian Locomotor Output Cycles Kaput protein

CNS – central nervous system

CRE - cAMP-responsive elements

CREB – cyclic-AMP response element binding protein

Cry – Cryptochrome

CSNK1E - casein kinase 1 epsilon

CT – circadian time

CVO - circumventricular organs

DA – dopaminergic

DD – constant darkness

DMH - doromedial hypothalamus

DSPS - delayed sleep phase syndrome

dSPZ - dorsal subparaventricular zone

DSS - dextran sodium sulfate

ELISA - enzyme-linked immunosorbent assay

ENK - encephalin

ERK - extracellular signal related kinase

FASPS - familial advanced phase sleep syndrome

fMRI - functional magnetic resonance imaging

FRP – free running period

GABA - γ-aminobutyric acid

GFAP - glial fibrillary acidic protein

GHT - geniculohypothalmic tract

GRP - gastrin releasing peptide

H_2O_2 - hydrogen peroxide

HDx1 – exon-1

Htt – huntingtin

IBD – inflammatory bowel disease

IEGs – immediate early genes

IGL - intergeniculate leaflet

IHC - immunohistochemistry

IκB - I-KappaB's

IκK – I-kappaB kinase

IKK - IκB kinase complex

IL-1 - interleukin-1

IOD - integrated optical density

i.p – intraperitoneal

ir – immunoreactive

IRAK1 - IL-1 receptor-associated kinase

JNK - c-Jun NH_2-terminal kinases

KO – knockout

LBP – LPS binding protein

LD – light/dark

LL – constant light

LPS - lipopolysacchaires

LT-β - lymphotoxin-β

LTP - long term potentiation

MAPKs - MAP kinases

MBH - mediobasal hypothalamus

MKK - mitogen-activated protein kinase kinase

MPOA - medial preoptic area

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

M.S – multiple sclerosis

MyD88 - myeloid differentiation primary response protein 88

NaCl - saline

NBD - NF-κB essential modifier-binding domain

NF-κB - Nuclear factor-kappa B

NGS – normal goat serum

NHS – normal horse serum

NiDAB - nickel-enhanced diaminobenzidine

NIK - NF-κB inducing kinase

NLS - Nuclear Localization signal

NMDA - N-methyl-D-aspartate receptor

NO – nitric oxide

NPAS2 – neuronal PAS domain protein 2

NPY - Neuropeptide Y

OC - optic chiasm

PACAP - pituitary adenylate cyclase-activating peptide

PAMPs – pathogen-associated molecular patterns

PB – phosphate buffer

PD - Parkinson's disease

Per – period

PFA – paraformaldehyde

PKC - protein kinase C

PMT - photomultiplier tube

PolyQ – polyglutamine

PRC – phase response curve

PRR – pathogen recognition receptors

PVN - paraventricular nucleus

RA - rheumatoid arthritis

REMS - rapid eye movement sleep

RHD - Rel Homology Domain

RHT - retinohypothalamic tract

RORE - Retinoid-related orphan receptor response element

RORα - Retinoid-related orphan receptor α

RT PCR – Real-time PCR

SCN - suprachiasmatic nucleus

SNpc - substantia nigra pars compacta

SP - substance P

SPZ - subparaventricular zone

SSRI - selective serotonin reuptake inhibitor

SWS - slow-wave sleep

TAD - transcriptional activation domain

TAK1 - Transforming growth factor-B-activated kinase 1

TBI - traumatic brain injury

TIRAP - TIR domain-containing adaptor protein

TNF-α - tumour necrosis factor-alpha

TLR – toll-like receptor

TRAF-6 - TNF receptor-associated factor 6

TRAM - TRIF-related adaptor molecule

TRIF - TIR domain-containing adaptor protein inducing IFNβ

UBC13 - ubiquitin-conjugating enzyme 13

UC – ulcerative colitis

UEVIA - ubiquitin-conjugating enzyme E2 variant 1 isoform A

VIP - vasoactive intestinal polypeptide

vLGN - ventral lateral geniculate nucleus

VLPO – ventrolateral preoptic nucleus

vSPZ - ventral subparaventricular zone

ZT - Zeitgeber time

8-OH-DPAT - 8-hydroxy-2-(di-n-propylamino) tetralin

Chapter One
General Introduction

1.1 What are circadian rhythms?

Chronobiology is the study of circadian and other biological rhythms. Chronobiology is a Greek word, and is derived from three words, 'chrono' meaning time, 'bios' meaning life and 'logos' meaning the study of (Cornélissen et al., 1990; Wetterberg, 1994). The term circadian arises from the Latin circa, meaning about; and dies, meaning day (Jud et al., 2005; Monk & Welsh, 2003). Circadian rhythms are endogenous cycles that recur around every twenty 24 hours in relation to behaviour, physiology and other parameters. They are entrained daily by Zeitgebers (from the German 'time giver') which are external cues such as photic stimuli, or non- photic stimuli such as feeding times and temperature, with light being the most powerful Zeitgeber (Challet, 2007). Circadian rhythms exist in all eukaryotic organisms from unicellular fungi to plants, animals and humans (Jud et al., 2005; Reppert & Weaver, 2002). In order for a rhythm to be circadian in nature a criteria of three properties must exist. The cell, tissue or organism must exhibit an endogenous self-sustaining rhythm that persists in the absence of external environmental cues. Secondly, it must be able to temperature compensate to maintain the period of rhythms. Finally, the pacemaker must also be capable of being reset by environmental cues, for example light or temperature (Butler & Silver, 2009).

A range of biological processes are regulated by circadian clocks including the sleep-wake cycle, body temperature, blood pressure and hormone secretion (Honma & Hiroshige, 1978). The first human circadian rhythm studies were carried out in the late 1930s by Nathaniel Kleitman and Bruce Richardson. Kleitman and Richardson retreated for 32 days to a cave in the U.S. state of Kentucky, and were deprived of all time cues. They maintained constant environmental conditions with regard to temperature, humidity and darkness, as they attempted to live on a non-24 hour day in the cave (Monk & Welsh, 2003). Jurgen Ashcoff was a crucial figure in the development of chronobiology as a field of study. He was the first to prove that humans have endogenous cycles, and that our circadian sleep cycles were not dependent on environmental cues, for example sunlight

or darkness. His studies consisted of an underground bunker, where he was able to research human subjects in isolation from time cues. Ashcoff and his colleague Wever carried out a range of experiments over 20 years and they established that human behaviour, cognition and physiology were controlled by endogenous circadian oscillators (Aschoff, 1960). Experiments carried out examined the human circadian system under constant conditions and under different entrainment. While experimenting on himself, Aschoff established that there was a difference in temperature over a 24-hour period in humans (Aschoff, 1965; Foster & Roenneberg, 2008). These pioneers of chronobiology were key in the development of chronobiology that followed and continues to the current day.

1.2 The Master Circadian pacemaker

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) (Moore, 1983). The SCN coordinates peripheral circadian clocks in individual cells and organs throughout the body and delivers coherent circadian rhythms. The SCN is a bilateral structure located in the anterior part of the hypothalamus at the base of the third ventricle, above the optic chiasm (Reghunandanan & Reghunandanan, 2006; Reppert et al., 1981). In the mouse SCN there is roughly 20,000 neurons, with 10,000 per SCN (Antle & Silver, 2005; Ramkisoensing & Meijer, 2015; Welsh et al., 1995; Welsh et al., 2010). Individual SCN cells are able to oscillate independently when placed in culture (Welsh et al., 1995). The human SCN contains approximately 100,000 neurons and is $1mm^3$ in volume (Hofman & Swaab, 2006).

Studies carried out have shown lesions of the SCN abolish circadian rhythmicity in various physiological and behavioural processes (Moore & Eichler, 1972; Stephan & Zucker, 1972; Wager-Smith & Kay, 2000; Yan, 2009). Studies have shown that SCN lesions can result in the loss of circadian rhythms in locomotor activity (Ralph et al., 1990; Stephan & Zucker, 1972) along with circadian adrenal corticosterone rhythms in rats (Moore & Eichler, 1972). Another vital experiment was a transplantation study performed in lesioned wild type (WT) SCN. Foetal SCN tissue from a Syrian hamster carrying the *tau* mutation was transplanted into the WT hamsters. The host animal took on the behavioural rhythm phenotype of the donor. The host hamster ran with a shortened

activity period of approximately 20 hours, characteristic of animals exhibiting the *tau* mutation compared to the previous WT period of roughly 24 hours. WT foetal SCN tissue can also be transplanted into tau mutant hamster, with the host now exhibiting a period of approximately 24 hours (Ralph et al., 1990; Silver et al., 1996).

If the SCN is detached from the rest of the brain and maintained in slice preparation *in vitro*, SCN neurons still manage to retain their rhythmic electrical activity (Shirakawa et al., 2000; Welsh et al., 1995; Welsh et al., 2010). However, outside the SCN rhythmic electrical activity was destroyed. This demonstrates the importance of SCN projections for conveying rhythmicity to neurons outside the SCN (Hofman & Swaab, 2006; Inouye & Kawamura, 1979). These early studies emphasised a circadian pacemaker function for the SCN.

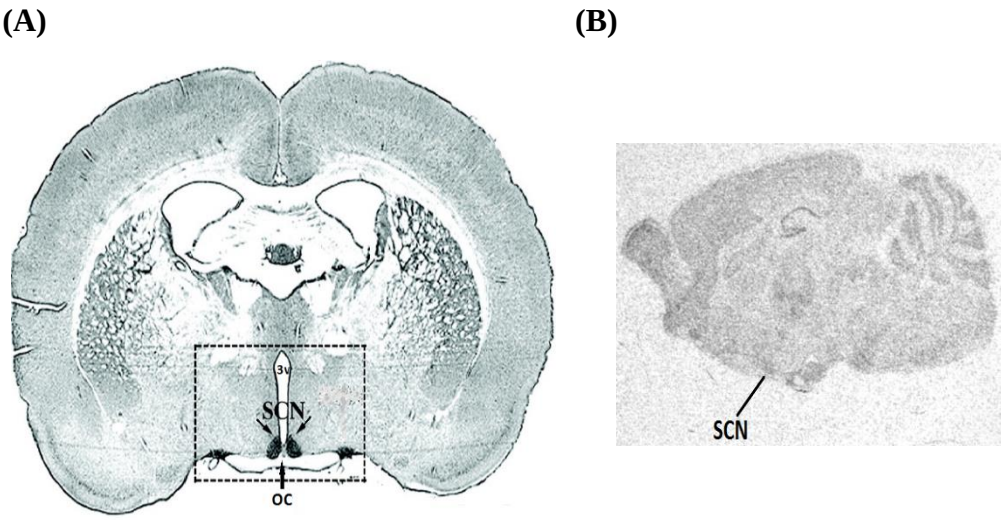


Figure 1.1: The location of the master circadian clock. (A) Coronal section of a mouse brain with the location of the SCN been depicted. The SCN sits directly above the optic chiasm (OC) and on either side of the third ventricle (3v). The two arrows represent the two nucleus of the SCN, adapted from (Mutsuga et al., 2004). (B) The approximate location of the SCN rosral-caudal, depicted in the parasagittal section, adapted from (Zylka et al., 1998).

The SCN is a heterogeneous structure and has been subdivided into two distinct anatomical regions which can be distinguished on the basis of their chemoarchitecture, the ventrolateral region/core and the dorsomedial region/shell (Abrahamson & Moore, 2001; Welsh, 2009). The core makes up 43% and the shell 57% of the total neuronal population of the SCN (Moore et al., 2002). The nuclei receive both direct and indirect

retinal input, helping to carry photic input for entrainment to the environmental photoperiod (Reppert & Weaver, 2002). The core region receives heavy retinal input, and both the core and the shell region shows high amplitude of circadian oscillations in clock gene expression (Morin & Allen, 2006).

The core and the shell are defined by the different neuropeptides and neuronal compositions they contain. The shell region is rich in arginine vasopressin polypeptide (AVP) neurons which makes up 20% of all SCN cells. Angiotensin II, calbindin (CALB) and met-enkephalin are found in neurons in the shells also (Abrahamson & Moore, 2001). The core region is rich in vasoactive intestinal polypeptide (VIP) which accounts for 10% of all SCN cells and gastrin releasing peptide (GRP) which contributes to 5% of cells (Welsh et al., 2010). Neurotensin (NT) (in the human SCN) and calretinin are also located in the core (Rosenwasser, 2009; Welsh, 2010; Yan, 2009). Additionally, the core contains CALB cells, which are known to effect responses to photic input in the SCN (Hamada et al., 2003; Moore et al., 2002).

Intercellular signalling between the core and the shell is vital in maintaining the order of SCN cellular oscillators and circadian function within the SCN (Yan, 2009). Signalling occurs from the SCN core to the SCN shell, however, less projections via the shell to the core exist (Moore et al., 2002). The core; projections have been found to terminate at shell neurons which display links between both regions (Abrahamson & Moore, 2001; Antle & Silver, 2005; Morin, 2007), with light information projected to the shell, which in turns projects to efferent regions (Kalsbeek & Buijs, 2002).

1.3 Afferent input and efferent pathways

Light information reaches the brains SCN via the optic nerves from the retina. There are three major input pathways identified in the SCN; the retinohypothalamic tract (RHT), the geniculohypothalmic tract (GHT) and projections from the raple nuclei.

1.3.1 The Retinohypothalmic Tract

The ventrolateral subdivision of the SCN receives retinal input from the RHT. The RHT is important for photic entrainment of the master clock in the SCN, this is displayed in studies showing that sectioning of RHT abolishes entrainment without

causing any visual difficulties. It has also been demonstrated in animals that ablation of all visual pathways resulting in blindness, still had normal circadian rhythm entrainment (Johnson et al., 1988). Light is perceived from ganglion cells in the retina, and then the photic information is transferred directly to the SCN clock directly via the RHT (Challet, 2007; Gooley et al., 2001). Intrinsically-photosensitive retinal ganglion cells (ipGRC) which contain photopigment melanopsin (Schmidt et al., 2011) are retinal ganglion cells that make up the RHT projections (Berson et al., 2002; Gooley et al., 2001; Hattar et al., 2002; Hattar et al., 2006; Rollag et al., 2003), and it is these cells that play a role in transmitting axons to both the intergeniculate leaflet (IGL) and SCN, to aid photic synchronisation of circadian rhythms (Hattar et al., 2006; Hirota & Fukada, 2004; Morin et al., 2003). Melanopsin KO mice display a reduced lengthening in period when placed in LL compared to WT mice. Furthermore, phase shifts caused by light are also attenuated in these KO mice (Rollag et al., 2003).

Glutamate is the major neurotransmitter contained in the RHT terminals involved in transferring information along the RHT to the SCN, (Hannibal & Fahrenkrug, 2002; Hannibal, 2006) which help control the entrainment process (Rosenwasser, 2009). Light stimulation of the retina produces secretion of glutamate from the RHT into the VIP part of the core SCN (Ding et al., 1994; Mikkelsen et al., 1995). Glutamate seems to require the activation of NMDA and non-NMDA receptors, the expression of immediate early genes (IEGs), along with nitric oxide (Rea, 1998). When glutamate receptor antagonists are applied *in vivo*, it blocks the effect of light on the SCN at certain time points inhibiting phase advances or delays in the subjective night (Colwell & Menaker, 1992; Ding et al., 1994; Shibata et al., 1994). Furthermore, it attenuates photically induced Fos expression in retinorecipient neurons of the SCN (Vindlacheruvu et al., 1992). Application of glutamate agonists to SCN slices *in vitro*, cause induced phase shifts in the rhythm of neuronal firing rate in a pattern that mimics the *in vivo* effect of light photic stimulus on behaviour rhythms (Ding et al., 1994; Ebling, 1996; Mintz et al., 1999). Therefore, these results indicate a role for glutamate in transferring photic information to the SCN.

PACAP can be found co-localised with glutamate in ganglion cells distributed throughout the retina and on terminals in the retinorecipient region of the SCN and may play a role in light signalling to the clock (Hannibal et al., 1997; Hannibal, 2002). PACAP

administration is dose and phase dependent on whether it effects circadian gene expression (Harrington et al., 1999). Administration of PACAP at high doses can increase glutamatergic signalling in the SCN during the early night. When PACAP and glutamate are administered at the same time, PACAP can inhibit the phase advance during the late subjective night usually induced by glutamate (Chen et al., 1999). PACAP6-38, a PACAP antagonist block the effects of glutamate phase advance (Nielsen et al., 2001) and also induce non-photic like phase advances during the subjective day (Hannibal et al., 1997). Whereas low dose PACAP results in a phase advance in the subjective night similar to light or glutamate (Harrington et al., 1999; Nielsen et al., 2001). This data represents PACAP as a modulatory component of nocturnal phase regulation of the SCN clock by light (Chen et al., 1999).

1.3.2 The Geniculohypothalmic Tract

The GHT is the second afferent photic projection from the IGL to the SCN. The IGL is the first indirect pathway, which receives input from the same retinal cells whose axons compose the RHT, and is involved in supplying retinal input to the SCN (Challet, 2007; Reppert & Weaver, 2001). The IGL is situated between the dorsal lateral (dLGN) and ventral lateral geniculate nucleus complex (vLGN) (Harrington, 1997; Moore & Card, 1994). The IGL contains neuropeptide Y (NPY), encephalin (ENK) and γ-aminobutyric acid (GABA) neurons, that project to the SCN via the GHT (Blasiak & Lewandowski, 2013; Harrington, 1997; Morin & Allen, 2006; Morin et al., 1992). The IGL plays a role in both photic and non-photic phase shifting (Abe & Rusak, 1992; Harrington, 1997).

Studies of non-photic phase shifting in the GHT displayed that phase shifts from a six hour dark pulse could be blocked by ablation of GHT (Harrington & Rusak, 1986). Administration of benzodiazepines triazolam and chlordiazepoxide which cause phase shifts at certain CTs were also blocked by GHT ablation (Biello et al., 1991). Furthermore, phase shifts can occur due to the introduction of a novel running wheel but GHT ablation prevented this (Wickland & Turek, 1994). Studies were carried out looking at the effects of photic phase shifting after GHT ablation occurred. In the early subjective night, phase delays that take place after a light pulse were either increased (Pickard et al., 1987) or unaffected in hamsters (Harrington & Rusak, 1986). Whereas in the late

subjective night following a light pulse, phase advances were decreased due to GHT ablations (Pickard et al., 1987). Another study demonstrated that the phase angle of entrainment was changed significantly by GHT ablations (Pickard, 1989). Therefore, the GHT appears to play a role in phase shifting of circadian rhythms caused by photic stimuli, but its key role may be its involvement in mediating non-photic phase shifts.

Recent electrophysiological studies in the rat, have shown that IGL neuron projecting to the SCN, differ from IGL neurons projecting to contralateral IGL. They have individual firing patterns and react different to light environments. Therefore, this suggests that IGL neurons play different roles in the brain (Blasiak & Lewandowski, 2013). Another study carried out displayed that dim night light caused changes to the photoperiod responses through the IGL in hamsters. The IGL is involved in transducing the effects of dim light to the SCN. When the IGL was damaged, the accelerated response to the L:D 10:14 photoperiod shown by control hamsters, were not demonstrated by ablated IGL's. This suggested further that the IGL is involved in facilitating the effects of dim light at night with regard to the expression of short photoperiod responses (Evans et al., 2012). Additionally, alterations as a result of transferring to a short photoperiod occurred in mice lacking NPY, with *npy*$^{-/-}$ mice exhibiting a significantly delayed onset of activity in comparison to control mice. Therefore, NPY plays a role in entrainment in the SCN (Harrington et al., 2007; Kim & Harrington, 2008).

1.3.3 The Midbrain raphe nuclei projections

The final afferent pathway is the midbrain raphe nuclei which is the second indirect pathway and which contains serotonergic projections to the retinorecipient SCN. Neurotransmitters in the raphe nuclei pathways appear to play a part in mediating non-photic phase shifts, such as those caused by behavioural arousal (Reppert & Weaver, 2001). Serotonin (5-HT) plays a role in regulating circadian rhythmicity and administration of 5-HT receptor agonists can reduce/block photic phase shifts by inhibiting the release of glutamate (Rea & Pickard, 2010; Selim et al., 1993; Weber et al., 1998).

Administration of 5-HT receptor agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT), during the mid to late subjective day can result in a small phase advance in wheel running hamsters in DD (Cuesta et al., 2008; Edgar et al., 1993).

Therefore, serotoninergic innervation to the SCN conveys non-photic information to the SCN clock. Furthermore, PER1 and PER2 expression is attenuated following 8-OH-DPAT administration (Cuesta et al., 2008). The 5-HT_{1A} agonist/antagonists, NAN-190, is known to significantly increase photic phase shifts (Gannon, 2003), as a result of reduced inhibition of the RHT and by also increasing the responsiveness of retinorecipient SCN cells (Sterniczuk et al., 2008). A recent study investigated what therapeutic effect a 5-HT_7 antagonist, mixed with a selective serotonin (5-HT) reuptake inhibitor (SSRI), would have on circadian rhythms. It was established that the combination of agents had a larger effect on circadian rhythms, than either individual agent separately, and may help in patients with clock dysfunction (Westrich et al., 2013).

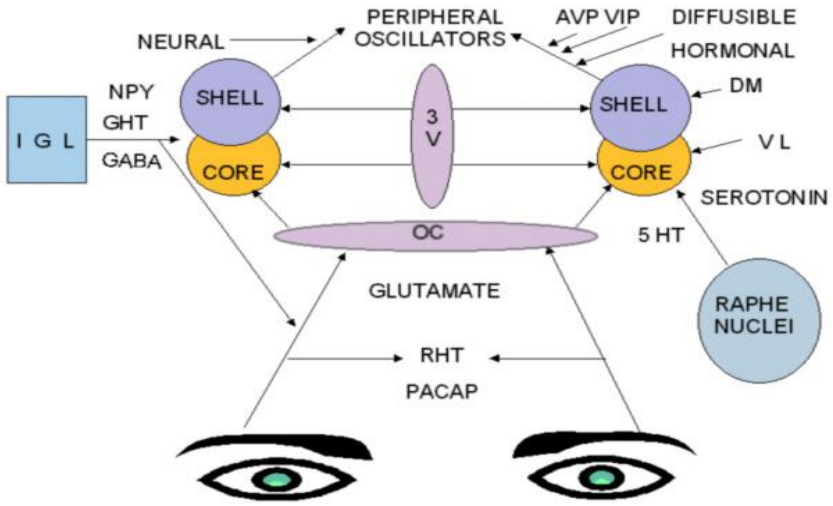


Figure 1.2: The afferent inputs and efferent pathways of the SCN, (Reghunandanan & Reghunandanan, 2006a)**.**

1.3.4 The efferent pathways

The main output of the clock is AVP expressing neurons which send efferents to the PVN, SPZ and DMH (Buijs & Kalsbeek, 2001; Dai et al., 1997; Reghunandanan & Reghunandanan, 2006). The SCN can communicate to the CNS and the periphery by SCN efferent projections which will allow SCN timing information throughout the nervous system (Deurveilher & Semba, 2005; Dibner et al., 2010). The SCN projections extends to the ventral and dorsal regions of the subparaventricular zone (SPZ). The ventral SPZ (vSPZ) transmit projections to dorsomedial hypothalamus (DMH). The DMH then sends

projections to the ventrolateral preoptic area (VLPO) important in sleep promoting, as well as the lateral hypothalamic area (LHA) which is important in feeding and wake promoting (Chou et al., 2003; Laposky et al., 2008; Saper et al., 2005). The dorsal SPZ (dSPZ) transmits to the medial preoptic area (MPOA) which is involved in regulating body temperature (Lu et al., 2001). The DMH contributes to circadian rhythms of sleep and wakefulness, corticosterone secretion and feeding (Chou et al., 2003). Lesions in the SPZ or the DMH will cause impairments to sleep and metabolic rhythms, hence they are important pathways in the output of the SCN (Laposky et al., 2008). Light is transmitted from the retina to the SCN, with output from the SCN occurring via the PVN which transmits to the pineal gland allowing for melatonin synthesis (Weaver & Emery, 2013). The medial parvicellular paraventricular nucleus (mPVH) and dorsal parvicellular PVH (dPVH) are involved in regulating adrenal rhythms and pineal melatonin secretion respectively (Saper et al., 2005; Weaver & Emery, 2013).

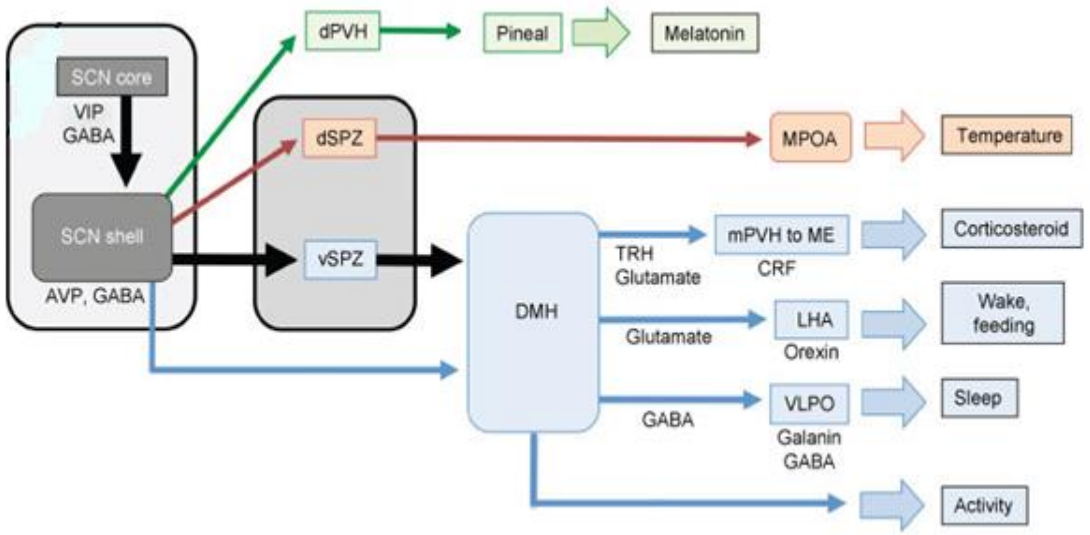


Figure 1.3: SCN output pathways in the hypothalamus. Environmental light: dark cues are transmitted to the SCN via the RHT, which is sent to the dPVH. The SCN sends efferent projections also to the vSPZ and dSPZ, which then transmits SCN signals to many nuclei involved in sleep/wake cycle, feeding, energy and autonomic regulation, adapted from (Weaver & Emery, 2013).

Humoral routes may also be involved in the SCN conveying information within the brain. Studies carried out by Silver and colleagues showed that a diffusible substance from transplanted fetal SCN tissue can restore weak circadian rhythmicity in locomotor

activity in SCN-lesioned hamsters (Silver et al, 1996). Various neurochemical outputs from the SCN have been highlighted to play a role in humoral output, including signalling via AVP, VIP, PK2 and cardiotrophin-like cytokines (CLC) (Kraves & Weitz, 2006; Reghunandanan & Reghunandanan, 2006b). PK2 plays a role in the SCN to help synchronise output (Cheng et al., 2002; Zhang et al., 2009).

1.4 Neurotransmitter in the SCN

Neurotransmitters are present in the SCN and play a role in the functioning of the pacemaker (Abrahamson & Moore, 2001; Card & Moore, 1984). Whether input is photic or non-photic, it can reach the circadian clock by neurotransmitters in nerve terminals. Neurotransmitters in the afferent and efferent projections of the SCN are vital for the entrainment of the clock and for the control of overt rhythms (Reghunandanan & Reghunandanan, 2006b). Neurotransmitters and neuropeptides such as GABA, NPY, NT, calretinin, somatostatin, MET-Enkephalin, prokineticin 2, serotonin, VIP, and AVP have all been implicated in the functioning of the SCN (Lall & Biello, 2003a, 2003b; Lall & Biello, 2002; Reghunandanan & Reghunandanan, 2006; Reppert & Weaver, 2002).

1.4.1 AVP

AVP is one of the principal neuropeptides in the SCN. AVP neurons occupy a large part of the SCN and are co-localised with somatostatin and GABA, where it is expressed in the shell of the SCN (Ingram et al., 1999). AVP is known to be synthesized and secreted by the SCN in a circadian pattern. AVP displays an excitatory role by activating V1a receptors (Ingram et al., 1998) which in turn increase the amplitude of electrical firing activity in the SCN through the subjective day (Mihai et al., 1994). *In vitro* application of AVP to brain slices resulted in increased SCN spontaneous electrical firing rate rhythms, whereas perfusion with V1a antagonist displayed decreased spontaneous electrical firing rate rhythms (Ingram et al., 1998). AVP is implicated in circadian time-keeping as it plays a role in controlling the circadian rhythm of food and water intake (Reghunandanan & Reghunandanan, 2006b). Administration of AVP antagonist at the beginning of the light and dark phases disrupted the circadian rhythm of food intake, whereas administration of AVP at the same time did not disrupt the circadian

rhythm. Therefore it displays that endogenous AVP has a regulatory role in circadian feeding rhythm (Reghunandanan et al., 1987). Another experiment carried out looked at the effect endogenous AVP and its antagonist had on water intake. Similar findings were discovered to that of food intake, with AVP antagonist disrupting circadian rhythms while AVP injections into the SCN did not disrupt the rhythms (Reghunandanan et al., 1992). Perturbations occurred in the sleep wake cycle due to reduced number of AVP neurons (Hofman & Swaab, 1994). AVP deficient rats contain reduced amplitude of circadian sleep rhythms (Brown & Nunez, 1989). A recent study has shown that mice deficient in vasopressin V1a and V1b receptor seem to be unaffected by jet lag, with mice instantly re-entrained to phase shifted light-dark cycle when monitoring both their behavioural locomotor activity and clock gene expression (Yamaguchi et al., 2013). Therefore these studies suggest that AVP plays a part in SCN output.

1.4.2 GABA

Another important neurotransmitter of the circadian timing system is GABA, which can be found in most SCN and IGL neurons (Moore & Speh, 1993). An important role of GABA involves the coupling between the core and shell of the SCN (Albus et al., 2005). Phase advances can occur in the subjective day when GABA receptors are activated in nocturnal species (Ehlen et al., 2006; Smith et al., 1989), while GABAergic stimulation during the subjective day results in a phase delay in diurnal species (Novak & Albers, 2004). GABA is connected to reduced Per1 and Per2 mRNA expression in the SCN of nocturnal animals, comparable with serotonin (Ehlen et al., 2006). Hence, GABA acts as a non-photic stimuli involved in the circadian clock.

In SCN, it is necessary for individual clock cells to become synchronized for a coordinated output signal. Communication through gap junction and neurotransmitter based interactions are mechanisms to help with synchronization. Studies have displayed functional gap junctions in the SCN. Studies presented the transfer of Lucifer Yellow between adult rat SCN neurons and inhibition of this transfer by the GABA receptor agonist muscimol (Shinohara et al., 2000). GABA activity is generally inhibitory, but a study reported that GABA excited SCN neurons during daytime, while at night GABA inhibited SCN electrical activity (Wagner et al., 1997). GABA is released from SCN terminals in the PVN region and appears to play a role also in regulating melatonin

synthesis from the pineal gland by switching off a stimulatory signal emitted from the PVN. When there is an increased SCN electrical activity, this leads to increased GABA release. However, further investigations must take place to link the circadian clock and the mechanisms that regulate GABA release from the SCN (Reppert & Weaver, 2001).

Research undertaken using electrical activity rhythms of SCN clock cells in culture displayed that GABA is an important neurotransmitter for synchronizing SCN neurons. A change in phase shifts and entrainment of clock cells in culture is caused by application of GABA (Liu & Reppert, 2000). $GABA_A$ and $GABA_B$ agonist application reduced phase advances caused by photic stimulation in the late subjective night. $GABA_B$ agonist and $GABA_B$ antagonist administration reduced and increased the phase delays respectively in the early subjective night (Gillespie et al., 1997; Novak & Albers, 2004). These findings represent that $GABA_A$ or $GABA_B$ activity within the SCN can alter the phase-shifting effects of light on circadian rhythms. It also may indicate a role for gating photic input to the circadian clock. GABAergic neurotransmission in the SCN can cause various changes throughout the day and night, therefore, there may be a time gating mechanism, which may enhance excitatory signals throughout the SCN during the night but causing inhibition of daytime excitation (De Jeu & Pennartz, 2002). Furthermore, a recent study has displayed how an extended length in day can affect GABA in the SCN, with an upsurge of GABAergic excitation (Farajnia et al., 2014). Lastly, another study exhibited how during the circadian day, 60% of GABA-dependent connections were inhibitory, whereas 40% were excitatory (Freeman et al., 2013).

1.4.3 VIP

The polypeptide VIP is an important neurotransmitter synthesised by 9-25% of SCN neurons and plays a role in SCN function. These SCN neurons are retinorecipient and can be stimulated by light (Cayetanot et al, 2005; Piggins & Cutler, 2003; Reghunandanan & Reghunandanan, 2006; Welsh et al., 2010). VIP is found in the core of the SCN (Welsh et al., 2010; Yan et al., 2007), and it is vital for intercellular signalling systems in the SCN and for cellular coupling (Hughes et al., 2015). The biological clock is synchronized to the light/dark cycle by photic information that is transferred from the retina to the SCN. These afferent fibres terminate in the core of the SCN, and synaptic contacts take place with VIP neurons. Therefore, VIP in the SCN is thought to play a vital

role in the facilitation of photic information to the circadian timing system (Hofman & Swaab, 2006).

Exogenous administration of VIP has the ability to reset the circadian clock in a manner comparable to that of light application, both *in vitro* and *in vivo* (Piggins et al., 1995). VIP has two important roles in the SCN. Firstly keeping circadian rhythmicity in a subset of neurons and, secondly, maintaining synchrony between intrinsically rhythmic neurons (Aton et al., 2005; Reghunandanan & Reghunandanan, 2006). Studies undertaken have shown that VIP is vital for the coordination of the daily rhythms in behaviour and physiology at the level of biological clock in mice. Loss of VIP, stopped circadian firing rhythms in roughly 50% of all SCN neurons and disrupted synchrony between rhythmic neurons. Administration of a $VPAC_2$ agonist re-established rhythmicity and synchrony to $VIP^{-/-}$ SCN neurons (Aton et al., 2005). Other studies demonstrate perturbations in gating of photic input to the SCN in *Vip2*$^{-/-}$ mice (Hughes et al., 2004). A recent study carried out has shown how constant light can actually improve behavioural locomotor rhythms and also SCN intercellular synchrony in *Vipr2*$^{-/-}$ mice, which is in contrast to the effect LL has on WT mice (Hughes et al., 2015). Phase shifts can arise with VIP application to SCN slices similar to those induced by light pulses (Reed et al., 2001). These findings represent the importance of VIP as a neurotransmitter of the SCN.

Circadian firing rhythms can be measured using a multielectrode dish. This allows for individual SCN neurons to be monitored for a few weeks (Shirakawa et al, 2000; Welsh et al, 1995). The circadian firing rate is important to the circadian pacemaker as both an input to and output of the molecular clockworks, as studies have shown that by manipulating the firing rate can reset circadian rhythms both *ex vivo* and *in vivo* (Jones et al, 2015).

1.5 Peripheral Clocks

Circadian clocks can be found in the brain outside the SCN and in peripheral tissues and organs throughout the body (Balsalobre et al., 2000; Brown & Azzi, 2013; Damiola et al., 2000; Yamazaki et al., 2000). There are eight known clock genes, *mBmal1*, *mNpas2*, *mRev-erbα*, *mDbp*, *mRev-erbβ*, *mPer3*, *mPer1* and *mPer2* expressed in almost every peripheral tissue. These genes have robust circadian expressions of mRNAs in

nearly all peripheral tissues (Yamamoto et al., 2004). Yamamoto and colleagues analysed mRNA expression of clock and clock-controlled genes in mouse peripheral tissues such as heart, lung, liver, stomach, spleen and kidney. Circadian mRNA expression patterns were similar in each tissue, suggesting, that there may be a unitary mechanism for resetting the peripheral clock. The peak transcript level of each circadian rhythm in the master clock was as follows, in *mBmal1* and *mNpas2* peaked at CT20-CT0, *mRev-erbα* the peak occurred between CT4-8, *mDbp* and *mRev-erbβ* at CT8, *mPer3* at CT8-12, *mPer1* at CT12; and *mPer2* biggest transcript level was at CT12-16. Whereas peripheral tissues mRNA peaks occurred roughly four hours later than those in the SCN (Yamamoto et al., 2004).

A study was carried out on mouse liver where the *bmal1* gene was knocked out. The liver was no longer able to produce sufficient amount of glucose needed in the blood circulation, which resulted in hypoglycaemia, as a result of $BMAL1^{-/-}$. This emphasises how vital the liver peripheral clock is in relation to the metabolic system (Lamia, et al., 2008). Apart from the master clock, peripheral oscillators have also been seen to be directly entrained by Zeitgebers for example body temperature alterations and feeding-fasting schedule (Buhr et al., 2010; Challet et al., 2003; Damiola et al., 2000).

Peripheral cells generally can sustain cellular rhythms independently. Studies have displayed that cell cultures have rhythmicity in their gene expression and functions in cells of peripheral tissues. The SCN plays a vital role in coordinating and synchronizing rhythmic behaviour all through the body, however, peripheral clocks can generate circadian oscillation in gene expression on their own. The central clocks light/dark cycle help regulate and retrain oscillations in the clocks in the peripheral cells, along with other factors such as food, stress and certain hormones (Sukumaran et al., 2010). The adrenal peripheral clock plays an important role in helping to generate glucocorticoid rhythms that synchronize peripheral clocks, as glucocorticoid modulates inflammation, immune function and lipid metabolism (Son et al., 2008; Son et al., 2011).

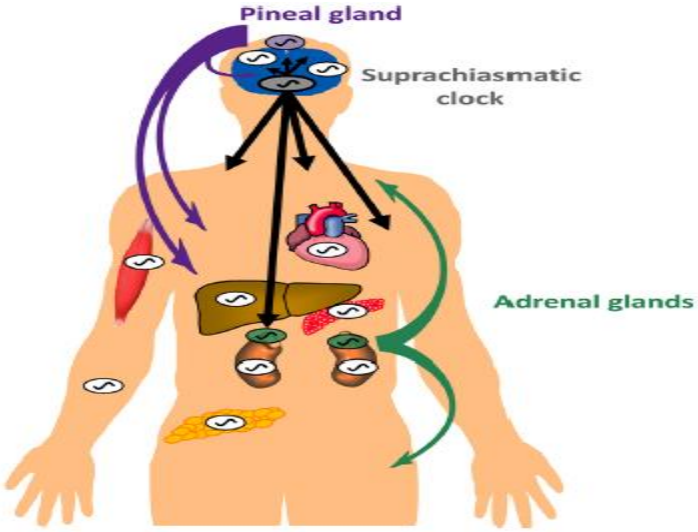


Figure 1.4: Interaction between the SCN and peripheral clocks, adapted from (Pevet & Challet, 2011). Nervous fibres can transmit circadian signals from the SCN to peripheral clocks, represented by the (black arrows). The signals reach peripheral organs including the liver, lung, heart and adrenal glands. Two hormonal outputs presented are melatonin (purple) and glucocorticoids (green), which are synthesized by the pineal gland and the adrenal gland respectively. These outputs distribute temporal cues produced by the SCN to numerous peripheral tissue targets which express the necessary receptors.

1.6 The Molecular Clock

1.6.1 Transcriptional feedback loops

In the early 1970's the first circadian rhythm mutants to be isolated were the *frequency* mutants in the fungi *neurospora* (Feldman & Hoyle, 1976) and the *period* mutants in the fruitfly *Drosophila* (Konopka & Benzer, 1971). Research undertaken on the fruit fly *Drosophila melanogaster* has assisted in finding out the mechanisms of the mammalian clock (Young & Kay, 2001). Genes in the circadian clock of this fruit fly have a similar transcriptional feedback mechanism loop that is in mice and humans (Reppert & Weaver, 2002).

The SCN endogenously generates circadian rhythm by a molecular oscillator with a periodicy of about a day, and consists of a transcription-translation feedback loops. This process involves a set of eight core clock genes and clock-controlled output genes that link the oscillator to clock-controlled processes (Hastings & Herzog, 2004; Ko & Takahashi, 2006; Reppert & Weaver, 2001). The transcription translation feedback loops

of the molecular clock drive expression of the core genes (Reppert & Weaver, 2002), and the oscillations of clock gene proteins are important for generating and regulating circadian rhythms (Takahashi, 2004). The following mammalian core clock gene involved in this process, circadian Locomotor Output Cycles Kaput protein (CLOCK), Brain and Muscle ARNT-like protein 1 (BMAL1), the period proteins PER1, PER2, PER3, and the cryptochromes CRY1 and CRY2 and casein kinase 1 epsilon (CSNK1E) (Ko & Takahashi, 2006).

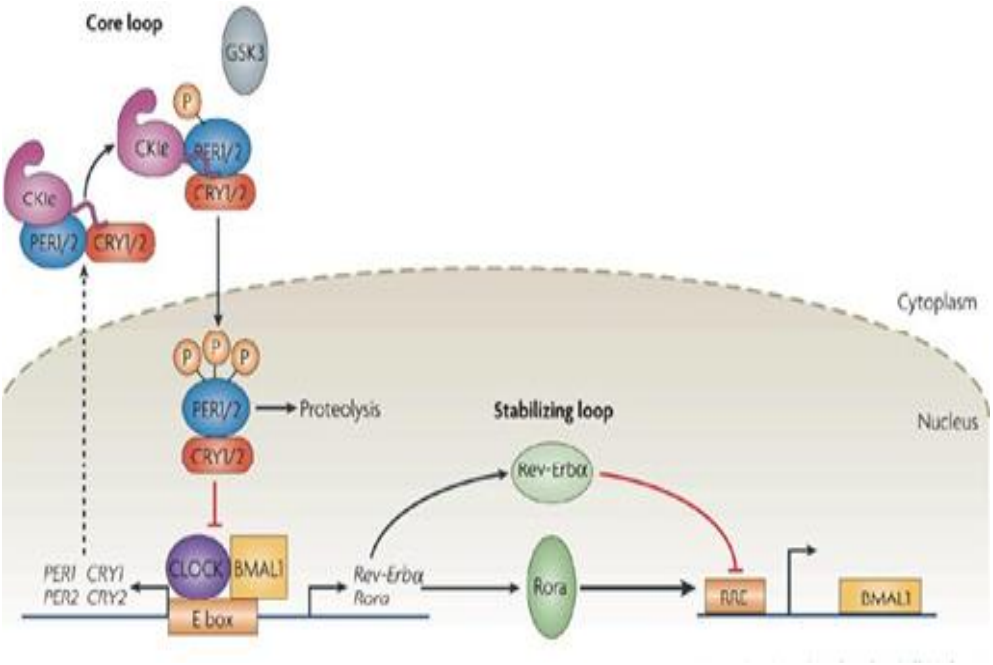


Figure 1.5: The molecular circadian clock network, (Gallego & Virshup, 2007). This diagram presents the action of CLOCK and BMAL1 proteins which dimerise to produce a complex which promotes the transcription of the *Per* and *Cry* genes. PER and CRY products in turn dimerise in the cytoplasm and inhibit CLOCK-BMAL1, which indirectly regulates their own transcription. This loop occurs over a period of 24 h and generates mammalian circadian rhythms. A secondary transcription mechanism exists between CLOCK-BMAL1 and the genes *Rev-erbα* and *Rorα,* the proteins of which respectively negatively or positively drive *BMAL1* transcription.

The clock genes are entrained to the light-dark cycle with CLOCK: BMAL peaking during the light period, in contrast to PER: CRY peaking during the dark period in diurnal and nocturnal animals (Sukumaran et al., 2010). The major transcriptional activator consists of a hetrodimer between CLOCK and BMAL1 (positive feedback loop), which binds to E-box sequences in the promoters of many genes including *per* and *cry* genes (negative feedback loop). Throughout the day, the PER and CRY proteins build up in the cytoplasm where they are phosphorylated by casein kinase 1 ε and glycogen synthase kinase-3 (GSK3) (Gekakis, 1998; Harms et al., 2003; Kurabayashi et al., 2006).

This results in mPER and mCRY proteins translocating into the nucleus and the mCRY represses the activity of CLOCK/BMAL and it prevents transcription which in turn closes the negative feedback loop (Kume et al., 1999; Sato et al., 2006; Stojkovic et al., 2014). Degrading occurs of the PER and CRY proteins in a CKI-dependent manner which releases the repression of the transcription and allows the next cycle to start.

A second regulatory loop is induced by CLOCK-BMAL1 heterodimers which activates transcription of the orphan nuclear receptor gene *Rev-Erbα* and *Rorα* (Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004). The REV-ERBα protein suppresses *Bmal1* transcription by the Rev-Erb/ROR response element in its promoter. This results in *Bmal1* RNA levels reducing and *mCry* and *mPer* RNA levels increasing. The mCRY proteins is then able to enter the nucleus to inhibit mPer and mCry transcription as it acts on the CLOCK-BMAL1, mCRY protein also blocks Rev-Erbα transcription which in turn causes activation of *Bmal1* transcription (Preitner et al., 2002). An additional core member of the mammalian circadian clock is neuronal PAS-domain protein 2 (NPAS2). NPAS2 is comparable to CLOCK, displaying similar activities but tissue distribution is different. NPAS2 can also heterodimerize with BMAL1, and attach to E-box motifs, transcribing and activating circadian genes (Reick et al., 2001). The positive and negative feedback loop described above are responsible for producing messenger ribonucleic acids (mRNAs) from the *Per*, *Cry*, *Rev-erbα* and *Bmal1* genes throughout the circadian phase. In the SCN, *Per, Cry* and *Rev-erbα* all show peak during the light phase, with *Bmal1* producing an opposite phase with peak approximately 12 hours later. Rhythms in other regions of the brain and peripheral tissues are delayed by several hours.

Since a simple transcriptional feedback loops leads to mRNA oscillations with a period less than 24 hours, other mechanisms are needed in addition to this simple loop model that allows the 24 hour environment period to be slowed down. These mechanisms include post-transcriptional processing of the mRNAs, translation, post-translational processing of the proteins and nuclear translocation. Each of these mechanisms is important for delaying activation and repression of transcription that is necessary to keep the period at about 24 hours (Harms et al., 2003).

A model of the SCN clockwork suggests that at the beginning of the circadian day (CT 0), *mPer* and *mCry* transcription is driven by the accumulation of CLOCK: BMAL1 heterodimers. *Clock* RNA levels are expressed constitutively across the 24 hour cycle

(Gekakis, 1998). The circadian oscillations of *mPer* and *mCry* RNA levels exhibit similar yet different temporal profiles in the SCN, with the *mPer1* RNA rhythm peaking from CT 4 to 6, *mPer3* peaks between CT 4 and 9 (Shearman et al., 2000), *mPer2* at CT 8 (Bae et al., 2001), and *mCry1* at CT 10. At CT 12 the mPER and mCRY proteins are synchronously expressed in the nucleus where the mCRY proteins stop CLOCK: BMAL1 mediated transcription. Simultaneously, mPER2 enhances *Bmal1* transcription, which then leads to peak *Bmal1* RNA levels at approximately CT18 (Bunger et al., 2000). It is suggested that the *Bmal1* RNA rhythm drives a BMAL1 protein rhythm after a four to six hour delay. The restoration of BMAL1 levels at the end of the night most likely increases CLOCK: BMAL1 heterodimers to drive *mPer/mCry* transcription, resulting in the cycle starting again. It appears that the availability of BMAL1 is rate limiting for heterodimer formation and is vital for the positive: negative feedback loop to reoccur for the start of a new circadian day (Reppert & Weaver, 2001). The expression of proteins are seen 4-6 hours following the expression of *mRNA* peak levels (Field et al., 2000).

1.6.2 Post-translation

Post-translational modifications of clock proteins are vital for the timing of the clock feedback mechanism and also play a role in providing regulatory fine-tuning (Duguay & Cermakian, 2009). Modifications by acetylation, phosphorylation, ubiquitination, methylation and sumoylation plays an important role in regulating the circadian clock during post-translation. Regulation of the core circadian proteins occurs by phosphorylation using , Casein kinase 1 epsilon (CK1ε), Casein kinase 1 delta (CK1δ), Casein Kinase 2 (CKII), glycogen synthase kinase-3 (GSK3) and adenosine monophosphate-activated protein kinase (AMPK) (Reghunandanan & Reghunandanan, 2006a). Phosphorylation is necessary for the recruitment of ubiquitin ligases, which help mediate the polyubiquitylation and the subsequent degradation of these proteins in the proteasome. In mammals, βTrCP1 or βTrCP2 regulate PER1 and PER2. CKI are involved in phosphorylating PER1 and PER2 and this phosphorylation leads to the recruitment of βTrCP which mediates the ubiquitylation and proteasomal degradation of these proteins (Eide et al., 2005; Shirogane et al., 2005).

In the last 10 years, sumoylation was discovered, which is an additional post translational modification involving regulation of various mechanism involved in the

circadian clock. It is a reversible post translational modification resulting in small ubiquitin-related modifier protein (SUMO) covalently linking to lysine residues. It is controlled by an enzymatic pathway similar to the ubiquitin pathway (Cardone et al., 2005; Reghunandanan & Reghunandanan, 2006). However, in contrast to ubiquitination, sumoylation does not directly target proteins for degradation instead it regulates other functions for example nuclear localization, protein–protein interactions, transcriptional activity and ubiquitination itself (Buschmann et al., 2000; Desterro et al., 1998).

Mutations in CK1ε and CK1δ emphasize how vital post translational modification occurring in the molecular clock is (Lee et al, 2009). There is a decrease in free running period (FRP) and an altered phase angle of entrainment seen in CK1ε mutated hamsters (Ralph & Menaker, 1988). Mutations occurring in CK1ε and CK1δ effect kinase activities and result in a shorter circadian period in mammals (Akashi et al, 2002; Eide et al, 2002; Lowrey et al., 2000). CK1ε and CK1δ mutations are of particular interest to humans as they are associated in the sleep disorder familial advanced sleep phase syndrome (FASPS) (Xu et al., 2005). Post translation modifications are necessary in order to have a self-sustaining cycle of core clock components oscillating every 24 hours per cycle (Guilding & Piggins, 2007).

1.6.3 Mutations or deletions of the core clock genes

Disruption or deletion of circadian clock genes in mice impair circadian behaviour and altered expression profiles of circadian genes. *mPER* and *mCRY* outputs of the negative feedback loop are essential for maintaining a functioning circadian clock (Lowrey & Takahashi, 2004). Diseases including diabetes, obesity, and vascular disease and accelerated ageing are linked to impaired clock gene expression (Bass & Takahashi, 2010; Kondratov et al., 2006; Paschos et al., 2012; Rudic et al., 2004). The studies described next, emphasis the effect perturbations to components of the molecular clock mechanisms can have on circadian output rhythms.

1.6.3.1 *Per* mutations

Per1$^{-/-}$ mice display a shorter circadian period, with the period less stable and precise (Cermakian et al., 2001; Zheng et al., 2001). Furthermore, *Per1*$^{-/-}$ mice displayed

disturbed locomotor activity rhythms when placed in DD for an extended period (Bae et al., 2001). When *Per1*$^{-/-}$ mice are placed in LL their period length is extended and become arrhythmic in light of >500 lux (Steinlechner et al., 2002). Therefore, *PER1* seems to be important in the regulation of the circadian clock and maintenance. However, a subsequent study has shown that C57BL/6J *Per1*$^{-/-}$ mice had a similar free running period (FRP) to *Per1*$^{+/+}$ and *Per1*$^{+/-}$ of approximately 23.5 hours. Additionally, analysis of the total wheel running activity, phase angle of entrainment and amplitude were comparable between all 3 mice groups. Finally, no arrhythmic wheel running behaviour was observed in this study following DD conditions (Pendergast et al., 2009). This was in contrast to the findings from Bae and colleagues, where some of the *mPER1*-deficient mice displaying arrhythmic behaviour in DD (Bae et al., 2001; Zheng et al., 2001). Circadian behaviour can be affected by genetic background (Ebihara et al., 1978; Schwartz & Zimmerman, 1990; Shimomura et al., 2001), therefore, the strain of mice used in each study may be the reason for different findings, with Pendergast using a C57BL/6J genetic background, in contrast to both Bae and Zheng using mixed or isogenic 129/sv backgrounds. When studies were carried out *in vitro*, the cultured SCN of *Per1*$^{-/-}$ mice exhibit weak or absent molecular rhythms, which is in contrast to *in vivo* results established (Pendergast et al., 2009; Takasu et al., 2013).

Per2$^{-/-}$ mice in DD also exhibit a shorter circadian period and eventually lose their circadian rhythmicity. This arrhythmicity in DD can be overturned by the application of a light pulse, re-establishing circadian rhythmicity (Zheng et al., 1999, 2001). *Per2*$^{-/-}$ mice placed in LL differ from *Per1*$^{-/-}$ mice, with shortened period less than 24 hours and display robust activity rhythms. *Per2* is important for SCN gene expression, with reduced levels in *Per2*$^{-/-}$ mice (Bae et al., 2001). *In vitro* studies also displayed a shortened circadian period when analysing *Per2*$^{-/-}$ SCN explants using the Per1-Luc reporter. However, the cultures were greatly reduced by approximately 1.5 hours when compared to *in vivo* studies (Pendergast et al., 2010). A *Per2* mutation can result in a sleep disorder known as FASPS in humans (Toh et al., 2001). Additionally, *Per3*$^{-/-}$ mice express continuous mPer2^{Luc} bioluminescence rhythms in SCN explants (Liu et al., 2007). *mPer3* does not appear to be necessary in the functioning of circadian rhythmicity with only a modest period alteration in DD (Bae et al., 2001; Shearman et al., 2000) and in SCN explants (Pendergast et al., 2012). However, mice deficit in *Per3* exhibit alterations to phase and period in both lung and pituitary explants (Pendergast et al., 2010). Double knockout of

Per1/Per2 results in immediate behavioural arrhythmicity if the knockout mice are put in DD (Zheng et al., 2001). The Per1 and Per2 genes are vital in regulating circadian rhythms as animals deficient in both these genes show no circadian rhythm.

1.6.3.2 *Cry* mutations

Cryptochromes play a key role in the negative feedback loop of the circadian timekeeping system. When in constant conditions of either DD or LL *in vivo*, the double knockout of *Cry1/Cry2* mice results in behavioural arrhythmicity. Whereas either *Cry1* or *Cry2* alone can maintain rhythmicity. *Cry1*$^{-/-}$ mice display a shortened period length, while *Cry2*$^{-/-}$ mice exhibit an extended period length in DD (van der Horst et al., 1999; Vitaterna et al., 1999). Therefore, the CRY proteins are necessary for the smooth running of the circadian timekeeping system. *In vitro* studies present similar findings with either *Cry1*$^{-/-}$ or *Cry2*$^{-/-}$ mice been able to maintain mPer2^{Luc} bioluminescence rhythms in SCN explants. Interestingly, neither *Cry1*$^{-/-}$ or *Cry2*$^{-/-}$ mice were able to maintain mPer2^{Luc} bioluminescence rhythms in lung explants (Liu et al., 2007).

1.6.3.3 *Clock* and *NPAS2* mutations

Peripheral *Clock*$^{-/-}$ liver and lung explants display long period locomotor activity that ultimately causes arrhythmicity in DD, hence, CLOCK is vital in peripheral oscillations (DeBruyne et al., 2007). Heterozygous *Clock* mutant mice display a FRP of approximately 24.5 hours, whereas homozygous *Clock* mutant mice exhibit a FRP of roughly 28 hours before becoming arrhythmic (Vitaterna et al., 1994; Young & Kay, 2001). Studies have revealed that *clock*$^{-/-}$ mice maintain SCN bioluminescence rhythmicity and demonstrate robust circadian rhythms in locomotor activity (Asher & Schibler, 2006; Debruyne et al., 2006), unlike peripheral oscillators which showed loss of rhythmicity without CLOCK (DeBruyne et al., 2007b). In LL conditions, *clock*$^{-/-}$ mice do not have a lengthened period, which conflicts with the Aschoff's Rule (Dallmann et al., 2011). Under LD conditions, *clock*$^{-/-}$ mice have an altered phase angle of entrainment. Furthermore, they exhibit a decrease in phase delays and greater phase advances, therefore, this might suggest that CLOCK is an important clock gene component with

regard to either transferring photic information to the SCN or in regulating SCN sensitivity to photic stimulation (Debruyne et al., 2006).

The transcription factor NPAS2 (which is also referred to as MOP4), is a paralog of Drosophila CLOCK (Hogenesch et al., 1997; Zhou et al., 1997) and can also heterodimerize with *bmal1* to drive gene expression (Hogenesch et al., 1998; Reick et al., 2001). NPAS4 can be used in place of CLOCK in *clock*$^{-/-}$ mice, as it is seen to regulate circadian rhythmicity in the absence of CLOCK. Arrhythmia occurs if mice are deficit in both CLOCK and NPAS4, therefore, indicating that one is necessary for functional circadian rhythms (DeBruyne et al., 2007a). Evidence for an overlapping role of both CLOCK and NPAS4 was also displayed in liver circadian oscillators (Bertolucci et al., 2008).

Studies have investigated what effect clock gene knockout has with regard to mood regulation and obesity in mice respectively (McClung, 2011; Turek, 2005). Reduced diurnal feeding rhythms, obesity and a hyperphagic condition are presented in homozygous *Clock* mutant mice. Further findings from this study also showed these mice developed a metabolic syndrome similar to diabetes (Marcheva et al., 2009; Turek, 2005). Another study investigating behavioural changes, showed that *Clock* mutant mice exhibited cocaine sensitization, hyperactive in a new environment and attenuated anxiety when compared to controls (Easton et al., 2003; McClung et al., 2005). *Npas4/Clock*$^{-/-}$ expressed marked astroglial activation throughout the brain, with the cortex greatest affected (Musiek et al., 2013).

1.6.3.4 *Bmal1* mutations

Bmal1 (Mop3) is essential for maintaining behavioural rhythms in both the SCN and peripheral clocks. It is also thought to be involved in the functioning of behavioural output. *Bmal1-/-* mice in DD are arrhythmic, have impaired circadian behaviour, display altered entrainment to LD cycle and contain impaired *mPer1* and *mPer2* rhythmicity in the SCN (Bunger et al., 2000). These KO mice presented with hyperlipidaemia and glucose intolerance (Marcheva et al., 2009; Rudic et al., 2004). *Bmal1*$^{-/-}$ mice display signs of impairment in learning and memory (Kondratova et al, 2010), along with early ageing and a reduction in their lifespan (Ali et al., 2015; Kondratov et al., 2006). A recent study found interesting findings with regard to astroglial activation in *Bmal1*$^{-/-}$ mice. At

2.5 months, glial fibrillary acidic protein (GFAP) exposed a high occurrence of astrogliosis throughout the brain, in *Bmal1*$^{-/-}$ mice. This increased further by six months, with the cortex most affected. These findings suggest that transcriptional regulation by BMAL1:NPAS2/CLOCK heterodimers, is necessary to avoid neuropathology in the brain (Musiek et al., 2013).

1.6.3.5 *Rev-erbα* mutations

REV-ERBα plays a role in regulating clock rhythmicity, as it represses *Bmal1* transcription (Liu et al., 2008). *Rev-erbα*$^{-/-}$ mice display changes in phase shifting of locomotor behaviour and there is also a decrease in their FRP when placed in constant conditions (Preitner et al., 2002).

Table 1: Summary of the effect of genetic mutation of core clock genes on behavioural phenotypes.

Gene	Mutation	Circadian Behaviour (DD)	Reference
Per1	*Per1*$^{-/-}$	Short	(Cermakian *et a*l. 2003)
Per2	*Per2*$^{-/-}$	Short/arrhythmic	(Zheng *et al*. 1999; Bae *et al*. 2001)
Per3	*Per3*$^{-/-}$	Short	(Bae *et al*. 2001)
Per1/Per2	*Per1*$^{-/-}$/*Per2*$^{-/-}$	Arrhythmic	(Bae *et al*. 2001)
Per2/Cry1	*Per2*$^{-/-}$ /*Cry1*$^{-/-}$	Arrhythmic	(van der Horst *et al*. 1999; Bae *et al*. 2001)
Cry1	*Cry1*$^{-/-}$	Short	(van der Horst *et al*. 1999)
Cry2	*Cry2*$^{-/-}$	Long	(van der Horst *et al*. 1999; Vitaterna *et al.* 1999)
Cry1/Cry2	*Cry1*$^{-/-}$/*Cry2*$^{-/-}$	Arrhythmic	(van der Horst *et al*. 1999;

			Vitaterna *et al.* 1999)
Bmal1	*Bmal1*$^{-/-}$	Arrhythmic	(Bunger et al. 2005)
Clock	*Clk*$^{-/-}$	Long/arrhythmic	(Dallman et al. 2011)
Rev-erbα	*Rev-erbα*$^{-/-}$	Short	(Preitner et al., 2002)

1.7 Entrainment of the SCN pacemaker

The circadian clock not only can generate its own rhythms but can also be entrained by Zeitgebers, which are external cues, allowing for photic entrainment using light or non- photic entrainment such as feeding time, temperature, exercise and chemical stimuli. Light is the most influential synchronizer of the SCN clock (Challet, 2007). Circadian rhythms vary between species since diurnal animals are active during the light phase and nocturnal animals are active during the night. Zeitgeber time (ZT), ZT0 is defined as the start of the light phase and ZT12 corresponds to the start of the dark phase. Phase response curves (PRC) is the time across the circadian cycle at which an external cue will effect circadian rhythmicity and cause phases shifts of the circadian locomotor activity, which will the oscillator to reset (Daan & Pittendrigh, 1976; Jud et al., 2005). Species differences are displayed in the form of the shape and amplitude of the PRC (Golombek & Rosenstein, 2010).

In constant light (LL) or constant darkness (DD) conditions, animals express a free running rhythm in the absence of external time cues. LL causes a lengthened period and presents a low amplitude of activity resulting in arrhythmic behavioural rhythms in mice (Aschoff, 1960; Ohta et al., 2005; Pittendrigh & Daan, 1976; Sudo et al., 2003). The lengthening period in LL is in proportion with the logarithm of the applied light intensity (Aschoff, 1952; Jud et al., 2005). Mice in DD usually have a shorter FRP due to their internal rhythms usually less than 24 hours, with one circadian unit typically less than 1 hour in constant darkness (Jud et al., 2005; Pittendrigh & Daan, 1976; Sudo et al., 2003). Since free running animals usually no longer have a period length of 24 hours, time is now represented in circadian time (CT) units. CT0 is defined as the onset of the resting

phase whereas CT12 is the onset of activity in free-running conditions. PRC's can occur either by photic stimuli or non-photic stimuli.

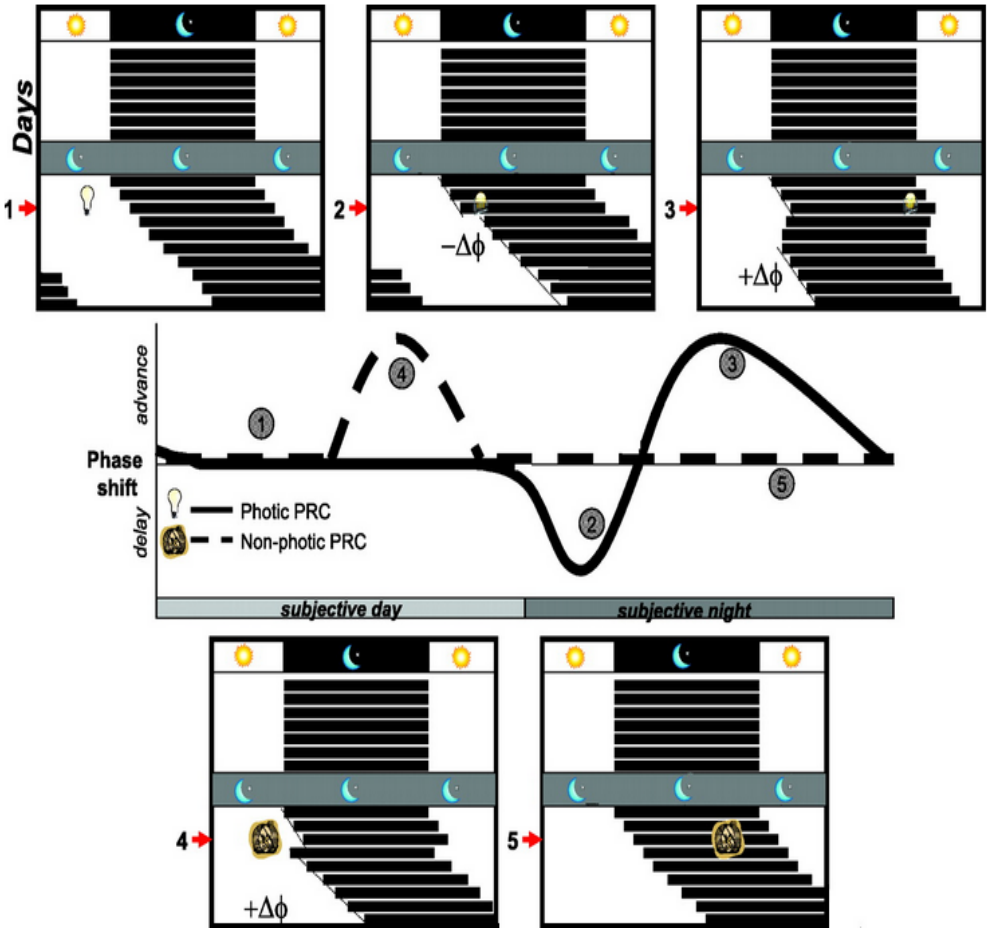


Figure 1.6: Actograms representing phase response curves of circadian rhythms. (1) displays no effect of photic stimulation during the subjective day, (2) a phase delay follows as a result of a light pulse in the early subjective night, (3) a phase advance occurs due to photic stimulation in the late subjective night, (4) a phase advance arises as a consequence of non-photic stimuli (novel running wheel) administered in the subjective day, (5) no phase shift was displayed in the subjective night as a result of non-photic stimuli. The middle graph emphasises the photic PRC (solid line) and the non-photic PRC (dashed line) with numbers 1-5 corresponding to the actograms (Golombek & Rosenstein, 2010).

1.7.1 Photic entrainment

1.7.1.1 Light pulses

Light is the most important cue in entraining the circadian clock. The photic PRC describes where photic stimuli at various circadian phases will result in a phase delay, a

phase advance or no change in the circadian phase (Jud et al., 2005; Marpegán et al, 2005). The phase shifting responses of the SCN clock to light depend on what time of the day light is administered. In nocturnal and diurnal animals, phase delays occur when light pulses are carried out in DD in the early subjective night, while phase advances take place when light pulses are administered in the late subjective night (Sumová & Illnerová, 2005). When a light pulse is administered during the day in nocturnal animals no phase shift occurs. This period where light has no resetting effect is called the dead zone (Daan & Pittendrigh, 1976; Takahashi et al., 1984).

Environmental light stimulates the photopigment melanospin found in retinal ganglion cells, which transmit light to the core of the SCN via the RHT (Hattar et al., 2002; Rollag et al., 2003; Ruby et al., 2002). The molecular mechanisms for entraining the circadian clock are believed to arise from a light pulse causing the release of the neurotransmitter glutamate (Reppert & Weaver, 2002). This brings about activation of glutamate receptors, which in turn induces phosphorylation of CREB resulting in its translocation to the nucleus where it binds to cAMP-responsive elements (CRE) on promoter regions of Per1 promoter, which leads to upregulation of Per1 transcription (Albrecht et al., 1997).

The extracellular signal related kinases (ERKs) are pathways also implicated in the phase shifts following photic stimulation. Activation of ERK leads to the phosphorylation of CREB, which results in the transcription of photically inducible genes. Activation of the ERK pathway is vital for photic entrainment of the circadian clock (Obrietan et al., 1998). Phosphorylated ERK (p-ERK) are rhythmic and peak during the subjective day in the SCN of Syrian hamsters (Coogan & Piggins, 2003; Webb et al., 2013). p-ERK is induced when light pulses are administered during the subjective night. Certain ERK pathway inhibitors or NMDA receptor channel blockers can cause reduce photic induction of p-ERK and attenuate phase advances of running wheel behaviour (Coogan & Piggins, 2003). Additionally, other pathways including CRTC1-SIK1 and protein kinase C (PKC) also are involved in regulating photoentrainment of the circadian clock (Bonsall & Lall, 2013; Jagannath et al., 2013). The CRTC1-SIK1 pathway may play a role in clock re-setting, with prompt re-entrainment displayed following jet lag experiments, therefore, SIK1 seems to be involved in reducing the effects of light on the SCN (Jagannath et al., 2013), whereas inhibition of PKC causes re-entrainment to be considerably slower (Bonsall & Lall, 2013).

Light induced phase shifts are noticeable by the induction of photically IEGs, which include c-Fos, egr-1 and jun-B. These genes contain CRE in their promoter regions, and bind to CREB which allow transcription to take place in response to photic simulation in the subjective night (Greenberg et al., 1992). c-Fos is the most studied IEG in response to photic stimulation. There is induction of c-Fos mRNA and immunoreactive Fos protein expression in the SCN of diurnal and nocturnal animals after administration of a light pulse at night and in response to lights on at dawn in the LD cycle (Hughes et al., 2004; Rea, 1989; Rusak et al., 1990). c-Fos expression is displayed in the core region of the SCN after even five minute light pulses in the subjective night, and can result in rapid induction of c-Fos within the hour of exposure to light (Colwell & Foster, 1992; Kornhauser et al., 1990; Schwartz et al., 1995). Therefore, c-Fos is thought to play a role in circadian function and photic entrainment.

There is induction of Per1 and Per2 protein expression in the SCN of diurnal and nocturnal animals after administration of a light pulse at night. This expression is not seen during the subjective day following administration of light pulse (Hamada et al, 2001; Shigeyoshi et al., 1997). The Per genes show that in the early subjective night there is upregulation of Per1 in the core and increased Per2 expression in the shell, following a light pulse. Following a light pulse in the late subjective night, Per1 expression was upregulated in the core and the shell. However, there was no increase in Per2 expression in the core or shell. This evidence presented here supports the idea that light acts on cells in the SCN core, resulting in the resetting of the pacemaker cells in the SCN shell from core output (Yan & Silver, 2004). Furthermore, the involvement of Per1 and Per2 in the synchronisation of the SCN clock to photic signals has been displayed by the fact that mice with a mutated *Per1* gene show no light induced phase advances, whereas mice with a *Per2* mutated gene display no light induced phase delays (Albrecht et al., 2001). It is established from these studies that at a molecular and behavioural level, circadian responses to light are not different between diurnal and nocturnal animals.

1.7.2 Non-photic entrainment

Non-photic entrainment can arise from chemical stimuli-scheduled food, scheduled exercise and temperature-but does not show responses to photic stimulation. The involvement of the IGL and the SCN, and serotonergic projections from the median

raphe to the SCN, seem to play a role in non-photic shifting (Meyer-Bernstein & Morin, 1996). There are two main chemical Zeitgebers which can be used to entrain animals to circadian rhythms-serotonin and melatonin-which will all be discussed.

1.7.2.1 Dark pulses

Animals kept in LL can have their SCN clock reset by a dark pulse. When dark pulses are administered in nocturnal rodents it results in phase advances during most of the subjective day, but during the late subjective night and early morning dark pulses cause phase delays (Boulos & Rusak, 1982; Canal & Piggins, 2006; Rosenwasser & Dwyer, 2002). Studies indicate that there is a down regulation of FOS, Per1 and Per2 expression in the SCN of hamsters (Coogan & Piggins, 2005; Mendoza et al., 2004). With regard to dark pulses, phase advance of circadian rhythms occurs in the resting phase of the animals. Therefore, nocturnal and diurnal species differ when dark pulses are administered (Mendoza et al., 2007). Studies have displayed that at a behavioural level, circadian responses to light differ between diurnal and nocturnal animals.

1.7.2.2 Serotonin

Serotonin is a neurotransmitter important in circadian entrainment, as it appears to be involved in non-photic phase shifting (Mintz et al., 1997). The SCN and the IGL obtain serotonergic projections from the midbrain raphe nuclei (Morin, 1999). Serotonin and 5-HT receptor agonists either *in vitro* or *in vivo*, can cause phase shifts of the SCN when administered at times where light does not cause any phase shifts in the circadian cycle (Challet et al, 1998; Ehlen et al., 2001; Reghunandanan & Reghunandanan, 2006a). Peripheral serotonin agonist injections which activate serotonin neurons cause phase advances in nocturnal animals when administered during the day in LL at CT8, whereas administration of serotonin agonist injections at any other time did not phase shift the SCN clock (Tominaga et al., 1992). In contrast, diurnal animals phase shift during the subjective night. Therefore, serotonin phase shifts the SCN clock in the animals resting phase (Cuesta et al., 2008).

Peripheral injection of the serotonin agonist, 8-OH-DPAT, administered in the mid subjective day in golden/Syrian hamster's results in phase advances (Challet et al.,

1998; Cutrera et al., 1994; Mintz et al., 1997). There has been some inconsistent findings with regard to direct serotonin agonist administration to the SCN directly *in vivo,* with phase shifts displayed in some studies and others finding no alterations (Antle et al., 2003; Prosser et al., 2006). However, *in vitro* studies have revealed that serotonin and serotonin agonists can act directly on the SCN to alter the phase of the circadian pacemaker in rats and mice (Medanic & Gillette, 1992; Prosser et al., 2006; Shibata et al., 1992; Sprouse et al., 2005). Administration to SCN explants during the subjective day results in a phase advance in the circadian pacemaker, whereas at night it causes phase delays (Prosser, 2000; Prosser, 2003).

1.7.2.3 Melatonin

Melatonin is a hormone secreted during the night by the pineal gland, which can also phase shift the master clock and amplify circadian rhythmicity (Reghunandanan & Reghunandanan, 2006a). The SCN controls circadian rhythmicity of the pineal gland to allow secretion of melatonin, hence melatonin is thought to be involved in a feedback role on the SCN (Challet, 2007). The highest amount of melatonin receptors such as MT_1 and MT_2 are located in the SCN (Ekmekcioglu, 2006; Reppert et al., 1988). The function of melatonin in the SCN has been recognised in several rodent studies. Administration of melatonin injections at subjective dusk in mice resulted in phase advances (Benloucif & Dubocovich, 1996). Furthermore, daily treatment of melatonin by oral administration, subcutaneous injection or infusion are able to entrain the locomotor rhythms of free running rodents (Pitrosky et al., 1999; Redman et al., 1983; Slotten et al., 1999; Slotten et al., 2002). Mice in DD present phase delays after morning administration, whereas phase advances occur after evening administration (Benloucif & Dubocovich, 1996). The effects of melatonin are independent of whether the animal is nocturnal or diurnal, as phase shifting occurs at similar times in both types (Slotten et al., 2002).

Timed administration of melatonin helps people readjust after acute phase shifts that take place from jet lag and shift work (Sack & Lewy, 1997). Another study carried out administered intermitting bright light by morning, melatonin by afternoon and a gradual advanced sleep schedule which resulted in an advance in circadian rhythms by one hour per day. This may be an effective treatment for passengers traveling eastwards to alleviate jet lag. It may also be a useful treatment in the condition delayed sleep phase

syndrome (DSPS) (Revell et al., 2006). Administration of melatonin can also entrain free running circadian rhythms in blind people and patients with dementia (Lockley et al., 2000; Mishima et al., 1994; Sack et al., 2000; Sack & Lewy, 1997).

1.7.2.4 Food and exercise entrainment

Feeding cues have only a weak influence over the SCN but are potent synchronizers of peripheral oscillators. Temporal restricted feeding together with calorie restriction, can alter phase angle of photic synchronisation in the SCN (Challet et al., 2003; Mendoza et al., 2005). A study was carried out to investigate if daily restricted feeding in CS mice was able to reset the circadian clock in the SCN. CS mice in DD were fed for 3-4 weeks at a fixed time of three hours per day, and then returned to having free access to food for 2-3 weeks. The outcome was that CS mice wheel running rhythms entrained to a restricted feeding schedule, whereas controls SCN did not entrain to a restricted feeding schedule. Therefore the circadian clock can be reset by food schedule in DD in CS mice (Abe et al., 2007).

Exercise cues can have an effect on the entrainment of the master clock (Mistlberger & Skene, 2005; Mrosovsky, 1996). In a study carried out by Mrosovsk, the effect of a three hour exercise pulse using a novel running wheel was investigated. This was given seven hours before the time hamsters became active in the LD cycle. The three hour exercise pulse caused a greater phase advance when compared to control mice. When hamsters were put into DD the following day after their exercise accelerated phase shift, their free-running activity rhythms started at a time nearer to the onset of darkness in the new LD cycle rather than the previous LD cycle, showing that scheduled exercise can entrain the SCN. An additional experiment carried out displayed that a three hour exercise pulse followed by a light pulse eight days after DD resulted in a phase advance, whereas controls who received a light pulse only phase delayed (Mrosovsky, 1989).

1.8 Circadian rhythms and disease

Environmental factors such as sleep restriction and shift work are known to impact on circadian rhythms which can lead to certain cancers and inflammation (Bechtold et al., 2010). Epidemiological studies have suggested a link between shift workers and an

increased risk of cancer (Logan & Sarkar, 2012). Shift work can cause altered circadian rhythms. Animal models have shown a link between circadian disruption and cancer. A higher incidence of breast cancer is associated with long-term shift workers (Schernhammer et al., 2006). In males, long-term shift workers face increased risk of prostate cancer (Kubo et al., 2006). By ablating the SCN or by manipulating the circadian cycle to cause chronic jet lag, alterations in circadian rhythms resulted in decreased lymphocytes numbers, which is related to increased rate of tumour growth (Filipski et al., 2006). An increase in the mortality of aged C57BL/6 mice can be seen with chronic circadian disruption (Davidson et al., 2006).

In relation to inflammation, chronic disruption in circadian timing using jet lag experiments in mouse models, resulted in altered innate immune responses. LPS challenge in chronic jet lag mice lead to an increased mortality rate compared to controls (Castanon-Cervantes et al., 2010). The potential long-term health consequences of shift workers and airline crew frequently crossing time zones may cause unnecessary health issues. Hence, there is a need for employers to work out a better working time schedule.

Genetic analyses of circadian rhythm sleep disorders allowed for the discovery of FASPS and DSPS sleep disorders. Intrinsic sleep disorders may be shown by DSPS, where patients have shifted circadian rhythms. Patient's falls asleep later at night, have more difficultly waking up in the morning and have reduced alertness in the morning. DSPS typically happens in adolescence and is the most common intrinsic circadian rhythm sleep disorder. Genetic studies identified extreme diurnal preference using the hPer3 gene, which has been implicated in the pathogenesis of DSPS (Archer et al., 2003; Ebisawa et al., 2001). The second intrinsic circadian rhythm sleep disorder discussed briefly earlier is FASPS, which occurs in patients who fall asleep early and wake earlier. This sleep disorder is associated with depression and has greater prevalence in elderly patients (Monk & Welsh, 2003; Xu et al., 2005). Genetic studies have revealed that missense mutation in the clock component *hPer2* gene causes alterations in the circadian period (Toh et al., 2001).

1.9 The innate immune system

The innate immune system is the first line of defence during infection and plays a vital role in recognising invading pathogens and activating a pro-inflammatory response

against them (Mogensen, 2009). When infection occurs, the cells of the innate immune system cause inflammation to arise. Macrophages and dendritic cells are pathogen recognition receptors (PRR's) which recognise pathogen-associated molecular patterns (PAMPs) that are present on the surface of invading microbes. This results in the activation of the innate immune system (Medzhitov & Janeway, 2000). The most commonly studied PPRs are toll like receptors (TLRs). There are 10 known TLRs studied in humans, and each TLR produces specific proteins that act as cell surface receptors which identify a group of ligands PAMPs in bacteria, fungi and viruses (Akira et al., 2006). The TLR4 binds to pathogen-associated molecular patterns in LPS which causes a signalling cascade via two pathways, NF-κB-dependent and NF-κB-independent pathways (Pålsson-McDermott & O'Neill, 2004).

There are two basic intercellular pathways which play a role in TLR signalling, firstly the MyD88 dependent pathway (NF-κB-dependent) and secondly the MyD88-independent pathway (NF-κB-independent) (Bagchi et al., 2007; Takeda & Akira, 2004). The MyD88 dependent pathway is important for the early activation of the NF-κB signalling pathway and is responsible for pro-inflammatory cytokines expression, whereas the MyD88-independent pathway is involved in the late phase NF-κB activation and is responsible for the induction of IFN-inducible genes, along with maturation of dendritic cells (Akira et al., 2006; Broad et al., 2007; Kawai & Akira, 2011). It is necessary for both the early and late phase activation of NF-κB to enable the induction of inflammatory cytokines (Kawai & Akira, 2011). The adaptors myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) are involved in the signal transduction of TLR4 (Akira, 2009; Horng et al., 2002; Yamamoto et al., 2003).

The early activation phase of NF-κB stimulation of TLR4 and LPS result in receptor recruits MAL/TIRAP and MyD88 which are TIR containing proteins. This complex then recruits IRAK4 and IRAK1/2 (IL-1 receptor-associated kinase) which contain both a death domain and a kinase domain (Lu et al., 2008). IRAK4 then phosphorylates IRAK1/2 resulting in recruitment of the adaptor protein TRAF-6 (TNF receptor-associated factor 6) (Kawai & Akira, 2010; Keating et al., 2007). TRAF-6 forms a complex with UEVIA (ubiquitin-conjugating enzyme E2 variant 1 isoform A) and UBC13 (ubiquitin-conjugating enzyme 13), this allows for the activation of Transforming

growth factor-B-activated kinase 1 (TAK1) to take place. TAK 1 is important for activating the downstream IKK pathway as well as the MAPK (mitogen-activated protein kinase) pathway. This IKK complex phosphorylates IκB proteins which leads to the translocation of NF-κB from the cytoplasm to the nucleus. NF-κB DNA binding and transcription of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α takes place (Mogensen, 2009; Moynagh, 2005b).

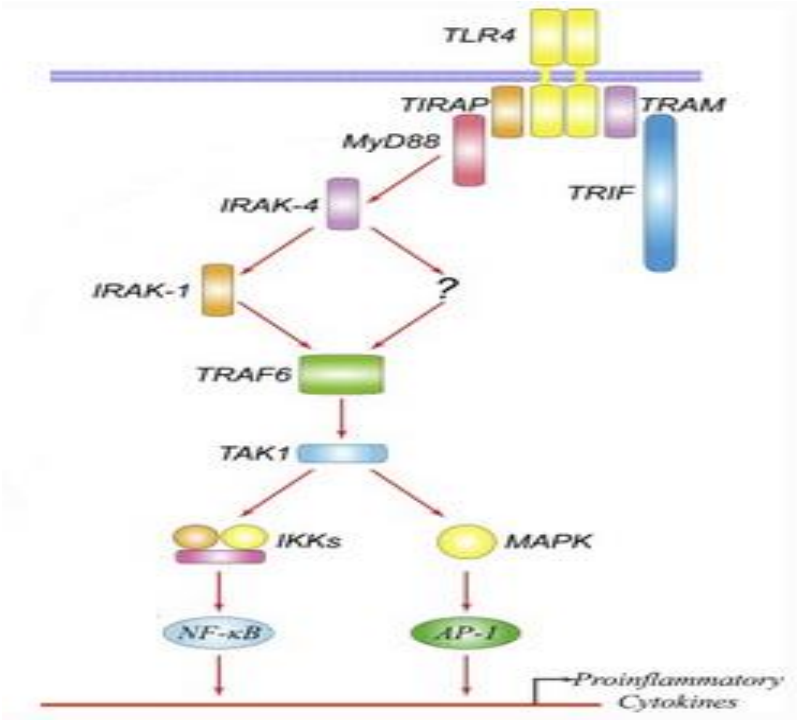


Figure 1.7: MyD88-dependent pathway- involves MyD88 activating IRAKs/TRAF which results in the activation of the transcription factor NF-κB and AP-1 further downstream, adapted from (Lu et al., 2008).

TLR4 triggers the MyD88 independent pathway via TRAM to induce IFNs. TRIF is an important TIR-containing adaptor protein which interacts with RIP and TRAF3 and activates MAPK, IRF3 and the late phase activation of NF-κB. TBK1/IKK-I phosphorylates IRF-3 which results in the translocation of IRF-3 into the nucleus which leads to the expression of the IFN inducible gene important for anti-viral and anti-bacterial responses. Mice lacking MyD88, RIP and TRIF result in reduced activation of NF-κB in response to TLR4 stimulation (Bohannon et al., 2013; Yamamoto et al., 2003).

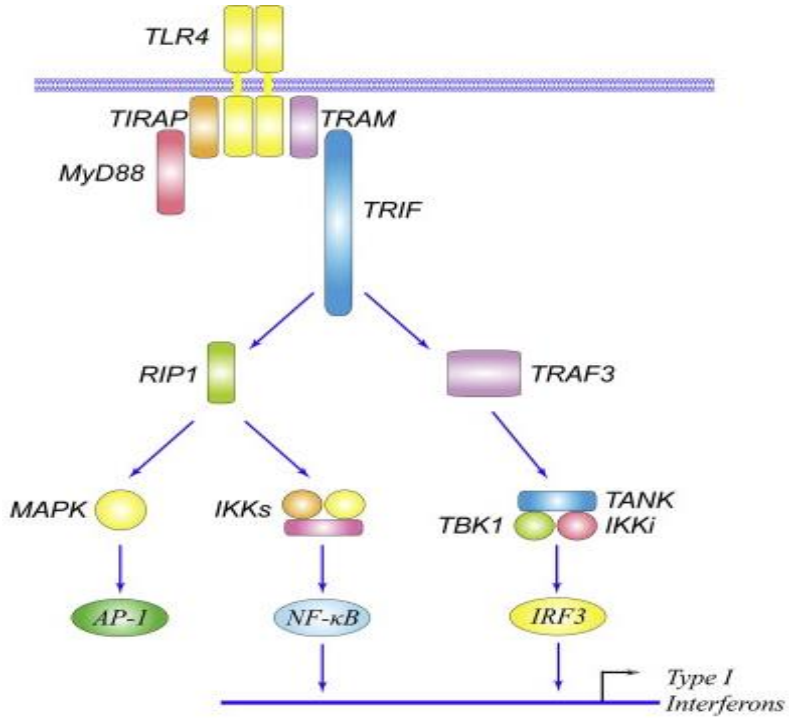


Figure 1.8: MyD88-independent pathway- TRIF recruits TRAF3 and RIP1 which result in activation of the transcription factor IRF3, NF-κB and AP-1.

LPS consists of lipid A which is a hydrophobic endotoxin and makes up the outer membrane of most gram negative bacteria. LPS stimulation occurs through a series of interactions with several proteins, such as TLR4, CD14, MD-2 and LBP (LPS binding protein). LBP forms a high affinity complex with the lipid A part of LPS which results in LPS forming a ternary complex with CD14. CD14 can only bind to LPS in the presence of LBP (Wright, 1999). CD14 directs the transfer of LPS to the TLR4/MD-2 receptor complex allowing for LPS-dependent responses in many cells (Juan et al., 1995; Palsson and O'Neill, 2004). MD-2 is a soluble protein that non-covalently associates with TLR4, but can directly form a complex with LPS in the absence of TLR4 (Shimazu et al., 1999). MD-2 is vital for LPS signalling to occur as mice lacking this protein are unresponsive to LPS (Bohannon et al., 2013; Nagai et al., 2002).

(A) **(B)**

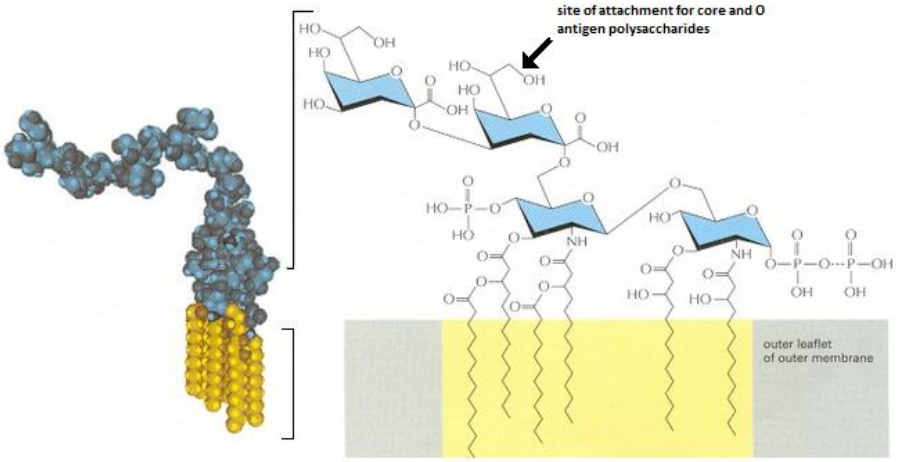


Figure 1.9: Simple LPS structure: (A) is a 3-dimensional structure of LPS, with sugars displayed in blue and fatty acids illustrated in yellow. **(B)** Represents the molecular base structure of LPS made up of lipid A, a core oligosaccharide, and an O side chain (Alberts et al., 2002).

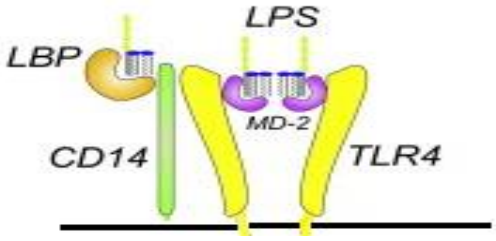


Figure 1.10: LPS interaction with TLR4, LBP, CD14 and MD-2, adapted from (Lu et al., 2008)**.**

1.10 Interaction between the circadian system and immune response

The crosstalk between the circadian system and the immune system is bidirectional (Coogan & Wyse, 2008; O'Callaghan et al., 2012). The circadian system can play a role in immune function, and likewise, the different immune cells can affect the circadian system by acting on the SCN clock and in peripheral clock tissues (Cermakian et al., 2013; Logan & Sarkar, 2012).

1.10.1 Interaction of the circadian systems and the immune system

The endocrine and autonomic pathways plays a role in communicating circadian timing information from the SCN to the immune system (Logan & Sarkar, 2012). Stress

and immune function are regulated by the SCN which communicates with the PVN and arcuate nuclei (ARC) through axonal connections (Saeb-Parsy et al., 2000).

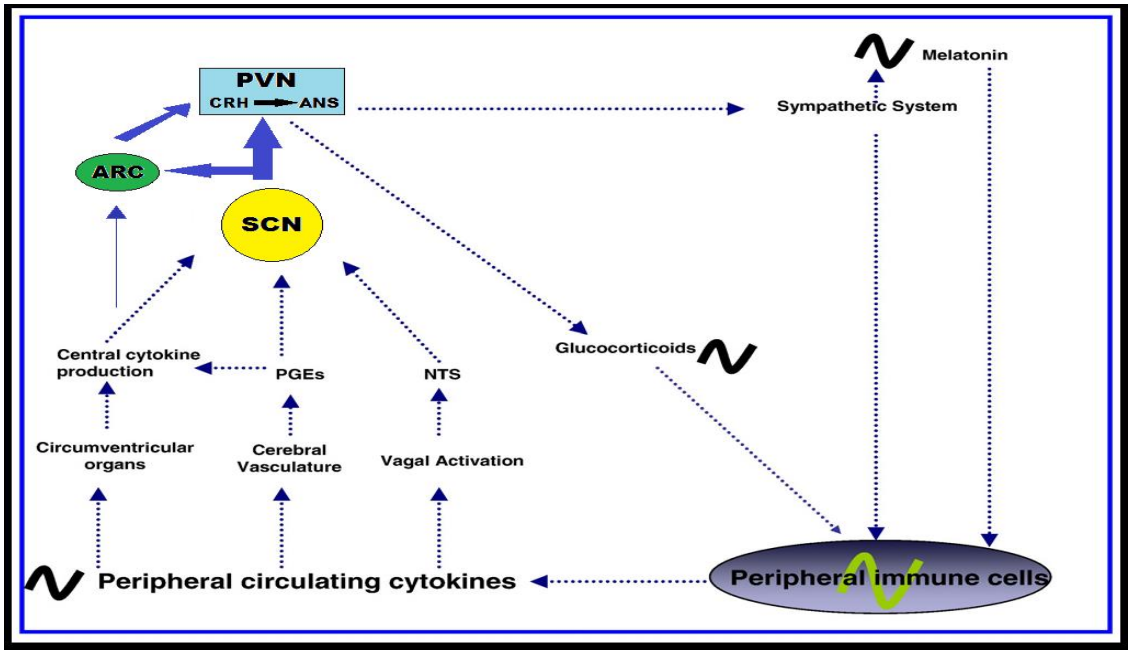


Figure 1.11: Bidirectional communication between the circadian and immune system. The SCN and the ARC send projections to the PVN which results in modulating the autonomic nervous system (ANS) endocrine and neural signalling to peripheral immune tissues. Activated cytokines can modulate clocks in the SCN and ARC, adapted from (Coogan & Wyse, 2008; Logan & Sarkar, 2012).

The innate immune system is under circadian control. In the brain, cytokines display circadian variations: in particular IL-1β peak in the hypothalamus at the onset of the activity phase (Taishi et al., 1997). TNF-α, glucocorticoids, noradrenaline and adrenaline also exhibit circadian variation in expression, similar to IL-1β (Scheiermann et al., 2013). The circadian regulation exhibited in the immune pathway components may serve to enhance immune responsiveness at the time of the circadian cycle at which infection is most likely to occur. Mice are highly sensitive and have reduced survival rates when exposed to endotoxins and cytokines in the early subjective night (active phase) (Scheiermann et al., 2013). A recent study has displayed how LPS administration can cause diurnal changes to microglia inflammatory responses, with the light phase displaying increased cytokine gene expression when monitored *ex vivo*, in isolated microglia from the hippocampus of rats (Fonken et al., 2015). Other studies have shown

that LPS administered in the late subjective day ZT11 can increase mortality, in comparison to ZT19, when mice are in their active phase (Marpegan et al., 2009).

IL-1β and TNF-α receptors of the immune system are expressed rhythmically in the SCN (Beynon & Coogan, 2010).The administration of lipopolysaccharides (LPS) can modulate circadian rhythms in the SCN, resulting in phase delays or phase advances depending on the time of administration (Marpegán et al., 2005). In addition, Per2 and Dbp expression is briefly repressed in the SCN following LPS application (Cavadini et al., 2007; Okada et al., 2008). Clock gene expression can be altered when IFN-α is administered (Koyanagi, 2002; Kwak et al., 2008). Firstly, CLOCK and BMAL1 levels are reduced in the SCN as a result of IFN-α application (Koyanagi, 2002). Secondly, it was shown that long term IFN-α application in SCN explants from Per1::Luc rats have attenuated Per1-luc rhythm aptitude in comparison to controls (Kwak et al., 2008). Astrocytes in the SCN have also been discovered to control the circadian clock as a result of TNF-α administration, with SCN astrocyte cultures having altered phase and amplitude of PER2 expression rhythms, however, it is phase dependent. This study suggests that astrocytes may play a role in the alteration of circadian timing as a result of an immune challenge (Duhart et al., 2013).

Application of TNF-α *in vitro* on SCN cultures, resulted a prompt increase in the spontaneous firing rate in most SCN neurons, with a nitric oxide (NO) inhibitor seen to block this effect. Therefore, NO application may play a role in controlling SCN electrical output (Nygård et al., 2009). Studies have shown how LPS-induced circadian responses are mediated by TLR4 and TNF respectively (Paladino et al., 2010; Paladino et al., 2015). In mice deficit in the TNF-α receptor Tnfr1, it was witnessed that LPS administration caused no phase delay of locomotor activity at CT15, and furthermore, it did not alter c-Fos or Per2 expression in the SCN when compared to controls (Paladino et al., 2015). When the role of TLR4 in the circadian system was investigated since it is known to initiate the LPS cascade, LPS administered to $TLR4^{-/-}$ mice also displayed no phase shifts at CT15 as a result of this immune challenge. Additionally, c-Fos and Per1 expression were significantly upregulated in the PVN of controls mice following LPS, however, no induction of c-Fos or Per1 expression were displayed in $TLR4^{-/-}$ mice (Paladino et al., 2010). The two studies emphasise the importance of TLR4 and TNF-α in circadian modulation as a result of an immune challenge.

1.10.2 The Circadian system and immune function and disease

Circadian disruption can have implications on health leading to sleep disorders, inflammation and cancers (Bechtold et al., 2010; Brooks & Canal, 2013; Evans & Davidson, 2013; Logan & Sarkar, 2012). Studies carried out found a link between cancer and shift work. Shift workers have a high prevalence in obesity, diabetes and cardiovascular disease (Logan & Sarkar, 2012). Several chronic inflammatory diseases are known to exhibit circadian variations. Rheumatoid arthritis (RA) patients have joint stiffness in the early morning which is associated with the increased serum levels of TNF and IL-6 (Cutolo, 2012).

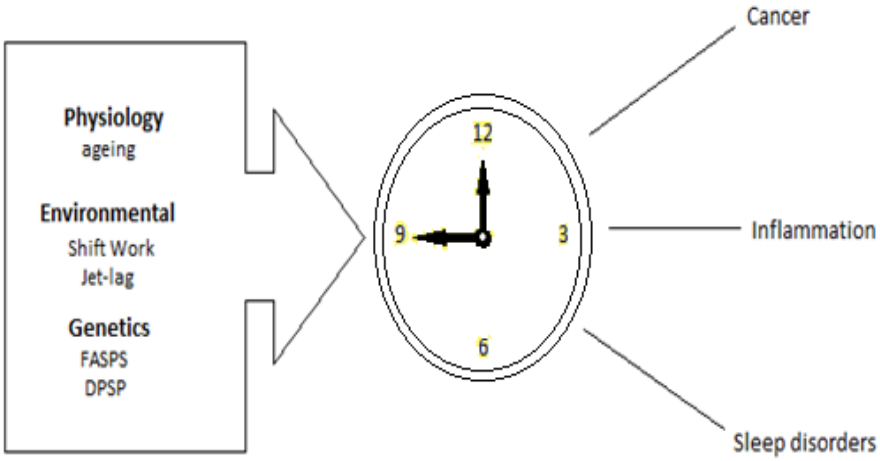


Figure 1.12: The circadian clock and disease: Disruption of the circadian clock due to physiology, environmental factors and genetics can lead to clinical and pathological conditions for example cancer, inflammation and sleep disorder, adapted from (Bechtold et al., 2010).

Sleep disorders can occur due to circadian dysfunction. In circadian rhythm sleep disorders there is a mismatch between circadian timing of sleep and the demands of the environment, which results in symptoms of disturbed sleep and impaired day time alertness. There are two types of circadian sleeping disorders - extrinsic and intrinsic disorders. Jet lag and shift work are examples of extrinsic disorders caused by environmental factors whereas delayed sleep phase syndrome (DSPS) and familial advanced phase sleep syndrome (FASPS) are examples of intrinsic disorders related to genetics.

1.11 The role ageing plays in the circadian timing system

Understanding the role neurobiology plays in normal ageing and disease is of vital importance. There are two types of ageing. Firstly, primary ageing which causes gradual changes over time which are generally not harmful. The other type of ageing is senescence or secondary ageing which is a more progressive form of ageing with deficiencies to metabolic, physiological and neurological functions causing a higher death rate. Dysfunctional circadian clocks in mouse models can result in attenuated lifespan. Therefore, a hypothesis needing investigation is that the ageing process can be slowed down by the re-organising of metabolic and physiological function by the circadian clock (Costa & Ripperger, 2015).

Studies have shown that ageing can cause dysfunction to the circadian timekeeping system in terms of behaviour and also at a molecular level (Crowley, 2011; Popa-Wagner et al., 2015; Weinert, 2000). The exact underlying mechanisms of this dysfunction are not clearly understood. Defects in the circadian clock can result in premature ageing, with *Bmal-1*$^{-/-}$ and *Clock-/-* mice having disrupted circadian behaviour along with reduced lifespans. *Bmal-1*$^{-/-}$ mice display sarcopenia and cataract development as a consequence of ageing (Dubrovsky et al., 2010; Kondratov et al., 2006). The average lifespan of *Bmal-1*$^{-/-}$ mice is 8 months is comparison to WT mice with an average of lifespan of 26 months (Kondratov et al., 2006). Furthermore, increased inflammation was observed in ageing *Bmal-1*$^{-/-}$ mice, along with increased astrocyte activation in the brain. This study also found chronic inflammation with elevated levels of *Ptghs2* and *Tnf-α* mRNAs and COX2 protein in the cortex of ageing *Bmal-1*$^{-/-}$ mice (Musiek et al., 2013). Physiological factors such as senescence causes circadian dysfunction in humans and mammals, as there is a decrease in amplitude of circadian rhythms in temperature and hormone secretion when analysed (Kondratova & Kondratov, 2012; Sellix et al., 2012). In the healthy ageing, dementia and neurodegenerative diseases neuropathological changes were found in the SCN when studying post-mortem brains (Hofman & Swaab, 2006).

As ageing results in circadian dysfunction, it can impact on certain neurodegenerative diseases for example Alzheimer's disease (AD) and Huntington's disease (HD). In neurodegenerative diseases, circadian rhythm and sleep dysfunction are some of the first clinical symptoms seen in AD, HD and PD. Circadian system and sleep

irregularities worsen as the diseases progress. One theory for the dysfunction is that there is degeneration of nuclei containing circadian clock regulation circuits in the SCN. Excitatory and inhibitory neurotransmitters are regulated by the SCN, therefore, disruption to the release and suppression of neurotransmitters may be contributing to circadian clock and sleep irregularities in ageing and neurodegenerative diseases (Wulff et al., 2010). Animal models in AD, HD and PD have all shown negative effects of neurodegeneration on the circadian clock (Kudo et al., 2011; Oakeshott et al., 2011; Sterniczuk et al., 2010).

AD is a neurodegenerative disease associated with cognitive and behaviour impairments. AD patients have a troubled diurnal and nocturnal rhythm, with increased nocturnal awakening while there is an increase in the amount of time one sleeps during the day (Coogan et al., 2013). Since CLOCK genes are an important factor in maintaining circadian rhythmicity, studies had investigated how alterations of the CLOCK genes may in fact play a part in the pathophysiology of AD, but no CLOCK gene variant has been identified as a risk factor in AD to date (Thome et al., 2011). The mouse model 3xTg-AD displayed alterations to their circadian pacemaker with a decline in nocturnal behaviour when compared to aged controls. There was also a decrease in VIP and AVP in the SCN of 3xTg-AD compared to controls (Sterniczuk et al., 2010). Since VIP and AVP are able to alter SCN neuronal function, their decrease in AD may play a role in the disease.

Having robust daily rhythms of the sleep and wake cycle are vital for good health. HD patients display disturbances in their daily cycle of the sleep and wake cycle which has an impact on a patient's cognitive function and quality of life (Cuesta et al., 2014). In the mouse model of BAC HD, studies have revealed circadian dysregulation of sleep, heart rate and body temperature (Kudo et al., 2011). BAC HD mice also displayed significant lengthening of their period, demonstrating that circadian regulation is a core feature in HD (Oakeshott et al., 2011). Another mouse model R6/2 which carries the HD mutation displays dysregulation of circadian rhythms that deteriorate as the disease progresses. Cognitive decline is also exhibited in R6/2 mice, and treatment with Alprazolam can improve cognitive function (Pallier et al., 2007). An additional study using the R6/2 mice used bright light therapy and voluntary exercise as treatment which resulted in positive effects, as there was improved behavioural synchronisation to the light–dark cycle (Cuesta et al., 2014). Trying to stabilise the sleep wake cycle may in turn

reduce the symptoms of neurodegenerative diseases such as cognitive decline, motor function difficulties and behavioural impairments.

Since there is circadian dysfunction in neurodegenerative diseases, a therapeutic approach to treating the disease might be beneficial to ameliorate circadian misalignments. Chronotherapeutics might involve light therapy, exercise and melatonin for treating AD and HD patients (Coogan & Thome, 2011; Dowling et al., 2008; Mishima et al., 1994). Current and future studies in chronopharmacology are aiming to correlate the time of administrating a drug according to their peak efficacy while exerting minimal side effects (Levi & Schibler, 2007). As knowledge expands even more on the relationship of the circadian clock and health and disease, future chronopharmacology and chronotherapeutics discoveries will be of immense importance.

1.12 NF-κB

1.12.1 What is NF-κB?

Nuclear factor-kappa B (NF-κB) is a transcription factor that is essential for immune and cell survival signalling pathway (Baeuerle & Henkel, 1994; Bhakar et al., 2002). NF-κB plays an important role in coordinating both innate and adaptive immunity and is also involved in regulating certain genes responding to different stimuli and infections (Pomerantz & Baltimore, 2002). Therefore a breakdown in this transcription factor can lead to inflammation and autoimmune diseases (Yamamoto & Gaynor, 2001) It was 29 years ago when the NF-κB transcription factor was discovered (Sen & Baltimore, 1986). NF-κB is composed of homo and hetro dimers of the Rel family, which consist of p65 (RELA), RelB, c-Rel, p50/p105 (NF-κB1), p52/p100 (NF-κB2) (Perkins, 2007). The NF-κB proteins play a part in specific biological responses as they can regulate target gene transcription differentially. Dimers that contain transcriptional activation domain (TAD) like p65, RelB or c-Rel are transcriptional activators, while p50 and p52 homodimers are repressors as they lack a TAD (Bonizzi & Karin, 2004; Hayden & Ghosh, 2008). The proteins exhibit various different binding specificities including p50/c-Rel, p52/c-Rel, p65/c-Rel, p65/p65, p50/p50, p52/p52, Rel-B/p50 and Rel-B/p52, with p65/p50 being the most common dimeric complex (Moynagh, 2005a). p65 allows for the strongest transcriptional activating potential of NF-κB, and the p50 subunit allows for the

strongest DNA binding affinity (Schmitz et al, 2004). Each dimer has different transcriptional functions, p65/p50 promotes transcription and is pro-growth and pro-survival. p52/RelB partakes in B-lymphocyte differention and while p50/p50 and p52/p52 are usually inhibitory (Ling & Kumar, 2012). Each Rel protein contains an N-terminal region, called the Rel Homology Domain (RHD) which contains a conserved 300 amino acid residue domain. The RHD has a DNA-binding domain on the N-terminal end, and it contains sequences necessary for dimerization, nuclear localization and inhibitor binding (Courtois & Gilmore, 2006; Yamamoto & Gaynor, 2001). The dimerization domain is located at the C-terminal of the RHD. Near the C-terminal, the Nuclear Localization signal (NLS) can be located. This is vital for transporting active NF-κB complexes into the nucleus (Ghosh et al., 1998).

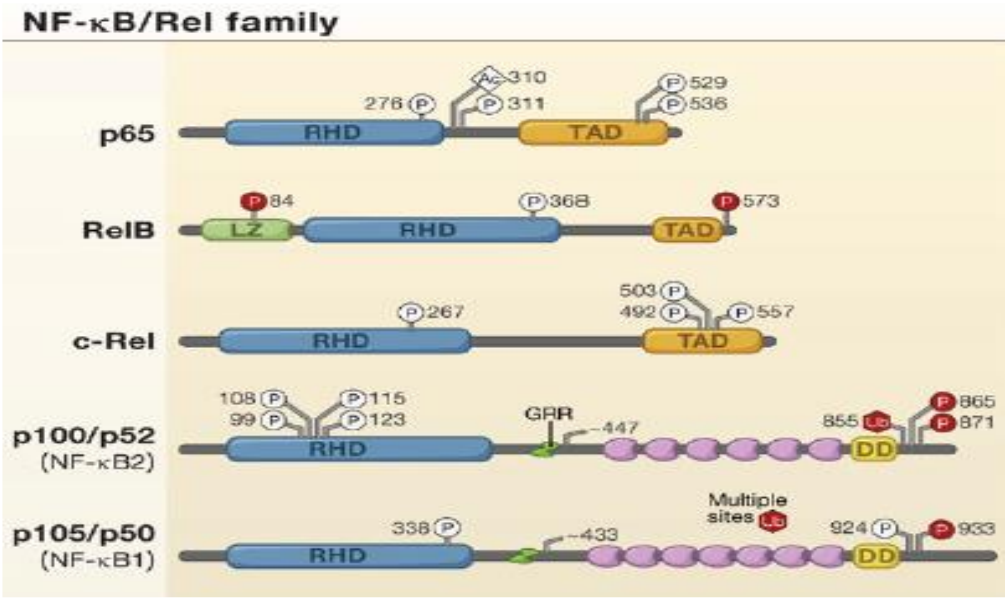


Figure 1.13: Members of the NF-κB family, (Hayden & Ghosh, 2008)

There are two other proteins that are vital for regulating the NF-κB pathway known as the IKB family and the IKK complex. There are seven known IκB's which have been identified so far, IκB-alpha, IκB-beta, IκB-gamma, IκB-epsilon, BCL3, P100 and p105 (Hayden & Ghosh, 2004). All forms of the inhibitor IκB comprise of multiple ankyrin repeat domain, which are multiple copies of around 30-33 amino acid sequences. The ankyrin repeats facilitate the association between the IκB and NF-κB dimers binding to the RHD (Courtois & Gilmore, 2006). Nuclear translocation is prevented when ankyrin repeats interact with a region in the RHD and this masks their NLS. IκBα can only mask

one nuclear localisation sequence, whereas IκBβ can mask both nuclear localisation sequences (Moynagh, 2005).

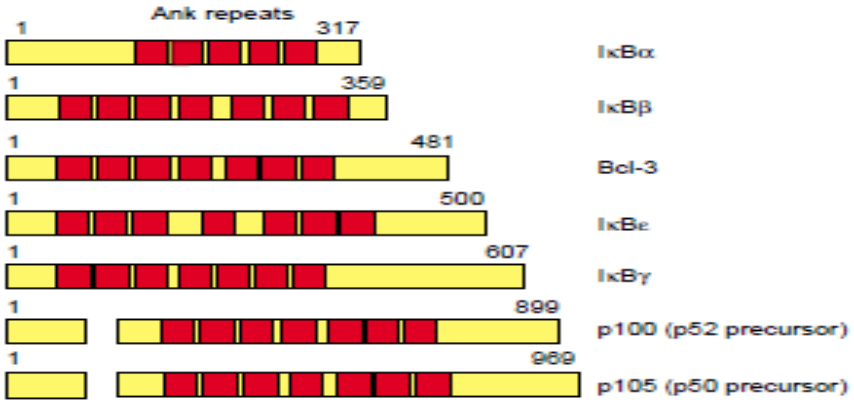


Figure 1.14: Members of the IκB family, (Bonizzi & Karin, 2004)

There are 3 main subunits of the IκB kinase (IKK) complex. IKKα (IKK1) and IKKβ (IKK2) are catalytic subunits, whereas the third subunit IKKγ (NEMO), has no catalytic activity (Karin & Ben-Neriah, 2000; Scheidereit, 2006).

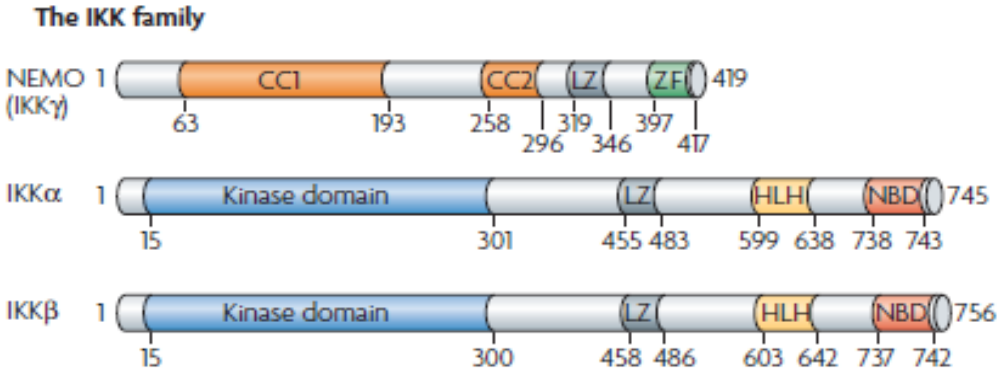


Figure 1.15: The IKK complex, (Perkins, 2007)

1.12.2 NF-κB Pathways

There are two main NF-κB pathways recognised-the canonical pathway and the non-canonical pathway (Bonizzi & Karin, 2004; Ghosh & Karin, 2002). The two pathways play different roles in immune function as they turn on different genes (Karin & Greten, 2005). The canonical and non-canonical pathway differ significantly in terms of signalling mechanisms (Sun, 2011). The canonical pathway is the better characterised pathway involved in activating NF-κB. This pathway is activated by physiological NF-κB inducing agents, inducing inflammatory cytokines, and antigen receptors. The

p65/p50 complex in its non-active form is held in the cytoplasm due to its association with members of the inhibitor IκB protein family. Activation of NF-κB in the canonical pathways depends on the degradation of IκB, and this only occurs when the IKK complex is activated (Moynagh, 2005; Perkins, 2007). NF-κB stimuli such as pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) and LPS can activate the IKK complex. These stimuli cause NEMO to ubiquitylaten at lysine 63 which recruits kinases which then phosphorylate IKKβ at serine 177/181. This results in IKK complex activation. In the canonical pathway IKKβ is the main IκB kinase (Perkins, 2007). Phosphorylation of IκBα occurs on two serine residues, S32 and S36, resulting in ubiquitination by SCF-βTrCP at Lys21 and Lys22 and then degradation by the 26S proteasome (Bonizzi & Karin, 2004; Courtois & Gilmore, 2006; Doyle et al., 2013). Once IκB is removed, translocation of NF-κB can now take place from the cytoplasm to the nucleus where it binds to a κB consensus sequence GGGACTTTCC, where transcription of specific cellular genes occurs (Yamamoto & Gaynor, 2001). A feedback pathway where new synthesized IκBα binds to the activated NF-κB in the cytoplasm results in down-regulation, and transportation out of the cytosol (Hayden & Ghosh, 2004). However modulation can also take place of NF-κB transcription complexes, by post-translational modifications and interacting with other proteins (Perkins, 2006).

The non-canonical pathway/alternative pathway has shown in genetic studies with mice that it is important in lymphoid organogenesis and B-lymphocyte function (Bonizzi et al., 2004). Dysregulation of this alternative pathway signalling is connected with lymphoid malignancies. Hence, it is important to understand the mechanism regulating this pathway (Sun, 2011). This pathway is activated in response to certain TNF family members, comprising of B-cell activating factor (BAFF), CD40 ligand, and lymphotoxin-β (LT-β) receptor (Claudio et al., 2002; Coope et al., 2002; Doyle et al., 2013). The main dimer involved is the p100-RelB heterodimer. When at rest this dimer is located in the cytoplasm. This pathway is based on regulated processing of NF-Kb2/p100 precursor protein (Xiao et al., 2001). p100 contains an N-terminal RHD, and an inhibitory IκB-like C-terminal domain. This C-terminal domain inhibits nuclear translocation of p100 (Bonizzi et al., 2004). It is strictly dependent on the IKKα subunit, whereas IKKβ and IKKγ play no role in this pathway (Bonizzi & Karin, 2004). NF-κB inducing kinase (NIK) activates IKKα, and phosphorylation takes place of p100 at two C-terminal sites which leads to its ubiquitation and proteasomal processing to p52 (Xiao et al., 2001). This in

turn causes activation and translocation of p52-RelB hetrodimers into the nucleus to activate certain genes that are vital for B-cell development and lymphoid organogenesis.

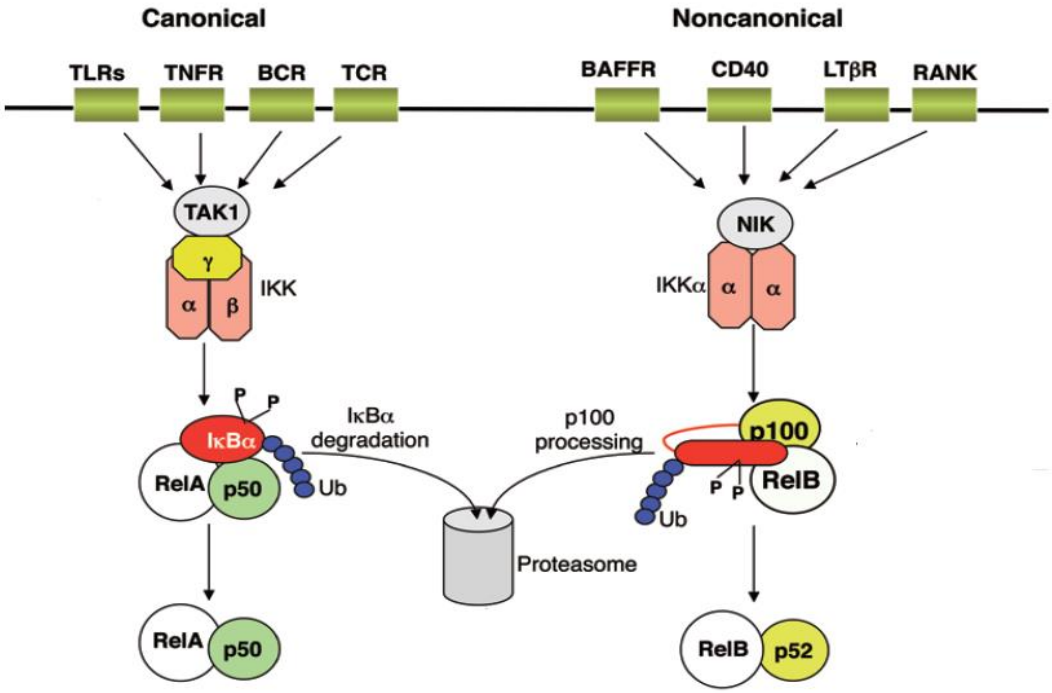


Figure 1.16: the canonical and non-canonical pathway, adapted from (Sun, 2011). The canonical pathway is induced by TLRs and TNFs and many other stimuli and is dependent on activation of IKKβ by Tak1. Activation of IKKβ results in phosphorylation of IκBα, this leads to ubiquitylation and degradation by proteasome. The heterodimer RelA/p50 can then translocate to the nucleus from the cytoplasm. The non-canonical pathway activates IKKα by NIK, p100 is then phosphorylated by IKKα. This results in proteasome processing of p100 to p52, forming the heterodimer p52/RelB. Translocation to the nucleus of p52/RelB occurs to activate certain genes.

1.12.3 NF-κB in diseases

The transcription factor NF-κB plays a key role in immune cell development, homeostasis and survival. NF-κB regulates the expression of inducers and effectors in response to pathogens in the immune system (Hayden & Ghosh, 2008). This pathway controls the expression of hundreds of human genes (Courtois & Gilmore, 2006). NF-κB is activated by pathogen recognition through either toll-like receptors of the innate immune system or from specific B and T cell receptors of the adaptive immune system (Kumar et al., 2004). Dysregulation of this pathway due to amplified Rel genes or aberrant activation of upstream regulators can lead to the development of autoimmune diseases,

chronic inflammation, and cancer (Schulze-Luehrmann & Ghosh, 2006). There has, so far, been 700 inhibitors of NF-κB discovered and these are vital due to the central role that this transcription factor plays in inflammation and cancer (Tafani et al., 2013).

Patients with chronic inflammatory diseases such as RA, asthma, and IBD have high levels of active nuclear NF-κB (Atreya et al., 2008). It is unknown what causes these diseases, though genetics and environmental factors are thought to be fundamental in disease process. In chronic inflammatory diseases, adhesion molecules recruit inflammatory cells to the area of inflammation such as neutrophils, eosinophils and T lymphocytes. NF-κB controls the expression of certain genes that encode adhesion molecules for example vascular-cell adhesion molecule 1, intercellular adhesion molecule and E-selectin. IL-1β, IL-6 and TNF-α are increased in patients suffering with chronic inflammatory diseases. Persistent activation of NF-κB from IL-1β and TNF-α may decide the severity of the disease. (Barnes & Karin, 1997). When a patient is suffering from chronic inflammation, treatment of glucocorticoid or immunosuppressive therapy may suppress the disease, however there is no curative treatment available to deal with any chronic inflammatory diseases (Barnes & Karin, 1997).

RA is an autoimmune disease that primarily targets synovial tissues, cartilage and bone. The cause of this disease is unknown and it is the most common form of immune-mediated arthritis. Studies have showed that pro-inflammatory chemokines and cytokines produced by diseased tissue is NF-κB dependent (Aupperle et al., 1999; Aupperle et al., 2001). Animal models of arthritis also demonstrate the role NF-κB plays. Joint inflammation is followed by NF-κB activation but this can be prevented by abolishing NF-κB function (Miagkov et al., 1998). In RA patients, levels of TNF-α (mediated by NF-κB) are elevated in the synovial fluid which result in chronic inflammatory changes in the joints. Symptoms of patients can improve when treated with either TNF-α inhibitors or a TNF-α receptor that will bind to TNF-α (Feldmann et al., 1996). However there are many patients who do not respond to TNF-α inhibitors or do not respond well to this treatment after time, leading to a need for an alternative therapy (Emery, 2012; Smolen & Keystone, 2012).

Idiopathic inflammatory bowel disease consists of two types of chronic intestinal disorders 1) crohn's disease and 2) ulcerative colitis. Crohn's disease mainly affects the ileum and colon, whereas in ulcerative colitis, inflammation usually occurs in the rectum and parts of the colon (Abraham & Cho, 2009). In inflammatory bowel diseases, patients

with active crohn's disease and ulcerative colitis show NF-κB activation in their tissue macrophages and mucosal biopsy specimens (Atreya et al., 2008; Pacifico & Leonardi, 2006). A study was carried out to examine the role of NF-κB activation during mucosal inflammation in situ. Activated NF-κB was discovered in biopsy specimens of inflamed mucosal whereas, in uninflamed mucosal, there was minute NF-κB activation. The activated NF-κB was similar in the inflamed mucosal of crohn's and ulcerative colitis patients (Rogler et al., 1998). An effective agent for treating inflammatory bowel diseases is sulfasalazine which is an NF-κB inhibitor (Wahl et al., 1998). Patients are also treated with steroids which lessen NF-κB activity and reduce clinical symptoms. This indicates that activation of the NF-κB pathway is concomitant with the enhanced inflammatory response that is connected with these diseases (Yamamoto & Gaynor, 2001).

Cancer causes normal cells to transform into highly malignant cells. NF-κB is activated in tumours and plays a role in tumour growth, progression and resistance to chemotherapy treatment (Tafani et al., 2013). Certain cancers have abnormalities in the regulation of the NF-κB pathway. These defects result in elevated levels of NF-κB in the nucleus of many tumours including breast, colon and prostate cancer (Pacifico & Leonardi, 2006; Yamamoto & Gaynor, 2001). Mutations that can result in tumours include those that inactivate IκB proteins, as well as the amplification and rearrangements of genes encoding the NF-κB transcription factor subunits. It is also thought that changes in the upstream pathways that lead to NF-κB activation become deregulated in cancer (Rayet & Gélinas, 1999). Constant nuclear NF-κB activity is thought to play a role in protecting cancer cells from apoptosis and, in certain cases, stimulate their growth. Many current anti-tumour therapies try to block NF-κB activity to attempt to inhibit tumour growth or to sensitize the tumours cells (Tafani et al., 2013). One problem in cancer therapy based on inhibition of NF-κB activity is the difficulty to find compounds which block the oncogenic activity of NF-κB without obstructing its physiological roles in immunity, inflammation and cellular homeostasis. With future research, hopefully several NF-κB inhibitors can increase the therapeutic efficiency of chemotherapy and radiotherapy which could be used effectively in the treatment of cancer patients.

Genetic studies have shown the importance of certain components of NF-κB. Studies from knockout mice have revealed that NF-κB proteins can have both pro- and anti-inflammatory roles (Lawrence, 2009). $p65^{-/-}$ mice show embryonic lethality during day 15/16 gestation caused by substantial apoptosis in the fetal liver due to TNF-α (Beg

et al., 1995). Similar to $p65^{-/-}$, mice deficit for IKKβ and IKKγ are also embryonic lethal due to massive liver apoptosis. There is a signalling link associated between p65, IKKβ and IKKγ subunits as cells resulting from these deficit embryos are unresponsive to classical NF-κB inducers such as TNF-α and Il-1β (Flood et al., 2011). Another study considered the effect of long-term treatment of TNF-α on $p65^{-/-}$ embryonic fibroblasts to establish if there were alterations in cellular function. Treatment with mouse TNF-α for eight hours on $p65^{-/-}$ embryonic fibroblasts resulted in cell death, unlike $p65^{-/-}$ and $p50^{-/-}$ where no substantial effect on viability resulted. This would suggest that p65 regulates a cellular protective mechanism against the cytotoxic effects of TNF-α (Beg & Baltimore, 1996). Lack of $p50^{-/-}$ component results in immune deficiencies and increased risk of infection, but does not demonstration developmental abnormalities shown in $p65^{-/-}$ mice. $p50^{-/-}$ mice displayed inhibition of B cells proliferation in response to LPS, and there were defects in basal and specific antibody production (Sha et al., 1995). Sleep irregularities are also found in these knockout mice (Jhaveri et al., 2006). $p52^{-/-}$ have inadequate humoral responses, reduced number of B cells and are impaired in antibody response to T-dependent antigens (Franzoso et al., 1998). c-$Rel^{-/-}$ mice fail to produce a productive humoral immune response and mature B and T cells are unresponsive to mitogenic stimuli (Köntgen et al., 1995). Rel-$B^{-/-}$ mice proliferates inflammatory infiltration in many organs and also have deficits in adaptive immunity (Hayden & Ghosh, 2004).

There has been a strong focus on the development of anti-inflammatory drugs targeting NF-κB (Lawrence, 2009). Drugs that inhibit NF-κB have side effects due to non-selectivity. In the future, the development of effective inhibitors of the NF-κB pathway that can act selectively with be of vital importance as it will evade the risk of unwanted side effects (Hayden & Ghosh, 2008).

1.12.4 NF-κB expression in the brain

Until 1994, NF-κB had not been reported in the brain. The first evidence exhibiting the expression of NF-κB activity was in the rat brain (Prasad et al., 1994). NF-κB activity is expressed in neurons and glia throughout the central nervous system (CNS) and the brain. NF-κB is important for mediating the immune and inflammatory response in the brain, learning and memory, neuronal plasticity and neurodegeneration (Meffert & Baltimore, 2005; O'Neill & Kaltschmidt, 1997).

NF-κB plays a role in normal physiological function of the CNS at both cellular and behavioural levels (Meffert & Baltimore, 2005). Basal levels of synaptic activity are able to activate NF-κB family members in a range of cultured neurons, located in the cerebellum and the hippocampus (Guerrini et al., 1995; Meffert et al., 2003). Constitutive NF-κB activity was found in glutamatergic neurons in the hippocampus and cerebral cortex using detection methods of western blotting, gelshift assays and antibody staining (Kaltschmidt et al., 2005; Kaltschmidt et al., 1994). As NF-κB is located in the synaptic regions it has been suggested that NF-κB might serve as a vital role in transducing synaptic events to the nuclear transcriptional machinery (Meberg et al., 1996). NF-κB in synaptic transmission is thought to be a factor due to the expression of COX-2 in neurons being regulated by synaptic activity, and COX-2 was discovered colocalised with active NF-κB in neurons of the cortex and hippocampus (Yamagata et al., 1993). Another study displaying evidence that NF-κB is important in regulating synaptic function was inhibiting NF-κB activation causing alteration in synaptic plasticity (Albensi & Mattson, 2000). There has been conflicting findings in recent years with regard to Kaltschmidt results, in relation to NF-κB activity in the brain. Studies have found that there is minimal NF-κB activity in neurons (Listwak et al., 2013; Zhang et al., 2013). Studies that found basal expression of NF-κB in the brain used non-specific antibodies (Herkenham et al., 2011).

Neurotransmitters such as glutamate and N-methyl-D-aspartate receptor (NMDA) can activate NF-κB in cerebellar granular neurons. Glutamate mediated, long-term potentiation (LTP) increased p65/p50 mRNA levels significantly in the hippocampus, suggesting its role as an activator of NF-κB during synaptic activity and neuronal plasticity (Meberg et al., 1996; O'Neill & Kaltschmidt, 1997). If NMDA receptors and L-type Ca^{2+} are blocked, basal synaptic activation of NF-κB is reduced (Guerrini et al., 1995; Meffert et al., 2003). This also suggests a role for Ca^{2+} signalling in NF-κB activation (Meffert & Baltimore, 2005; Meffert et al., 2003). In double knock out mice $TNFRI^{-/-}/P65^{-/-}$, there was no expression of p65, p50, IκBα and IκBβ in the synapses. This suggests the vital role of p65 as the driving subunit for synapses localisation and transport (Kaltschmidt & Kaltschmidt, 2009).

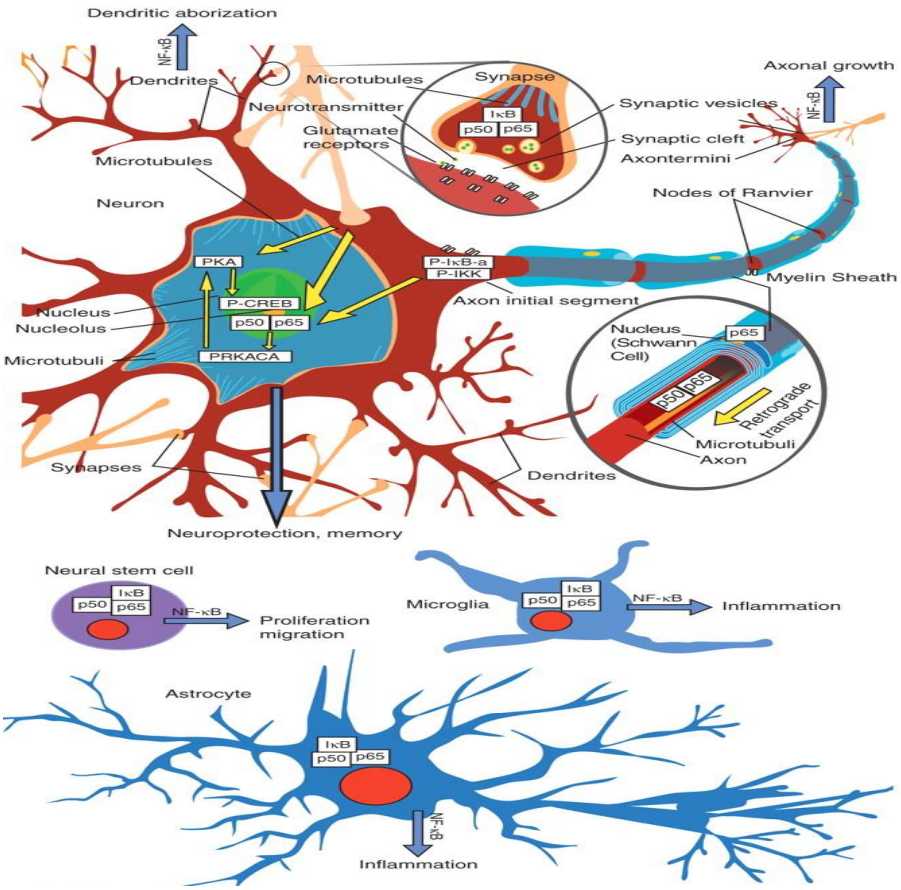


Figure 1.17: The role of NF-κB within the cellular context of the nervous system, adapted from (Kaltschmidt & Kaltschmidt, 2009).

NF-κB plays a role in cognitive functions such as learning and memory. Inhibition of NF-κB by pharmacological or genetic methods results in impaired inhibitory avoidance of long-term memory and spatial navigation learning in mice (Meffert & Baltimore, 2005). $p65^{-/-}$ and $p50^{-/-}$ mice exhibit deficits in specific cognitive tasks (Kassed & Herkenham, 2004). p65 deficient mice have defects in learning and memory when compared to controls. In p50 deficient mice, learning deficits were not as severe as p65 deficient mice. p65 deficient mice showed a discerning learning deficit in the spatial version of the radial arm maze when compared to controls. (Kaltschmidt & Kaltschmidt, 2009; Meffert & Baltimore, 2005; Meffert et al., 2003). $p50^{-/-}$ mice display low level of anxiety-like behaviour in experiments such as the open field test, novel option and the elevated plus maze compared to controls. This would draw the conclusion that the p50 subunit plays a role in normal expression of anxiety (Kassed & Herkenham, 2004). The investigation of double knock out mice $TNFR^{-/-}/p65^{-/-}$ compared to $TNFR^{-/-}/p65^{+/+}$ tested

on spatial and cue versions of RAM took place. $TNFR^{-/-}/p65^{-/-}$ made more errors on the spatial RAM compared to $TNFR^{-/-}/p65^{+/+}$. There was no difference observed on the cue RAM. With extra space training, the $TNFR^{-/-}/p65^{-/-}$ were able to implement the task similar to $TNFR^{-/-}/p65^{+/+}$, again emphasising the requirement of p65 for learning (Meffert & Baltimore, 2005). NF-κB transcription factors play a part in learning and memory and p65 inhibition of NF-κB function can cause a variety of learning paradigms (Meffert & Baltimore, 2005).

NF-κB is involved in sleep regulation under normal conditions. The greatest amount of NF-κB activation occurs during the light phase in the cerebral cortex. During sleep loss NF-κB increases in the cerebral cortex of mice and an upregulation of p65 immunoreactivity in the hypothalamus in sleep deprived rats arises. IL-1β and TNF-α are involved in sleep regulation and activates NF-κB, whereas IL-4 is associated with sleep inhibition and inhibits NF-κB activation (Brandt et al., 2004; Chen et al., 1999; Krueger, 2008). Sleep inhibition can also happen from administering glucocorticoids which can also inhibit NF-κB (Krueger et al., 2001; Unlap & Jope, 1995). Infectious and inflammation alter normal patterns of sleep in certain species and this may be due to activation of NF-κB. A study carried out looked at p50 KO mice and controls and assessed sleep under basal conditions-after 6 hours of sleep deprivation and after challenge with LPS or influenza virus. The p50 knockout (KO) mice demonstrate more slow-wave sleep (SWS) and rapid eye movement sleep (REMS) under normal conditions and have a heightened homeostatic recovery of sleep after sleep loss compared to controls. In addition, they have altered immune responses to administration of LPS, producing increased SWS and suppression of REMS and temperature. However, p50 KO mice do not display increased SWS during infection with influenza virus. Therefore the p50 subunit of NF-κB seems to play a role in modulating sleep under physiological conditions and after immune challenge in mice (Jhaveri et al., 2006).

The NF-κB transcription factor is activated within the CNS in apoptosis and in neurological diseases (Meffert & Baltimore, 2005). NF-κB has been associated with neurodegenerative diseases. Abnormal regulation of NF-κB can underlie neurological disorders concomitant with neurodegeneration (Pizzi & Spano, 2006). Inflammation occurs in most neurodegenerative diseases. The severity of these diseases proliferates over time and neuronal death accumulates (Block & Hong, 2005). Neurodegenerative diseases are either acute neurodegeneration such as stroke and traumatic brain injury or

chronic neurodegeneration, for example Parkinson's disease (PD), HD and multiple sclerosis (MS) (Mattson & Camandola, 2001a).

Brain injury results in increased p65 levels in axons one to two hours after injury. After 24 hours and up to seven days later, activated NF-κB levels were detected in cytoplasm and nucleus of neurons. Activated NF-κB in microglial and astrocytes were also discovered in the cerebral cortex after 24 hours and, importantly, could be activated for up to one year in the cortex following injury. This finding suggests that NF-κB activation may play a part in long-term inflammatory processes following traumatic brain injury (Nonaka et al., 1999). This may occur due to increased production of pro-inflammatory cytokines IL-1 or TNF which leads to activation of NF-κB ensuing the induction of a range of pro-inflammatory genes.

One study carried out investigated whether NF-κB was activated *in vivo* global ischemia in the hippocampal CA1 neuron. Elevated levels of NF-κB were discovered in degenerating CA1 neurons. Other areas in the hippocampus did not display nuclear NF-κB localization (Clemens et al., 1997). IκBα is involved in neuroprotection-it inhibits the abnormal NF-κB activation caused by severe ischemia preventing brain damage. p65 deficient mice have defects in loss of neuroprotection.

PD is a progressive degenerative disorder of the nigro-striatal DA resulting in the impairment of motor skills and speech (Jellinger, 2002; Olanow & Tatton, 1999). There is selective loss of dopaminergic neurons in the nigrostriatal pathway. Neuronal death may occur due to oxidative stress, mitochondrial defeats and protein mishandling in the nigrostriatal pathway (Mogi et al., 2007). In PD patients, there is an increase 70-fold in DA neurons in the substantia nigra which exhibit nuclear p65 immunoreactivity when compared to controls (Hunot et al., 1997; Mogi et al., 2007). PD may also play a neuroprotective role as the increase of NF-κB activity may be due to dysfunction in oxidative stress and mitochondria in neurons (Mattson & Meffert, 2006). Studies have revealed that the canonical pathway is activated significantly in the substantia nigra (SN) of mice undergoing dopaminergic (DA) neurodegeneration, and also seen in the brains of deceased PD patients (Hunot et al., 1997). The non-canonical pathway has been shown to play a role in the regeneration of DA neurons within the SN by treating rats with glial-derived neurotropic factor (GDNF) (Cao et al., 2008). Standard agents and natural anti-inflammatory compounds to treat inflammation such as sulfasalazine, corticosteroids, Il-10 and glutamate, are effective inhibitors of microglial activation and

play a role in neuroprotection to DA neurons *in vitro* and in *vivo*. Inhibiting NF-κB activity in microglial cells more effectively should lead to improved therapies for PD patients, aiming to end and reverse DA neuron loss. (Flood et al., 2011). Using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD, a study to determine if therapeutic administration of selective NF-κB inhibitors could halt the progression of neurodegeneration induced by the neurotoxin MPTP, was investigated. Administration of MPTP resulted in an up regulation of NF-κB activation within the midbrain of animals undergoing neurodegeneration due to MPTP administration. There was also increased NF-κB activation in the SNpc of PD patients. Activation was seen in astrocytes, microglial and TH^+ DA^- neurons. When the NF-κB essential modifier-binding domain (NBD) peptide was administered prior to MPTP intraperitoneal (i.p), it was shown to inhibit MPP^+ induced NF-κB activation *in vitro* in microglial and astrocytes, and it also inhibited the activation *in vivo* in the mid brain region.

Another finding was that the mice showed highly significant production of nigrostriatum from MPTP induced neurodegeneration of the Th^+ neurons and the loss of dopamine production, and improvements in their locomotor function in contrast with MPTP mice injected with the mutant peptide. Since treatment is given after a disease has begun, treatment with NBD peptide was given two days after the injection of MPTP. There was still significant protection of Th^+ neurons, proposing that the NBD peptide can be used therapeutically to halt the development of DA neurodegeneration in MPTP treated mice and in turn suggests that NF-κB is a viable target for treatment in PD patients (Ghosh et al., 2007). Other neurological disorders as a result of ageing, such as AD, will be discussed in chapter four in detail.

HD is another incurable progressive degenerative disorder resulting in the impairments of motor function, speech and cognitive loss (Martin & Gusella, 1986). It is caused by polyglutamine (polyQ) repeat expansion in htt. There is degeneration of neurons in the striatum. Administration of mitochondrial toxin 3-nitropropionic acid in $p50^{-/-}$ results in increased damage to the striatal neurons and enhanced motor impairments. The effect of this treatment gives rise to an increase in NF-κB activity and Mn-SOD levels in striatal cells of wild-type, but not in $p50^{-/-}$ mice. Therefore, NF-κB activation seems to serve a neuroprotective role in this animal model of HD (Yu et al., 2000). Mutant Huntingtin, expressed from an inducible promoter, was found to activate NF-κB in a

neuronal cell line. Inhibiting the NF-κB activation pathway reduced the toxicity of mutant Huntingtin (Khoshnan et al., 2004).

MS is an idiopathic inflammatory demyelinating disease, with symptoms deteriorating over time but there are many periods of remission. Neurodegeneration appears in the form of lesions in the CNS. Early symptoms occurring can range from loss of optic neuritis, fatigue and vertigo. As the disease progresses cognitive impairment, ataxia and limb spasms can ensue. Nuclear magnetic resonance imaging and the MS animal model show an increase in microglial activity around MS lesions (Block & Hong, 2005). Immunohistochemistry was carried out to examine the expression in situ of molecules of NF-κB pathways in chronic MS, to investigate if it had a function in oligodendrocyte pathology. There was up regulation of NF-κB signals found on microglial and oligodendrocytes in chronic MS plagues in contrast to control and silent MS results (Bonetti et al., 1999).

1.12.5 NF-κB and Circadian Interaction

Links have been established between the immune system and the circadian clock due to the fact that many clock controlled genes contain NF-κB binding sites on their promoters. Establishing the relationship between the circadian clock and NF-κB may be of important benefit to therapeutic treatments used in a wide variety of diseases, including cancer, immunodeficiency and inflammation (Bozek et al., 2007; Marpegán et al., 2005; Silver et al., 2012). The circadian-immune interaction operates in two directions. The SCN drives circadian variations in several immune variables, whereas humoral signals can also have an affect the molecular mechanism of the circadian clock and its entrainment (Nicolas Cermakian et al., 2013; Guerrero-Vargas et al., 2014). There is evidence for neuroimmune regulation of circadian rhythms, since the SCN is regulated by an immune challenge of either TNF-α, LPS and interferon, which was discussed in (section 1.10.1) (Kwak et al., 2008; Nygård et al., 2009; Okada et al., 2008).

NF-κB activation displays a diurnal rhythm in mouse cerebral cortex. There is higher activiation during the resting phase compared to the active phase in the dark (Chen et al., 1999). Also in the rat pineal gland, NF-κB expresses a diurnal rhythm and it is significantly reduced when DD occurs (Cecon et al., 2010). A study carried out on the rat liver shows the opposite effect with peak levels of NF-κB in the active phase (Luna-

Moreno et al., 2009). Another interesting study was carried out looking at DD inducing IL-6- dependent depression like behaviour through the NF-κB signalling pathway (Monje et al., 2011). An increase in IL-6 is displayed in depressed patients (Anisman et al., 2005; Chourbaji et al., 2006; Maes et al., 1995), with DD shown to cause depression in rats (Gonzalez & Aston-Jones, 2008). There was an increase in IL-6 plasma and protein levels (in the hippocampus) also as a result of four weeks in DD, however, the NF-κB inhibitor PDTC significantly reduced the effects of DD in this experiment. Furthermore, DD caused alterations of PER2 and NPAS2 expression in their hippocampus (Monje et al., 2011), with both circadian clock genes thought to contribute to SAD disease (Partonen et al., 2007). These alterations of PER2 and NPAS2 expression did not occur as a result of PDTC. Finally, NF-κB activity was elevated in hippocampus tissue as a result of mice placed in DD (Monje et al., 2011).

Another study carried out has shown how the circadian clock protein CRY can regulate the expression of pro-inflammatory cytokines. Mice deficient in the protein CRY have shown an increase in pro-inflammatory cytokines, with the NF-κB signalling pathway seen to be constantly activated in CRY KO fibroblasts. When the NF-κB pathway was blocked, a reduction in the activation of IL-6 was observed in CRY KO fibroblasts (Narasimamurthy et al., 2012). It was also found that a mutation in CRY in p53-null mice resulted in the onset of cancer been delayed, with apoptosis been regulated by the circadian clock through NF-κB signalling (Lee & Sancar, 2011). These studies further add to linking the NF-κB signalling pathway and circadian interaction.

There is correlation between time of day and illness or immune activity. Administration of LPS, as well as of pro-inflammatory cytokines IL-1β and TNF-α result in changes in locomotor activity rhythms. At the molecular level, this effect occurs by activation of NF-κB which leads to the expression of specific clock genes, resulting in regulation of rhythmic output (Coogan & Wyse, 2008). Administration of LPS in hamsters caused induce phase-delays in free-running hamsters and also altered photic induction of c-Fos in the SCN of mice in the early subjective night, but not at any other CT. Therefore, showing a link between the time of LPS challenge and immune response. Administration of an NF-κB inhibitor sulfasalazine blocked LPS, resulting in no phase shift (Marpegán et al., 2005). Activation of the NF-κB pathway also result from IL-1β, TNF-α and LPS treatment of SCN derived astrocytes (Leone et al., 2006).

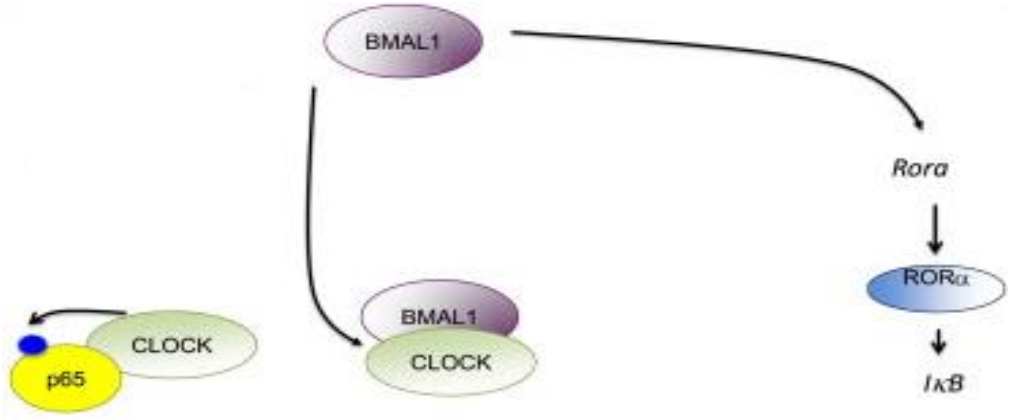


Figure 1.18: Circadian clock and immune interaction, adapted from (Curtis et al., 2014). BMAL-1 sequesters CLOCK and prevents it from acetylating and activating p65, which results in reduced active NF-κB, which in turn leads to less induction of specific genes such as cytokines necessary for survival and proliferation. BMAL-1 also drives the expression of *Rora* that can increase the expression of IkB, which is a major negative regulator of NF-kB. This may give rise to the delay of the NF-kB complex in the cytoplasm, translocating into the nucleus and activating a range of genes including cytokines.

CLOCK and the NF-κB subunit p65 can be found concomitant in protein complexes. Overexpression of CLOCK results in increased phosphorylation and acetylation of p65, which in turn leads to higher transcriptional activity of the NF-κB complex ensuing over production of inflammatory cytokines (Spengler et al., 2012). BMAL-1 reduces the effect of CLOCK on NF-κB by sequestering CLOCK. This mechanism by BMAL-1 reduces inflammation. Therefore CLOCK and BMAL-1 are inter-reliant on each other in their role in the immune system (Curtis et al., 2014). BMAL-1 is also involved in controlling the expression of retinoid-related organ receptor α (RORα) which is a member of the nuclear receptor family. RORα can induce the transcription of IκBα which in turn restricts NF-κB translocating to the nucleus. Therefore, BMAL1 has an anti-inflammatory role to play in limiting NF-κB (Delerive et al., 2001).

Another component of NF-κB subunit RelB interacts directly with BMAL1 to supress the circadian gene *Dbp* which is CLOCK controlled. Therefore circadian transcription can be supressed by RelB. Alterations of circadian genes expression were seen in $RelB^{-/-}$ fibroblasts (Bellet et al, 2012). These findings provide evidence of a molecular link between CLOCK:BMAL-1 transcription pathway and the NF-κB pathway, therefore establishing a relationship between the inflammatory response and the

circadian clock. NF-κB plays vital roles in the immune and inflammatory response. If NF-κB plays a part in the intracellular integration of circadian and neuroimmune system, then understanding its exact role will be important in understanding the effects of age, neuroinflammation and neurodegeneration on circadian rhythms. Recent studies have uncovered that mice deficit in $CRY^{-/-}$ protein display an upregulation of NF-κB activation and pro-inflammatory cytokines, consequently highlighting an association between an arrhythmic circadian clock and a higher inflammatory response (Narasimamurthy et al., 2012).

The NF-kB signalling system has been shown to be under circadian influence and to impact on circadian function, in a variety of peripheral cell types. Outside of neurodevelopment, neuroinflammation and pathological states, the physiological role of the NF-kB system in neuronal function has however been controversial. Additionally, little is known about NF-κB in the SCN clock, but given the potential for this pathway to modulate circadian processes, we set out to examine whether there might be a role for the NF-kB system in the master suprachiasmatic clock.

1.13 The aims of this study were:

- To investigate the expression of constituents of the NF-κB pathway across the circadian cycle in the SCN. Photic resetting was also examined on NF-κB components. Furthermore, pharmacological inhibition of the NF-κB pathway was examined *in vivo* (Chapter Two).

- To examine the effects of acute low and high dose LPS treatment on the levels on expression of NF-κB in the SCN and PVN (Chapter Three).

- To assess the effect ageing has on the master circadian clock in relation to the NF-κB pathway. This will be investigated by behavioural experiments and immunohistochemistry, using non-treated aged mice, acute and chronic LPS treated mice (Chapter Four).

- To examine PER2::LUC and NF:κB::LUC mice using luciferase reporter system which will examine circadian regulation of NF- κB *in vitro*. Blockage of the NF-κB pathway or LPS administration will be carried out *in vitro* (Chapter Five).

Chapter Two

Analysing the effects of light pulses and PDTC on NF-κB in the circadian timing system

2.1 Introduction

Research into understanding the signalling input pathways that convey actions at the SCN neuronal membrane to core nuclear clockworks have been investigated greatly over the years. Signalling pathways involving mitogen activated protein kinases (Coogan & Piggins, 2003) and protein kinase C (Lee et al., 2007), have been recognised as playing vital roles. These signallers are thought to change the activity of transcription factors, which result in them binding to promoter sequences on clock genes to alter their expression (Obrietan et al., 1999). The MAPK pathways can be utilised as an input pathway into the SCN for the purpose of entraining the endogenous clock to a 24 hour environmental cycle, along with playing a role as an output in the circadian clock (Bennett et al., 2013; de Paula et al., 2008; Dziema et al., 2003; Goldsmith & Bell-Pedersen, 2013; Wang, 2002). Activation of the MAPK signalling cascade in the SCN is vital in coupling light to circadian clock entrainment (Antoun et al., 2012). The families of the MAPK pathways have been elucidated, JNK, p38 and ERK, and these pathways have been associated directly to circadian clock mechanism (Goldsmith & Bell-Pedersen, 2013). MAPK is an important intermediate pathway between photic stimulation and transcriptional activation which is essential to allow for the resetting of the circadian clock (Dziema et al., 2003). Therefore, our aim in this study was to establish if the NF-κB pathway played a similar role between photic stimulation and transcriptional activation in relation to the resetting of the circadian clock.

This NF-κB pathway has not been systemically examined in terms of regulation of the SCN clock function. The NF-κB pathway can be activated when bound to TLRs and pro-inflammatory cytokines (Kaltschmidt et al., 2005; Lawrence, 2009; Perkins, 2007). Some studies have confirmed that NF-κB is expressed in neurons and glia throughout the brain (Kaltschmidt et al., 2005; Mattson & Camandola, 2001). Learning and memory defects can be displayed in mice with neuronal NF-κB inhibition, whereas, in glia cells, NF-κB is involved in the inflammatory processes that intensifies diseases for

example autoimmune encephalomyelitis, ischemia, PD and AD (Kaltschmidt & Kaltschmidt, 2009). Studies in recent years have suggested a role for NF-κB in the CNS under basal conditions, and NF-κB has been implicated in such processes as synaptic transmission, plasticity and sleep (Meffert & Baltimore, 2005). Therefore, we want to investigate whether the NF-κB pathway plays any role in the SCN.

Findings have emerged that the neuroimmune system regulates circadian rhythms, with TNF-α (Cavadini et al., 2007; Nygård et al., 2009), interferon-γ (Kwak et al., 2008) and LPS (Leone et al., 2012; Marpegán et al., 2005; Okada et al., 2008) shown to cause alterations to the SCN and to behavioural locomotor activity. Photic induction at time points where light appears to have maximal effect, can also causes behavioural and molecular changes (Marpegán et al., 2005; Meijer & Schwartz, 2003; Shibata & Moore, 1993). Studies discussed previously, reiterate the link between the NF-κB pathway and the circadian clock (Bellet et al., 2012; Curtis et al., 2014; Delerive et al., 2001; Spengler et al., 2012). Since this pathway appears to play a role in modulating circadian processes, the current study sets out to investigate whether there might be a role for the NF-kB system in the master circadian clock.

2.1.1 Immediate Early Genes

IEGs are used as neuronal activity makers (Guzowski et al., 2005) and have an established role in learning and memory (Kovács, 2008) along with circadian rhythms entrainment (Guido et al, 1999; Kornhauser et al, 1990). There are two groupings of IEG, the regulatory transcription factor (RTF) IEGs and effector IEGs. The RTF IEGs regulate the expression of downstream genes, while effector IEGs directly influence cell functions. The expression of IEGs in neurons of control animals is very low, however, IEG mRNA and proteins can be expressed rapidly, due to certain extracellular stimulation (Sheng & Greenberg, 1990). This chapter will focus on the IEGs, c-Fos and activity-regulated cytoskeletal-association protein (Arc) in relation to circadian rhythm entrainment. Stimuli such as nocturnal light, which cause phase shifts in the circadian pacemaker, can also cause alterations in the expression of various IEGs in the retinorecipient area of the SCN (Aronin et al., 1990; Rea, 1989) and PVN (Beynon & Coogan, 2010; Sagar et al., 1988). Light stimulus results in IEGs such as c-*fos* and *jun*-B being transcriptionally activated, in the ventrolateral part of the SCN (Schwartz et al, 1995). The role of these genes are

thought to function in coupling short-term signals stimulated by extracellular events to long-term changes in cellular phenotype by arbitrating subsequent alterations in gene expression (Hughes & Dragunow, 1995; Morgan & Curran, 1991). Once translation occurs in the cytoplasm, c-Fos, re-enter the nucleus to form a heterodimeric complex with a member of the Jun family, such as c-Jun, JunB or JunD, which results in the production of a functional transcription factor termed activating protein-1 (AP-1). This binds certain genes, which regulate the transcription of late response genes, affecting cell structure and function (Chaudhuri et al., 2000).

c-Fos proto-oncogene was one of the first IEGs to be discovered (Greenberg & Ziff, 1984; Müller et al., 1984). This IEG is one of the RTF and is expressed at very low levels throughout the brain, with low levels expressed in the SCN and PVN in control rodents. Since c-Fos has low basal levels of expression, it makes it suitable for research, due to the fact that administration of certain stimuli, may cause upregulation in certain brain regions (Chaudhuri et al., 2000; Kaczmarek & Chaudhuri, 1997). The *c-Fos* gene is linked with playing a role in circadian function (Guido et al, 1999; Kornhauser et al, 1990), however, a better understanding is needed in relation to the exact role of c-Fos in the SCN in the generation of circadian oscillation. When examining the role *c-fos* plays in the circadian system, it was observed that when this gene was knockout in mice, that the magnitude of light induced phase shifts in DD lessened, however, the times of phase delays and advances still remained the same. Therefore, *c-fos* is essential for normal entrainment in the circadian timing system (Honrado et al., 1996).

The second IEG examined in this chapter was Arc, which is an effector IEG. *Arc* mRNA quickly travels to dendrites and gathers close to synapses that have been stimulated (Steward & Worley, 2001) and it is promptly induced by synaptic activity in the hippocampus and the cerebral cortex (Lyford et al., 1995). Arc expression is dependent on NMDA receptor activation (Steward & Worley, 2001) and intracellular MAPK signalling (Waltereit et al., 2001). Furthermore, Arc is a key protein in neurobiology as it is an important marker for synaptic plasticity changes in the brain (Korb & Finkbeiner, 2011). Arc is also vital in sustaining long term memory formation and LTP, with Arc KO mice displaying lack of late phase LTP (Guzowski et al., 2000; Plath et al., 2006). Arc is involved in homeostatic plasticity, by increasing or decreasing neuron activity in order to stabilise extremes in activity caused by LTP and LTD, leading to dysfunction to the network (Korb & Finkbeiner, 2011; Turrigiano, 2008).

2.1.2 Inhibition of the NF-κB signalling pathway

The NF-κB signalling pathway has established itself to be a possible target for pharmacological intervention in various diseases. There are many NF-κB inhibitors within the nervous system (Kaltschmidt et al., 2005), with approximately 700-800 inhibitors of NF-κB signalling discovered to date (Gilmore & Herscovitch, 2006; Gupta et al., 2010). The NF-κB pathway is vital in the body's immune defence system, with IKK acting as a crucial regulator of the NF-κB pathway, with ubiquitination and proteasome of IκB also necessary for NF-κB activation. Disease can result in overproduction of NF-κB activity, therefore, inhibition of the this pathway has being investigated in terms of therapeutic treatments for inflammation and cancer (Yamamoto & Gaynor, 2001).

There are many genes controlled by NF-κB including transcription factors, immunoreceptors, early response genes, cytokines and chemokines. Since there is several levels of regulation in this pathway, inhibition of NF-κB can be targeted at certain levels such as kinases, phosphatases, ubiquitination, nuclear translocation, and DNA binding (Gupta et al., 2010). Studies have investigated blocking signals such as cytokines (which can activate NF-κB) upstream of IKK, this results in inhibiting the activation of IKK (Song et al., 2002). Molecules such as calagualine and Anti CD146 antibody AA98 can block NF-κB by causing inhibition also upstream from IKK (Bu et al., 2006; Manna et al., 2003). By upregulating IκB in the cytoplasm it inhibits NF-κB nuclear translocation to the nucleus (Ehrlich et al., 1998; Lentsch et al., 1997). Another approach is to use ubiquitination (Swinney et al., 2002; Zhou et al., 2005) or proteasome inhibitors (Grisham et al., 1999).

Anti-inflammatories such as aspirin (Kopp & Ghosh, 1994) and glucocorticoids for example prednisone have been reported to inhibit NF-κB activity by unsettling the interaction of p65 with the basal transcription machinery (De Bosscher, 2000; Unlap & Jope, 1997; Unlap & Jope, 1995), with other reports suggesting that glucocorticoids increase IκB expression which results in a decrease in NF-κB translocating to the nucleus for the purpose of DNA binding (Auphan et al., 1995; Quan et al., 2000; Scheinman et al., 1995). Furthermore, more recent studies have shown that small molecules inhibitors of the NF-κB pathway may be utilised as anti-inflammatory therapeutics (Ivanenkov et al., 2011).

The NF-κB inhibitor, sulfasalazine, is an anti-inflammatory agent used in the treatment of IBD and RA, and is vital in treating chronic inflammation, as it restricts NF-κB signalling (van Vollenhoven et al., 2009; Wahl et al, 1998). NF-κB activation caused by either LPS or TNF-α can be blocked by sulfasalazine, for that reason it seems that this inhibitor affects an early common signal in both signal transduction cascades (Wahl et al., 1998). This inhibitor supresses NF-κB activity by direct inhibition of IκB kinase α and β, prohibiting the translocation of NF-κB into the nucleus (Wahl et al., 1998; Weber et al., 2000). Sulfasalazine is thought to be a specific inhibitor of NF-κB, due to the fact that administration of this inhibitor did not impact on other DNA-binding activities for example AP1 (Wahl et al., 1998).

Sulfasalazine has being shown to block phase shifts in mice as a consequence of an immune challenge of LPS in the early subjective night (Marpegán et al., 2005). This is similar to PDTC blocking photic phase shifts in hamsters (Marpegan et al., 2004). Marpegán and colleagues wanted to see if there was an interaction between LPS and light, due to the fact that LPS administration induced a photic-like PRC (Schwartz & Zimmerman, 1990). Accordingly, investigations were carried out to decide if both stimuli shared a common pathway, no additive effects were found as a result of administration of both light pulse and LPS on phase shifts, therefore leading us to believe that there is some interaction between both pathways. As previously discussed, at a molecular level administration of LPS at CT15 induced c-Fos expression in the dmSCN region only. In contrast a light pulse administered at CT15 resulted in c-Fos expression exhibited throughout the dmSCN and vlSCN (Marpegán et al., 2005) and displayed in this current study at CT22. It would be of interest in future studies to ascertain if sulfasalazine can inhibit phase shifts caused by photic induction displayed in both the early and late subjective night, and also to establish if this inhibitor can block induced IEG expression as a result of a light pulse to see if the NF-κB pathway is involved in entrainment and regulation of circadian rhythms and also IEG induction in the SCN of mice.

2.1.3 The NF-κB inhibitor PDTC

PDTC is a metal chelating compound, which can be used for anti-oxidant and anti-inflammatory purposes (Ivan et al., 2014; Yadav et al., 2011) however, it is also known for its pro-oxidant effects (Brennan & O'Neill, 1996; Burkitt et al., 1998; Riera et

al., 2015). PDTC was first discovered as an inhibitor of the NF-κB pathway in 1992. NF-κB inhibition can result from using micromolar concentrations of PDTC (Schreck et al., 1992), and it is thought that this arises from inhibiting IκBα degradation (Cuzzocrea et al., 2002), which in turn prevents the translocation of NF-κB dimers to the nucleus and preventing pro-inflammatory gene transcription to take place (Henkel et al., 1993; Liu et al., 1999). Studies have shown that for inhibition of NF-κB to occur, PDTC administration of between 25-50 mg/kg is required in animals (Cuzzocrea et al., 2002; Siegel et al., 2009; Zhang et al., 2014). Toxic effects were established at an LD_{50} i.v of 282.4mg/kg in both the central and nervous system of mice (Chabicovsky et al., 2010), therefore, PDTC is a modulator of the inflammatory response at particular doses.

PDTC administration has been shown to affect different pathways in the immune system including casein kinase 2 (CK2), ERK and JNK pathways (Chung et al., 2000; Min et al., 2003). Studies carried out have shown how PDTC administration can inhibit cell death caused by various factors (Hockenbery et al., 1993; Kane et al., 1993; Rothstein et al., 1994), however, it can also induce cell death (Chung et al., 2000; Kim et al., 1999; Wu et al., 1996). Acute inflammation in the form of pleurisy (Cuzzocrea et al., 2002) and acute kidney injury (Kumar et al., 2015) were reduced by PDTC. Furthermore, acute administration of PDTC has been shown to attenuate focal brain ischemic injury (Nurmi et al., 2004), decrease impairment of learning and memory (Zhang et al., 2014) and reduce microglial activation as a result of neuroprotection in the hippocampus (Lv et al., 2014).

2.1.4 Aims of this chapter are to

- Examine whether the NF-κB components, p65, p-IκB, p-IκK and c-Rel express a circadian rhythm across a 24 hour cycle in DD in the SCN under basal conditions.

- Observe what effect light pulses during the subjective night have on NF-κB signalling.

- Investigate if NF-κB inhibitors play a role in behavioural alterations from photic induction.

- Examine if induced c-Fos or Arc expression caused by photic induction in the SCN and PVN can be blocked by the NF-κB inhibitor PDTC.

- Study the effect of chronic long term administration of PDTC on behavioural locomotor activity, the SCN and PVN

2.2 Materials and Methods

2.2.1 Ethical approval

All protocols in this thesis were approved by the Research Ethics Committee at Maynooth University and were licensed by the Department of Health and Children, Ireland. Animals were treated in agreement 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 taken to minimize the number of animals used and any potential suffering in each procedure. To reduce the number of animals used for experimentation, all animals used for behavioural analysis were also used for immunohistochemical analysis.

2.2.2 Animals and housing

The animal model that was used in this study is the C57BL/6 strain of mouse (*Mus musculus*). This study requires the use of an animal model as we monitor rhythmicity at the whole animal level and C57BL/6 mice are well studied from a chronobiological aspect, extensive studies have been undertaken on their circadian parameters. The adult C57BL/6 male aged 6 weeks (24-30g) were grouped house on arrival from Charles Rivers, UK or Harlan, UK both designated breeding establishments. Animals were 8-10 weeks old when experiments were carried out.

When behavioural analysis was undertaken, mice were individually housed in polypropylene cages (33cm long x 15cm wide x 13cm high) along with steel running wheels (11.5cm diameter) with food and water available *ad libitum* and also wood-chip bedding and shredded paper for environmental enrichment. Lighting conditions for the cage were adjusted using a timer which was placed on the outside of the cabinet, therefore, the cabinet door did not have to be opened disturbing the mice without need. The light in each individual cage was roughly 150 lux luminance level. The inside of the cabinets

were black and non- reflective in order to minimise any reflection of light and guarantee similar lighting conditions for each cage.

In relation to group housed mice, 10 were in each cage (44.5cm long x 28.5cm wide x 10.2cm high) and again equipped with food and water available *ad libitum* and the same environmental enrichment as single housed mice. Animals were maintained in LD 12:12 cycle prior to any experiment. When mice were housed in DD conditions, daily checks were done with the assistance of a dim red safety light (<1 lux). Furthermore, temperature and humidity were recorded daily to make sure they were both remaining constant, with average temperatures of 21 ± 1°C and humidity 50± 10%. Ventilation of cages took place via axial fans to avoid a build-up of pheromones and these fans produced white noise at the level of 50dB.

2.2.3 Treatments

2.2.3.1 NF-κB inhibition by i.p injection

Mice were treated with ammonium pyrrolidinedithiocarbamate (PDTC) ($C_5H_{12}N_2S_2$), which is a potent antioxidant and pharmacological inhibitor of NF-κB (Ivan et al., 2014; Schreck et al., 1992; Zhao et al., 2013), 30 minutes before a light pulse at CT15 or CT22. Mice were administered a single dose of 200mg/kg i.p (0.12ml) injection of PDTC dissolved in 100mM DMSO. This dose was previously displayed in rats to maximally inhibit NF-κB activation (Liu et al., 1999). Control animals were treated at the same time with an equal volume of sterile saline (0.9% NaCl) to DMSO i.p (0.12ml).

2.2.3.2 NF-κB inhibition by oral administration

To study the effects of chronic treatment with PDTC, mice were administered fresh PDTC every 3 days in their drinking water available *ab libitum*. PDTC (50mg/kg) was dissolved in water along with 0.1% DMSO (Gu et al., 2009; Hirata et al., 2007; Luo et al., 2015; Malm et al., 2007; Mariappan et al., 2010; Zhao et al., 2013), while controls were administered with water containing 0.1% DMSO.

2.2.4 Transcardiac perfusion and tissue preparation

An i.p injection of sodium pentobarbital (Euthanal, Merial Animal Health, UK) was used to terminally anesthetise mice. When anesthetised, the chest cavity was opened and mice perfused transcardially with roughly 30mls of 0.9% saline (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 removed, post fixed overnight in 4% PFA for 48 hours at 4°C, followed by putting all brains into 30% sucrose (Sigma) in 0.1M PB for a further 24-48 hours at 4°C, so they were cryoprotected. Brains were then removed from 30% sucrose for cutting. Each brain was cut caudally removing the cerebellum first, and rostrally to remove the preoptic region using a single edged blade. Brains were mounted on a microtome (Leica) using mounting medium and brains were frozen using dry ice. The caudal end was attached to the microtome stage, while the ventral end faced the blade. Brain slices were cut at 30µm thick throughout the rostrocaudal extent of the SCN. All sections of the SCN were collected and divided into 4 different wells, which contained 5-6 SCN sections. The sections were placed in wells and stored in 0.1M PB, pH 7.4 (Sigma) along with 0.1% sodium azide at 4°C. Sodium azide prevents microbial growth prior to being processed for immunohistochemistry.

2.2.5 Immunohistochemistry protocol

This protocol method used the avidin-biotin-peroxidase complex (ABC)/Nickel DAB colorometric technique (Coogan & Piggins, 2003). The SCN sections in the wells were washed twice with 0.1M PB (pH 7.4) for 10 minutes at room temperature. This was followed by a 10 minute wash of PBX (0.1M PB + 0.03% Triton X-100 (Sigma)). PBX's function is to perforate the cell membrane of these sections. Sections were then incubated for 20 minutes in 0.1% PB and 1.5% hydrogen peroxide (H_2O_2). H_2O_2 plays an important role by inactivating endogenous peroxidases and reduces non-specific background staining. Another round of 2 X PB washes and a PBX wash for 10 minutes each was carried out. The next step was using a non-specific antibody block with 0.1M PBX, which was either 5% normal goat serum (NGS- used for rabbit and rat polyclonal) or normal horse serum (NHS- used for goat polyclonal), which mixed for 60 minutes at room

temperature. The final part of the first day immunohistochemistry protocol was that sections were incubated with primary antisera raised in either rabbit or goat diluted in 0.1M PBX and 2% NGS or NHS (blocking solution) for 24-48 hours at 4°C.

Table 2: Primary antisera used for Immunohistochemical analysis

Antibody	Dilution	Raised in	Supplier	Product co
c-Fos (4)	1:2000	Rabbit	Santa Cruz Biotechnology	sc-52
Arc (H-300)	1:500	Rabbit	Santa Cruz Biotechnology	sc-15325
Per1 (N20)	1:500	Goat	Santa Cruz Biotechnology	s-7724
p65	1:500	Rabbit	Santa Cruz Biotechnology	sc-372

The second part of the protocol, begins with sections being put through a serious of washes as above, two 0.1M PB and one 0.1M PBX wash. The sections were then incubated with biotinylated secondary antibody (1:400 biotinylated goat anti-rabbit Jackson Immuno research labs, 1:400 anti-goat) which was diluted in 0.1M PBX and 2% NGS or NHS (blocking solution) for 70 minutes at room temperature. Biotinylated secondary antibody is photosensitive, therefore the well plates were covered from light for the remainder of the experiment. After the 70 minutes, 3 washes were carried out as previous with PB and PBX. This was followed by sections been incubated with avidin-biotin method (0.4%) in 0.1M PBX with a Vectastain Elite Universal Kit (Vector Laboratories) for 90 minutes, again at room temperature. Sections were washed twice with 0.1M PB, followed by 0.1M sodium acetate (pH6, Sigma) for 10 minutes. Light sensitive nickel-enhanced diaminobenzidine (NiDAB) (pH6) can visualise immunoreactivity of the antigen. 1ml of NiDAB and 60µl of catalyst, glucose oxidase (5mg/ml) per well was added for immunoreactivity. When the staining was satisfactory,

sections were placed in PB to stop the reaction. The sections went through 3 final washes in 0.1M PB.

The sections were mounted on to slides coated in gelatine (1% gelatine and 0.05% chromium potassium sulphate) and were placed to dry for 2-3 days before being put through dehydrating and delipifying steps. Dehydrating steps were carried out by putting the slides through 70%, 90% and 100% ethanol for 3 minutes each, and were then cleared using two 3 minute delipifying steps in 2% histoclear washes (National Diagnostics, UK). Finally the slides were coverslipped using mounting media (Eukitt, Fluka Analytical). All sections followed this protocol at all stages of tissue processing to minimise any interassay variability. Sections were reacted for standardised lengths of time to make sure similar staining intensity across each experimental group.

2.2.6 Quantitative Analysis

Photomicrographs of the mid rostral-caudal level of the SCN were taken using a digital camera connected to an Olympus BX-51 light microscope which was equipped with an image analysis digital system (ImageJ 1.43, NIH, USA). The same camera and magnification settings were used for every single image. Brain sections were examined under the microscope using the 40 X objective lens. Roughly 3-5 sections were analysed and evaluated for each animal, and a mean value was calculated. Photomicrographs are presented in this thesis with scale bars representing 100μm unless otherwise noted.

Mid-rostrocaudal levels of SCN were analysed by two ways. The first analysing method was quantification of immunoreactive (ir) cell numbers by an observer by manually counting for the purpose of analysing immunoreactive nuclear staining. The sections were counted from the rostral through to caudal of the SCN. However, the SCN sections were measured at the mid rostral-caudal level. This included counts of both the core and shell regions individually too. The observer was blinded to the treatment during immunoreactive cell counts.

The second analysing method was carried out using an image analysis software, and evaluated using integrated optical density (IOD). This method was carried out by taking the SCN images and using ImageJ for analysing the immunostaining in the SCN (Schneider et al., 2012). ImageJ allows for the automatic quantification of numbers of cells, this can eliminate experimenter bias. The light intensity was kept constant while

taking all the images to standardise IOD measurements. Results were taken as a mean value (IOD or cell number count) ± standard error of mean. Statistical significance results were $P<0.05$.

2.2.7 Circadian Behavioural Analysis

Mice were singly housed in cages with running wheel for behavioural analysis. Each cage contained a micro-switch on the outside which was connected to a data acquisition system computer using the Chronobiology Kit by Stanford System (Santa Cruz, California) for the recording of daily rhythms of locomotor activity. The data acquisition system is able to record each wheel revolution and collects data every 5 minutes, which in turn produces actograms or actigraphs of each mice activity rhythms. The onset of activity is ZT12 when lights are turned off. Mice are usually entrained in a light:dark (LD) (12:12) cycle for two weeks, before they are released into constant darkness (DD), where they begin to free run. Mice are kept in DD for two weeks before any behavioural experiment takes place. Using the actogram data for all mice, photic-induced phase shifts are calculated by fitting a line of best fit through activity onsets for one week prior to treatment and then another line for one week post treatment (Figure 2.1). Two to three researchers calculated the difference between the two lines and take the average to exclude experimenter bias. The FRP and rhythm amplitude were calculated using the programme Chronobiology Kit Chi Squared periodogram (Figure 2.1) (Stanford software system).

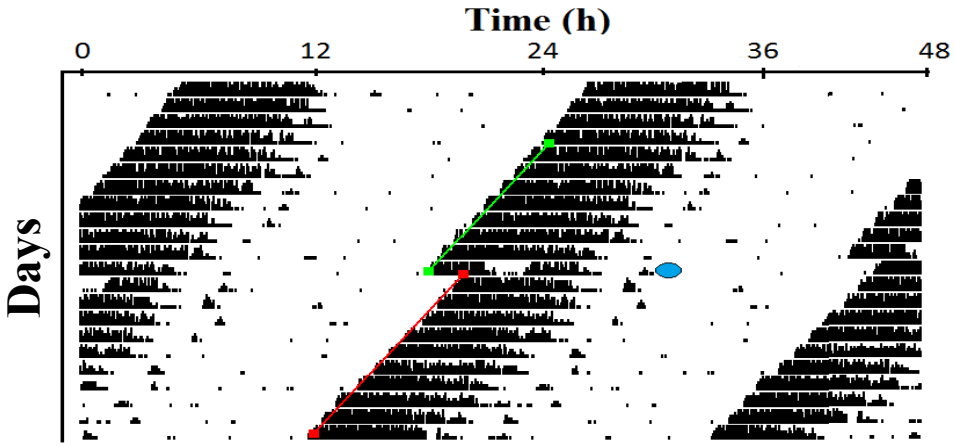


Figure 2.1: Line of best fit analysis. A sample double plotted actogram displaying the line of best fit method used to assess locomotor activity onsets following applications of a phase shifting

stimulus to a subject free running in DD. The application of the stimulus is displayed by the blue circle. The lines demonstrate the lines of best fit-through activity onsets. The green 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 there is omission of transient days from analysis of the locomotor phase resetting response.

2.2.8 Statistical Analysis

All the data was analysed using IBM SPSS Statistics version 20. The type of test used is indicated in experimental and results section for each experiment. Mean values are presented ± the standard error of the mean (SEM). P levels below 0.05 were significant for all tests. All data is presented as means ± SEM. * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$.

2.2.9 Analysis of NF-κB and clock gene expression across the 24 hour circadian cycle

For the purpose of this experiment, adult male mice were group housed in cages and allowed to habituate to a 12:12 LD cycle (150 lux, lights on 0700h) for two weeks prior to release into DD where they establish FRPs. Mice continued in DD for two weeks before experimental procedures took place. Mice were kept in DD to ascertain if expression was circadian in nature, over a 24 hour period. Mice (n=5) were sampled in dim red light (<1 lux) at time points over a twenty four hour period ranging from CT2, CT6, CT10, CT14, CT18 and CT22. Mice (n=30) were perfused (outlined in section 2.2.3) and brains were removed and analysed by immunohistochemistry (outlined in section 2.2.4). Brains were fixed in 4% paraformaldehyde and after a day placed in 30% sucrose. Immunohistochemistry was carried out to analysis glia activation in the mice for the expression of components of NF-κB including p65, IκK, IκB and c-Rel and also nuclear expression of the clock gene PER1. The mean was calculated for each animal and grouped by location and each time point was compared and analysed by one way AVOVA. Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

2.2.10 Cosinor Analysis

To Evaluate the individual 24-h cycle of expression of the clock gene protein PER1 and components of NF-κB in SCN and to discover if the rhythm was indeed circadian in nature, a cosinor method was applied (Nelson et al., 1979). Using the CircWave 1.4 analyses software (established by Dr. R.A. Hut, 2007, http://www.euclock.org) it uses a forward linear harmonic regression in order to evaluate the profile of the wave with a 24 hour period for independent samples using 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, representing the fundamental wave, the first harmonic, the second harmonic, such as $(i-1)^{th}$ harmonic; p_i is the sine coefficient of the $(i-1)^{th}$ harmonic and q_i signifies the coefficient of the $(i-1)^{th}$ harmonic; t=timepoint value (modulo τ); f(t) is the calculated function value at time point t. Additionally, y is the fitted value for time point t; a and b denote 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 recognised if the null amplitude hypothesis was rejected from an F test creating a significant value $P<0.05$. Values for the amplitude, acrophase and rhythms adjusted mean level through the 24 hour cycle were also provided during the cosinor analysis.

2.2.11 Analysing the effect photic regulation has on the behavioural circadian locomotor activity in the early and late subjective night

Mice (n=6-7) were placed in 12:12 L:D conditions for two weeks before being placed in DD for two weeks. Mice received a 30 minute light pulse in the early subjective night at CT15. There was a control crossover carried out two weeks later. In the second experiment, mice received a 30 minute light pulse in the late subjective night at CT22. Again, a control crossover was carried out two weeks later. The mean was calculated for each animal and each time point was compared and analysed by one way AVOVA.

Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

2.2.12 Analysing photic regulation of an early immediate clock gene and NF-κB p65 expression in the SCN

In order to assess photic regulation of the NF-κB pathway, adult male mice were maintained in DD for 2 days and then received a 30 minute light pulse at C15 (the early subjective night) or CT22 (the late subjective night). Mice (n=8) were sampled, 4 taken before the light pulse, another 4 immediately after the light pulse, 4 mice 30 minutes after, and finally 4 mice 60 minutes after a photic induction. Light appears to have maximal effect at these two time points, therefore helping to investigate the temporal nature of any regulation. Sections from these animals were stained for the component of NF-κB, p65, and the early immediate gene, c-Fos at CT22. The mean was calculated for each animal and each time point was compared and analysed by one way AVOVA. Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

2.2.13 Acute PDTC treatment prior to light pulse

Mice were entrained to a 12:12 cycle for two weeks before undergoing two weeks in DD. Application of either DMSO (n=4) or the NF-kB inhibitor PDTC (200mg/kg Tocris Bioscience, USA) (n=4) was given i.p 30 minutes prior to a 30 minutes light pulse administered to mice. Mice were treated in the late subjective night CT22. A cross-over design between DMSO and PDTC was carried out two weeks later. This data was recorded by the Chronobiology Biology Kit to analyse any phase shifts that may have occurred. Statistical analyses was carried out using repeated measures t-tests.

Finally, mice were treated again when two weeks had passed, and were given either DMSO or PDTC at CT22 with a 30 minute light pulse following. The mice were sampled at CT23.5-24. Brains were removed and fixed in 4% PFA for 2 days. Immunohistochemistry was carried out to analysis mice for the expression of c-Fos and ARC. The mean was calculated for each animal's nuclei per SCN/PVN and analysing of

data was carried out using an independent t-test. Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

2.2.14 Chronic PDTC oral treatment

It was also examined whether chronic treatment of PDTC caused any changes in core circadian locomotor activity or at a molecular level. Actogram data and the core circadian locomotor behaviour rhythm parameters of free running period and rhythm amplitude were assessed under DD conditions following administration of either PDTC (50mg/kg) or DMSO (0.005%) vehicle in drinking water for a period of 42 days. Additionally, total wheel running activity was assessed under 12:12 LD cycle across a 42 day period with either PDTC or vehicle being administered. In order to assess the integrity of the retinohypothalamic tract (RHT) transmission from it, the phase angles of entrainment were examined for each animal in both treatment groups under the LD photoperiod, since alterations in RHT transmission impact upon the phase angle of entrainment. Total wheel running activity, the phase angle of entrainment, the FRP and the rhythm amplitude for animals treated with either PDTC or DMSO were analysed. Statistical analyses was carried out using repeated measure t-tests.

After 84 days, mice administered PDTC (n=8) and DMSO (n=8) were sampled at ZT6 and ZT12. Brains were removed and fixed in 4% PFA for 2 days as outlined in 2.2.3. Immunohistochemistry (2.2.4) was carried out on SCN sections to analysis expression of c-Fos, PER1 and p65. The mean was calculated for each animal's nuclei per SCN/PVN and analysing of data was carried out using a two way Anova test. Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

2.3 Results

2.3.1 Analysis of NF-κB and clock gene expression across the 24 hour circadian cycle

2.3.1.1 PER1 expression across the 24 hour circadian cycle

One way ANOVA was utilised to assess statistically significant differences in the mean expression of the clock gene protein PER1 across the 24 hour circadian cycle. There was an effect of time found in the expression of PER1 in the SCN across 24 hours ($F_{5,19}$=9.87, P=0.01). Post-hoc analyses revealed significant elevation of PER1 expression at CT10 and CT14. A subdivision of the SCN was also analysed, investigating core and shell expression of PER1 across 24 hrs. There was an effect of time found in the core, (P<0.05, ANOVA), with post-hoc analyses revealing significant elevation of PER1 expression at CT14. Furthermore, the shell had a significant difference in the mean expression of PER1 at CT10 compared to all other time points excluding CT14, (P<0.01, ANOVA), (Figure 2.2).

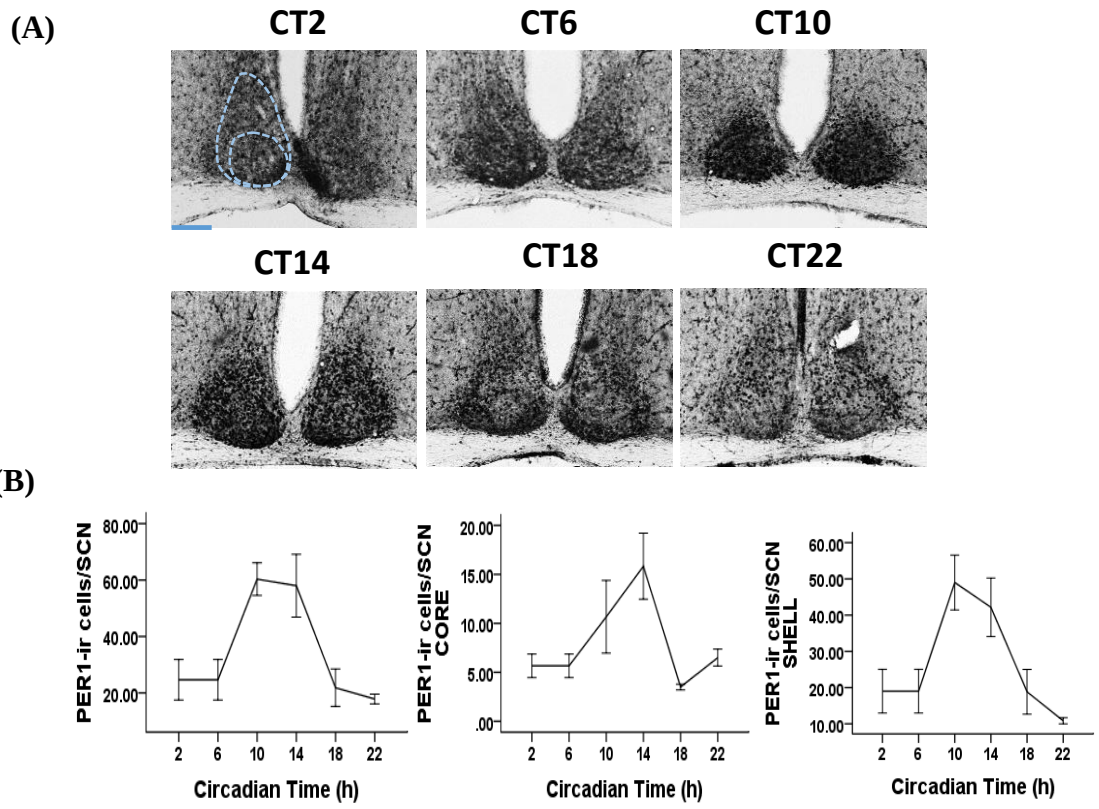


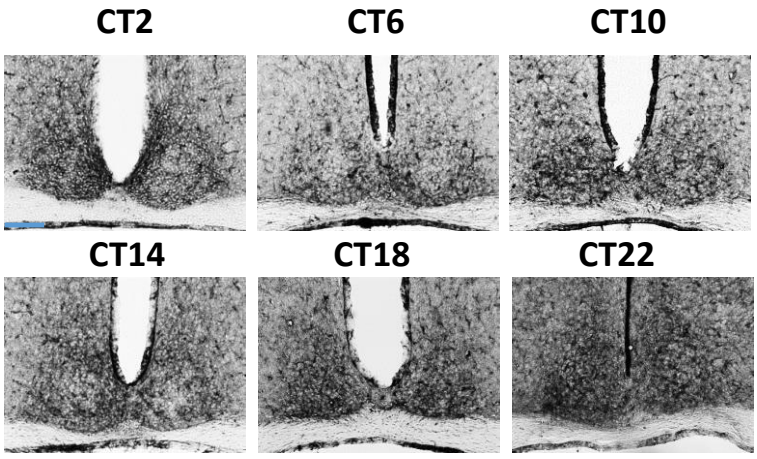
Figure 2.2: Effect of time on the expression of the clock gene product, PER1 in the SCN across the 24 hour circadian cycle. Circadian regulation expression of PER1 was examined in

the SCN of animals sampled every 4 hours across the 24 hour circadian cycle in DD. (A) Photomicrographs of PER1 expression in the SCN (n=4 per time point) at CT2, CT6, CT10, C14, CT18 and CT22 (scale bar = 100μm). Dashed lines outlines the SCN and delineates the core and shell subdivisions (B) Graph illustrating quantification of levels of PER1 immunoreactive nuclei in the whole, core and shell of the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and showing the expression pattern across the circadian cycle. ANOVA and Co-sinor analysis revealed effect of time on PER1 expression across the 24hr circadian cycle ($P<0.01$). Error bars represent ±1 SEM.

2.3.1.2 p65 expression across the 24 hour circadian cycle

Immunohistochemistry coupled with Integrated Optical Density (IOD) measurements and one way ANOVA analysis was used to evaluate statistically significant differences in the mean expression of the p65 NF-κB immunoreactivity in the SCN across the 24 hour circadian cycle. There was no significant effect of time detected by ANOVA ($P>0.05$) and no significant co-sinor fit was found in the expression of p65 in the SCN across 24 hours. A separate subdivision of the SCN was analysed, investigating the core and shell expression of p65 across 24 hrs. Again there was no significant effect of time found in the core or the shell ($F_{5,17}=1.236$, $P=0.351$), in the mean expression of p65. (Figure 2.3).

(A)



(B)

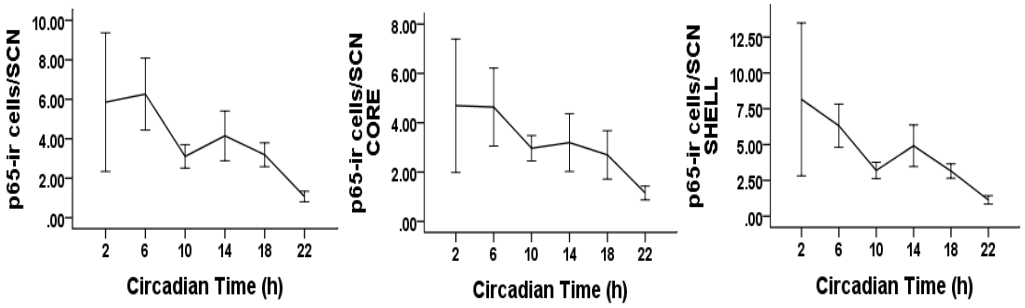
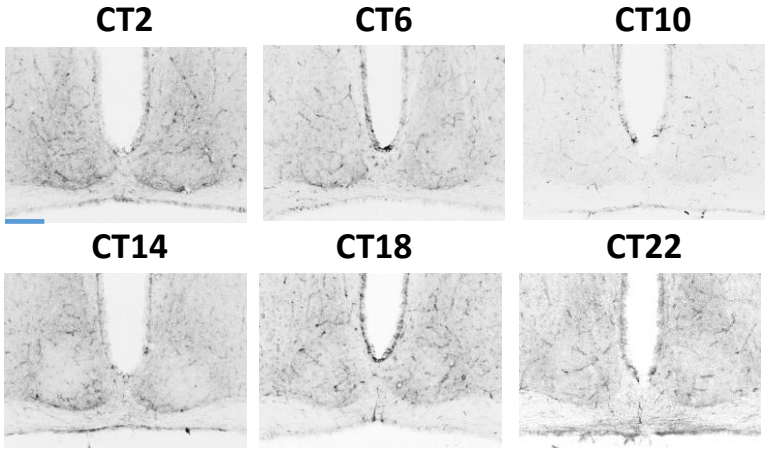


Figure 2.3: Effect of time on the expression of the p65 NF-κB in the SCN across the 24 hour circadian cycle. Circadian regulation expression of p65 was examined in the SCN of animals sampled every 4 hours across the 24 hour circadian cycle in DD. (A) Representative photomicrographs of p65 expression in the SCN (n=4 per time point) at CT2, CT6, CT10, C14, CT18 and CT22 (scale bar = 100μm). (B) Graph illustrating quantification of levels of p65 immunoreactive glial in the whole, core and shell of SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and showing the expression pattern across the circadian cycle. There was no significant effect of time on p65 expression across the 24hr circadian cycle (P>0.05). Error bars represent ±1 SEM.

2.3.1.3 p-IκB expression across the 24 hour circadian cycle

Immunohistochemistry coupled with IOD measurements and one way ANOVA analysis was used to evaluate statistically significant differences in the mean expression of the p-IκB immunoreactivity in the SCN across the 24 hour circadian cycle. There was no significant effect of time detected by ANOVA (P>0.05) and no significant co-sinor fit was established in the expression of p-IκB in the SCN across 24 hours. Investigations of the core and shell expression of p-IκB in the SCN took place across 24 hrs. Again, there was no significant effect of time found in the core or the shell ($F_{5,18}$=1.228, P=0.351), when analysing the mean expression of p-IκB. (Figure 2.4).

(A)



(B)

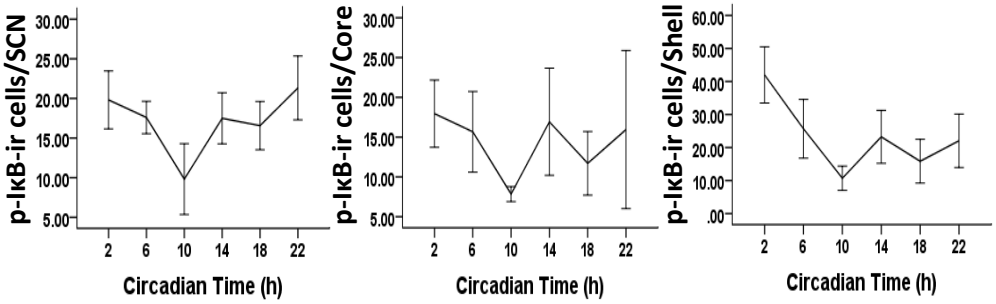


Figure 2.4: Effect of time on the expression of the p-IκB in the SCN across the 24 hour circadian cycle. Circadian regulation expression of p-IκB was examined in the SCN of animals sampled every 4 hours across the 24 hour circadian cycle in DD. (A) Representative photomicrographs of p-IκB expression in the SCN (n=4 per time point) at CT2, CT6, CT10, C14, CT18 and CT22 (scale bar = 100μm). (B) Bar graph illustrating quantification of levels of p-IκB immunoreactive glial in the whole, core and shell of the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and showing the expression pattern across the circadian cycle. ANOVA and Co-sinor analysis revealed no significant effect of time on p-IκB expression across the 24hr circadian cycle ($P>0.05$). Error bars represent ±1 SEM.

2.3.1.4 p-IκK expression across the 24 hour circadian cycle

Immunohistochemistry coupled with IOD measurements and one way ANOVA analysis, was used to assess statistically significant differences in the mean expression of the p-IκK immunoreactivity in the SCN across the 24 hour circadian cycle. There was no significant effect of time detected by ANOVA ($P>0.05$) and no significant co-sinor fit was found in the expression of p-IκK in the SCN across 24 hours. Investigation of the

core and shell took place of p-IκK expression across 24 hrs, finding no significant effect of time found in the core or the shell ($F_{5,19}$=0.220, P=0.948), (Figure 2.5).

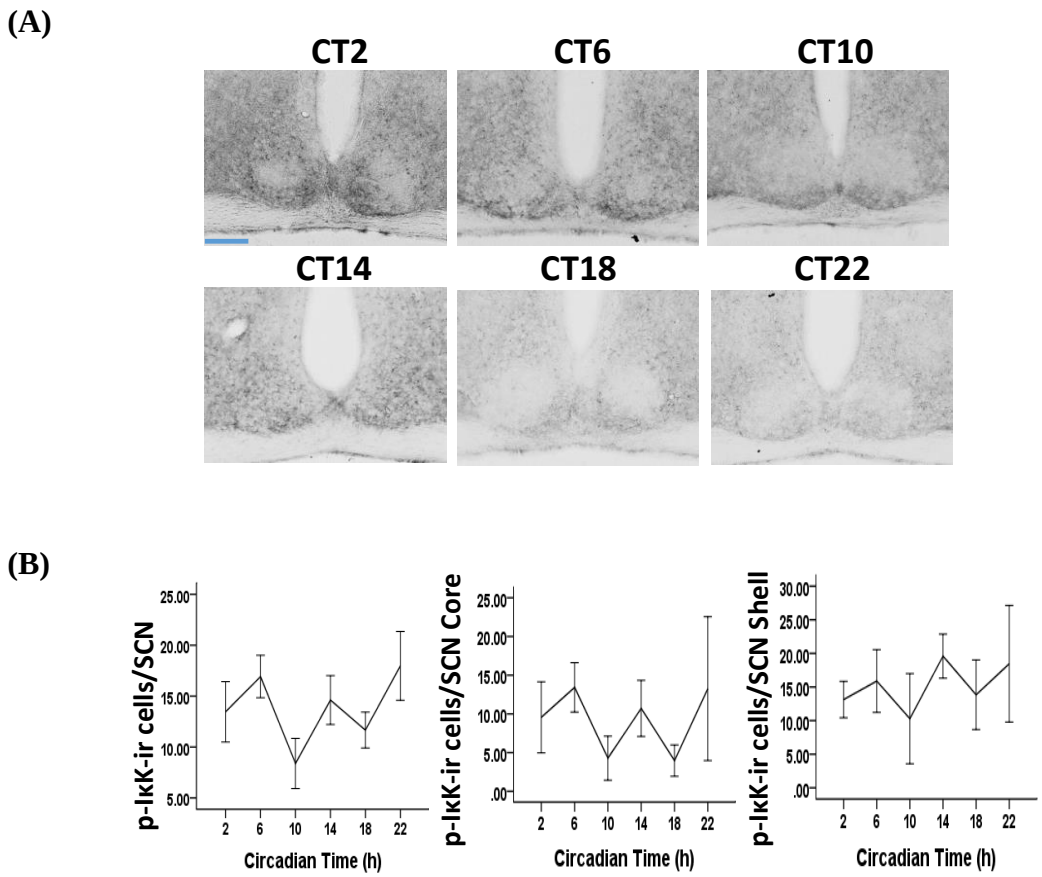


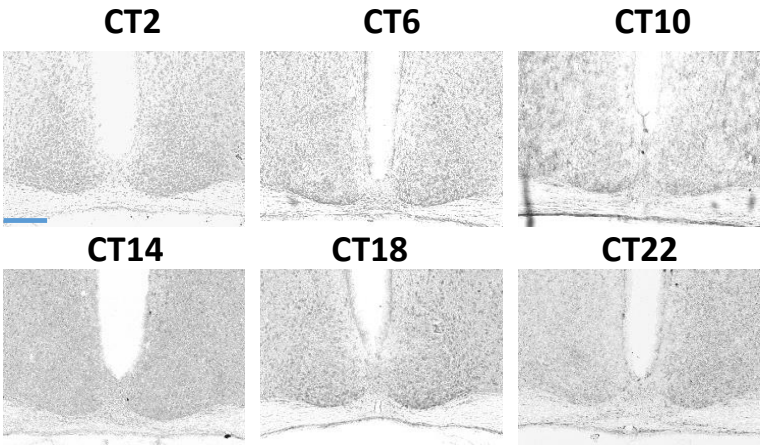
Figure 2.5: Effect of time on the expression of the p-IκK in the SCN across the 24 hour circadian cycle. Circadian regulation expression of p-IκK was examined in the SCN of animals sampled every 4 hours across the 24 hour circadian cycle in DD. (A) Representative photomicrographs of p-IκK expression in the SCN (n=4 per time point) at CT2, CT6, CT10, C14, CT18 and CT22 (scale bar = 100µm). (B) Graph illustrating quantification of levels of p-IκK immunoreactive glial in the whole, core and shell of the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and showing the expression pattern across the circadian cycle. ANOVA and Co-sinor analysis revealed no significant effect of time on p-IκK expression across the 24hr circadian cycle (P>0.05). Error bars represent ±1 SEM.

2.3.1.5 c-Rel expression across the 24 hour circadian cycle

Immunohistochemistry coupled with IOD measurements and one way ANOVA analysis, was used to assess statistically significant differences in the mean expression of the c-Rel immunoreactivity in the SCN across the 24 hour circadian cycle. There was no significant effect of time detected by ANOVA (P>0.05) and no significant co-sinor fit

was found in the expression of c-Rel in the SCN across 24 hours. A subdivision of the SCN was analysed, investigating the core and shell expression of c-Rel across 24 hrs. This resulted in no significant effect of time found in the core or the shell ($F_{5,18}$=0.893, P=0.513), in the mean expression of c-Rel. (Figure 2.6).

(A)



(B)

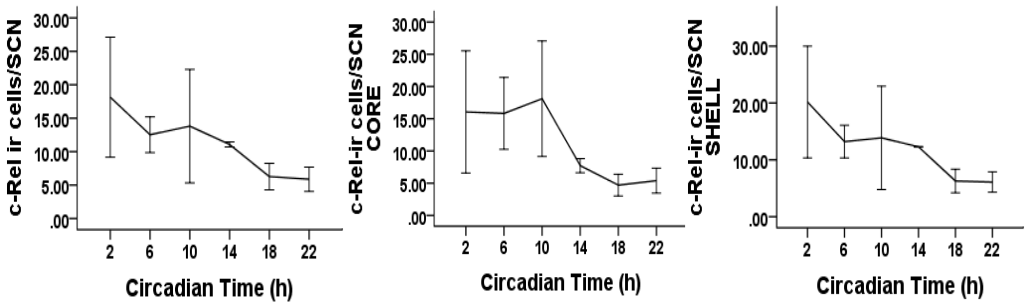


Figure 2.6: Effect of time on the expression of the c-Rel in the SCN across the 24 hour circadian cycle. Circadian regulation expression of c-Rel was examined in the SCN of animals sampled every 4 hours across the 24 hour circadian cycle in DD. (A) Representative photomicrographs of c-Rel expression in the SCN (n=4 per time point) at CT2, CT6, CT10, C14, CT18 and CT22 (scale bar = 100µm). (B) Graph illustrating quantification of levels of c-Rel immunoreactive glial in the whole, core and shell of the SCN at CT2, CT6, CT10, CT14, CT18 and CT22 and showing the expression pattern across the circadian cycle. ANOVA and Co-sinor analysis revealed no significant effect of time on c-Rel expression across the 24hr circadian cycle (P>0.05). Error bars represent ±1 SEM.

2.3.2 Analysing behaviour phase shifts as a result of photic induction

2.3.2.1 Analysing the effect a light pulse has on behaviour phase shifts in the early subjective night

A 30 minute light pulse administered at CT15 induces phase delays of -1.69 ± 0.34 hrs when compared to control mice in DD 0.18 ± 0.03 hrs (T_7= 5.349, P=0.002) (Figure 2.7).

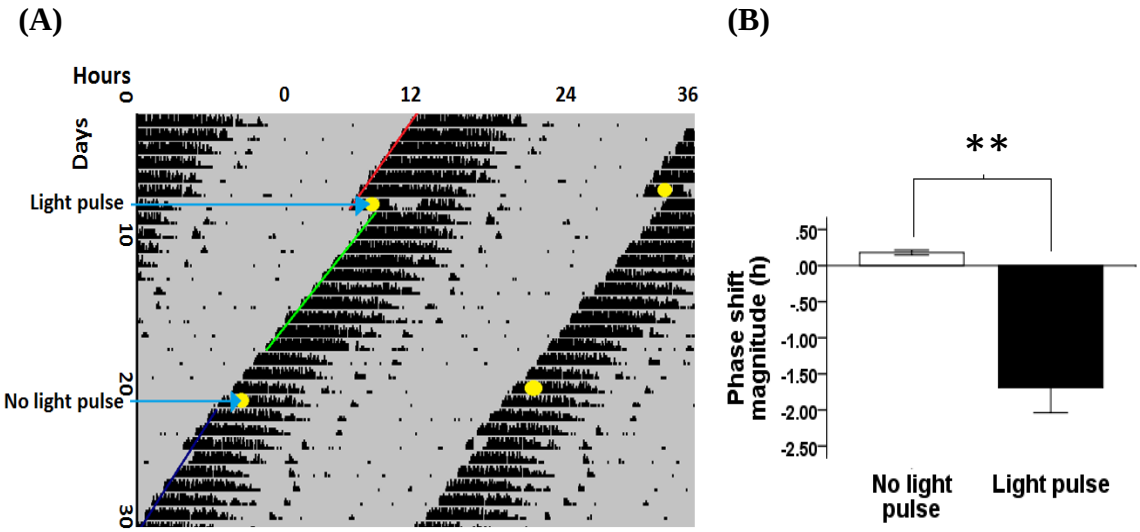


Figure 2.7: the effect photo induction causes on circadian locomotor activity at CT15.
(A) Mice were initially entrained to an LD cycle for two weeks before been placed in DD for a further two weeks. They were then administered a light pulse at CT15, and a crossover was carried out two weeks later with no photic induction administered. Light pulse is indicated by yellow circles, with lines indicating line of best fit through activity onsets for 7 days before and 10 days after the light pulse. Note the exclusion of transient days from analysis. Phase delays were displayed in the double plotted actograms. (B) Bar graph representing the magnitude of the phase delay a light pulse causes at CT15. **<0.01.

2.3.2.2 Analysing the effect a light pulse has on behaviour phase shifts in the late subjective night

A 30 minute light pulse administered at CT22 produced an average phase advances of 0.72 ± 0.11 hrs when compared to control mice in DD which displayed 0.15 ± 0.02 hrs (T_6=4.746, P=0.004) (Figure 2.8).

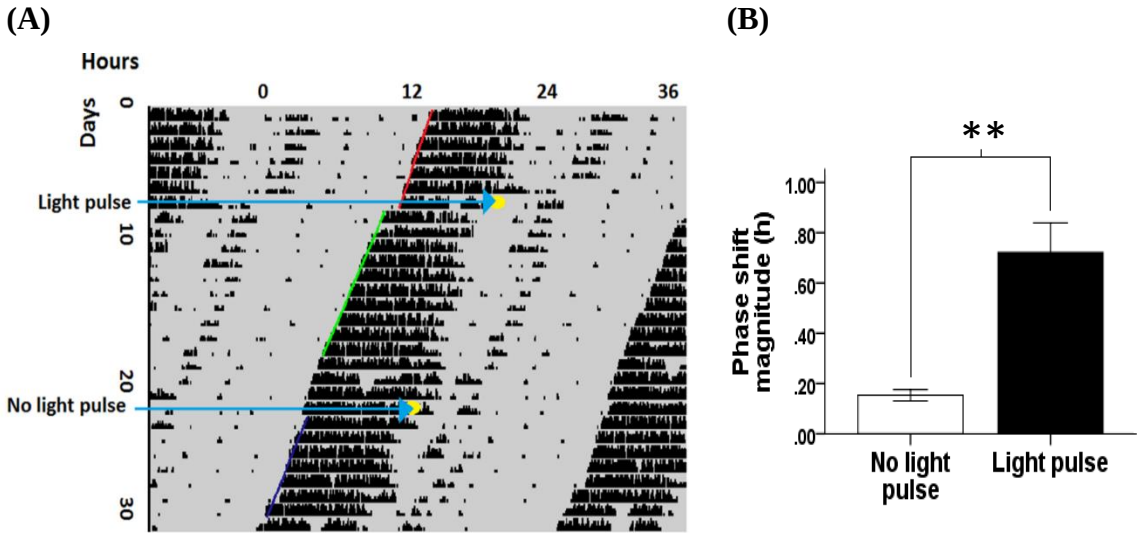


Figure 2.8: the effect photo induction causes on circadian locomotor activity at CT22. (A) Mice were initially entrained to an LD cycle for two weeks before been placed in DD for a further two weeks. They were then administered a light pulse at CT22, and a crossover was carried out two weeks later with no photic induction administered. The light pulse is indicated by both yellow circles, with lines indicating line of best fit through activity onsets for 7 days before and 10 days after the light pulse. Note the exclusion of transient days from analysis. Phase advances were displayed in the double plotted actograms. (B) Bar graph representing the magnitude of the phase advance a light pulse causes at CT22. **<0.01.

2.3.3 Analysing photic regulation of an early immediate clock gene and NF-κB p65 expression in the SCN

2.3.3.1 Analysing photic regulation of c-Fos expression in the SCN in the early and late subjective night

c-Fos expression was assessed following a 30 minute LP at CT22 by immunohistochemistry with 4 mice being sampled before the LP, another 4 mice 5 minutes into the LP, 4 mice 30 and 60 minutes post LP. Therefore, analysing levels of c-Fos immunoreactivity between the different time points by manual quantification took place, followed by statistical analysis using one way ANOVA which was used to assess statistically significant differences in the mean expression of the c-Fos immunoreactivity in the SCN across the different time points. One way ANOVA analysis at CT22 displayed altered expression of c-Fos in the SCN ($F_{3,11}$=33.58, P=0.001). Post Hoc analysis (Tukey) revealed that 30 minutes after a light pulse c-Fos expression (72.000 ± 9.54) was

significantly higher compared to samples taken in DD 8.000 ± 0.57 (P<0.001) and after a 5 minute light pulse 12.333 ± 1.92 (P<0.01).

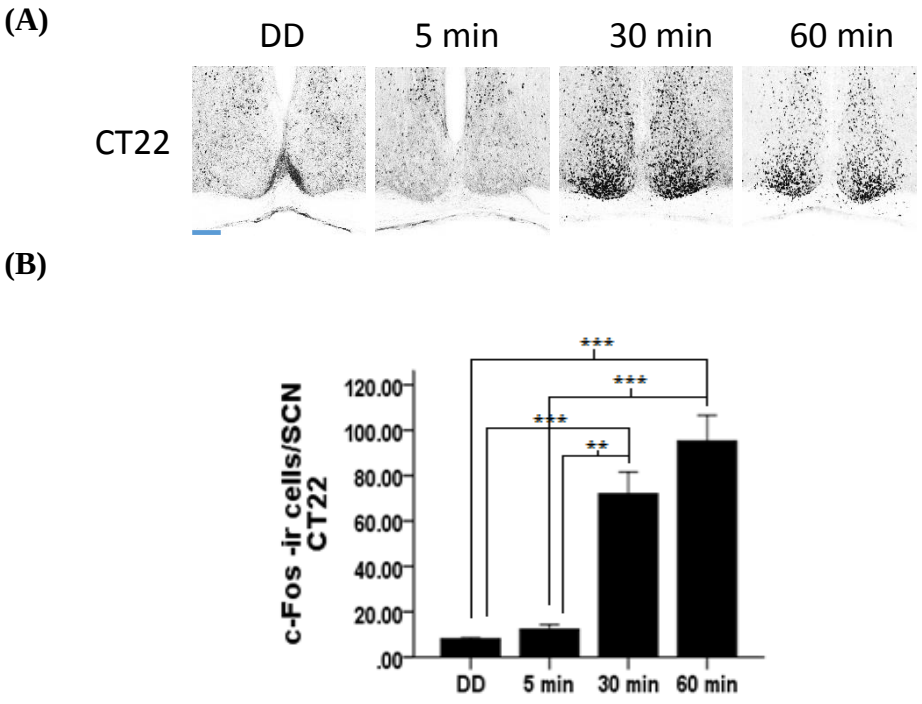
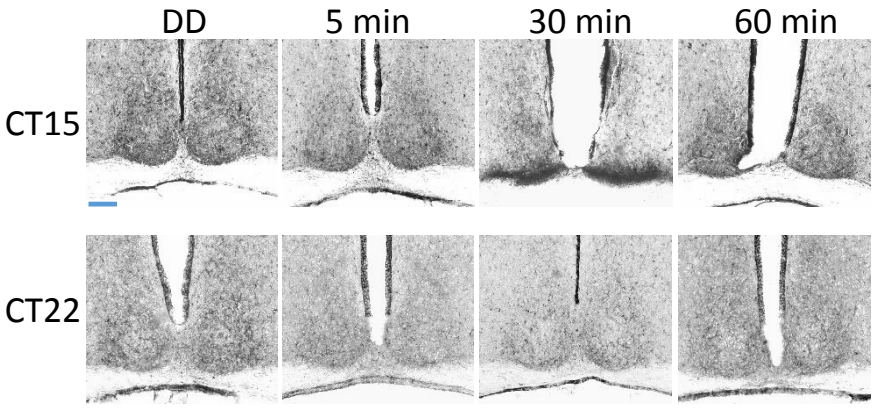


Figure 2.9: c-Fos expression after photic induction in the early and late subjective night. (A) Representative photomicrographs and (B) bar charts illustrating the effects at different time points DD and post light pulse 5min, 30 min, & 60 min at CT22 on the expression of c-Fos in the SCN. Scale bar = 100µM.

2.3.3.2 Analysing photic regulation of p65 expression in the SCN, in the early and late subjective night

p65 immunoreactivity between the different time points was under taken by IOD, followed by statistical analysis using one way ANOVA which was used to assess statistically significant differences in the mean expression of the p65 immunoreactivity in the SCN across the different time points. One way ANOVA test showed that a light pulse administered at CT15 did not alter p65 expression in the SCN before a light pulse, 5, 30, or 60 minutes after a light pulse ($F_{3,11}$=1.949, P>0.200). Similarly, a light pulse at CT22 did not alter p65 expression in the SCN at any time point ($F_{3,11}$=1.971, P=0.197).

(A)



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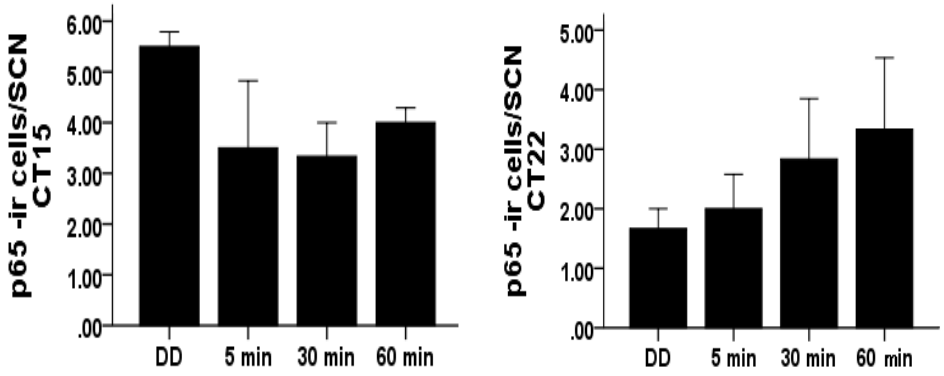


Figure 2.10: p65 expression after photic induction in the early and late subjective night. (A) Representative photomicrographs and (B) bar charts illustrating the effects at different time points after a light pulse at either CT15 or CT22 on the expression of p65 in the SCN. Scale bar = 100μM.

2.3.4 Acute PDTC treatment prior to light pulse

2.3.4.1 Behavioural analysis using PDTC treatment prior to light pulse in the early and late subjective night.

In order to examine the role of NF-κB in resetting circadian rhythms, mice free running in DD received PDTC (200mg/kg, i.p) or saline, 30 minutes before administration of a 30 minute light pulse at CT15. Pre-treatment with the NF-κB inhibitor did not attenuate the behavioural phase delay elicited by a light pulse in the early subjective night. Treatment with PDTC and light pulse displayed delays of -1.77 ± 0.22 hrs which was a similar phase delays as control and light pulse which resulted in -1.99 ± 0.08 hrs (T_{10}=-0.967, P=0.085). Similarly, administration of PDTC 30 minutes before a 30 minute light

pulse at CT22 also did not reduce the behavioural phase advance stimulated by a light pulse in the late subjective night. Treatment with PDTC and light pulse produced a phase advances of 0.56 ± 0.09 hrs and control and light pulse 0.92 ± 0.09 hrs (T_{11}=1.982, P=0.073).

(A) **(B)**

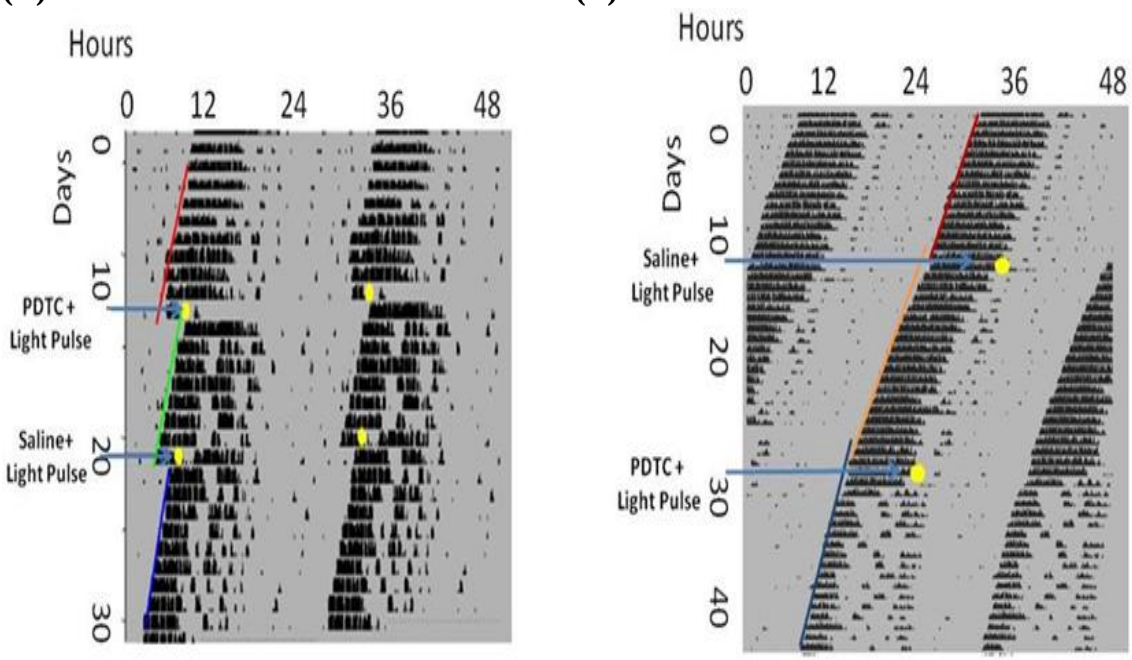
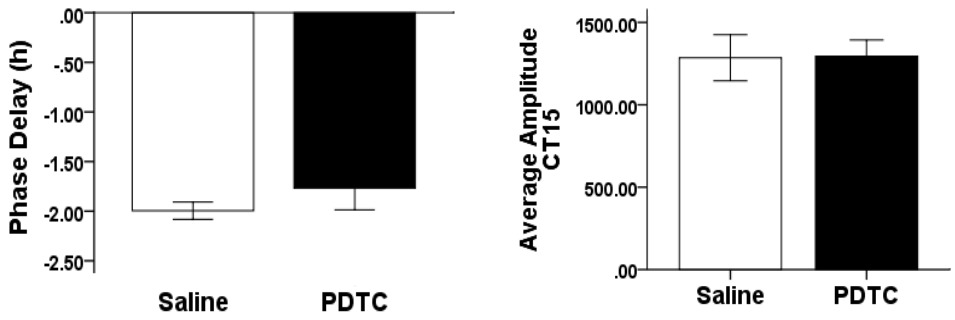


Figure 2.11: (A) and (B) are sample double plotted actograms. Mice were initially entrained to an LD cycle for two weeks before been placed in DD for a further two weeks. They were then administered PDTC/saline followed by a light pulse at CT15 or CT22, and a crossover was carried out two weeks later. Light pulse is indicated by yellow circles, with lines indicating line of best fit through activity onsets for 7 days before and 10 days after the light pulse. Note the exclusion of transient days from analysis. Phase delays and advances were not inhibited by a peripheral PDTC injection at either circadian time.

(C)



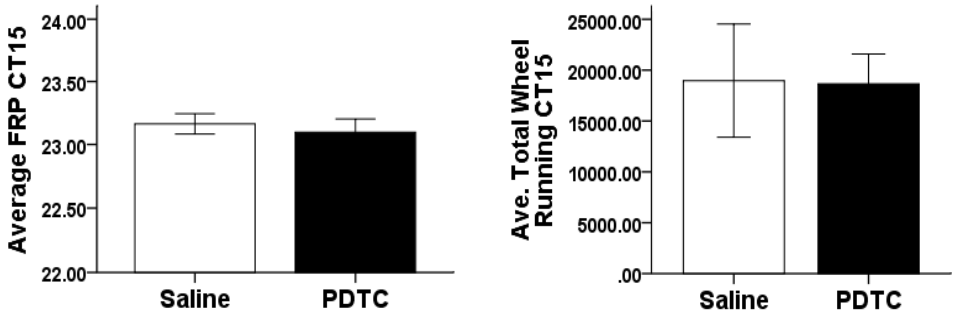


Figure 2.12: (C) Bar charts illustrating the average phase shift magnitude, amplitude, FRP and the total wheel running activity of each group following treatment and light pulse. There was no significant at CT15 (phase delay) when administered PDTC compared to saline.

(D)

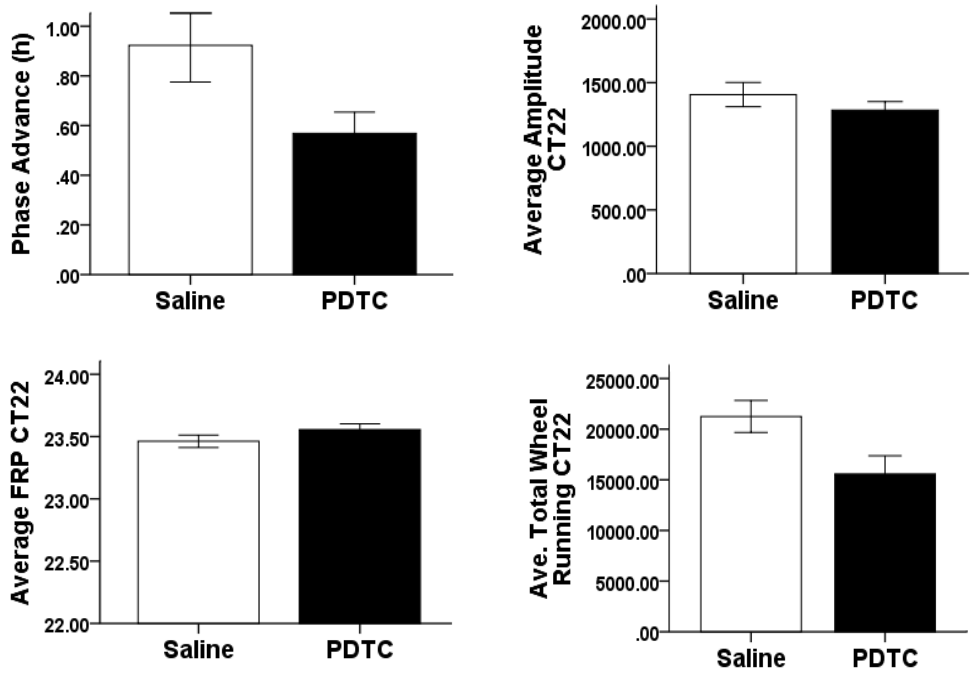
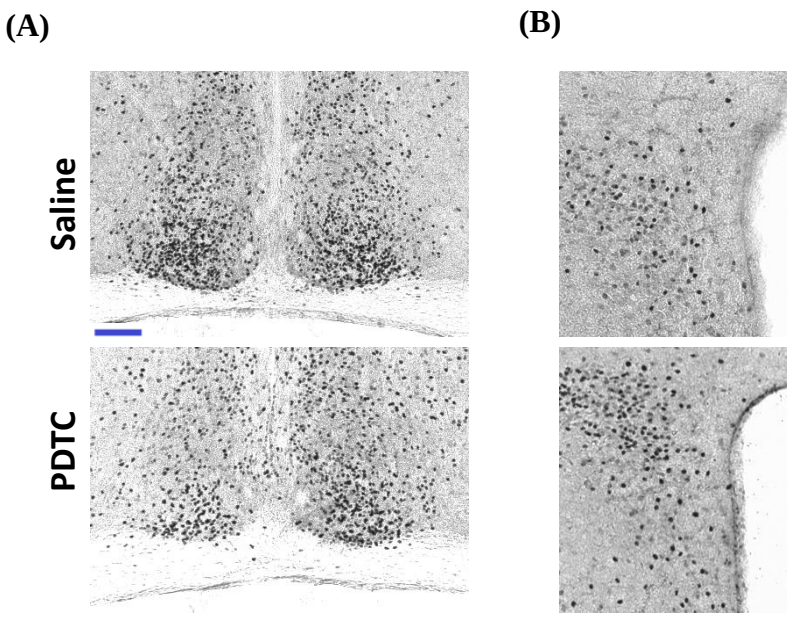


Figure 2.13: (D) Bar charts illustrating the average phase shift magnitude, amplitude, FRP and the total wheel running activity of each group following treatment and light pulse. There was no significant difference at CT22 (phase advance) when administered PDTC compared to saline.

2.3.4.2 Immunohistochemisty analysis using the immediate early gene following PDTC treatment prior to light pulse in the late subjective night.

Mice that received either PDTC or saline treatment prior to a 30 minute light pulse at CT22, were sampled for immunohistochemistry 1.5-2 hours after at CT23.5-24. The neuronal activation marker c-Fos was examined first for expression in the SCN and the PVN, since photic induction is known to cause significant upregulation of c-Fos in the SCN (Kornhauser et al., 1990; Rusak et al., 1990). Therefore, PDTC a NF-κB inhibitor was examined to see if it could attenuate c-Fos upregulation. PDTC did not lessen photic induction of c-Fos in the SCN. Analysing levels of c-Fos immunoreactivity between the treatments was carried out by manual quantification, followed by statistical analysis using an independent t-test to assess statistically significant differences in the mean expression of PDTC and controls. Independent t-tests found the mean, PDTC, 112 ± 8.42 vs. saline, 123 ± 7.52 (T_6=0.975, P=0.367). PDTC caused no alterations of c-Fos in the PVN, PDTC, 17 ± 1.87 compared with controls, 10.25 ± 3.28 (T_6=1.335, P=0.124).



(C)

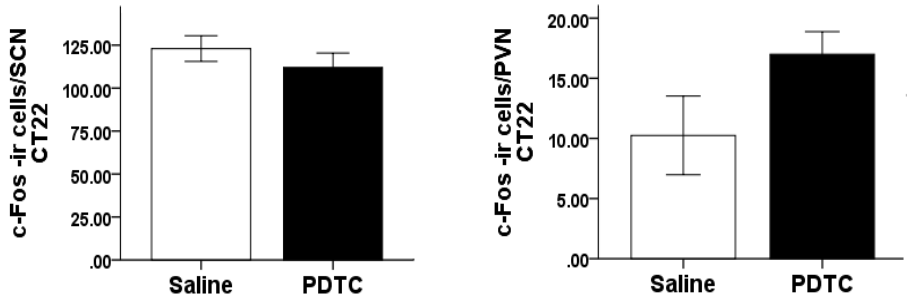


Figure 2.14: Photomicrographs of the (A) SCN and (B) PVN illustrating the expression of c-Fos after 30 minute light pulse prior to saline and PDTC treatment. Scale bar = 100μM. (C) Bar graphs representing c-Fos expression in both the SCN and PVN following administration of either PDTC or Saline prior to a 30 minute light pulse.

2.3.4.3 Immunohistochemistry analysis using ARC following PDTC treatment prior to light pulse in the late subjective night.

The ability of the NF-κB inhibitor, PDTC to alter the expression of ARC in the SCN and PVN prior to a light pulse was also investigated. The outcome was that PDTC did not lessen photic induction of ARC in the SCN. Analysing levels of ARC immunoreactivity between the different treatments was carried out by manual quantification, followed by statistical analysis using an independent t-test which was used to evaluate statistically significant differences in the mean expression of PDTC and controls. Independent t-tests found that there was no significant ARC expression between the two treatment groups. The mean PDTC was 15.00 ± 2.55 vs. saline 15.87 ± 1.30 (t_4=2.121, P=0.101). Furthermore, analysis using independent t-tests found no variation of ARC following treatment of PDTC (4.66 ± 0.33 when compared to control 6.66 ± 0.88, t_4=2, P=0.116).

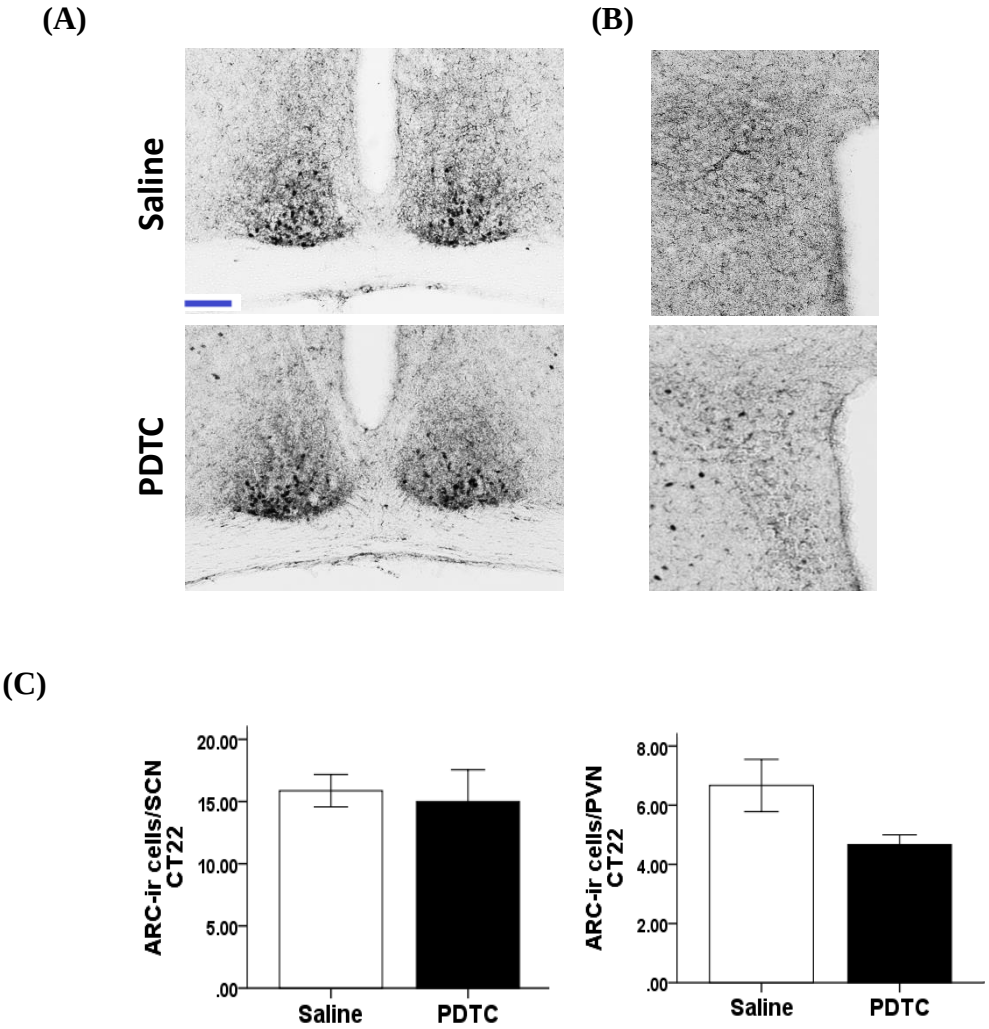


Figure 2.15: Photomicrographs (A) SCN and (B) PVN illustrating the expression of ARC after 30 minute light pulse prior to saline and PDTC treatment. Scale bar = 100µM. (C) Bar graphs representing ARC expression in both the SCN and PVN following administration of either PDTC or Saline prior to a 30 minute light pulse.

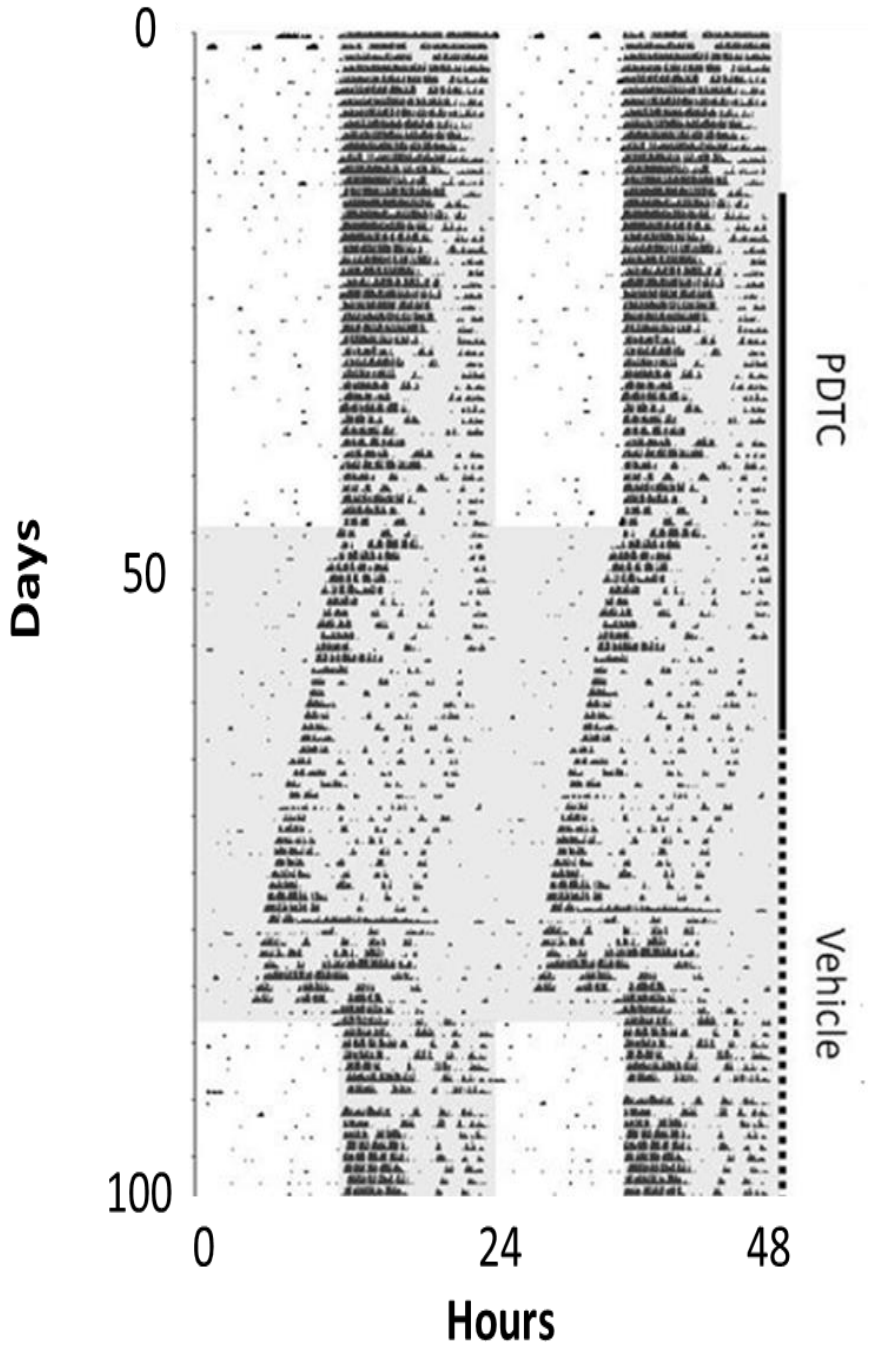
2.3.5 Chronic PDTC treatment

2.3.5.1 Behavioural analysis of chronic PDTC treatment by oral administration

Investigating the effects of chronic PDTC treatment by oral administration in mice drinking water resulted in no altering of the phase angle of entrainment, PDTC was 1.757 ± 0.05 hrs compared to DMSO 0.081 ± 0.14 hrs (F_{26}=3.279, P=0.526). Repeated measures analysis also indicated that there is no significant differences in FRP values between the treatment groups (PDTC, 23.79 ± 0.03 hrs vs. DMSO, 23.81 ± 0.03 hrs, F_{30}=0.385, P=0.516), nor was there with amplitude (PDTC, 1909.5 ± 197.02 vs. DMSO, 2126.3 ±

267.62, F_{30}=1.67, P=0.519). Repeated measures analysis was also used to examine whether there was statistically significant differences between the total wheel running activity between treatment groups (PDTC, 7.701.1 ± 1585.16 vs. DMSO, 12189.25 ± 1698.5, F_{24}=0.086, P=0.065). (Figure 2.16).

(A)



(B)

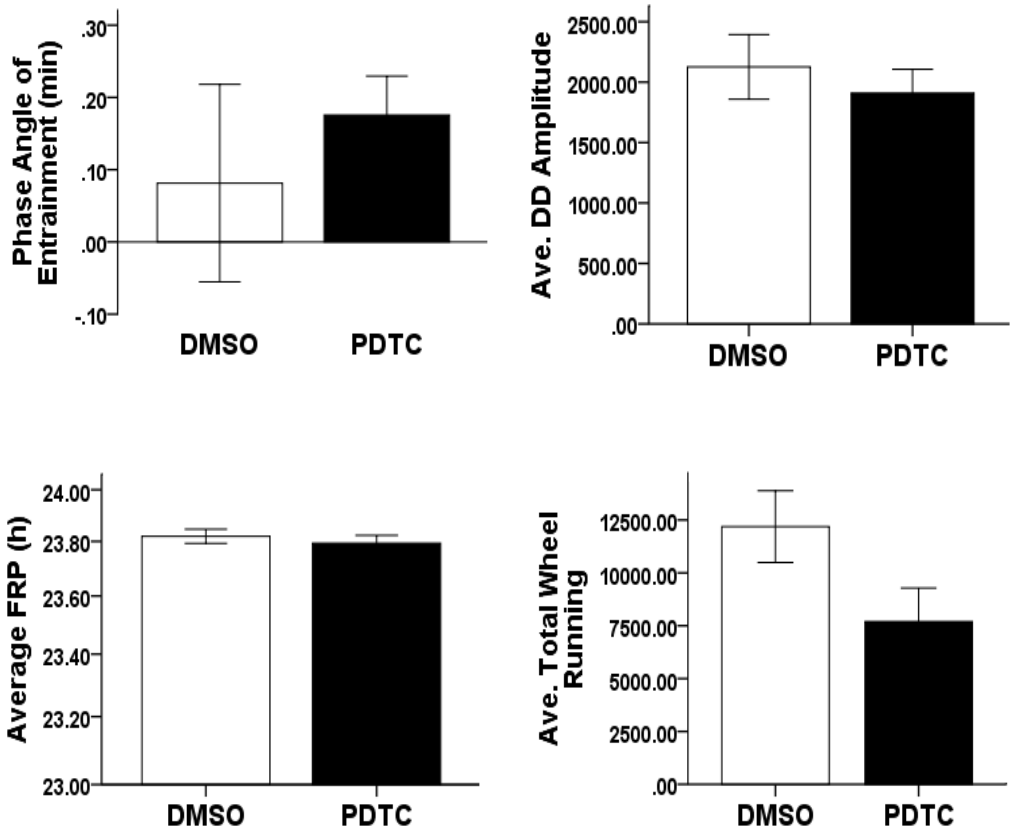


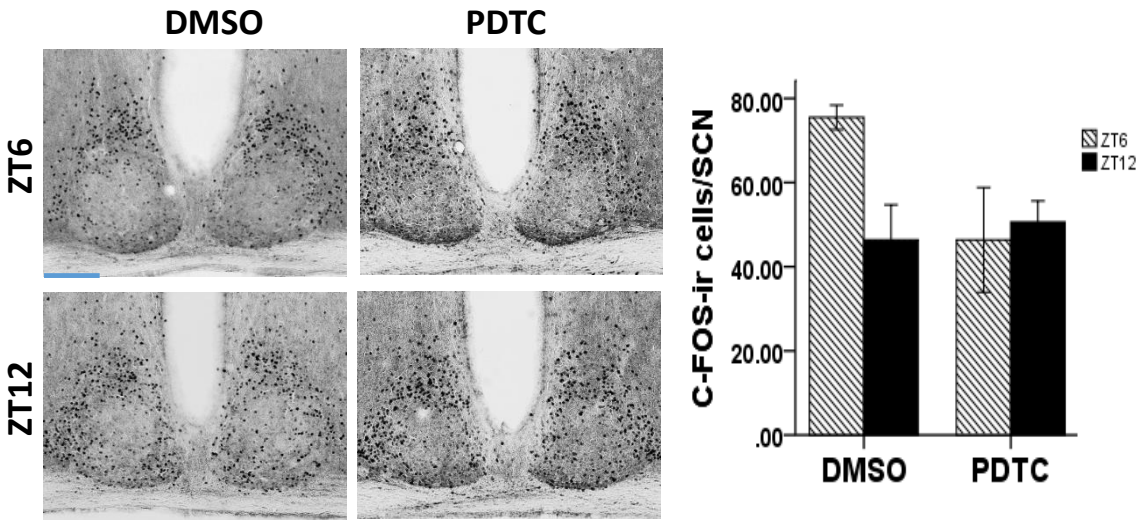
Figure 2.16: (A) Sample of a double plotted actogram from mice wheel running in LD 12:12 cycle for 6 weeks and DD for 6 weeks, while treated with chronic PDTC or DMSO in their drinking waters, with a crossover design carried out. (B) Bar graphs representing the phase angle of entrainment, amplitude in DD, FRP and the total wheel running activity.

2.3.5.2 Immunohistochemistry analysis of chronic PDTC treatment by oral administration

Chronic treatment with PDTC (50mg/kg daily via drinking water) does not alter the diurnal rhythm in c-Fos expression in the SCN. Analysing levels of Fos immunoreactivity between the different treatments and time were carried out by manual quantification, followed by statistical analysis using two way ANOVA analysis used to assess whether there was alterations in the expression of c-Fos in the SCN and PVN, following treatment of either PDTC or control at two different time points and whether there was a significant interaction effect between time and treatment. There was no main effect of time ($F_{1,16}$=2.39, P=0.147) or main effect of treatment ($F_{1,16}$=2.39, P=0.147) in the SCN. There was no statistically significant effect between time and treatment

($F_{1,16}$=4.316, P=0.060) on c-Fos expression levels in the SCN. No main effect of time ($F_{1,14}$=0.034, P=0.852) or main effect of treatment ($F_{1,14}$=1.327, P=0.276) were found in the PVN. There also was no statistically significant effect between time and treatment in the PVN ($F_{1,14}$=0.590, P=0.460) (Figure 2.17).

(A)



(B)

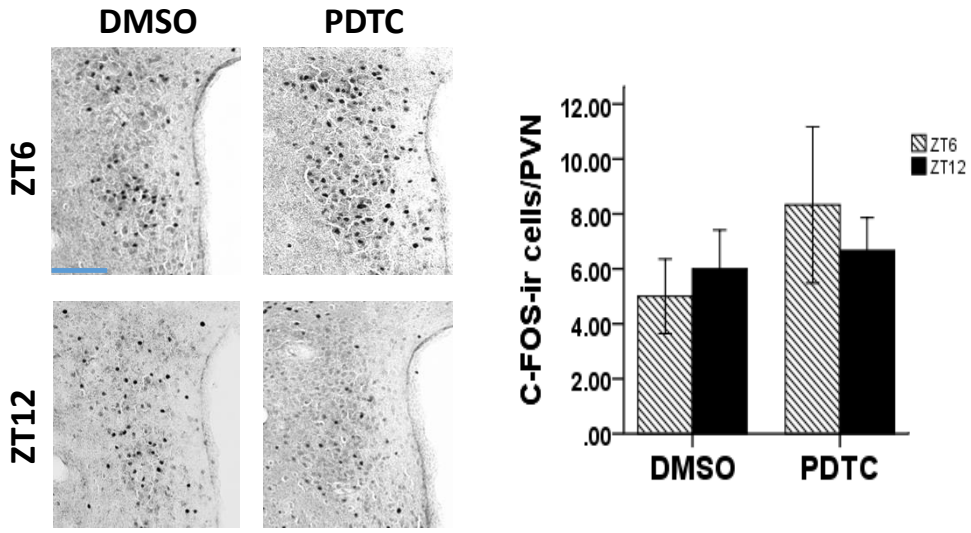
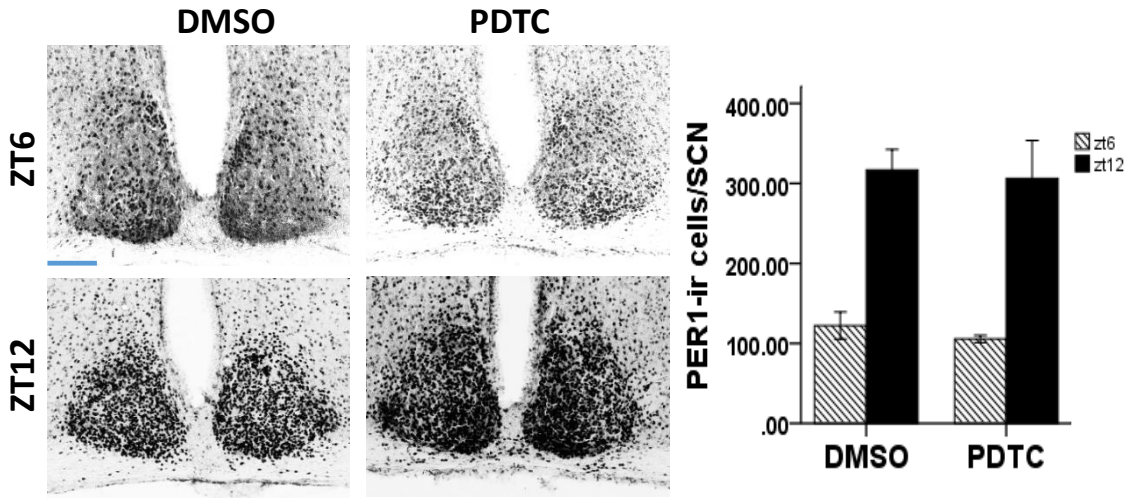


Figure 2.17: Photomicrographs and bar charts of (A) SCN and (B) PVN illustrating the expression of the neuronal marker c-Fos after treatment with either PDTC or control at two different timepoints, ZT6 and ZT12.

Chronic treatment with PDTC does not alter the diurnal rhythm in PER1 expression in the SCN. Analysing levels of PER1 immunoreactivity between the different

treatments and time were carried out by manual quantification. This was followed by statistical analysis using two way ANOVA analysis used to assess whether there was alterations in the expression of PER1 in the SCN and PVN following treatment of either PDTC or control at ZT6 and ZT12, and whether there was an effect of time*treatment. There was a main effect of time ($F_{1,16}$=48.603, P=0.001), however, there was no main effect of treatment ($F_{1,16}$=0.231, P=0.639) in the SCN. Results indicate that there was no statistically significant effect of time*treatment on PER1 expression levels in the SCN ($F_{1,16}$=0.012, P=0.914) and in the PVN ($F_{1,13}$=0.015, P=0.905). Additionally, no main effect of time ($F_{1,13}$=0.600, P=0.458) or main effect of treatment ($F_{1,13}$=1.212, P=0.300) were found in the PVN (Figure 2.18).

(A)



(B)

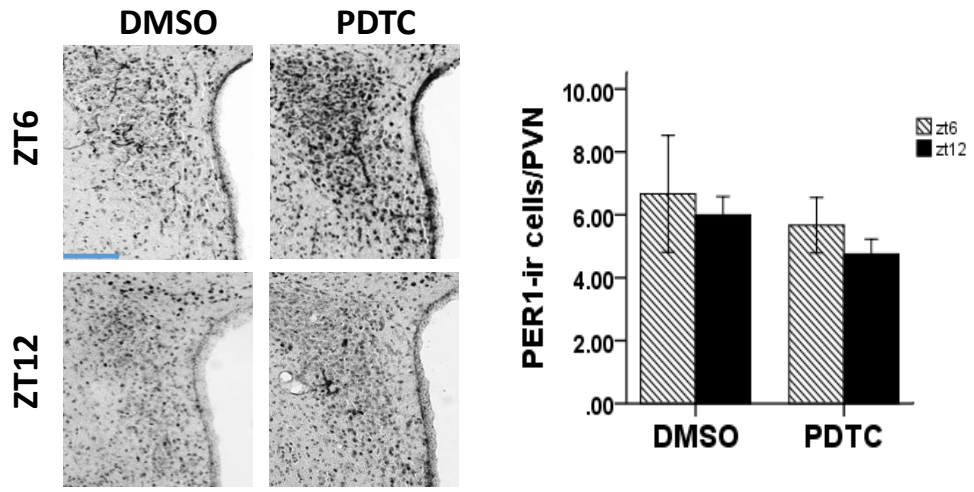


Figure 2.18: Photomicrographs and bar charts of (A) SCN and (B) PVN illustrating the expression of the clock gene protein PER1 after treatment with either PDTC or control at two different timepoints, ZT6 and ZT12.

Chronic treatment with PDTC does not alter p65 expression in the SCN. Analysing levels of p65 immunoreactivity between the different treatments and time were carried out by IOD. Statistical analysis using two way ANOVA analysis was used to assess whether there was alterations in the expression of p65 in the SCN and PVN following treatment of either PDTC or control at ZT6 and ZT12, and whether there was an effect of time*treatment. No main effect of time ($F_{1,15}$=0.044, P=0.837) or main effect of treatment ($F_{1,15}$=0.667, P=0.431) in the SCN was found. There was no statistically significant effect of time*treatment on p65 expression levels in the SCN ($F_{1,15}$=2.53, P=0.625), or in the PVN ($F_{1,13}$=3.60, P=0.563). Furthermore, no main effect of time ($F_{1,13}$=5.994, P=0.037) or main effect of treatment ($F_{1,13}$=0.360, P=0.563) were found in the PVN (Figure 2.19).

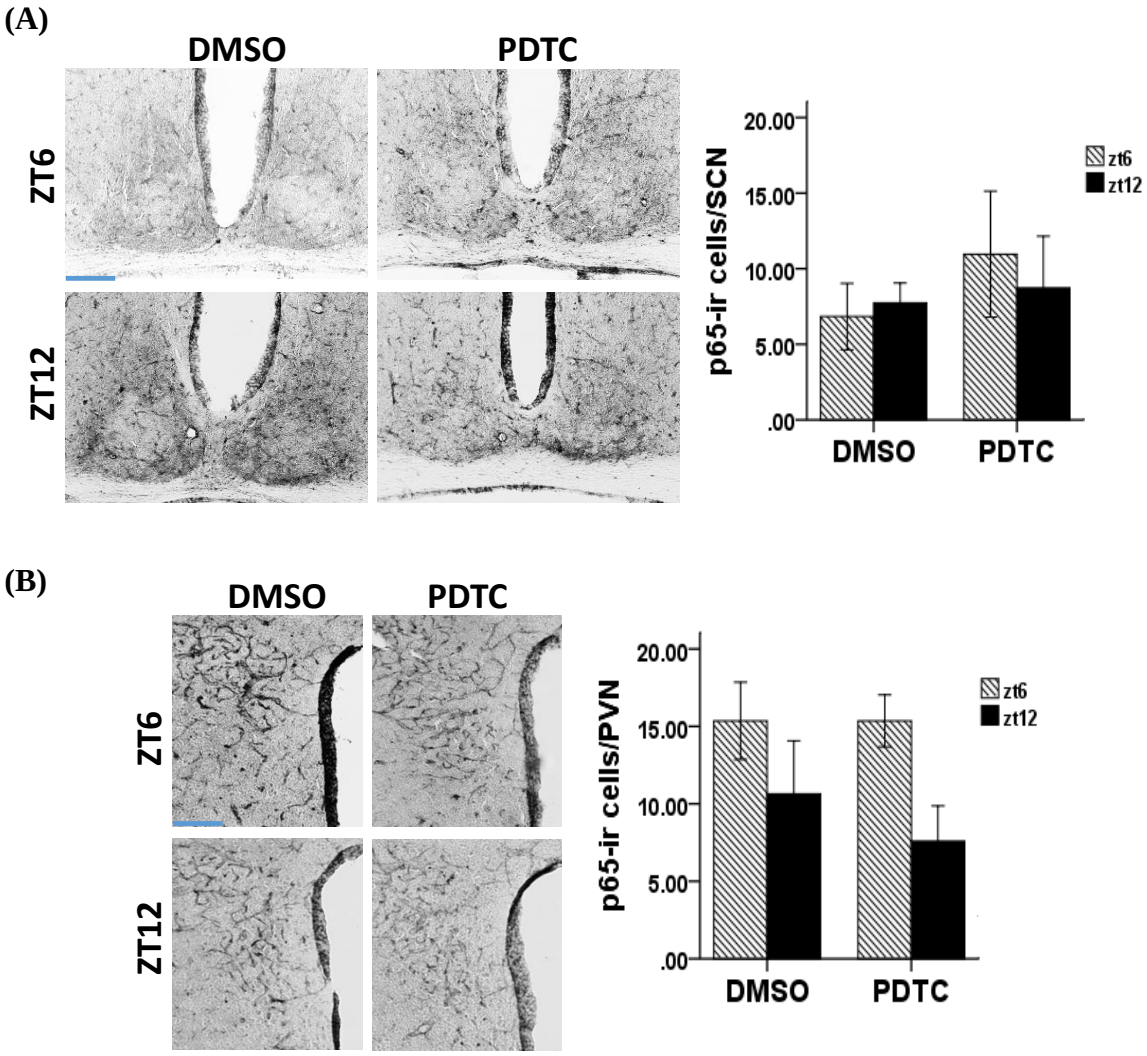


Figure 2.19: Photomicrographs and bar charts of (A) SCN and (B) PVN illustrating the expression of the NF-κB subunit p65 after treatment with either PDTC or control at two different timepoints, ZT6 and ZT12.

2.4 Discussion

The NF-κB pathway plays an important role in the body's immune system in relation to inflammation and disease. Since activation of NF-κB in glia seems to exacerbate certain diseases, it is thought by inhibiting NF-κB in glia cells, that it might result in reducing disease in some cases (Kaltschmidt & Kaltschmidt, 2009). In control animals, no constitutive NF-κB activity was found in glia cells (Bhakar et al., 2002; Schmidt-Ullrich et al., 1996). Past studies have found low levels of basal expression of NF-κB in neurons in brain areas such as the cerebellum, frontal cortex (Korhonen et al., 1997), hippocampus, cerebral cortex and basal forebrain observed using immunohistochemistry, western blotting and gel shift assays (Kaltschmidt et al., 1995, 1994; Toliver-Kinsky et al, 1997). Furthermore, using transgenic reporter mouse models, the previous data was corroborated, and this model also displayed constitutive NF-κB activity in additional brain regions, for example the hypothalamus, amygdala and olfactory lobes (Bhakar et al., 2002; Schmidt-Ullrich et al., 1996).

In this study there was no differentiation carried out between neurons, astrocytes and microglial in terms of NF-κB expression patterns. Current research has discovered that there is minimal NF-κB activity in neurons even after LPS treatment (Listwak et al., 2013), concluding that there is lack of evidence that NF-κB does play a role in the circadian clock. Extra care must be taken in interpreting results of neuronal NF-κB activity in the CNS, with numerous studies using antibodies that are not selective for p65. Herkenham and colleagues used selective and non-selective frequently used commercial and non-commercial p65 and p50 antibodies, using western blot assays to analyse the study of NF-κB activity in the CNS (Herkenham et al., 2011). Studies established that there is an inconsistency with the different antibodies used in western blot analysis, with some antibodies recognising the target proteins at the precise molecular weight, however, there are others that also mark in additional protein bands, therefore, making them unsuitable for immunohistochemistry, such as p65 (MAB3026; Chemicon) and p50 (sc-114; Santa Cruz) (Herkenham et al., 2011; Pereira et al., 1996). In this study however, we used the p65 antibody from Santa Cruz Biotechnology (sc-372), which Herkenham and colleagues found to be accurate and precise in their study. In conclusion, since antibodies can lack efficacy and specificity; experimental results can suffer as a consequence, in terms of perpetuating flawed findings, which may be the case in studies which had found

constitutive NF-κB in neurons using non-specific antibodies (Kaltschmidt et al., 1995, 1994; Toliver-Kinsky et al., 1997). A recent study carried out revealed that there is minimal hypothalamic NF-κB in young mice (Zhang et al., 2013).

2.4.1 The expression of clock genes and NF-κB components in the SCN

In this current study, we first looked at the expression of the clock gene PER1 protein in the SCN across the 24 hour cycle in DD under basal conditions. Previous studies have shown that *Per1* mRNA displays peak expression in the SCN between ZT/CT 4 and 6 (Bae et al., 2001; Guilding & Piggins, 2007; Ripperger et al., 2011). PER1 protein is rhythmic throughout the 24 hours circadian cycle, with peak expression displayed 4-6 hours after mRNA levels, roughly between CT10 and 12 (Guilding & Piggins, 2007). We also established similar findings using immunohistochemistry, with PER1 rhythmic expression displayed during the 24 hour cycle and peak expression exhibited at CT10. This protein was tested to confirm expected circadian expression profiles in our animals.

With regard to the expression of NF-κB components in the SCN of WT mice, studies were undertaken to examine the 24 hour circadian cycle in DD conditions, again under basal conditions, to ascertain if certain components of the NF-κB pathway are rhythmic in the circadian clock. Immunostaining exhibited no significant temporal regulation of p65, p-IκB, p-IκK and c-Rel in the basal functioning of the SCN clock. Low levels of p65 expression in the SCN may be due to the fact that NF-κB is usually in its inactive state in the cytoplasm, and when active after binding to IκB it is continuously shuttling between the nucleus and cytoplasm. This may play a factor in the low levels of nuclear p65 and p-IκB staining exhibited in the SCN under basal conditions. This corresponds to findings of Zhang and colleagues (Zhang et al., 2013), highlighting that there is minimal hypothalamic NF-κB in young mice. A previous study was carried out by Marpegan to investigate if p65 expression was diurnal or circadian in nature using western blots. SCN cell extracts from hamsters were analysed at various time points over a 24 hour cycle with findings stating that p65 expression had no diurnal or circadian variation (Marpegan et al., 2004).

2.4.2 The effects of photic induction on circadian behaviour, IEGs and the NF-κB pathway

In this current study, investigation of stimuli such as light pulses in the subjective night were analysed to see if it could activate the NF-κB pathway. The SCN is responsible for photic entrainment in nearly all mammals. From previous studies, light pulses administered during the night can result in phase shifts. Light induction in the early subjective night cause phase delays, in contrast to light pulses that are administered in the late subjective night, which result in phase advances (Meijer & Schwartz, 2003; Shibata & Moore, 1993). When photic stimuli occurs, the internal oscillator resets within one cycle, though overt behaviour shows transient cycles until a steady state of entrainment is attained (Johnson et al., 2003). Findings from our wheel running experiments found similar results, with light pulse administration causing phase delays at CT15 of approximately -1.69 hours, and phase advances at CT22 of 0.72 hours. Studies have displayed interesting findings, with NMDA administration causing similar effects with photic induction in the SCN. NMDA given at CT13.5 and CT19 cause phase delays and advances respectively (Mintz et al., 1999). Administration with NMDA receptor antagonists and non-NMDA antagonists were able to attenuate circadian phase shifting effects of light on wheel running behaviour in rodents (Colwell & Menaker, 1992; Mintz et al., 1999).

The expression of IEGs have been researched to understand the physiological mechanisms responsible for entrainment by stimuli such as light, and also for the endogenous generations of circadian rhythms (Guido et al., 1999; Rusak et al., 2002). Light pulses during the subjective day show no alteration in IEGs expression. However, light pulses that induce phase shifts in rodents in the early and late subjective night can induce upregulation of the IEGs such as c-Fos and ARC expression in the vlSCN (Guido et al., 1999; Mahoney et al., 2001; Rusak et al., 1992; Rusak et al. , 1990). Photic induction of the *arc* gene was displayed in the SCN at CT16 after a 30 minute light exposure. Under DD conditions, *Arc* mRNA and ARC protein were not seen at CT16, therefore, emphasising the role photic induction plays in the early subjective night. Furthermore, a 30 minute light pulse did not result in *Arc* mRNA signals during the subjective day, which were tested at different time points throughout this period. The expression of *Arc* mRNA is circadian phase-dependent, with light exposure producing

increased *Arc* mRNA at CT12, CT16 and CT20. There were significant increases in *Arc* mRNA and ARC protein expression observed 30-60 minutes after a light pulse, seen exclusively in the retinorecipient ventrolateral part of the SCN (Nishimura et al., 2003).

c-Fos is the most researched IEG in response to photic stimulation (Aronin et al., 1990; Kornhauser et al., 1990; Rea, 1989; Rusak et al., 1990). c-Fos protein expression is extremely faint throughout basal conditions (Herdegen et al., 1995; Sagar et al., 1988). *c-Fos* mRNA and c-Fos protein expression can be induced in the SCN of diurnal and nocturnal animals after administration of a light pulse at night or in response to lights on at dawn in the LD cycle (Hughes et al., 2004; Rea, 1989; Rusak et al., 1990). c-Fos induction can result from photic induction of as little as 5 minutes (Kornhauser et al., 1990), however the highest c-Fos expression can be seen within 30 minutes to one hour following exposure to light (Colwell & Foster, 1992; Kornhauser et al., 1990; Schwartz et al., 1995). Therefore, c-Fos protein expression can detect if photic induction is involved in photic entrainment and circadian function. The SCN itself is intrinsically rhythmic in its response to stimuli that produce c-Fos expression, with *in vitro* studies using SCN slices, displaying how electrical stimulus could stimulate c-Fos protein expression only during the subjective night (Bennett et al., 1996). Unlike ARC expression (Nishimura et al., 2003), spontaneous c-Fos expression is exhibited in the dmSCN and is independent of the effects caused by light pulses (Sumová et al., 1998).

Our results displayed a similar outcome to other studies with an increase in immunoreactivity for c-Fos occurring when a light pulse was administered in the late subjective night CT22 (Earnest & Olschowka, 1993; Ebling et al., 1991; Rusak et al., 1990). We found a statistically significant upregulation of c-Fos in the SCN at 30 minutes and 1 hour following a 30 minute light pulse before been culled. However, when investigating if the NF-κB component p65 was affected by photic induction, it was discovered that p65 expression was not altered in the SCN at either CT15 or CT22 when a light pulse was administered. The timeframe after the light pulse which we examined, 1 hour, should be long enough to observe any such change in photic resetting. This was not unusual since low levels of nuclear p65 expression are known under basal conditions in the SCN. Therefore, this seems to highlight that this NF-κB protein is not be involved in photic resetting, with other pathways such as MAPK key to photic stimulation in the circadian clock.

2.4.3 Acute PDTC treatment

We wanted to investigate further if the NF-κB pathway is linked with phase shifts following light pulses at a behavioural and molecular level since disruption to pathways such as MAPK can lead to a decrease in light induced phase shifts (Butcher et al., 2002). Inhibitors of the ERK pathway can reduce phase shifts of running wheel locomotor activity and attenuate IEGs expression including c-Fos and Egr1 (Dziema et al., 2003). Therefore, a study was carried out using the NF-κB inhibitor PDTC to establish if it could block the phase shifts previously displayed in this chapter. In this study we report on findings in relation to the NF-κB specific inhibitor PDTC using *in vivo* methods (Riera et al., 2015). An investigation took place to see if this inhibitor had any effect on photic stimulation at CT22, in relation to behavioural phase shifts and IEG expression in the SCN and PVN.

Administration of PDTC prior to an immune challenge was found to inhibit LPS-induced IκB degradation, along with an attenuation of neutrophils in certain peripheral organs (Liu et al., 1999). Additional studies found that PDTC reduces inflammation caused by LPS by inhibiting the NF-κB signalling pathway (Ohta et al., 2002). PDTC has to be administered prior to an immune challenge, because if given in conjunction with LPS it will preclude in blocking NF-κB (Cvek & Dvorak, 2007). Therefore, in this current study, administration of the NF-κB inhibitor, PDTC, was administered at 200mg/kg (Shu Fang Liu et al., 1999), 30 minutes prior to a light pulse, to establish if this inhibitor blocks photic phase shifting in wheel running rodents.

The effects of acute pharmacological inhibition of the NF-κB pathway on circadian processes *in vivo* have been investigated in Marpegans studies. Administration of NF-κB inhibitor PDTC in hamsters prevented phase advances caused by light pulses at CT18, whereas saline treated hamsters administered with a light pulse resulted in a phase advance (Marpegan et al., 2004). We investigated the effects of PDTC administration prior to a light pulse in mice in the early and late subjective night, CT15 and CT22 respectively. In contrast we found that PDTC did not inhibit photic induced phase shifts. The contrasting results found between Marpegans study and this study may be due to the fact that different rodents were used in both studies. Also in the two studies there were different injection times, therefore, the participation of NF-κB in photic induction may be dependent on the time of photic stimulation. Another vital reason for

contrasting results might be due to how PDTC was administered to the rodents. In our study, injections were administered i.p, while (Marpegan et al., 2004) used i.c.v injections in hamsters. Administration of PDTC i.c.v into the CNS could have a more potent and immediate effect then that of i.p injections. I.p injections may have a longer time course, resulting in PDTC been unable to prevent photic phase shifts.

We looked at the IEGs expression in the SCN and PVN following the administration of PDTC and a light pulse. The PVN was also investigated since it is one of the main target areas of the SCN. The SCN communicates with the PVN in order to regulate stress and immune function (Kalsbeek & Buijs, 2002). Circadian information is transferred to the immune system as a result of SCN projections to the PVN which is involved in the regulation of endocrine and autonomic neuronal activity (Kalsbeek et al., 2006). We hypothesised perhaps NF-κB may be in fact regulating c-Fos and ARC, since a recent study has displayed that there is a κB-binding site in the *c-Fos* promoter region, which was discovered in mouse embryonic fibroblasts (MEFs). This study found that NF-κB plays a role in PMA-induced *c-fos* mRNA and c-Fos protein expression in MEFs, since IKK-null MEFs have attenuated c-Fos expression compared to controls. Additionally PMA-induced c-Fos expression was supressed in MEFs deficit in p65, therefore, it would seem that NF-κB regulates c-fos expression. Finally, it was established the p65 homodimer can bind to the mouse *c-Fos* promoter region, associating the two transcriptional systems together (Tu et al., 2013).

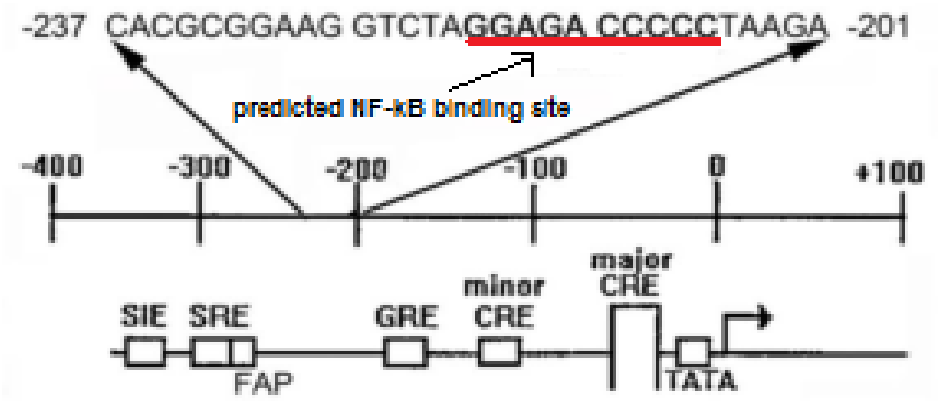


Figure 2.20: Displays the predicted NF-κB binding site in the mouse c-Fos promoter region. Adapted from (Tu et al., 2013).

It was discovered that there was no difference in neuronal activation of either c-Fos or ARC, in both the SCN and PVN, in mice administered with either PDTC or control before receiving a light pulse. This was not surprising since there was no difference seen in the behavioural experiment, with photic phase shifts comparable between PDTC and vehicle treated subjects. Therefore, it would seem that NF-κB is not essential in regulating c-Fos or ARC with regard to photic induction, as blocking this pathway using PDTC does not hinder neuronal activation.

2.4.4 Chronic PDTC treatment

Chronic pharmacological inhibition of the NF-κB pathway has being carried out in many studies using oral administration of PDTC, in relation to various diseases such as breast cancer (Gu et al., 2009), diabetes (Luo et al., 2015; Mariappan et al., 2010; Zhao et al., 2013), ulcerative colitis (UC) (Hirata et al., 2007) and AD (Malm et al., 2007). PDTC ameliorated each of these diseases by being administered in rodent's drinking water, varying from 20-200mg/kg daily. Patients with IBD, display higher expression of the p65 protein in their mucosal tissues (Neurath, 1998; Schreiber et al., 1998). The animal model of UC is known as (dextran sodium sulfate) DSS-induced colitis (Okayasu et al., 1990). Hirata and colleagues were able to alleviate DSS-induced colitis by oral administration of PDTC in mice, by suppressing NF-κB activity in the intestines, and reducing the production of IL-1β and TNF-α (Hirata et al., 2007). Other forms of chronic PDTC administration through injection have also been shown as attenuating NF-κB activation (Rangan et al., 1999). Chronic inflammation in the form of collagen-induced arthritis was reduced by an i.p injection of PDTC every two days (Cuzzocrea et al., 2002).

The next step in this study was to examine if chronic pharmacological inhibition of the NF-κB pathway on circadian processes *in vivo* caused any changes in core circadian locomotor activity or at a molecular level. As discussed previously, this was investigated by oral administration of PDTC in mice drinking water. In LD there was no difference in the period, amplitude and the total wheel running activity in PDTC and vehicle subjects. In order to assess the integrity of the retinohypothalamic tract or RHT transmission from it, the phase angles of entrainment were examined for each animal in both treatment groups under the LD photoperiod, since alterations in RHT transmission impact upon the phase angle of entrainment. Under the LD photoperiod, the phase angle of entrainment in

both treatment groups displayed no variation. When transferred into DD there was no masking difference seen between PDTC and vehicle treated mice. Furthermore, in DD there was no alterations in endogenous clock properties such as their FRP and amplitude.

Lastly we examined the effects of chronic pharmacological inhibition of the NF-κB pathway on circadian processes *in vivo* at a molecular level. We examined the effect of chronic PDTC versus vehicle at two different time points, ZT6 and ZT12, for expression of the NF-κB subunit p65, the IEG c-Fos and for clock gene protein expression using PER1. There was no alterations in the SCN or PVN in p65 expression, again perhaps due to the low levels of nuclear p65 expression displayed under basal conditions in the SCN. There are clock controlled genes known to contain NF-κB binding sites on their promoters which highlights a link between the immune and circadian system (Bozek et al., 2007). Therefore, we decided to see if blocking the NF-κB pathway using chronic oral treatment of inhibitor PDTC, had any effect on the molecular clock with regard to PER1 expression. There was no alterations in PER1 protein expression between treatment groups in the SCN or PVN. This would be expected as it ties in with the fact there are no behavioural alterations. When investigating the c-Fos expression there was a slight diurnal difference in control mice, with higher levels of expression displayed during the middle of the day in comparison to the middle of the night. This was not observed in mice treated with PDTC, therefore perhaps chronic PDTC treatment somewhat blocks NF-κB regulation of c-Fos expression in mice during the day, since NF-κB has been shown to regulate c-Fos expression (Tu et al., 2013).

Chapter Three

Analysing the effects of acute administration of LPS on NF-κB signalling in the SCN

3.1 Introduction

The aim of this chapter was to investigate the role acute LPS treatment has on NF-κB components in the SCN and PVN. LPS is a component of the outer membrane of Gram-negative bacteria which bind to TLR4 and CD14 on the surface of various cell types (Bohannon et al., 2013; Lu et al., 2008; Singh & Jiang, 2004; Van Amersfoort et al., 2003; Wright et al., 1990). It is an important target for recognition by the immune system as it results in septic shock in humans (Ulevitch & Tobias, 1995). LPS application is known to induce in a systemic inflammatory response, increasing the level of TNF-α, IL-6 and IL-1β in the brain, causing sickness behaviour (Dantzer, 2009; Raetz & Whitfield, 2002; Skelly et al., 2013). This sickness behaviour is displayed in the following manner, reduced social activity, lethargy, hypersomnia, fever, piloerection, grooming and hyperalgesia (Dantzer, 2004). LPS-induced sickness behaviour has been the subject of intense research, from molecular to behavioural studies. Strokes and traumatic brain injury (TBI) are two acute neurodegenerative diseases, with acute inflammation a key factor in both diseases. There is increased pro-inflammatory cytokines for instance, TNF-α, IL-6 and IL-1β in the brain, after strokes and TBI (Berti et al., 2002; Buttini et al., 1994; Hang et al., 2004; Pan et al., 2006).

The circadian system may be involved in the syndrome of sickness behaviour, since the time that LPS is applied can cause different effects. Studies have shown that administration of LPS injections near the end of the resting phase, can result in increased cytokines production for example IL-1β, along with a higher mortality rate in rodents. However, this increase in IL-1β production and mortality in rodents after LPS injection was not displayed at ZT19 (Halberg et al., 1960; Marpegan et al., 2009). Another study also investigated the circadian system with regard to what effect LPS has on sickness behaviour. I.P injections of LPS were administered to rats at either ZT0 or ZT12. It was established that this immune challenge altered temperature and behavioural parameters,

but LPS was time dependent (Mathias et al., 2000). This modulation of inflammatory response throughout the day is also displayed in humans, after LPS and TNF-α administration (Hrushesky et al., 1994; Petrovsky et al., 1998). Furthermore, mortality in humans as a result of sepsis and higher cortisol levels is more common in the early morning (Hrushesky et al., 1994; Sam et al., 2004). The dose received and how LPS is applied whether intraperitoneal (i.p), intravenous (i.v), subcutaneous or intracerebroventricular injection is also critical with regard to what effects the immune challenge has in rodents and humans (Biesmans et al., 2013).

3.1.1 The effect of LPS treatment on the CNS

The CNS consists of the brain and the spinal cord. Between the CNS and the peripheral immune system lies the blood brain barrier (BBB). The brain was once viewed as an immunological privileged organ, not susceptible to immune activation or inflammation. However, it is now established that the brain contains different immune components and is sensitive to inflammatory mediators. Peripheral immune mediators for example cytokines and chemokines are synthesized in the CNS by neurons, astrocytes and microglial. The two pathways involved in peripheral immune messages transferring to the brain are the neural and humoral pathway (Dantzer, 2009; Konsman et al., 2002).

A number of mechanisms are thought to be involved in how LPS may affect the brain. Firstly studies have shown that LPS may cross the BBB and activate cells within the CNS such as microglia (Marzolo et al., 2000), astrocytes and cells that express TLR4 (Chakravarty & Herkenham, 2005). Other studies have displayed that minimal amounts of peripherally LPS application actually cross the BBB, therefore, this immune challenge should not induce the neuroimmune reactions exhibited in the brain. This would indicate that acute peripheral LPS administration causes this neuroimmune reaction using LPS receptors located outside the BBB (Banks & Robinson, 2010). The LPS receptor TLR4 is known to be expressed in the leptomininges, choroid plexus and the circumventricular organs (CVO), which are close to the BBB (Laflamme & Rivest, 2001). Initially, peripheral LPS administration into the abdominal cavity causes inflammation of the peritoneum (Dantzer, 2009). Peripheral cytokines do not enter the brain, however, they manage to induce the expression of other cytokines in the brain resulting in sickness behaviour (Johnson, 2002). The neural route involves the vagus nerves which contain

TLRs, and these vagus nerves plays a major role in conveying messages to the brain in response to LPS i.p administration (Goehler et al., 1999). Furthermore, if vagus nerve is severed in rats, LPS administration does not cause sickness behaviour or expression of cytokines in the brain usually displayed in control rats (Bluthé et al., 1994; Laye et al., 1995).

Other forms of periphery immune messages transferring to the brain include the humoral pathway involving, IL-1β to the CVO (Dantzer, 2001; Konsman et al., 1999; Konsman et al., 2002), and active transport (Banks & Kastin, 1991; Gutierrez et al., 1993) after peripheral LPS administration. The TNF-α receptor is also involved in mediating the effects LPS has on the brain, since TNF-α receptor KO mice have exhibited that systemic LPS administration, does not produce TNF-α mRNA in the brain that is displayed in WT mice (Qin et al., 2007). Peripheral LPS permeates the CNS and activates microglia within brain regions including the cortex, hippocampus and SN, again reliant on the TNF-α receptor (Qin et al., 2007; Rivest, 2003). When microglia become activated there is distinct difference displayed compared to those in their resting state, as they appear hypertrophic and display a phagocytic cell (Kreutzberg, 1996).

Studies have shown that administration of a single dose of 5mg/kg LPS can increase the level of cytokines in the CNS for up to 8 weeks, including TNF-α and IL-1β in the prefrontal cortex. Furthermore, this immune challenge also results in an upregulation of microglial expression in the frontal cortex (Weberpals et al., 2009). Other studies have displayed chronic inflammation for up to 10 months following a single i.p dose of 5mg/kg LPS in young adult rodents, with upregulation of TNF-α in brain regions such as the hippocampus and frontal cortex (Bossù et al., 2012; Qin et al., 2007). Microglia and astrocytes also significantly increased in the cortex 8 hours post LPS treatment (Jacob et al., 2007). Another study showed that peripheral LPS treatment in rats caused significant increase of NOS2 in the cerebellum, hippocampus, midbrain and striatum, 24 hours following the immune challenge, while there was also a significant upregulation of astrocytes displayed in the cortex, striatum and hippocampus (Semmler et al., 2005). A recent study showed an upregulation of Iba1-positive microglial cells in the striatum, medial septum, frontal cortex, and hippocampus following LPS administration (Noh et al., 2014).

3.1.2 The effects of LPS treatment on the SCN and the circadian system

Activating the peripheral innate immune system using LPS produces an exaggerated neuroinflammatory response and extends sickness behaviour (Godbout et al., 2005). Unlike peripherally injected high dose LPS it remains to be confirmed whether peripherally injected low doses of LPS directly stimulate microglia or if they induce microglial activation through downstream messengers that are produced by immune cells or brain microvascular endothelial cells (Chen et al., 2012). Studies have shown that peripheral LPS administration whether high or low dose, can both effect both behavioural and molecular activity in the circadian timing system (Beynon & Coogan, 2010; Guerrero-Vargas et al., 2014; Marpegán et al., 2005; O'Callaghan et al., 2012; Okada et al., 2008; Paladino et al., 2010, 2015).

LPS treatment results in an increase in pro-inflammatory mediators which in turn can impact on clock gene expression, for example *in vitro*, IL-1β effects the expression of *dbp* and *Per3* in fibroblasts, with TNF-α treatment causes changes to SCN *dbp* expression (Cavadini et al., 2007). Furthermore, TNF-α treatment resulted in alterations to electrophysiological properties of SCN neurones (Nygård et al., 2009). IL-6 administration to a human hepatoma cell line (HuH-7) is shown to cause a significant increase of *Per1* gene expression (Motzkus et al., 2002). LPS administration to WT mice at the beginning of their active phase (CT12) causes a significant increase in IL-6 levels, when monitoring macrophages in mice, with lower levels of expression displayed at the beginning of their rest phase (CT0). Additionally, mice deficit in *bmal1*$^{-/-}$ or *rev-erb*α$^{-/-}$, showed no significant alterations between time points, CT0 and CT12, following LPS administration with regard to IL-6 expression. Therefore, showing the importance of the clock genes *bmal1* and *rev-erb*-α following an immune challenge, in relation to regulating a cytokine time of day effect, with IL-6 showing that it is a clock-regulated cytokine (Gibbs et al., 2012). This cytokine time effect of day was also exhibited in a Marpegan study, where IL-6 levels were increased at ZT11 following LPS treatment, when compared to lower levels displayed at ZT19 (Marpegan et al., 2009).

A transient suppression of locomotor wheel running activity was exhibited when monitoring circadian behaviour following low dose peripheral LPS treatment (Marpegán et al., 2005; O'Callaghan et al., 2012). When analysing the effect of low dose acute peripheral LPS treatment has on circadian locomotor behaviour, studies have shown that

administration of peripheral low dose LPS in the early subjective night CT15 induced photic like phase delays, which was not displayed at any other time point (Leone et al., 2012; Marpegán et al., 2005; Paladino et al., 2015). The cytokines TLR4 and TNF-α are both vital for LPS-induced circadian modulation in circadian locomotor behaviour, since mice deficit in either cytokine showed no significant alterations in phase shifts as a result of LPS (Paladino et al., 2010, 2015).

There are several studies displaying how acute peripheral LPS administration also modulate clock genes in the SCN and peripheral tissues (Murphy et al., 2007; Okada et al., 2008; Paladino et al., 2015). Both septic and low dose treatment of LPS results in upregulation of expression of the IEG's c-Fos and EGR-1 in the SCN respectively, 24 hours post treatment (Beynon & Coogan, 2010; Marpegán et al., 2005; O'Callaghan et al., 2012). Acute treatment of LPS also resulted in a significant increase in F4/80 expression in the SCN, when examined 24 hours after the immune challenge, showing microglia with activated morphology (O'Callaghan et al., 2012). Another study displayed how LPS administration resulted in suppressing the clock gene *Per2* in the SCN post treatment, along with suppression of *Per1* and *Per2* in the liver, with rhythmic expression returning to normal 48 hours post treatment (Okada et al., 2008). Furthermore, PER2 expression at CT14, was significantly lower in the SCN following LPS administration when examined one month post-treatment when compared to controls (O'Callaghan et al., 2012). Mice deficit in TNF-α display no alteration in c-Fos or Per2 expression in the SCN which was in contrast to WT mice post LPS treatment. This highlights that any alterations to the SCN neurochemistry as a result of LPS is mediated by TNF-α (Paladino et al., 2015). However, acute i.p LPS administration was not seen to significantly increase TNF-α in the SCN (O'Callaghan et al., 2012). Finally, the administration of LPS to SCN slices of rats *in vitro* resulted in the upregulation of the neurotransmitter AVP in the SCN, therefore suggesting that TLR4 might be expressed in the SCN (Nava et al., 2000). Peripheral organs under circadian control such as the heart and liver exhibited a phase dependent reduction in the expression of the clock genes Per1 and Per2 following LPS administration (Yamamura et al., 2010). LPS application caused a significant increase in *Per2* and *Bmal1* expression in equine blood (Murphy et al., 2007).

3.1.3 The effects of LPS treatment on the PVN

The SCN projects to the PVN through axonal connections, therefore, analysis of the PVN might give additional information into the effects of high and low dose LPS on the circadian system in young mice. The PVN plays a role in the regulation of autonomic (Ferguson et al., 2008) and endocrine outputs (Swanson & Sawchenko, 1980), including production of glucocorticoids and due to this the circadian system may be involved in the modulation of the immune system (Coogan & Wyse, 2008). The PVN contains the LPS receptor TLR4 (Laflamme & Rivest, 2001), therefore, the PVN may be directly stimulated by and respond to an LPS immune challenge in the ageing brain. IL-1 is thought to be a vital mediator in translating peripheral LPS into neuronal activation in the PVN (Quan et al., 2003). Studies carried out in rats, displayed how peripheral LPS administration resulted in a significant increase of IL-1β and iNOS mRNA levels in the hypothalamic PVN (Singh & Jiang, 2004), and significant upregulation of TNF-α and IL-6 in this brain region following i.v or i.p LPS treatment (Kakizaki et al., 1999). LPS administration has also been shown to modulate the expression the clock gene *per1* in the PVN (Takahashi, 2001). Similar to the SCN, the PVN also is reliant on the cytokines TLR4 and TNF-α for inducing LPS circadian modulation with regard to the expression of the clock gene Per1, since mice deficit in either cytokine showed no effect of LPS treatment with regard to alteration of c-Fos or Per1 expression in this region (Paladino et al., 2010, 2015).

In this chapter immunohistochemistry was carried out to investigate the effects of acute peripheral LPS treatment on young adult mice. The NF-κB pathway was investigated since studies have associated that the pathway is involved in the effect LPS has on the circadian timing system, shown by the role it plays in LPS induced phase shifts (Marpegán et al., 2004, 2005; Paladino et al., 2010). Additionally, a septic dose of peripheral LPS induced upregulation of the NF-κB component, p65 (Beynon & Coogan, 2010), therefore, components of NF-κB were analysed p65, p-IκB, p-IKK and c-Rel in this study. Furthermore, IEG proteins including c-Fos and EGR-1 to assess if acute peripheral LPS treatment induced alterations to expression of the IEG proteins in the SCN or PVN. Lastly, microglial activation was investigated using the ionized calcium binding adapter molecule 1 (IBA-1) immunoreactive microglial marker. Finally, we investigated

the effect of LPS administration on the molecular clockwork by analysing PER1 expression, along with behavioural analysis investigation in relation to low dose LPS treatment.

3.2 Materials and Methods

3.2.1 Animals and housing

The animal models that were used in the following studies were male C57BL/6 strain of mice and the B6.129S6-*Per2*^{tm1Jt}/J. The adult C57BL/6 male aged 6-8 weeks old (24-30g) were group housed on arrival from Charles Rivers, UK or Harlan, UK both designated breeding establishments. Homozygous B6.129S6-*Per2*^{tm1Jt}/J aged 6-8 weeks old (24-30g) were obtained from JAX mice (USA) via Charles River (U.K), and a breeding colony was established in Maynooth University. Homozygous B6.129S6-*Per2*^{tm1Jt}/J mice display no alterations in their circadian behaviour or in entrainment parameters (Yoo et al., 2004). One B6.129S6-*Per2*^{tm1Jt}/J male and female mice were housed together when they became sexually mature between 6-8 weeks (Phifer-Rixey & Nachman, 2015). Female mice are in estrus every 4-5 days (Byers et al., 2012), with gestation time of mice usually 3 weeks (Phifer-Rixey & Nachman, 2015). The litter sized between 4-9 (Singleton and Krebs, 2007) needs to be weaned at 21 days (Kikusui, Isaka, & Mori, 2005), housing male and female separately, with meticulous care that all records are in order. Behavioural analysis was undertaken by single housed mice or group housed mice 3 per cage as outlined in section 2.2.2. To examine the SCN neurochemistry in acute treatment of LPS mice were in a 12:12 L: D cycle. For behavioural analysis single housed mice were kept in DD.

3.2.2 Treatments

This chapter involves mice being treated with the endotoxin Lipopolysaccharide (LPS) from gram negative bacteria, Escherichia coli (Sigma, Ireland).

3.2.2.1 Acute high grade inflammatory response

LPS administration onto the abdominal cavity is used in the animal model of sepsis (Nemzek et al., 2008). Mice were treated with a single (5mg/kg i.p dissolved in 0.9% sterile saline) injection of LPS or with an equal volume of sterile saline control (0.9% NaCl, 0.12ml). LPS treatment elicited the full spectrum of sickness behaviour evident 1 hour after treatment. Roughly, 10% of LPS 5mg/kg treated mice display a significant moribundity requiring euthanasia or mortality in the first 72 hours following treatment.

3.2.2.2. Acute low grade inflammatory response

Mice were treated with a single (100µg/kg i.p dissolved in 0.9% sterile saline) injection of LPS or with an equal volume of sterile saline control (0.9% NaCl, 0.12ml).

3.2.3 Sepsis scoring

A sepsis score was calculated for LPS and control animals immediately after and 1 hour post treatment, and subsequently at regular intervals for 24 hours post treatment. Scores were assigned on a five-point scale assessing alterations on the parameters of behaviour, appearance, dehydration and respiration. Each parameter was then graded on a scale of 0-4; 0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe.

Table 3: A sample animal welfare scoring sheet

NUI MAYNOOTH

ANIMAL WELFARE SCORING SHEET

Animal ID

DATE & TIME	SCORE					
Weight						
≥ starting weight	0					
90-100% starting weight	1					
80-90% starting weight	2					
<80% starting weight	4					
Activity						
Normal	0					
Moves round cage spontaneously, but reduced	1					
Moves to stimulus, but not spontaneously	2					
Huddled, not moving to stimulation, failure to take food and water	5					
General appearance						
Normal	0					
Evidence of poor grooming/ridging	1					
Staring coat, shivering, matted/ruffled fur	3					
Hunched, badly matted fur	5					
Behaviour						
Normal	0					
Less mobile but alert	1					
Restless or very still, not alert	3					
Clinical signs						
No abnormal signs	0					
Slight changes in breathing, increased rate only	1					
Marked changes in breathing	3					
Marked abdominal breathing with cyanosis	4					
TOTAL (max=0-22)						

Judgement: ***Any animal scoring 4 or more in 1 section will be culled by schedule 1 method.***

0: no evidence of symptoms

***1-4**: Continue to monitor closely, on at least a daily basis.*

***5-6**: Consider culling.*

***7 or above**: cull*

3.2.4 The effects of acute LPS treatment on SCN and PVN neurochemistry in wild type mice and mPer2^{Luc} knock-in mice in LD

C57/BL6 mice were group housed and allowed to habituate for two weeks in L: D 12:12 conditions prior to experiment. In order to examine the effects acute LPS (5mg/kg) treatment causes in IEGs, clock genes and components of NF-κB expression in the SCN and PVN, C57/BL6 mice and mPer2^{Luc} knock-in mice, were treated with an acute dose of 5mg/kg LPS (n=6) or saline (n=6) i.p at ZT4, were perfused 24 hours later. This time was taken as the sampling time point because (Beynon & Coogan, 2010) showed effects of LPS treatment with regard to p65 expression in the SCN 24 hours post treatment, whereas 6 hours post treatment, there was no significant alteration of p65 expression.

All brains were subsequently processed for immunohistochemistry staining (section 2.2.4) for IEGs-c-Fos and Egr-1, the clock gene, PER1, and components of NF-

κB-p65, p-IκB, p-IKK and c-Rel. Quantitative analysis was carried out simultaneously (section 2.2.5). The mean of each group was confirmed and analysed by using an independent t-test. Results are stated by IOD or cell number mean value ± standard error of mean, with $P<0.05$ accepted as statistical significant.

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
p-IκB-α (Ser32/36)	1:200	Rabbit	Santa Cruz Biotechnology	sc-101713
Per1 (N20)	1:500	Goat	Santa Cruz Biotechnology	s-7724
p65 (C-20)	1:200	Rabbit	Santa Cruz Biotechnology	sc-372
p-IKKα/β 180/Ser181)	1:200	Rabbit	Santa Cruz Biotechnology	sc-23470-R
c-Rel	1:200	Rabbit	Santa Cruz Biotechnology	sc-71
Egr-1	1:3000	Rabbit	Santa Cruz Biotechnology	sc-189
IBA-1	1:000	Rabbit	Wako, Denmark	019-1974

3.2.5 The effects of acute low dose LPS treatment on circadian locomotor behaviour in wild type mice

This experiment was carried out to investigate if there were any altered behavioural responses between lose dose LPS treated animals compared to saline controls. C57/BL6 mice were firstly entrained to a 12:12 L:D cycle for two weeks before being placed into DD for a further two weeks and allowed free run. After two weeks in DD, animals received LPS 100µg/kg i.p injection (n=6) at CT15, the early subjective

night. Saline treatment (n=6) was administered two weeks later at CT15. Low dose LPS treatment at CT15 had been previously shown to cause phase delays of locomotor behaviour (Marpegán et al., 2005). The phase shifts magnitudes were calculated by fitting a line of best fit through activity onsets for the one week prior to treatment, and then another line for one week post treatment as seen in (Figure 2.1). Two to three researchers calculated the difference between the two lines and took the average to exclude experimenter bias. The FRP and rhythm amplitude were calculated for each animal using the programme Chronobiology Kit Chi Squared periodogram.

3.2.6 The effects of acute low dose LPS treatment on SCN and PVN neurochemistry in wild type mice

In order to establish whether altered neurochemical responses would be seen in the SCN in acute low dose LPS treatment, C57/BL6 mice were administered with 100µg/kg LPS i.p injection (n=8) or saline (n=8). Animals were single housed and were placed in DD for two weeks before treatments at CT15 were carried out and all 16 mice were perfused at CT19 (section 2.2.3). Immunohistochemistry (section 2.2.4) was carried out for NF-κB components p65 and p-IκB, the IEG c-Fos and the clock gene protein PER1 (details of primary antisera is outlined in table 2). Each animal and each antibody represented approximately 3-6 SCN images. They were examined by either IOD or manual quantification for the number of immunoreactive glial and nuclei per SCN (section 2.2.5). The mean of each group was confirmed and analysed by an independent t-test. Results are stated by IOD or cell number mean value ± standard error of mean, with $P<0.05$ accepted as statistical significant.

3.2.7 The effects of acute LPS treatment on SCN and PVN neurochemistry in mPer2^{Luc} knock-in mice in LD in the subjective day and night

B6.129S6-Per2^{tm1Jt}/J mice were entrained to a 12:12 L: D cycle for two weeks before the experiment was carried out. The expression of IEGs and components of NF-κB in the SCN and PVN were examined. Administration of acute LPS (5mg/kg) treatment was carried out in mice, at ZT6 and ZT18. Mice were perfused 24 hours later (section 2.2.3). All brains were subsequently processed for immunohistochemistry staining

(section 2.2.4) for c-Fos, Egr-1, p65 and p-IκB. The mean was calculated for each animal's nuclei per SCN/PVN and analysing of data was carried out using a two way Anova test. Statistical significance was accepted at $P<0.05$, and results are given as mean values ± standard error of the mean.

3.2.8 The effects of acute LPS treatment on SCN expression in mPer2^{Luc} knock-in mice LD using double immunostaining

B6.129S6-Per2^{tm1Jt}/J mice were entrained to a 12:12 L: D cycle for two weeks before experiment was carried out to habituate to their surroundings. The expression of a microglial marker and a NF-κB component in the SCN and PVN were examined. Administration of acute LPS (5mg/kg) treatment was carried out in mice at ZT8. Mice were perfused 24 hours later (section 2.2.3).

All brains were subsequently processed for immunohistochemistry staining for IBA-1 and p65 expression. Immunohistochemistry protocol differs to section 2.2.4. This protocol method used the avidin-biotin-peroxidase complex (ABC)/NovaRED technique firstly, followed by the alkaline phosphatase streptavidin/Vector Blue (Coogan & Piggins, 2003). The protocol takes 3 days in total. Day one follows the instructions of section (2.2.4) with SCN washes using PB, PBX and H_2O_2. The next step carried out used a non-specific antibody block NGS with 0.1M PBX, which was mixed for 60 minutes at room temperature. The final part of the first day of the immunohistochemistry protocol involved sections being incubated with primary antisera raised in rabbit, p65 diluted in 0.1M PBX and 2% for 24 hours at 4°C.

Day two began with sections being put through a serious of washes as above, two 0.1M PB and one 0.1M PBX wash. The sections were then incubated with biotinylated secondary antibody (1:400 biotinylated goat anti-rabbit, Jackson Immuno research labs) which was diluted in 0.1M PBX and 2% NGS or NHS (blocking solution) for 70 minutes at room temperature. After the 70 minutes, 3 washes were carried out as previous with PB and PBX. This was followed by sections being incubated with avidin-biotin method (0.4%) in 0,1M PBX with a Vectastain Elite Universal Kit (Vector Laboratories) for 90 minutes, again at room temperature. Sections were washed with PB (twice) and 0.1M sodium acetate (pH6, Sigma) for 10 mins. Light sensitive NovaRED (Vector Laboratories) was then used to visualise immunoreactivity of p65. 1ml of NovaRED and

60µl of catalyst, glucose oxidase (5mg/ml) per well was added for immunoreactivity. When the staining was satisfactory roughly after 8 minutes, sections were placed in dH_2O to stop the reaction. The sections went through 2 final washes in dH_2O for 10 minutes. The same protocol was carried out on the same SCN sections similar to day one previously mentioned, the only difference was IBA-1 primary antisera replaced p65 and was incubated overnight.

The final day of staining sees the SCN sections being put through another serious of washes and incubated with biotinylated goat anti-rabbit, as above. After the 70 minutes, 3 more washes were carried out as previous stated with PB and PBX. This was followed by SCN sections being incubated with alkaline phosphatase streptavidin (1:500) (Vector Laboratories) in 0.1M PBX for 90 minutes, again at room temperature. Sections were washed twice with 0.1M PB, followed by 0.1M sodium acetate for 10 mins. Light sensitive Vector Blue (Vector Laboratories) was then used to visualise immunoreactivity of the IBA-1. 1ml of Vector Blue and 60µl of catalyst, glucose oxidase (5mg/ml) per well was added for immunoreactivity for up to thirty minutes. When the staining was satisfactory, sections were placed in PB to stop the reaction. The sections went through 3 final washes in 0.1M PB before mounting took place, followed by dehydrating and delipifying steps twenty four hours later. Finally, the slides were cover slipped and examination of immunoreactive hypertrophic microglia in the SCN took place by qualitative methods.

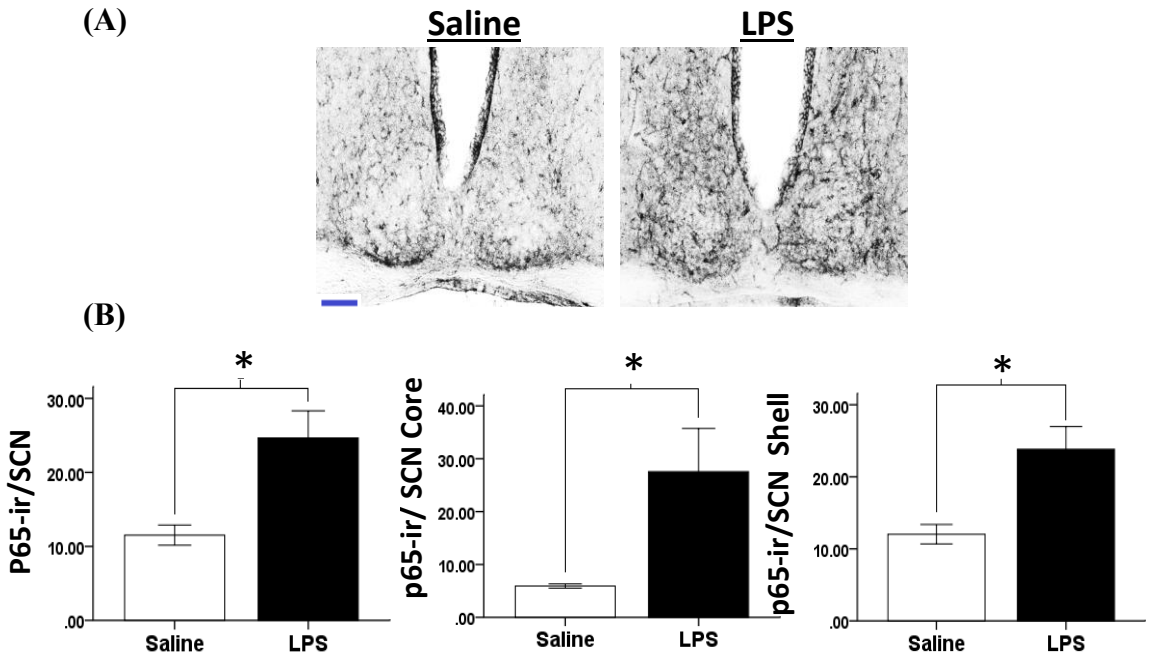
3.3 Results

3.3.1 Examination of NF-κB components in the SCN and PVN 24 hour post LPS treatment in wild type mice

Immunohistochemical analysis was carried out to examine the effects of acute LPS (5mg/kg) treatment on the expression of NF-κB components in the SCN. These components were investigated to see were they identified as playing a role in mediating the effects of LPS on the circadian system. The PVN, a target area of the SCN and one which is implicated in the response to LPS treatment was also investigated for any alterations in expression of NF-κB components.

3.3.1.1 p65 expression in the SCN and PVN following acute LPS treatment.

The SCN and PVN were both examined for p65 expression 24 hours post LPS or saline treatment. Analysing levels of p65 immunoreactivity was carried out by IOD, followed by statistical analysis using an independent t-test to calculate whether there was alterations in the expression of p65 in the SCN and PVN following treatment of either LPS or control at ZT4. Results indicate that there was statistically significant effect of treatment LPS (24.65 ± 3.66) versus saline (11.51 ± 1.36, $P<0.05$; Figure 3.1) on p65 expression levels in the SCN. Further assessment was carried out of p65 expression in the SCN subdivisions-core and shell. Significant differences were found in p65 expression in the core of LPS mice (27.57± 8.17) when compared to controls (5.93 ± 0.42, $P<0.05$). There was a statistical significant upregulation p65 of expression in the shell of LPS mice (23.83 ± 3.15) when compared to controls (12.03 ± 1.35, $P<0.05$). There was also a statistically significant effect of treatment of LPS (38.34 ± 6.92) and saline (20.12 ± 1.94, $P<0.05$; Figure 3.1) on the level of p65 expression in the PVN.



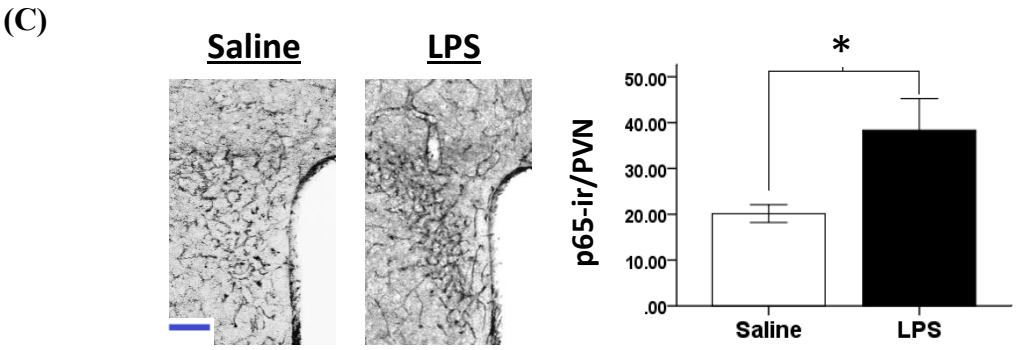


Figure 3.1: p65 expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of p65 expression in the SCN (scale bar = 100μm) and (B) Bar graphs illustrating quantification of levels of p65 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the NF-κB components p65 in all regions of the SCN in LPS treated animals vs. controls. (C) Photomicrographs (scale 100μm) and bar graph showing upregulations of p65 immunostained cells in the PVN. * P<0.05.

3.3.1.2 Phosphorylated (p)-IκB expression in the SCN and PVN following acute LPS treatment.

The SCN and PVN were both examined for p-IκB expression 24 hours post LPS or saline treatment. Analysing levels of p-IκB immunoreactivity was carried out by IOD, followed by statistical analysis using an independent t-test to calculate whether there was alterations in the expression of p-IκB in the SCN and PVN following treatment of either LPS or control at ZT4. Results indicate that there was statistically significant effect of treatment LPS (11.44 ± 3.22) versus saline (2.36 ± 0.47, P<0.05; Figure 3.2) on p-IκB expression levels in the SCN. Further assessment was carried out of p-IκB expression in the SCN subdivisions-core and shell. No significant differences were found in p-IκB expression in the core of LPS mice (8.70 ± 3.55) when compared to controls (2.41 ± 0.62, P>0.05). There was statistically significant upregulation p-IκB of expression in the shell of LPS mice (13.57 ± 4.09) when compared to controls (3.17 ± 0.56, P<0.05). There was no statistically significant effect of treatment of LPS (19.30 ± 4.10) and saline (12.62 ± 1.63, P>0.05; Figure 3.2) on the level of p-IκB expression in the PVN.

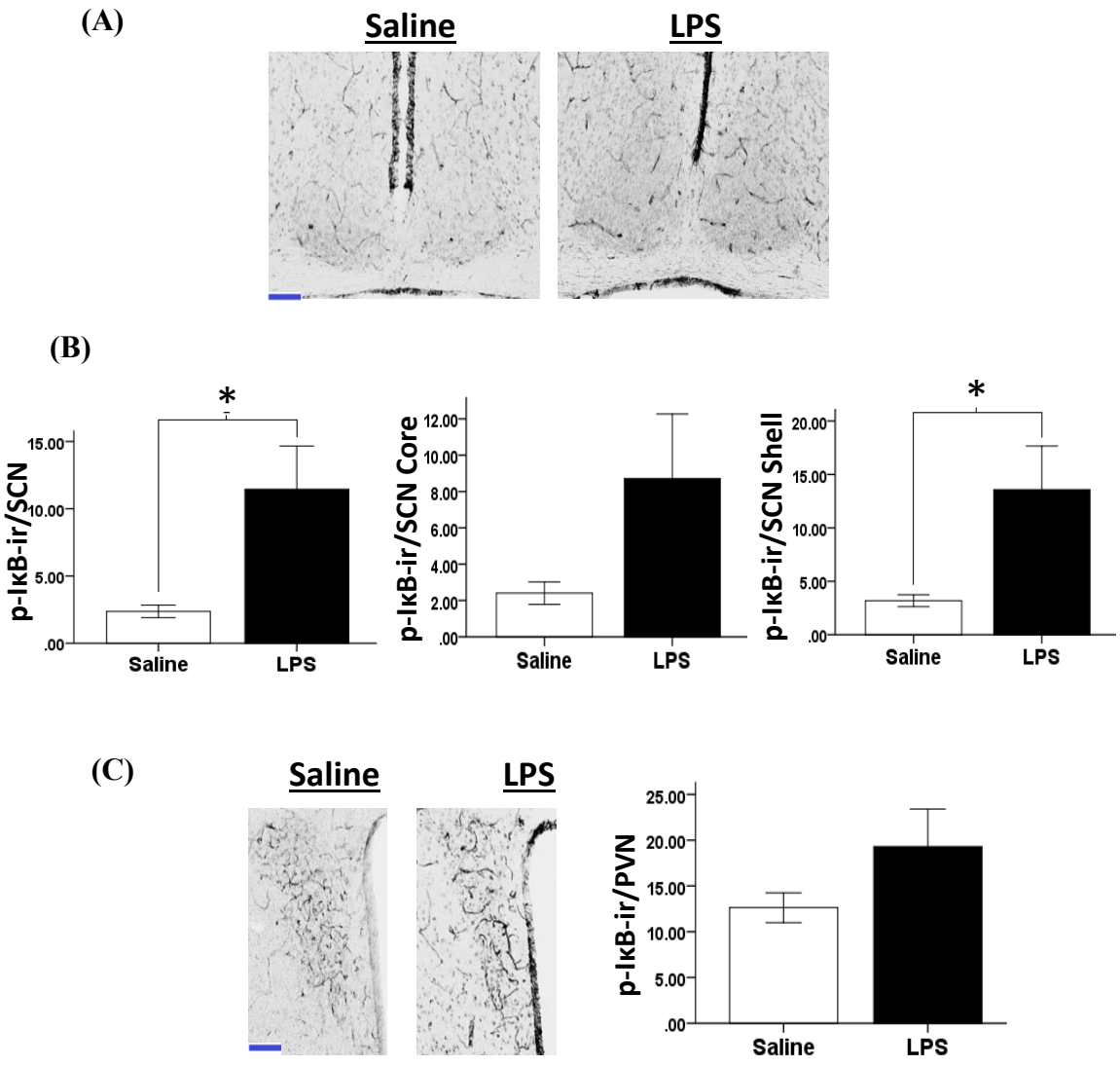
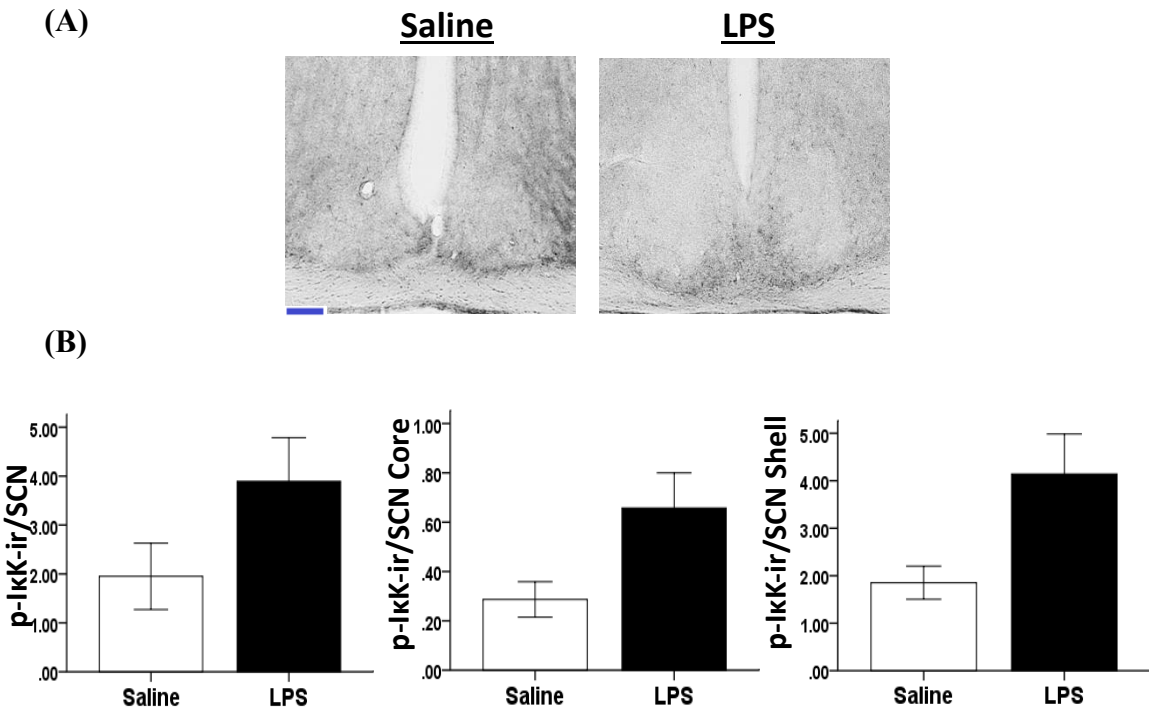


Figure 3.2: p-IκB expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of p-IκB expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of p-IκB immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of p-IκB in the mid rostro-caudal level of the SCN and the SCN shell in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing p-IκB immunostained cells in the PVN. * P<0.05.

3.3.1.3 p-IκK expression in the SCN and PVN following acute LPS treatment.

The SCN and PVN were both analysed for p-IκK expression 24 hours post LPS or saline treatment. Immunochemistry was carried out and IOD measurements, followed

by statistical analysis using an independent t-test to calculate whether there was alterations in the expression of p-IκK in the SCN and PVN, following treatment of either LPS or control at ZT4. Results indicate that there was no significant effect of treatment LPS (3.89 ± 0.89) compared to saline (1.95 ± 0.67, P>0.05; Figure 3.3) on p-IκK expression levels in the SCN. Additional assessment was carried out monitoring p-IκK expression in the SCN subdivisions-core and shell. There was no statistically significant differences in p-IκK immunosignal throughout the core of LPS mice (0.65 ± 0.09) when compared to controls (0.53 ± 0.56, P>0.05). There was statistically significant upregulation p-IκK of expression in the shell of LPS mice (4.14 ± 0.84) when compared to controls (1.52 ± 0.56, P<0.32). Finally, when analysing the level of p-IκK expression in the PVN, no significant alterations between LPS (2.16 ± 0.32) and saline treatment (1.57 ± 0.13 P>0.05; Figure 3.3).



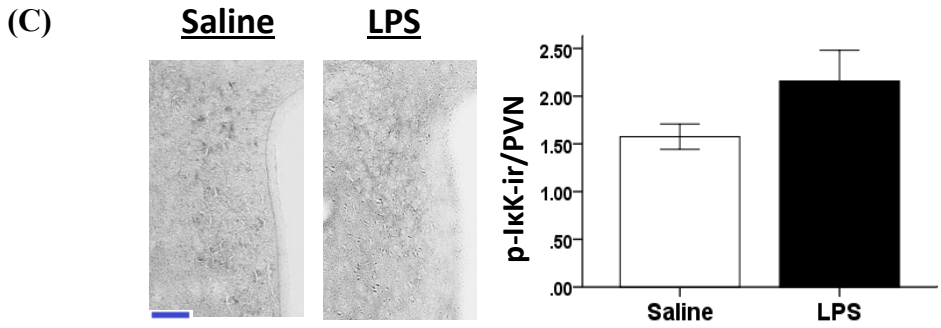


Figure 3.3: p-IκK expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of p-IκK expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of p-IκK immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment. (C) Photomicrographs (scale 100µm) and bar graph showing p-IκK immunostained cells in the PVN.

3.3.1.4 c-Rel expression in the SCN and PVN following acute LPS treatment.

c-Rel expression 24 hours post LPS or saline treatment were analysed in the SCN and PVN. Immunochemistry was combined with IOD measurements, and assessed by an independent t-test to calculate whether there were alterations in the expression of c-Rel in the SCN and PVN, following both treatments at ZT4. The expression of c-Rel in the SCN of LPS treated mice were analogous to controls, with mean values of (7.95 ± 0.74) and (7.52 ± 1.68) immunoreactive cells/SCN respectively (P>0.05; Figure 3.4). Further assessment was carried out monitoring c-Rel expression in the SCN subdivisions in both the core and shell. There was no statistically significant differences in c-Rel immunosignal throughout the core of LPS mice (6.20 ± 1.13) when compared to controls (6.80 ± 1.73, P>0.05). There was no statically significant alterations c-Rel of expression in the shell of LPS mice (8.55 ± 1.25) when compared to controls (8.35 ± 1.99, P<0.32). In addition, when assessing the PVN, c-Rel expression between LPS (5.86 ± 1.06) and saline treatment groups were comparable (5.03 ± 1.07 P>0.05; Figure 3.4).

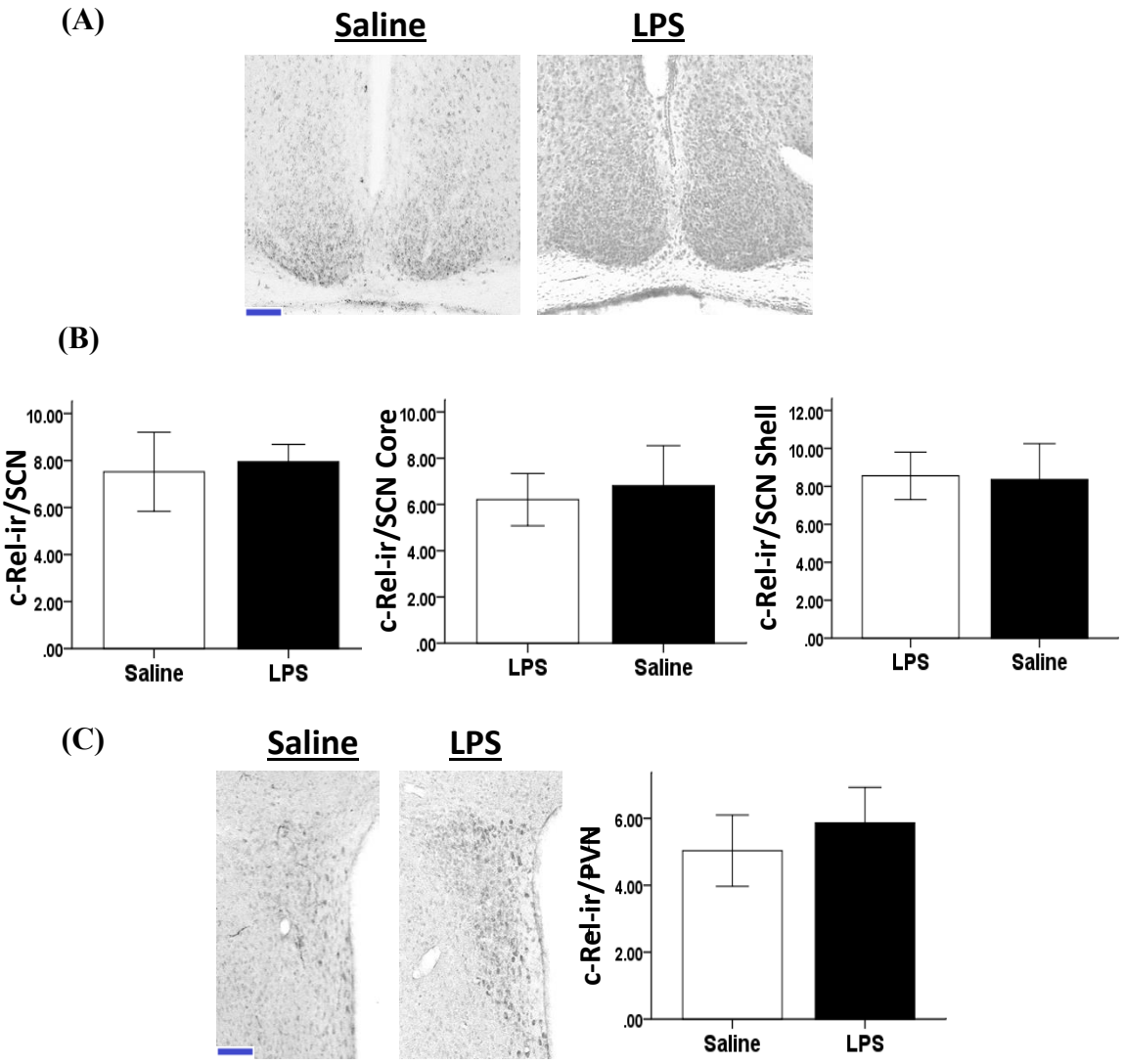


Figure 3.4: c-Rel expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of c-Rel expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of c-Rel immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment. (C) Photomicrographs (scale 100µm) and bar graph showing c-Rel immunostained cells in the PVN.

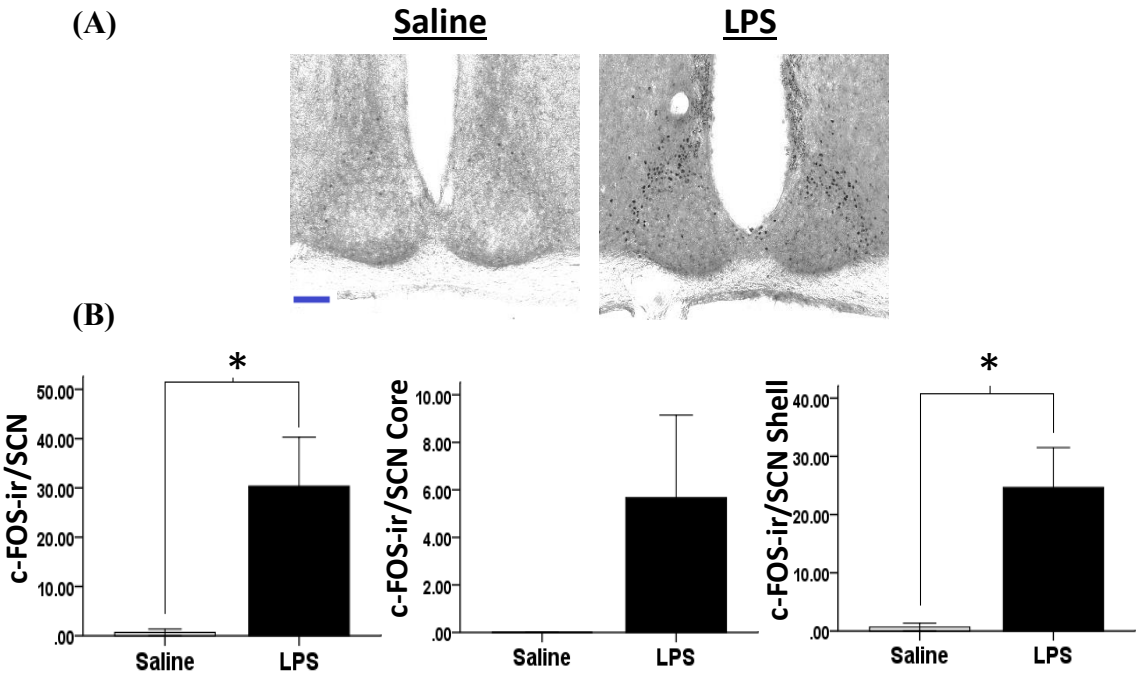
3.3.2 Analysing immediate early gene expressions in the SCN and PVN 24 hour post LPS treatment in wild type mice

Immunohistochemical analysis was carried out to examine the effects of acute LPS (5mg/kg) treatment on the expression of immediate early gene (IEG) expression in the SCN. These IEGs-c-Fos and egr-1-were investigated to see if they are a factor in mediating the effects of LPS on the circadian system. The PVN was also targeted for any

alterations in the expression of IEGs, since it is implicated in the response to LPS treatment.

3.3.2.1 c-Fos expression in the SCN and PVN following acute LPS treatment.

c-Fos expression was evaluated in the SCN and PVN 24 hours post LPS or saline treatment. Analysing levels of c-Fos immunoreactivity was carried out by manually quantification of immunoreactive nuclei, followed by statistical analysis using an independent t-test to assess whether there was alterations in the expression of c-Fos in the SCN and PVN following treatment at ZT4. Results indicate that there was statistically significant effect of treatment LPS (30.33 ± 9.93) versus saline (0.67 ± 0.67, $P<0.05$; Figure 3.5) on c-Fos expression levels in the SCN. When examining the subdivisions of the SCN, the mean number of immunoreactive c-Fos cells in the core of the SCN was found not significant between treatment groups, with LPS (5.66 ± 3.48) when compared to saline (0.00 ± 0.00, $P>0.05$). In the shell, mice treated with LPS (24.66 ± 6.83) displayed an upregulation of c-Fos in contrast to control mice (0.66 ± 0.66, $P<0.05$). Furthermore, there was also a statistically significant effect of treatment of LPS (37.00 ± 9.86) and saline (0.333 ± 0.333, $P<0.05$; Figure 3.5) on the level of c-Fos expression in the PVN.



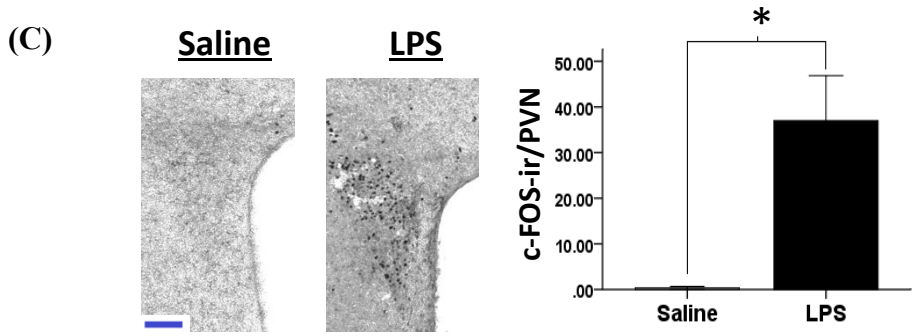


Figure 3.5: c-Fos expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of c-Fos expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of c-Fos immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the IEG c-Fos in the mid rostro-caudal level of the SCN and the SCN shell in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing c-Fos immunostained cells in the PVN. * $P<0.05$.

3.3.2.2 EGR-1 expression in the SCN and PVN following acute LPS treatment.

EGR-1 expression was evaluated in the SCN and PVN 24 hours post LPS or saline treatment. Analysing levels of EGR-1 immunoreactivity was carried out by manually quantification of immunoreactive nuclei, using an independent t-test to assess whether there was significant alterations in the expression of EGR-1 in the SCN and PVN following treatment at ZT4. Results show that there was statistically significant effect of LPS treatment (21.00 ± 6.51) compared to saline (0.70 ± 0.37, $P<0.05$; Figure 3.6) on EGR-1 expression levels in the SCN. When examining the subdivisions of the SCN, the mean number of immunoreactive EGR-1 cells in the core of the SCN displayed no significant alterations between treatment groups, with LPS (4.33 ± 1.72) when compared to saline (0.20 ± 0.20, $P>0.05$). In the shell, mice treated with LPS (16.66 ± 5.06) displayed an upregulation of EGR-1 in contrast to control mice (0.90 ± 0.45, $P<0.05$). Furthermore, there was also a statistically significant effect of treatment of LPS (14.00 ± 5.06) and saline (1.000 ± 0.46, $P<0.05$; Figure 3.6) on the level of EGR-1 expression in the PVN.

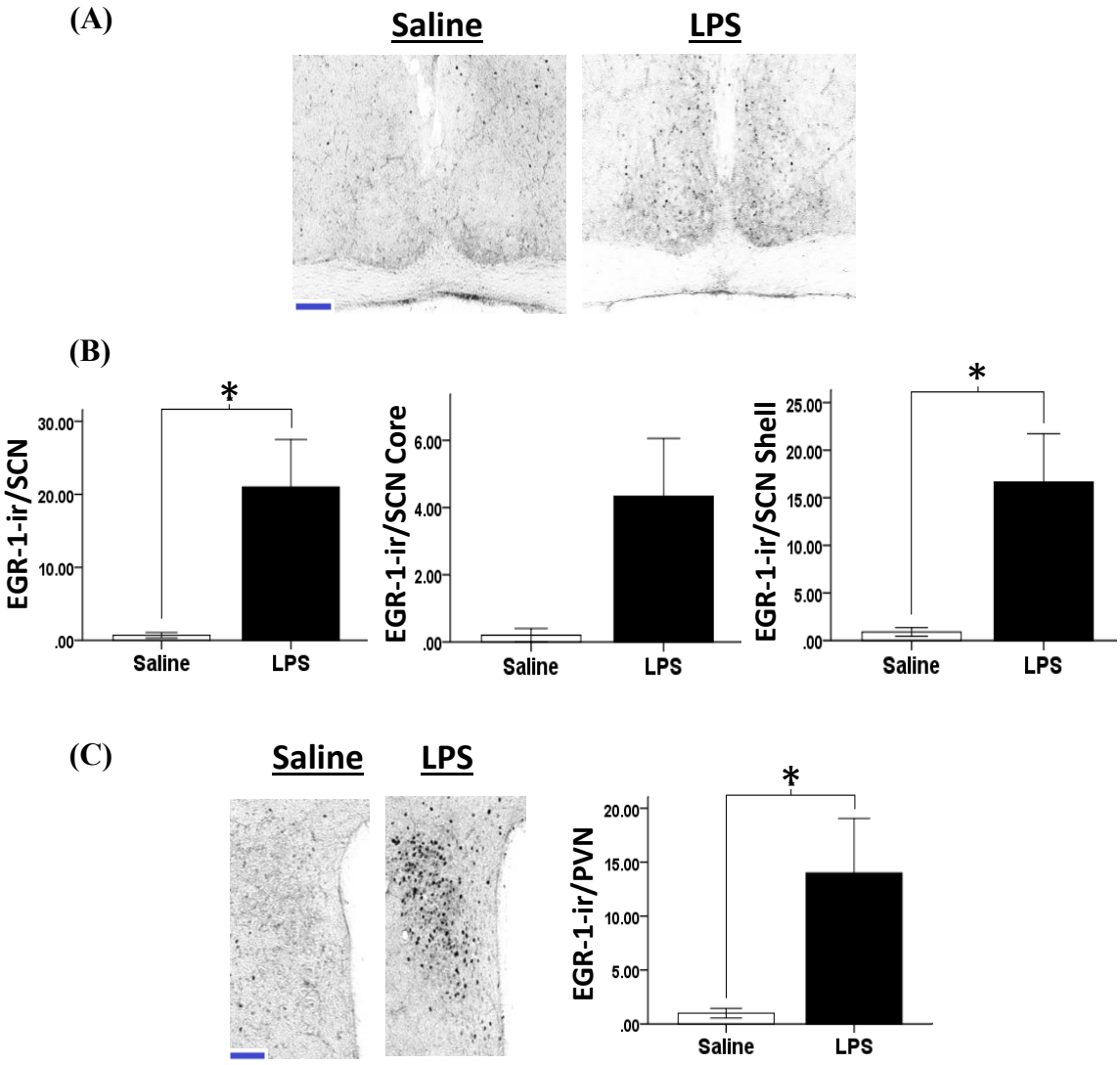


Figure 3.6: EGR-1 expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of EGR-1 expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of EGR-1 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the IEG ERG-1 in the mid rostro-caudal level of the SCN and the SCN shell in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing EGR=1 immunostained cells in the PVN. * $P<0.05$.

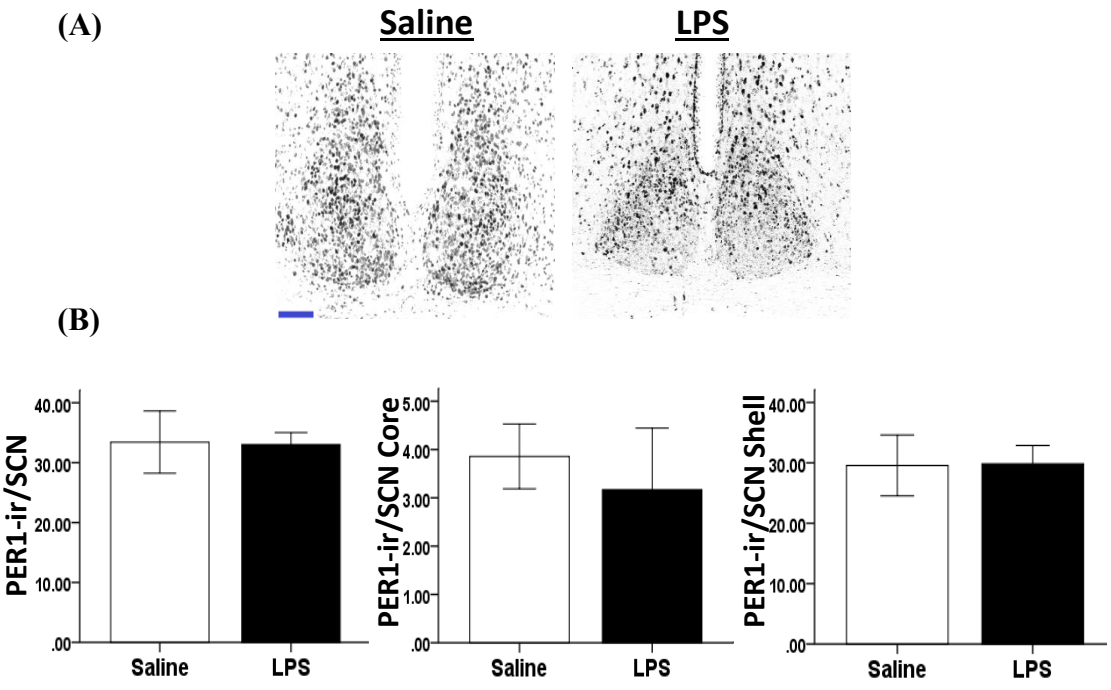
3.3.3 Analysing clock gene and NF-κB components expression in the SCN and PVN 24 hour post LPS treatment in B6.129s6-Per2^{tm1Jt}/J mice

For assessment of clock gene and NF-κB expression in the SCN and PVN immunohistochemical analysis was carried out to examine the effects of acute LPS

(5mg/kg). The clock gene protein PER1 and components of the NF-κB pathway-p65 and IκB-were investigated to see if they are a factor in mediating the effects of LPS on the circadian system. Another area investigated for any alterations in expression was the PVN, a target area of the SCN and one which is known to response to LPS treatment.

3.3.3.1 PER1 expression in the SCN and PVN following LPS 24 post treatment.

PER1 expression was assessed in the SCN and PVN 24 hours post LPS or saline treatment. Analysing the levels of PER1 was carried out by manually quantification of immunoreactive nuclei, followed by statistical analysis using an independent t-test to assess whether there was alterations in the expression of PER1 in the SCN and PVN following treatment at ZT4. There were comparable results displayed with no statistically significant effect of treatment LPS (33.00 ± 2.01) versus saline (33.42 ± 5.20, P>0.05; Figure 3.7) on PER1 expression levels in the SCN. When examining the subdivisions of the SCN, the mean number of immunoreactive PER1 cells in the core of the SCN was similar between treatment groups, with LPS (3.16 ± 1.27) when compared to saline (3.86 ± 0.67, P>0.05). The expression of PER1 in the SCN of LPS treated mice (29.83 ± 3.04) were analogous to controls mice (29.57 ± 5.04, P>0.05) in the shell. Furthermore, there was no statistically significant alterations in PER1 expression in the PVN of LPS treated mice (20.60 ± 3.50) and saline (22.40 ± 4.06, P>0.05; Figure 3.7).



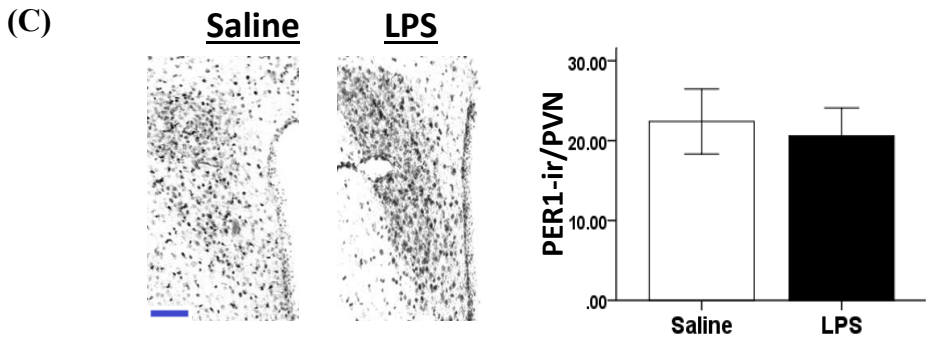


Figure 3.7: PER1 expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of PER1 expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of PER1 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment. (C) Photomicrographs (scale 100µm) and bar graph showing PER1 immunostained cells in the PVN.

3.3.3.2 p65 expression in the SCN and PVN following LPS 24 post treatment.

p65 expression was assessed in the SCN and PVN 24 hours post LPS or saline treatment. Analysing levels of p65 immunoreactivity was carried out by IOD, followed by statistical analysis using an independent t-test to assess whether there was alterations in the expression of p65 in the SCN and PVN following treatment at ZT4. There was statistically significant upregulation of p65 expression after LPS treatment (14.35 ± 1.70) in the SCN compared to saline controls (8.40 ± 1.74, $P<0.05$; Figure 3.8). When assessing the subdivisions of the SCN, there was no alterations in the mean number of immunoreactive p65 cells in the core of the SCN between treatment groups, with LPS (18.14 ± 5.18) when compared to saline (9.00 ± 2.12, $P>0.05$). Furthermore, there was similar expression of p65 in the shell of the SCN of LPS treated mice (12.64 ± 2.51) versus controls mice (7.69 ± 1.31, $P>0.05$). There was upregulation of p65 results expression in the PVN of LPS treated mice (22.60 ± 3.50) and saline (11.40 ± 4.06, $P>0.05$; Figure 3.8).

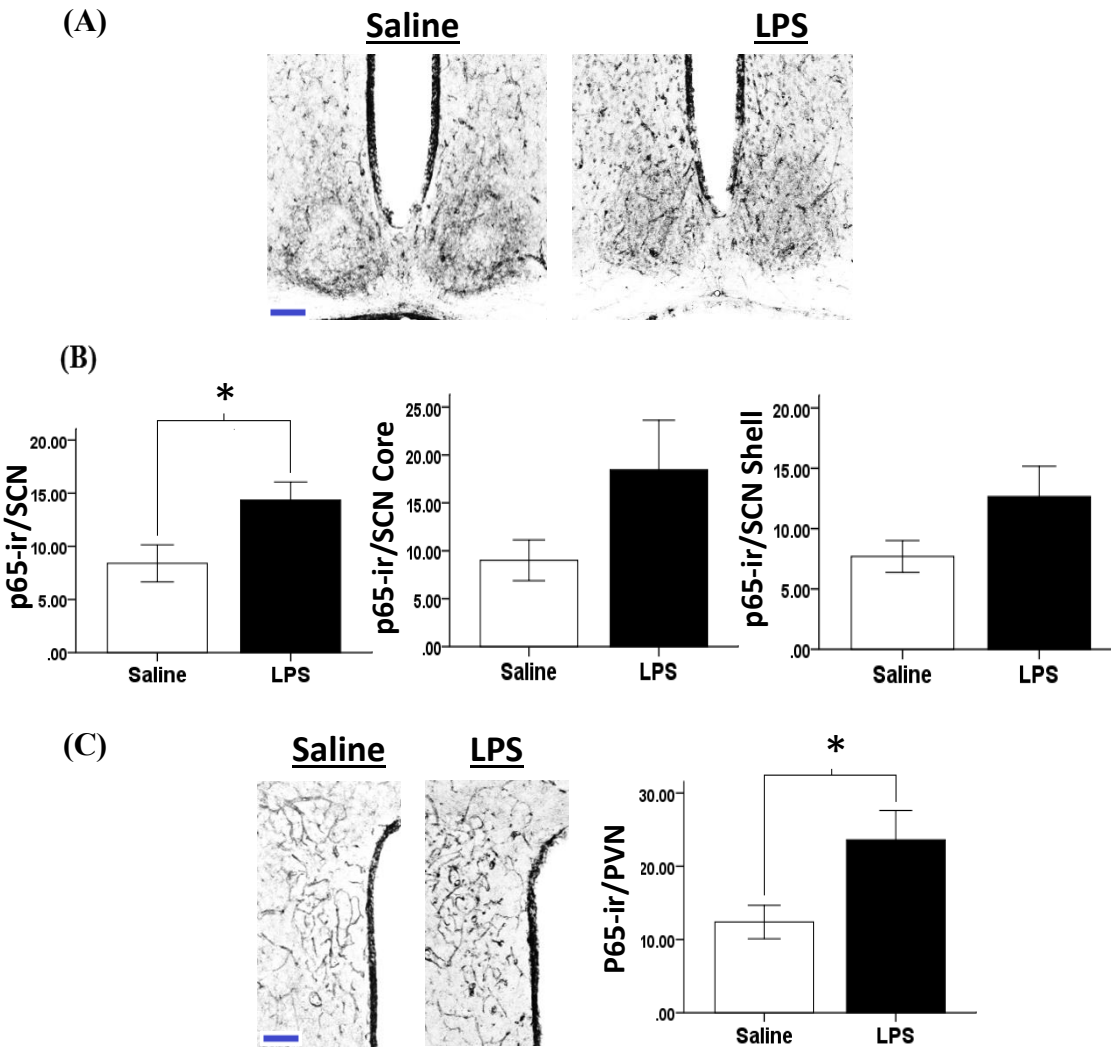


Figure 3.8: p65 expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of p65 expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of p65 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the NF-κB components p65 in the mid rostro-caudal level of the SCN in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing upregulations of p65 immunostained cells in the PVN. * P<0.05.

3.3.3.3 p-IκB expression in the SCN and PVN following LPS 24 post treatment.

p-IκB expression was assessed in the SCN and PVN 24 hours post LPS or saline treatment. Analysing levels of p-IκB immunoreactivity was carried out by IOD, followed by statistical analysis using an independent t-test to assess whether there was alterations in the expression of p-IκB in the SCN and PVN following treatment at ZT4. There was

significant results displayed when analysing the effect of treatment LPS (11.03 ± 1.98) versus saline (4.34 ± 0.90, P<0.05; Figure 3.9) on p-IκB expression levels in the SCN. When examining the subdivisions of the SCN, the mean number of immunoreactive p-IκB cells in the core of the SCN was similar between treatment groups, with LPS (4.78 ± 1.23) when compared to saline (2.03 ± 0.51, P>0.05). The expression of p-IκB in the SCN of LPS treated mice (11.97 ± 3.31) were statistically significant to controls mice in the shell (3.31 ± 0.69, P<0.05). Furthermore, there was no statistically significant alterations in p-IκB expression in the PVN of LPS treated mice (17.49 ± 3.10) and saline (12.41 ± 1.19, P>0.05; Figure 3.9).

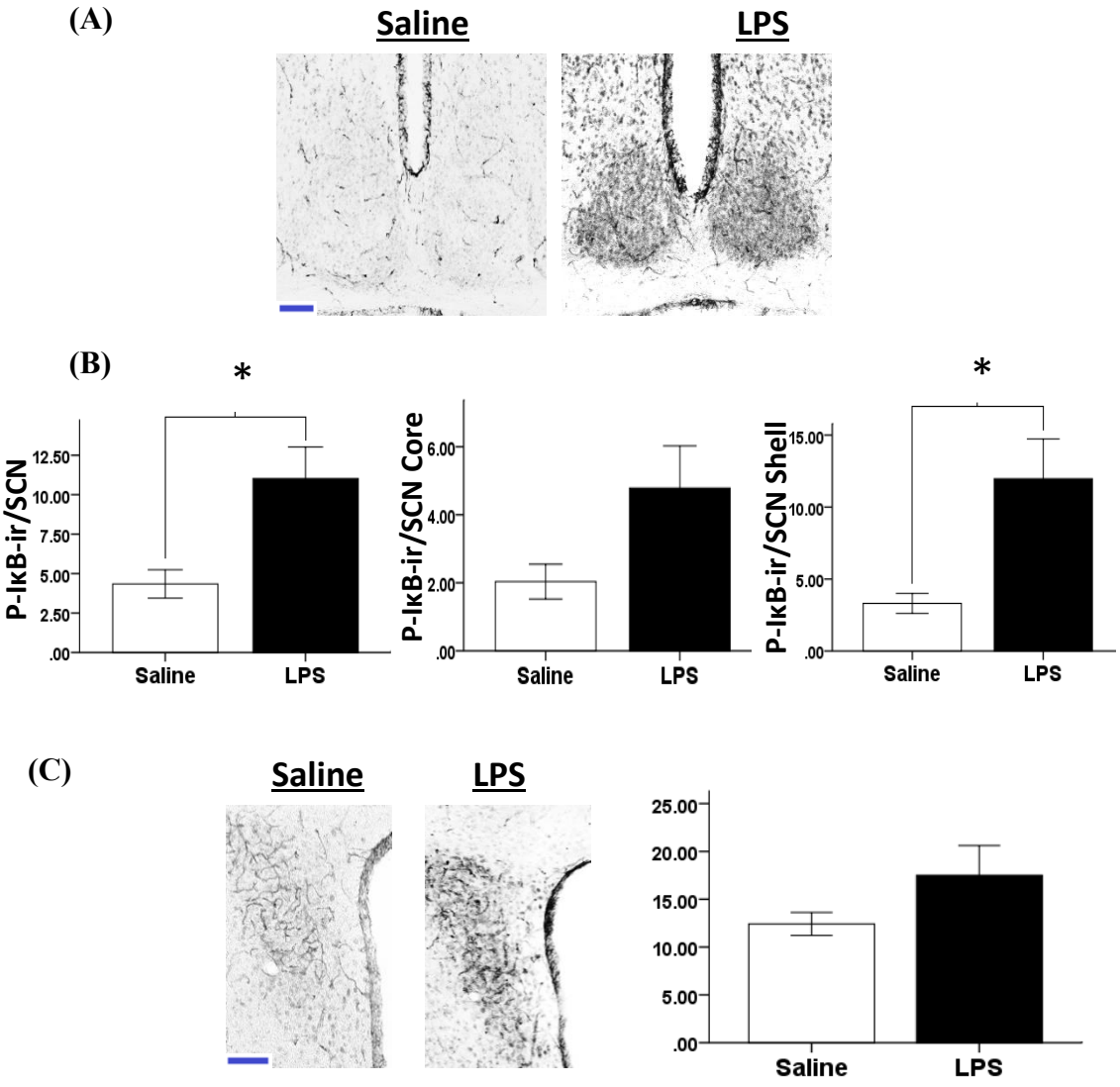
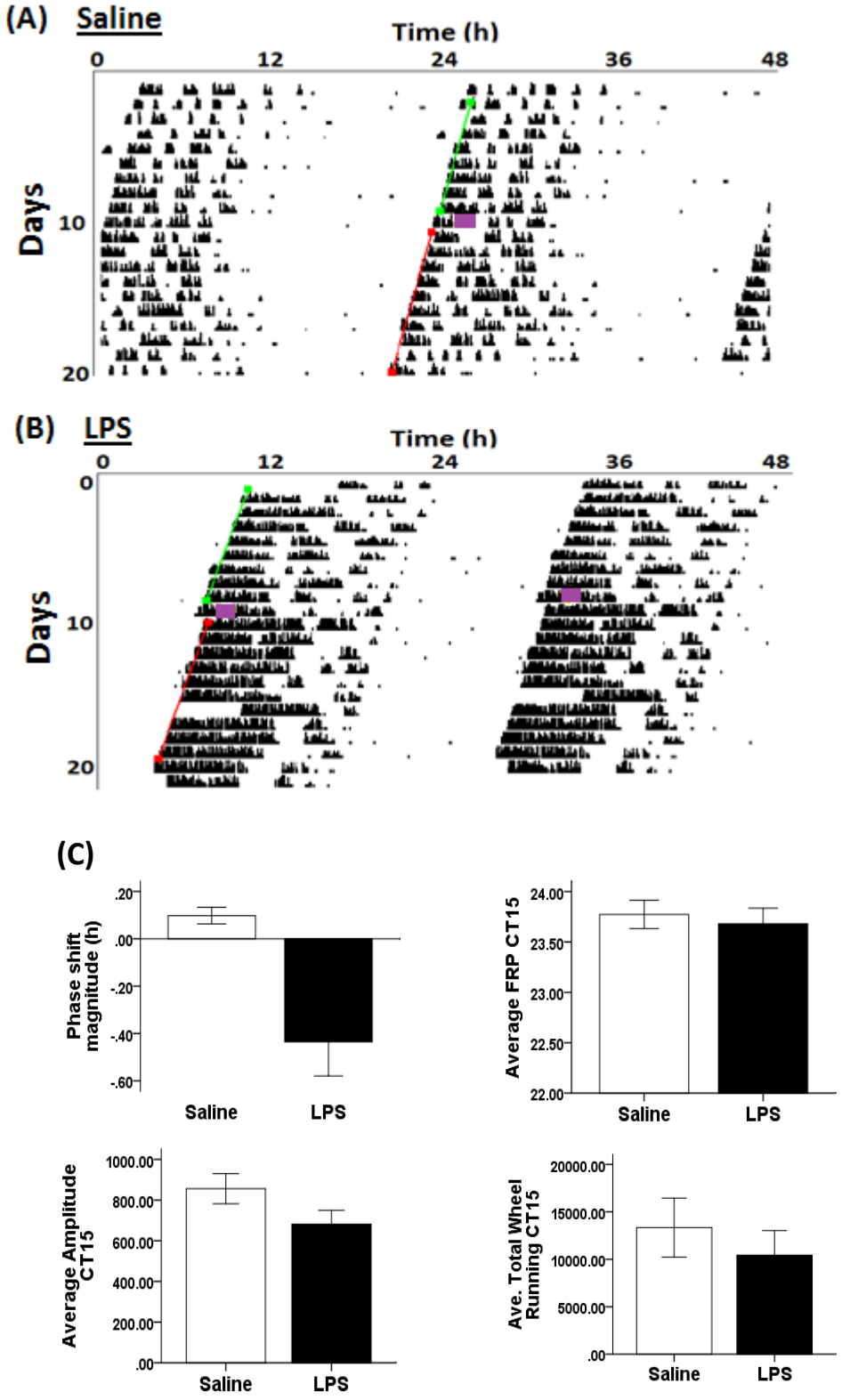


Figure 3.9: p-IκB expression after 24 hours post LPS treatment. (A) Representative photomicrographs illustrating of p-IκB expression in the SCN (scale bar = 100μm) and (B) Bar graphs illustrating quantification of levels of p-IκB immunostained cells at the mid rostro-caudal

level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the NF-κB components p-IκB in the mid rostro-caudal level of the SCN and in the dorsomedial shell of the SCN in LPS treated animals vs. controls. (C) Photomicrographs (scale 100μm) and bar graph showing no significance of p-IκB immunostained cells in the PVN between treatment groups. * $P<0.05$.

3.3.4 The effects of acute low dose LPS treatment on circadian locomotor behaviour in wild type mice.

In order to examine what effect low dose LPS has on circadian locomotor behaviour, mice free running in DD received either LPS (100μg/kg) or saline treatment at CT15. Statistical analysis was carried out using an independent t-test to assess whether there was a phase shift magnitude. Our results are similar to (Marpegán et al., 2005) displaying a statically significant phase delay when animals were administered low dose LPS (-0.45 ± 0.14 hrs) compared to saline controls (0.10 ± 0.04 hrs, $P<0.05$) at CT15. There was no alterations in circadian parameters between the LPS and saline control treatment groups (Figure 3.10). The mean free running periods was comparable between LPS and saline treated controls 23.68 ± 0.15 hrs and 23.77 ± 0.17 hrs respectively ($p>0.05$). There was no significant difference of amplitude between LPS (681.14 ± 68.62) and saline treated controls (856.20 ± 73.75, $P>0.05$). Furthermore, there was little variation between the total wheel running activity between the two groups with LPS animals displaying activity of (10438.53 ± 2598.08) and saline controls (13345.52 ± 3111.02, $P>0.05$).



3.10: Phase resetting following application of low dose LPS treatment. (A) and **(B)** are sample double plotted actograms of a control animal and a LPS treated animal free running in DD and treated with either saline or LPS at CT15. CT15 treatment is indicated by a purple square, with lines indicating the line of best-fit through activity onsets for the 7 days before and 10 days post treatment. Note exclusion of transient days from analysis. **(C)** Bar charts illustrating the average

phase shift magnitude, FRP, amplitude and the total wheel running activity of each group following saline or LPS treatment. There was a statically significance phase delay at CT15 when administered LPS compared to saline.

3.3.5 The effects of acute low dose LPS treatment on SCN and PVN neurochemistry in wild type mice

For assessment of clock gene, immediate early gene and NF-κB expression in the SCN and PVN immunohistochemical analysis was carried out to examine the effects of acute low dose LPS (100µg/kg). The clock gene protein-PER1, IEG-c-Fos and components of the NF-κB pathway-p65 and IκB-were investigated to see if low dose LPS alters neurochemical responses in the SCN. In addition, we investigated the PVN for any alterations in expression, which is a target area of the SCN and one which is known to response to LPS treatment.

3.3.5.1 p65 expression in the SCN and PVN following acute low dose LPS treatment.

The SCN and PVN were both examined for p65 expression at CT19, 4 hours post LPS or saline treatment, at CT15. Analysing levels of p65 immunoreactivity was carried out by IOD. Statistical analysis was calculated using an independent t-test to determine were there any alterations in the expression of p65 in the SCN and PVN. Results indicate that there was statistically significant effect of treatment LPS (15.27 ± 3.21) versus saline (7.29 ± 1.71, $P<0.05$; Figure 3.11) on p65 expression levels in the SCN. Further assessment was carried out of p65 expression in the SCN subdivisions-core and shell. There was no significant differences found in p65 expression in the core of LPS mice (22.66 ± 5.88) when compared to controls (13.01 ± 4.04, $P>0.05$). There was no statically significant alterations of p65 expression in the shell of LPS mice (14.22 ± 3.46) in comparison to saline controls (6.09 ± 1.05, $P>0.05$). Finally, there was no statistically significant effect of treatment of LPS (19.11 ± 2.09) and saline (13.50 ± 2.08, $P<0.05$; Figure 3.11) on the level of p65 expression in the PVN.

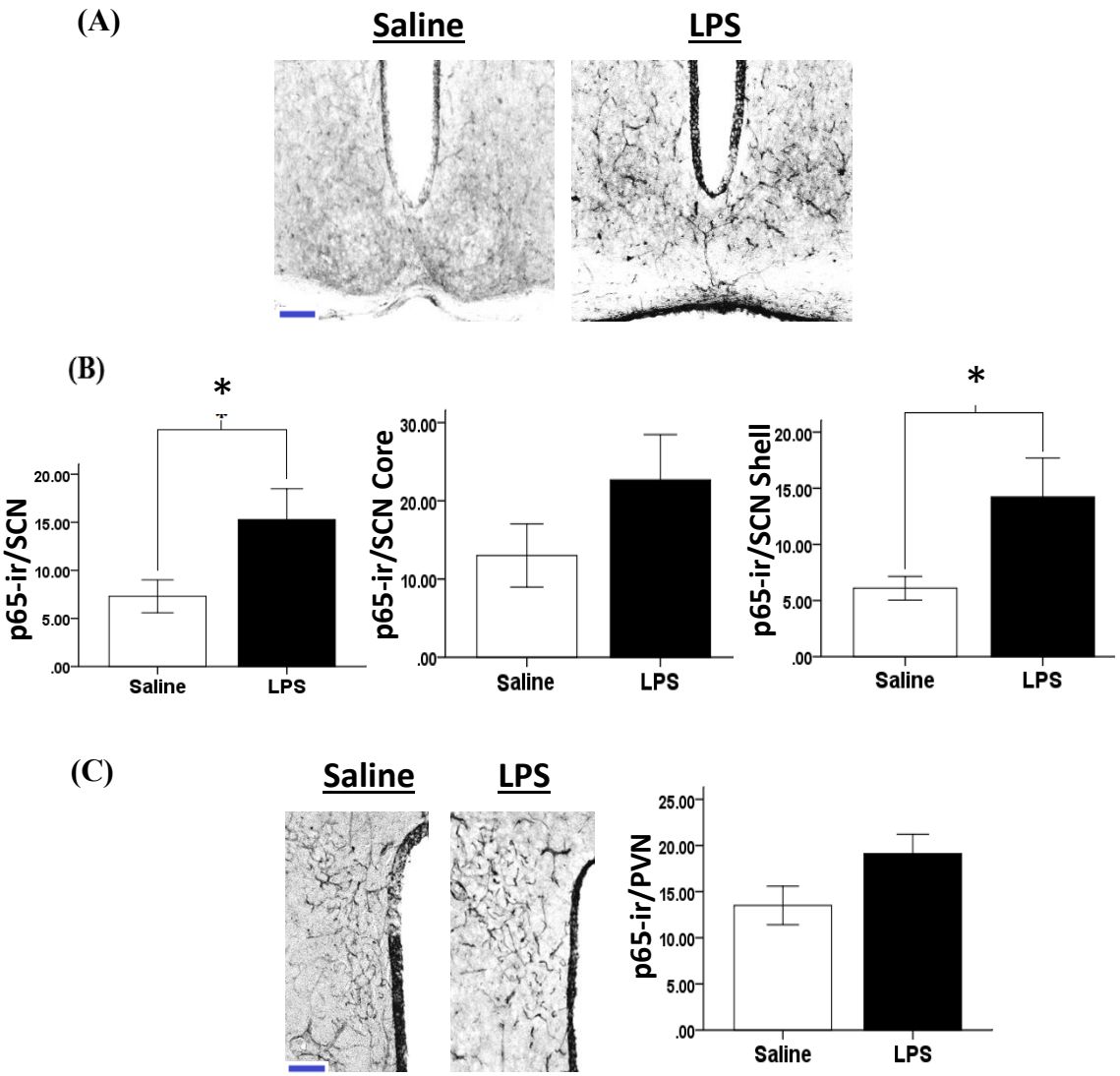


Figure 3.11: p65 expression 4 hrs after low dose LPS treatment. (A) Representative photomicrographs illustrating of p65 expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of p65 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the NF-κB components p65 in the mid rostro-caudal level of the SCN and in the dorsomedial shell of the SCN in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing upregulations of p65 immunostained cells in the PVN. * P<0.05.

3.3.5.2 p-IκB expression in the SCN and PVN following acute low dose LPS treatment.

The SCN and PVN were both examined for p-IκB expression 4 hours post LPS or saline treatment at CT15. Analysing levels of p-IκB immunoreactivity was carried out by IOD. Statistical analysis was calculated using an independent t-test to determine were there any alterations in the expression of p-IκB in the SCN and PVN. Results indicate

that there was no statistically significant effect of treatment LPS (13.48 ± 2.96) versus saline (9.10 ± 1.89, P>0.05; Figure 3.12) on p-IκB expression levels in the SCN. Further assessment was carried out of p-IκB expression in the SCN subdivisions-core and shell. There was no significant differences found in p-IκB expression in the core of LPS mice (19.46 ± 5.59) when compared to controls (15.51 ± 3.54, P>0.05). There was no statically significant alterations of p-IκB expression in the shell of LPS mice (13.94 ± 3.60) in comparison to saline controls (7.49 ± 2.26, P>0.05). Finally, there was no statistically significant effect of treatment of LPS (19.16 ± 2.38) and saline (14.92 ± 3.87, P>0.05; Figure 3.12) on the level of p-IκB expression in the PVN.

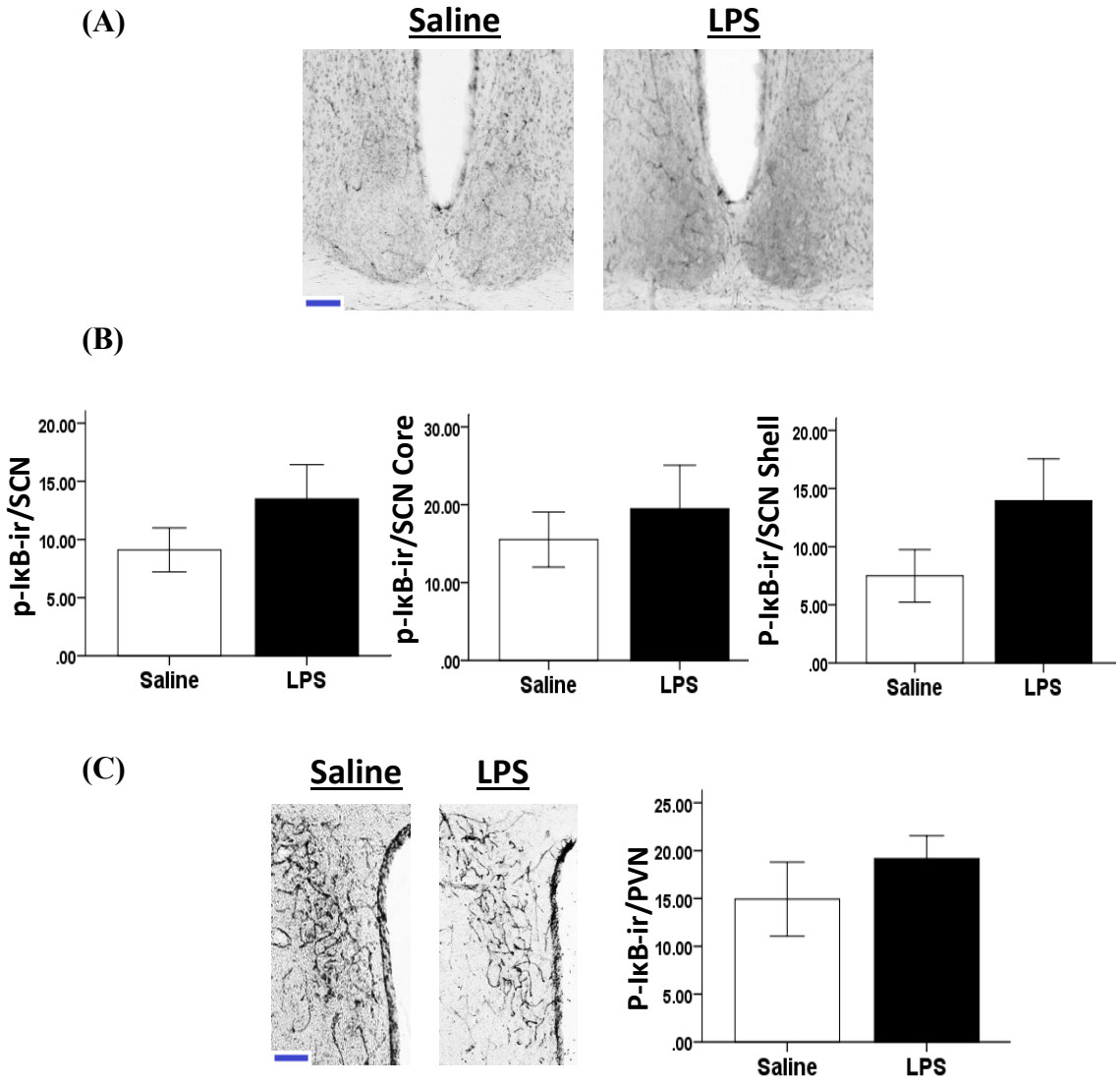
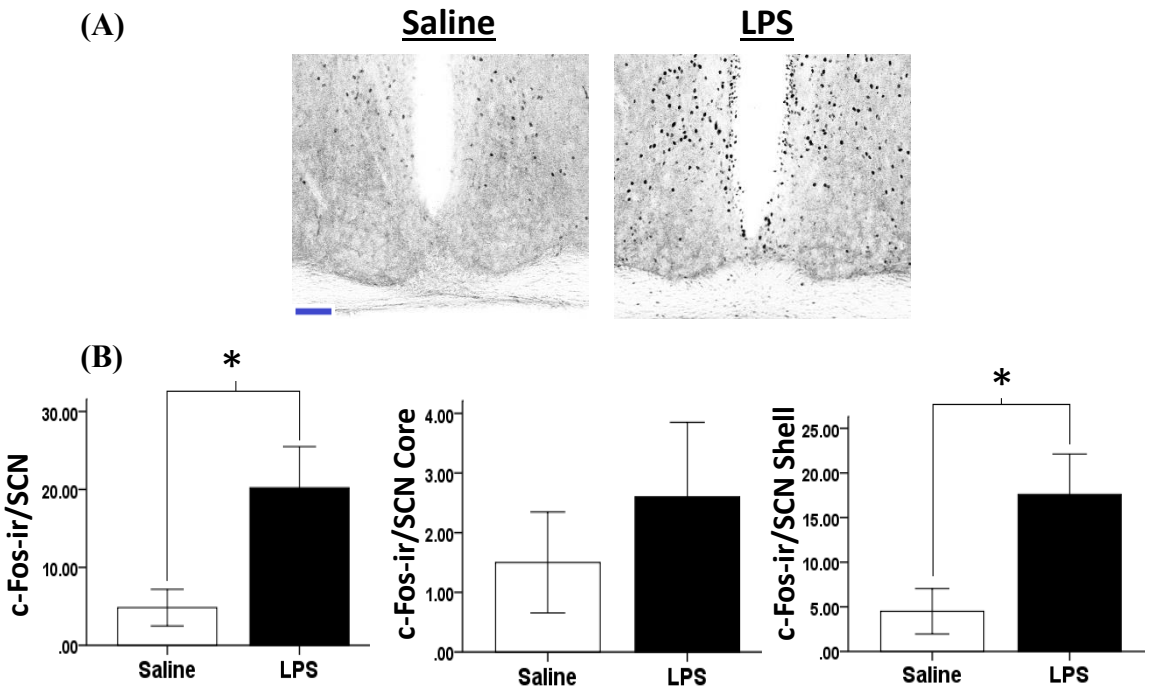


Figure 3.12: p-IκB expression 4 hrs after low dose LPS treatment. (A) Representative photomicrographs illustrating of p-IκB expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of p-IκB immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control

and LPS treatment groups, 24 hours post treatment in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing upregulations of p-IκB immunostained cells in the PVN. * P<0.05.

3.3.5.3 c-Fos expression in the SCN and PVN following acute low dose LPS treatment.

The SCN and PVN were both examined for c-Fos expression 4 hours after administration of LPS or saline treatment at CT15. Analysing levels of c-Fos immunoreactivity was carried out by manual quantification. Statistical analysis was calculated using an independent t-test to determine was there any changes in expression of c-Fos in the SCN and PVN. Results indicate that there was statistically significant upregulation of c-Fos expression after LPS treatment (20.20 ± 5.28) versus saline (4.83 ± 2.36, P<0.05; Figure 3.13) in the SCN. Additional assessment was carried out of c-Fos expression in the SCN subdivisions-core and shell. There was no alterations found in c-Fos expression in the core of LPS mice (2.600 ± 1.24) when compared to controls (1.500 ± 0.84, P>0.05). There was statically significant upregulation of c-Fos expression in the shell of LPS mice (17.60 ± 4.52) in comparison to saline controls (4.50 ± 2.54, P<0.05). There was statistically significant effect of treatment of LPS (49.40 ± 13.44) and saline (4.33 ± 1.82, P<0.05; Figure 3.13) on the level of c-Fos expression in the PVN. LPS treatment results in c-Fos expression occurring in the dorsal region of the SCN expanding into the peri-SCN, displayed below LPS photomicrograph in Figure 3.13.



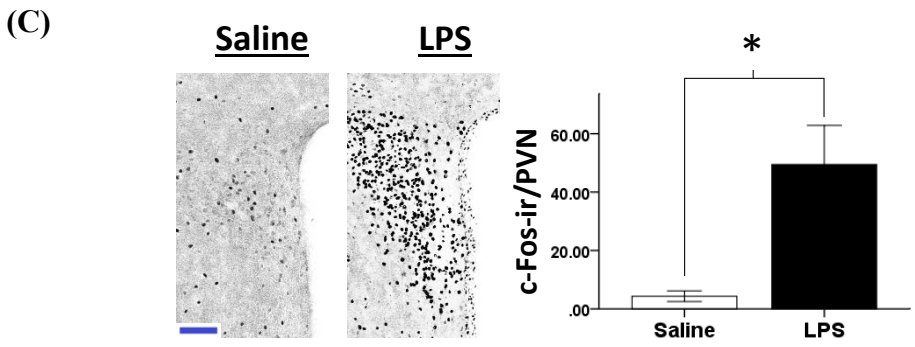


Figure 3.13: c-Fos expression 4 hours post LPS treatment. (A) Representative photomicrographs illustrating of c-Fos expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of c-Fos immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment, showing a significant upregulation of the IEG c-Fos in the mid rostro-caudal level of the SCN and in the dorsomedial shell of the SCN in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing upregulations of c-Fos immunostained cells in the PVN. * P<0.05.

3.3.5.4 PER1 expression in the SCN and PVN following acute low dose LPS treatment.

The SCN and PVN were investigated for PER1 expression 4 hours after LPS or saline treatment. The levels of PER1 expression was manually counted for immunoreactive nuclei, followed by statistical analysis using an independent t-test to evaluate whether there was variations in the expression of PER1 in the SCN and PVN following treatment at ZT4. There was comparable results displayed with no statistically significant effect of treatment LPS (159.26 ± 16.14) versus saline (126.70 ± 16.66, P>0.05; Figure 3.14) on PER1 expression levels in the SCN. When examining the subdivisions of the SCN, the mean number of immunoreactive PER1 cells in the core of the SCN was similar between treatment groups, with LPS (137.03 ± 12.10) when compared to saline (107.06 ± 0.67, P>0.05). The expression of PER1 in the shell of LPS treated mice (143.83 ± 3.04) were analogous to controls mice (129.57 ± 19.19, P>0.05). Finally, there was statistically significant alterations in PER1 expression in the PVN of LPS treated mice (71.06 ± 9.13) and saline (31.61 ± 5.78, P>0.05; Figure 3.14).

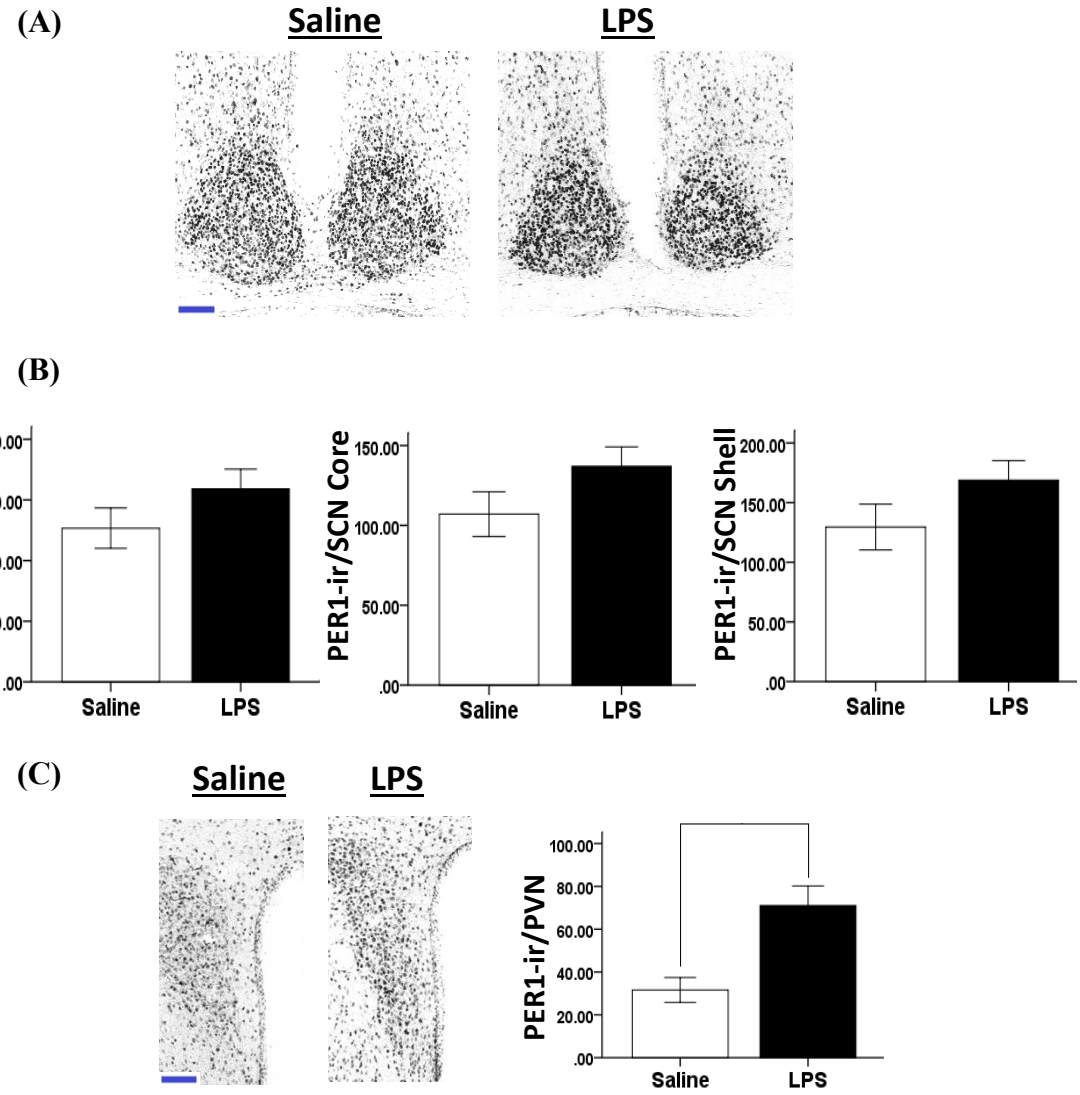


Figure 3.14: PER1 expression 4 hours post LPS treatment. (A) Representative photomicrographs illustrating of PER1 expression in the SCN (scale bar = 100µm) and (B) Bar graphs illustrating quantification of levels of PER1 immunostained cells at the mid rostro-caudal level of the SCN, in the SCN ventrolateral core and in the dorsomedial shell of the SCN of control and LPS treatment groups, 24 hours post treatment in LPS treated animals vs. controls. (C) Photomicrographs (scale 100µm) and bar graph showing upregulations of PER1 immunostained cells in the PVN.

3.3.6 The effects of constant darkness on mPer2^{Luc} knock-in mice

When investigating a locomotor wheel running experiment using mPer2^{Luc} knock-in mice, unusual FRP's were observed when mice were transferred from their L:D light cycle into DD. As we know mice transferred from L:D into DD usually have a shorter FRP daily, with a the FRP usually shorter than 24 hours (Jud et al., 2005). In this study

using 16 mice, it was discovered that half the mice had a shorter FRP, while the remaining half had a longer FRP of over 24 hours (Figure 3.15). Therefore, behavioural locomotor wheel running experiments undertaken in this chapter was carried out using WT mice.

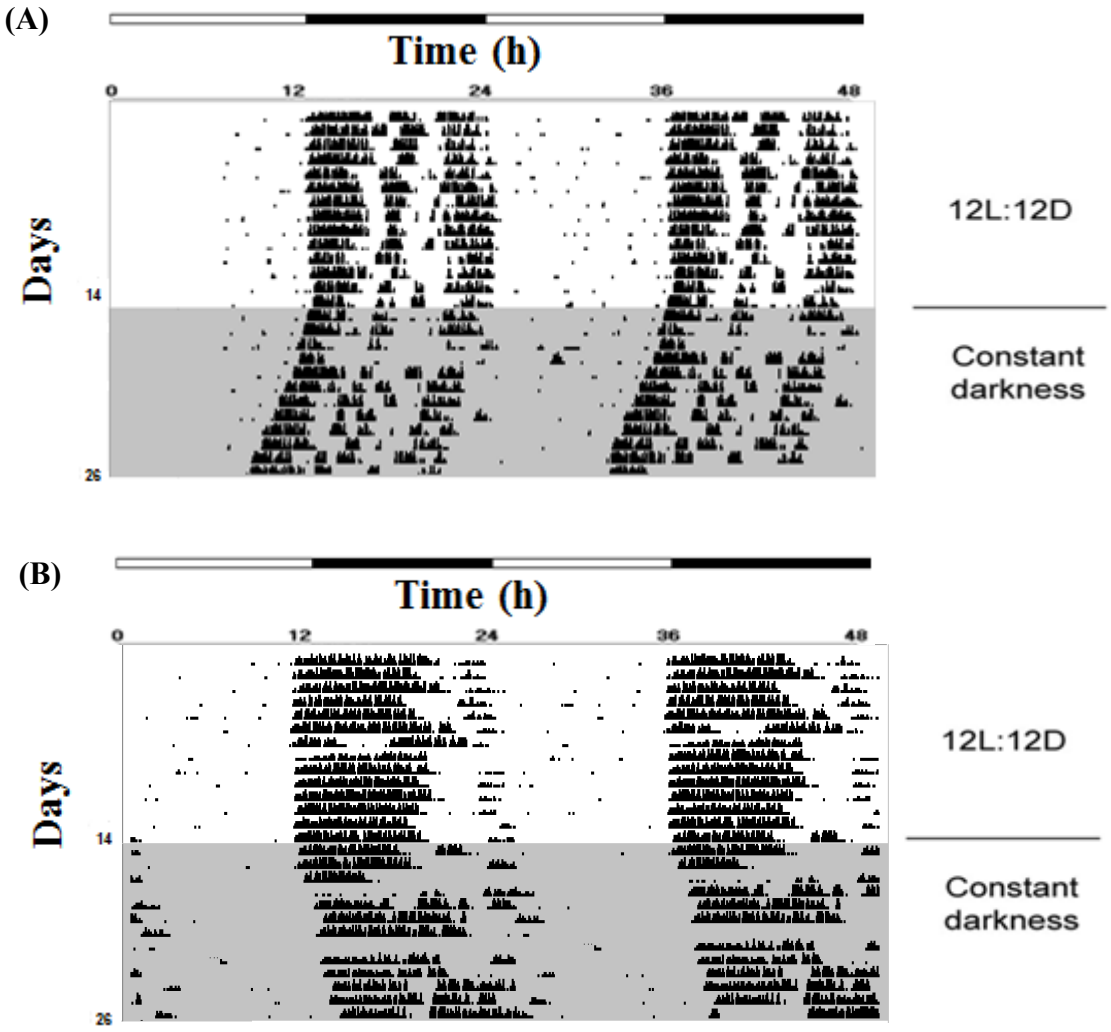


Figure 3.15: Double plotted actograms, (A) displaying that B6.129S6 mice have shorter FRP daily when in constant darkness, while (B) uncovers that constant darkness causes their FRP to be longer. The shaded area represents DD conditions.

3.3.7 The effects of acute LPS treatment on SCN and PVN neurochemistry in mPer2^{Luc} knock-in mice in the subjective day and night

The IEG-c-Fos and EGR-1-and components of the NF-κB pathway-p65 and IκB- were investigated to see if acute LPS (5mg/kg) altered neurochemical responses in the SCN during the subjective day (ZT6) compared to the subjective night (ZT18).

Furthermore, the PVN was examined for any alterations in expression, since it is known to response to LPS treatment.

3.3.7.1 p65 expression in the SCN and PVN following LPS treatment at either ZT6 or ZT18.

A two way ANOVA was used to assess whether there was a main effect of either treatment or time on p65 expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1, 17}$=0.255, P=0.622). There was a main effect of treatment ($F_{1, 17}$=6.55, P=0.024). When data was spilt by time, independent t-tests revealed a significant difference of p65 expression levels between treatments, at ZT6 (LPS, 11.88 ± 1.86 vs. saline, 6.49 ± 1.13, P=0.048) and at ZT18 (LPS, 9.86 ± 2.06 vs. saline, 6.84 ± 0.70, P=0.252). There was no interaction effect between the two variables ($F_{1, 17}$=0.523, P=0.483, Figure 3.16) on p65 expression levels in the SCN.

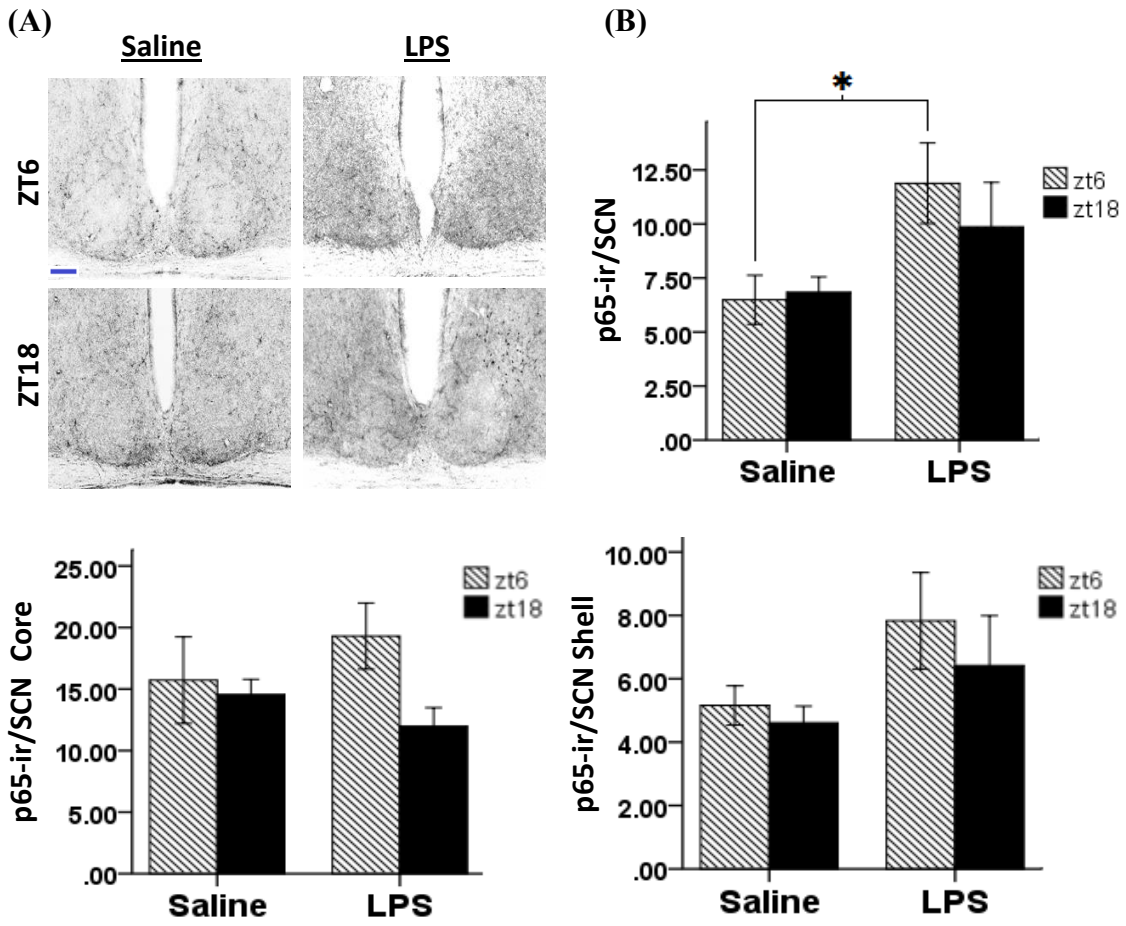


Figure 3.16: (A) Photomicrographs of the mid rostro-caudal level of the SCN and **(B)** bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell

of the SCN, illustrating the expression of the p65 after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There is significant p65 upregulation in the mid rostro-caudal level of the SCN, after LPS treatment compared to control at ZT6. * denotes P<0.05.

Furthermore, there was no interaction effect between the two variables ($F_{1, 13}$=0.727, P=0.416, Figure 3.17) on p65 expression levels in the PVN. No main effect of time was displayed ($F_{1, 13}$=1.01, P=0.321), however, there was a main effect of overall treatment (LPS, 18.74 ± 2.31 v saline, 11.41 ± 0.90, $F_{1,13}$=8.32, P=0.018) resulting in upregulation of p65 expression levels. ZT6 (LPS, 16.57 ± 2.97 vs. saline, 11.15 ± 1.70) and at ZT18 (LPS, 21.64 ± 3.51 vs. saline, 11.68 ± 1.05) both result in (P>0.05).

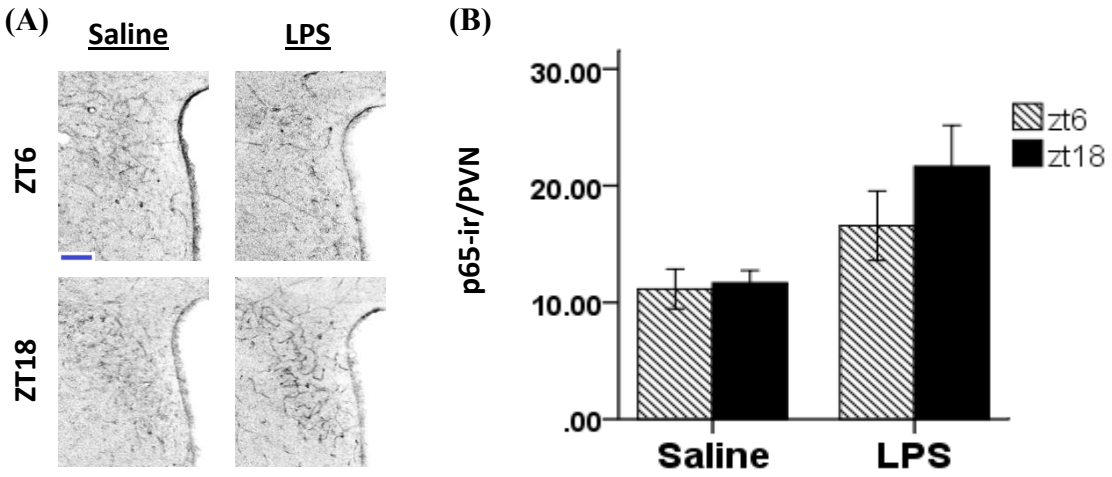


Figure 3.17: (A) Photomicrographs and **(B)** bar chart of the PVN illustrating the expression of the p65 after treatment with either LPS or control at two different timepoints, ZT6 and ZT18.

3.3.7.2 p-IκB expression in the SCN and PVN following LPS treatment at either ZT6 or ZT18.

Two way ANOVA analysis was used to assess whether there were alterations in the expression of p-IκB in the SCN and PVN following LPS 5mg/kg administration at two different time point. It was assessed whether there was a main effect of either treatment or time on p-IκB expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1, 16}$=0.03, P=0.957), nor was there a main effect of treatment ($F_{1, 16}$=2.61, P=0.132). There was significant upregulation of p-IκB expression levels post LPS treatment at ZT6 (LPS, 16.96 ± 6.99 vs. saline, 6.58 ± 1.96, P<0.05). Even though there was upregulation at ZT18 with LPS,

14.39 ± 6.50 vs. saline, 8.60 ± 2.23 it was non-significant (P>0.05). There was no interaction effect between the two variables ($F_{1, 16}$=0.21, P=0.654, Figure 3.18) for p-IκB expression levels in the SCN.

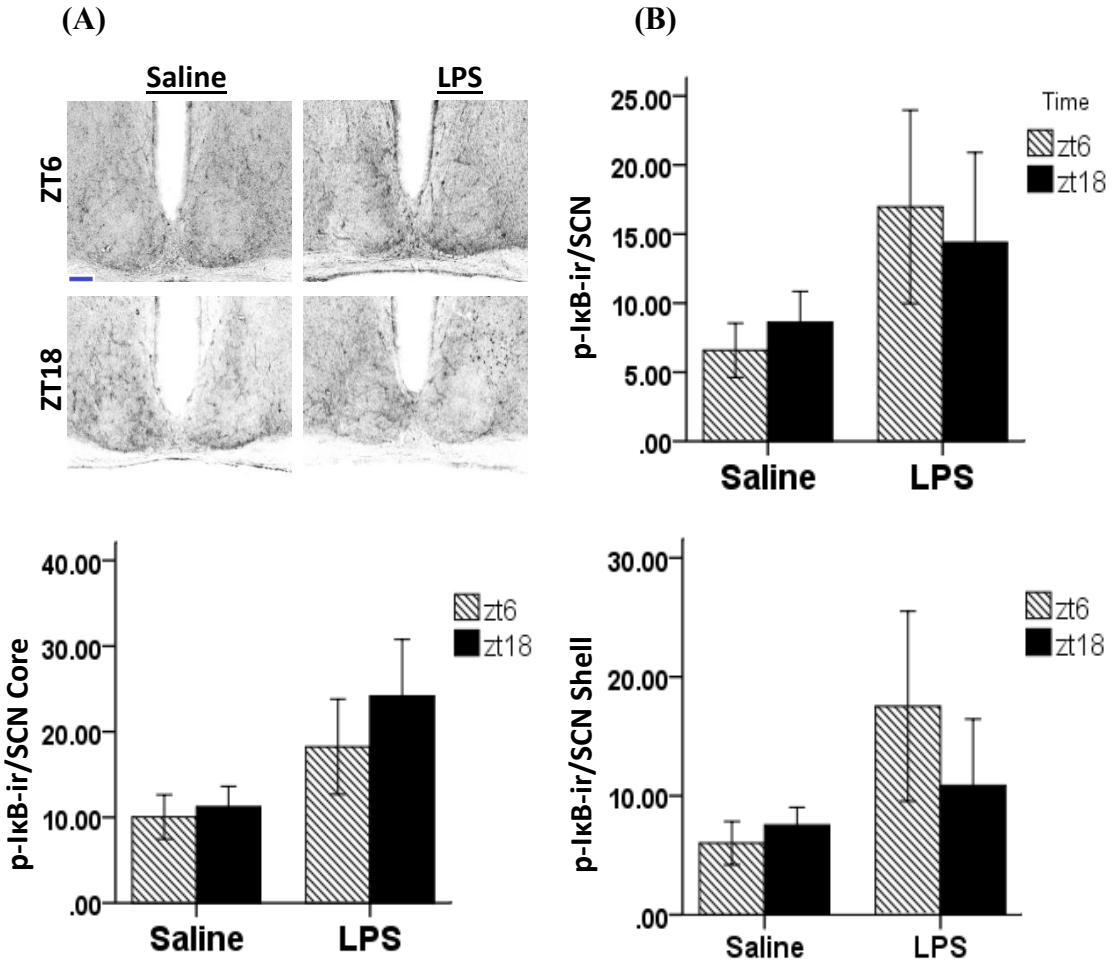


Figure 3.18: (A) Photomicrographs of the mid rostro-caudal level of the SCN and **(B)** bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of the p-IκB after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There is significant p-IκB upregulation in the mid rostro-caudal level of the SCN, after LPS treatment compared to control at ZT6. * denotes P<0.05.

In addition, it was assessed whether there was a main effect of either treatment or time on p-IκB expression in the PVN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1, 16}$=1.70, P=0.688), nor was there a main effect of treatment ($F_{1, 16}$=1.48, P=0.247). Furthermore, there was no interaction effect between the two variables ($F_{1, 16}$=0.01, P=0.991, Figure 3.19) for p-IκB expression levels in the PVN.

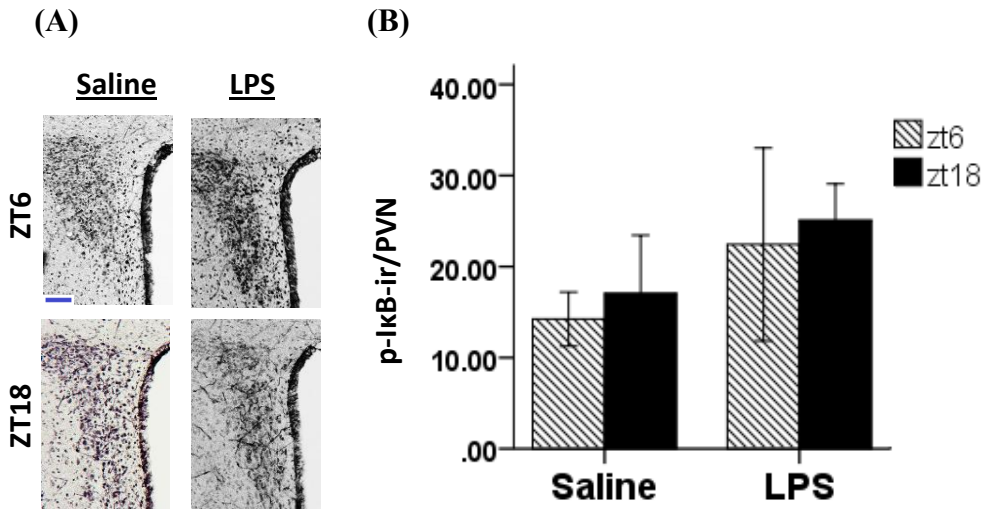


Figure 3.19: (A) Photomicrographs and **(B)** bar chart of the PVN illustrating the expression of the p-IκB after treatment with either LPS or control at two different timepoints, ZT6 and ZT18.

3.3.7.3 c-Fos expression in the SCN and PVN following LPS treatment at either ZT6 or ZT18.

A two way ANOVA was used to assess whether there was a main effect of either treatment or time on c-Fos expression in the SCN, or if there is an interaction effect between the two variables. There was a main effect of time ($F_{1, 12}$=11.42, P=0.008). There was no overall significant effect of treatment ($F_{1, 12}$=4.98, P=0.052). The interaction effect between the two variables was ($F_{1, 12=}$1.30, P=0.284, Figure 3:20) for c-Fos expression levels in the SCN. When data was spilt by time, independent t-tests revealed a significant difference of c-Fos expression levels between treatments at ZT18, with upregulation of c-Fos expression following LPS treatment (LPS, 31.33 ± 3.38 vs. saline, 11.00 ± 1.52, P=0.005). Furthermore, there was differences displayed at both time points in relation to treatment. Firstly with saline treatment, there was greater c-Fos expression exhibits at (ZT6, 38.25 ± 8.62 vs. ZT18, 11.00 ± 1.52, P=0.045). Following LPS treatment, there was also significant c-Fos expression during the subjective day when compared to the subjective night, (ZT6, 44.83 ± 3.44 vs. ZT18, 31.33 ± 3.38, P=0.049).

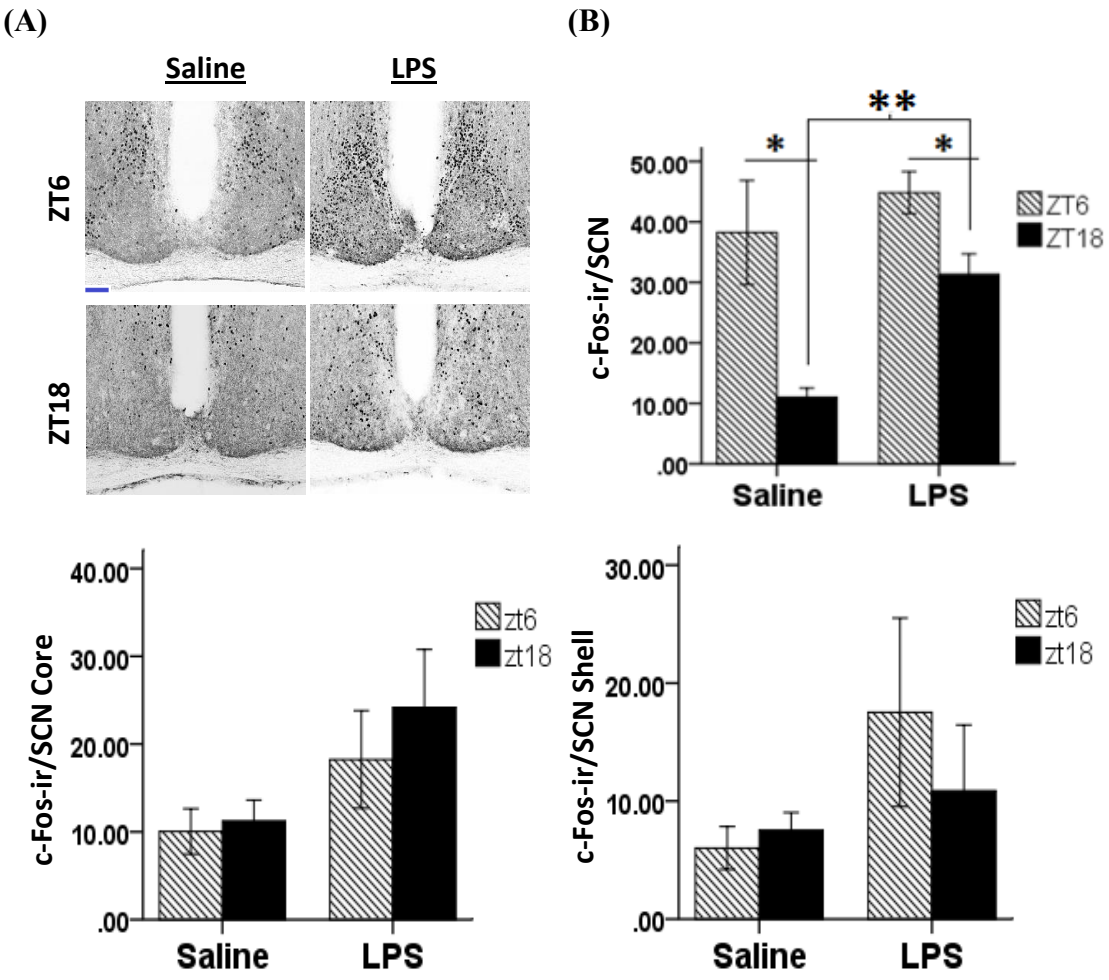


Figure 3.20: (A) Photomicrographs of the mid rostro-caudal level of the SCN and **(B)** bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of the IEG c-Fos after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There is significant upregulation of c-Fos in the mid rostro-caudal level of the SCN, after LPS treatment compared to control at ZT18. There is also significant differences of c-Fos expression between timepoints of treatment *denotes, P<0.05 and **denotes, P<0.01.

There was no main effect of time displayed in the PVN ($F_{1, 13}$=0.159, P=0.699). There was a main effect of treatment ($F_{1, 13}$=22.23, P=0.001). When data was spilt by time, independent t-tests revealed a significant upregulation of c-Fos expression levels between both treatment groups at ZT6 (LPS, 30.66 ± 4.25 vs. saline, 14.25 ± 4.17, P=0.043) and ZT18 (LPS, 34.00 ± 7.23 vs. saline, 11.00 ± 3.78, P=0.011). Finally, no interaction effect between the two variables were exhibited ($F_{1, 13}$=0.215, P=0.654, Figure 3:21) for c-Fos expression levels in the PVN.

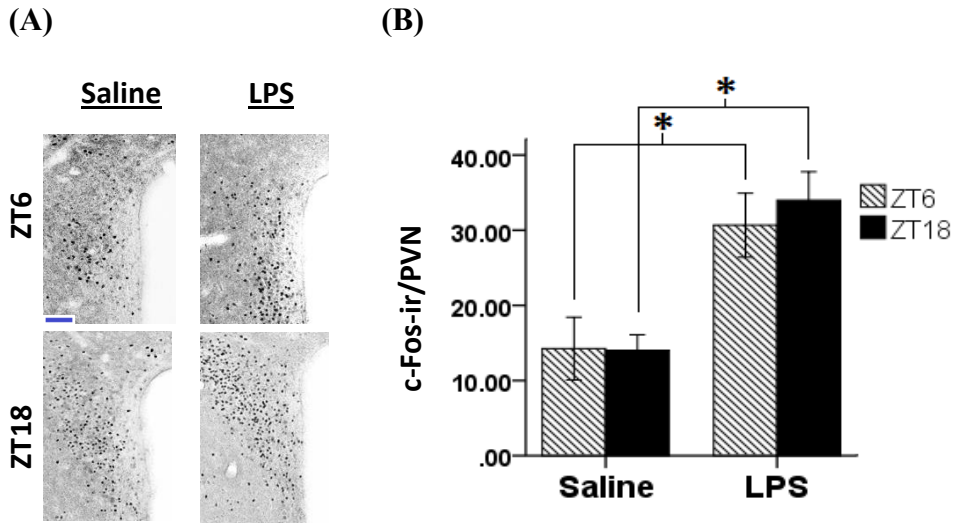


Figure 3.21: (A) Photomicrographs and **(B)** bar chart of the PVN illustrating the expression of the c-Fos after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There was upregulation of c-Fos expression after administration of LPS at both timepoints. * denotes, P<0.05.

3.3.7.4 EGR-1 expression in the SCN and PVN following LPS treatment at either ZT6 or ZT18.

A two way ANOVA was used to evaluate whether there was a main effect of either treatment or time on EGR-1 expression in the SCN, or if there is an interaction effect between the two variables. There was a main effect of time ($F_{1, 17}$=33.76, P=0.000). When data was spilt by treatment, independent t-tests showed a significant difference of EGR-1 expression levels between time points for both treatment group, saline (ZT6, 64.50 ± 6.6 vs. ZT18, 26.60 ± 4.4, P=0.003) and LPS (ZT6, 82.80 ± 14.46 vs. 9.00 ± 1.87, P=0.003). No main effect of treatment ($F_{1, 17}$=0.01, P=0.972) was exhibited, however when data was spilt between time, an independent t-test displayed down regulation of EGR-1 expression following LPS treatment at ZT18 (LPS, 9.00 ± 1.87 vs. saline, 26.60 ± 4.44, P=0.013). The interaction effect between the two variables measured ($F_{1, 17}$=3.48, P=0.085, Figure 3:22) for EGR-1 expression levels in the SCN.

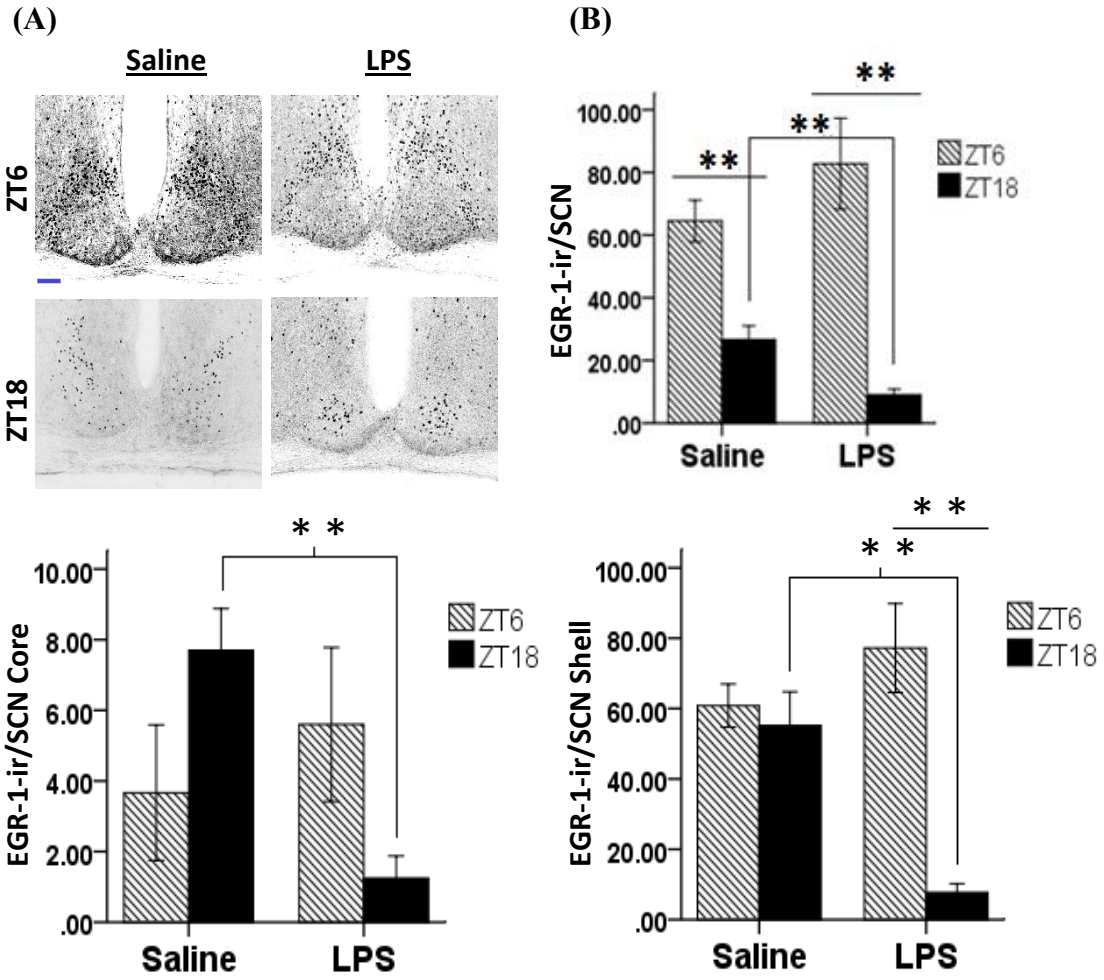


Figure 3.22: (A) Photomicrographs of the mid rostro-caudal level of the SCN and **(B)** bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of the IEG EGR-1 after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There is significant upregulation of EGR-1 in the mid rostro-caudal level of the SCN, after LPS treatment compared to control at ZT18. There is also significant differences of EGR-1 expression between timepoints of treatment. **denotes, P<0.01.

Further analysis showed that there was no main effect of time displayed in the PVN ($F_{1,\ 14}$=0.070, P=0.797). There was a main effect of treatment ($F_{1,\ 14}$=18.74, P=0.001). When data was spilt by time, independent t-tests revealed a significant upregulation of EGR-1 expression levels between both treatment groups at ZT6 (LPS, 66.75 ± 12.02 vs. saline, 5.75 ± 1.75, P=0.014) and ZT18 (LPS, 57.75 ± 17.00 vs. saline, 8.00 ± 1.47, P=0.027). Finally, no interaction effect between the two variables were exhibited ($F_{1,\ 14}$=0.193, P=0.669, Figure 3:23) for EGR-1 expression levels in the PVN.

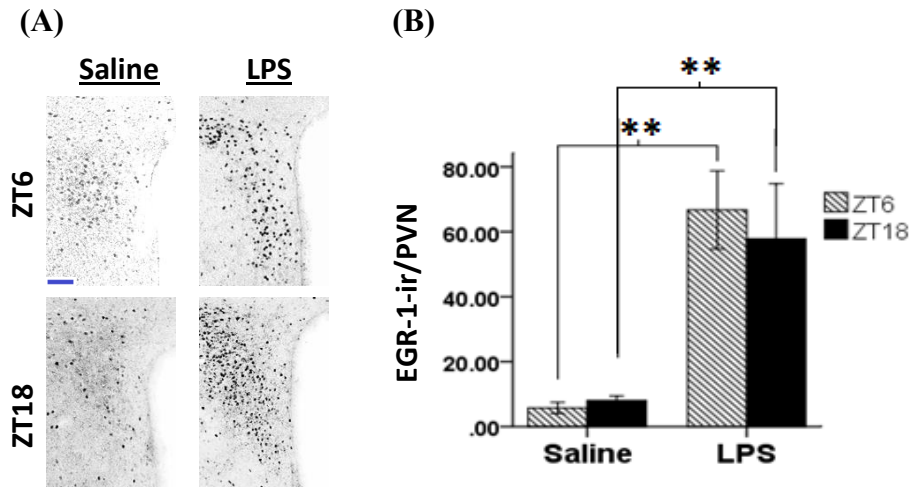


Figure 3.23: (A) Photomicrographs and **(B)** bar chart of the PVN illustrating the expression of the EGR-1 after treatment with either LPS or control at two different timepoints, ZT6 and ZT18. There was upregulation of EGR-1 expression after administration of LPS at both timepoints. ** denotes, P<0.01.

3.3.8 The effects of acute LPS treatment on SCN expression in mPer2^{Luc} knock-in mice using double immunostaining

A qualitative method was used to analysis the double immunostaining technique looking at the microglial marker-IBA-1 and a component of the NF-κB pathway-p65 expression post LPS (5mg/kg) treatment at ZT8. IBA-1 is represented by blue staining microglia, whereas p65 is displayed by red staining microglia. There is clear upregulation of IBA-1 and p65 post LPS treatment compared to saline when observing the photomicrographs (Figure 3.24).

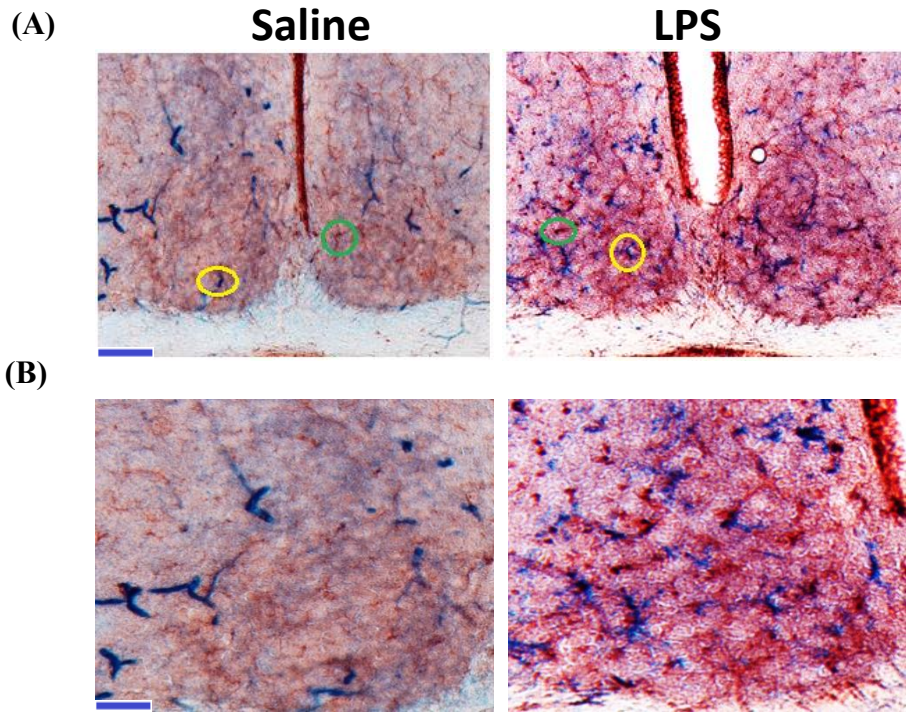


Figure 3.24: (A) photomicrographs of the SCN illustrating the immunoreactive microglia expression of IBA-1 and p65 expression after LPS or saline treatment at ZT8 (scale bar = 100µm). IBA-1 is represented by blue glial staining marked by a yellow circle and p65 is shown by red glial staining, with a green circle displaying microglia expression. **(B)** IBA-1 and p65 immunostained microglia are shown in higher magnification in the SCNs from saline and LPS treated animals (scale bar 50µm).

3.4 Discussion

The SCN expresses cytokine receptors and regulatory molecules (Beynon & Coogan, 2010; Beynon et al., 2009; Lundkvist et al., 1999; Sadki et al., 2007) which means that inflammatory mediators induced by peripheral LPS may impact upon the SCN causing functional changes and alterations in clock gene expression following this immune challenge (Marpegán et al., 2005). LPS causes a fast systemic inflammatory response, which in turn results in increased systemic levels of pro-inflammatory cytokines such as TNF-α, Il-6 and IL-1β (Benson et al., 2012; Hang et al., 2004; Martich et al., 1993). Systemic LPS, TNF-α and IL-1β challenges have all been shown to induce robust and comparable systemic and brain inflammation (Skelly et al., 2013). Upregulation of pro-inflammatory cytokines are displayed in the SCN and hippocampus brain regions as a result of prenatal stress or an immune challenge such as LPS administration (Diz-

Chaves et al., 2013; Leone et al., 2012; Paladino et al., 2015; Rossol et al., 2011). As cytokine production increases in the brain, it results in sickness behaviour. When LPS is administered at the start of the subjective night (active phase) it induces a greater response to both temperature and cytokines in comparison to LPS treatment administered in the subjective day. Interaction between the SCN and the immune system is displayed when lesions occur to the SCN, as it results in a higher increase to immune response in relation to LPS, compared to control SCN (Guerrero-Vargas et al., 2014).

As discussed in the previous chapter, Bellet et al (2012) established a molecular link between the NF-κB pathway and the clock-driven transcriptional pathway. However, protein levels of the different NF-κB subunits examined -p65, RelB, p50, and p52- in both WT and *Clock* mutant fibroblast showed no significant upregulation as a result of LPS treatment (Bellet et al., 2012). NF-κB activation was understood to be under circadian control when studying the NF-κB reporter mice, as an immune challenge resulted in daily variations of NF-κB activation during the 24 hour cycle. Investigations carried out in this study also showed that BMAL1 is anti-inflammatory via clocks interaction with NF-κB, since CLOCK upregulated p65-mediated transcription in fibroblasts following an immune challenge independent of BMAL1, however, the co-expression of CLOCK/BMAL1/p65 caused BMAL1 to reduce the NF-κB activation by sequestering CLOCK (Spengler et al., 2012). Recent studies further add to the fact that BMAL1 has anti-inflammatory properties, with mice deficient in Myeloid BMAL1 displaying an upregulation of pro-inflammatory microRNA Mir-155 and NF-κB activity following LPS treatment (Curtis et al., 2015). Other studies have displayed how the master clock is affected by LPS treatment in mice, however, further investigations need to be carried out on the pathway involved in the circadian LPS responses, with NF-κB and the LPS receptor TLR4 thought to play a role in this circadian effect (Marpegán et al., 2005; Marpegan et al., 2004; Paladino et al., 2010).

This chapter investigated the effect LPS treatment has on the NF-κB pathway, IEG's and clock genes in relation to the neurochemistry of the SCN and PVN. Behavioural analysis was also carried out to monitor the effect low dose LPS administration plays on locomotor rhythms. Peripheral administration of LPS (5mg/kg) lead to acute, evident sickness behaviour. Sepsis scoring was carried out to confirm effective induction of sepsis in LPS treated animals when compared to vehicle treated controls in this study.

3.4.1 The effect acute LPS administration has on the SCN and PVN

3.4.1.1 Expression of NF-κB components in the SCN and PVN post 24 hour LPS treatment.

NF-κB is vital for the body's immune system. The levels of expression of components of NF-κB -p65, p-IκB, p-IKK and c-Rel- were studied in the SCN and PVN after administration of an acute peripheral immune challenge using LPS. In this study it was established that administration of acute peripheral LPS at ZT4 resulted in significant upregulation of the p65 subunit of NF-κB in the SCN when compared to controls, 24 hours post treatment. Other studies also found comparable findings due to immune stimulation using peripheral LPS causing alterations in expression of p65 in the SCN (Beynon & Coogan, 2010). This upregulation of p65 expression does not last over time, this was displayed in post-sepsis mice, 3 months after LPS treatment mice had similar p65 expression levels as controls (O'Callaghan et al., 2012). Furthermore, we discovered upregulation of p65 expression in the PVN as a result of LPS challenge. Beynon also discovered that there was upregulation of p65 in the PVN 6 hours post LPS treatment compared to controls, but this significant increase in p65 levels after 6 hours was not displayed in the SCN. Therefore, the autonomic/endocrine response to LPS immune challenge seems to occur before the circadian clock (Beynon & Coogan, 2010).

IκB controls the activity of NF-κB, which is responsible for the transcription of many immune signal molecules. The detection of IκB induction would reveal the extent and the cellular location of brain-derived immune molecules in response to peripheral immune challenges. *In vivo,* LPS i.p injections were administered to rats, and caused upregulation in IκBα mRNA levels in the SFO, choroid plexus and meninges, two hours post LPS treatment. After 24 hours IκBα mRNA levels had attenuated in these brain areas, till it subsided towards basal levels (Quan et al., 1997). It was found in this study, that p-IκB was sensitive to an immune challenge using LPS. Levels of p-IκB were significantly upregulated in the SCN as a result of LPS treatment. There are no alterations in p-IκB expression in post-sepsis mice, 3 months post LPS, in either the SCN or the CA1 (Anderson et al., 2015; O'Callaghan et al., 2012). Therefore, it would seem that some component of the NF-κB pathway response to an acute immune challenge, however, the effects are not long term. It was established that acute LPS administration did not cause

alterations to the expression of p-IκB in the PVN. Other studies examining the PVN have discovered that LPS induced strong IκBα mRNA expression when compared with vehicle (Quan et al., 2003; Quan et al., 2000).

Two other signalling components of the NF-κB pathway, p-IκK and c-Rel, were analysed for sensitivity to a LPS immune challenge. There was minimal p-IκK and c-Rel expression in the SCN and PVN at ZT4 in both treatment groups. Therefore, both components were not sensitive to this immune signal. No long-term effect was established either, with post-sepsis mice also showing similar p-IκK expression with controls in the SCN and CA1 (Anderson et al., 2015; O'Callaghan et al., 2012).

3.4.1.2 Expression of IEG'S in the SCN and PVN post 24 hour LPS treatment.

The IEG –c-Fos- is a transcription factor which is frequently used as a marker of neuronal activation in neuroscience research (Benito & Barco, 2015; Cruz et al., 2013). c-Fos is a useful marker of neuronal activation due to the fact that it is rapid and specific to a variety of stimuli such as photic or noxious stimuli. It is also easy to quantify using immunohistochemistry techniques (Gao & Ji, 2009). A study reported how c-Fos induction is reliant on NF-κB (Nadjar et al., 2005). Stimuli such as LPS (which activate the NF-κB pathway) is known to upregulate c-Fos expression in neurons of rodents 2-6 hours post treatment, in various brain regions such as the central amygdala and nucleus of solitary tract (Elmquist et al., 1993; Marvel et al., 2004), and this expression is still upregulated 24 hours post treatment in the SCN (Beynon & Coogan, 2010) and PVN (Beynon & Coogan, 2010; Ogilvie et al., 1998). However, there is no long term modulation of c-Fos expression displayed in the SCN one month after LPS treatment in comparison to control mice (O'Callaghan et al., 2012). In this current study, there was significant upregulation of c-Fos expression in the vlSCN as a result of an LPS immune challenge when compared with vehicle, highlighting the effect of the peripheral immune challenge on the SCN. The study of c-Fos induction after LPS administration has been examined widely in the PVN (Bienkowski & Rinaman, 2008; Rivest & Laflamme, 1995), with the MAPK inhibitor, PD98059, able to attenuate c-Fos upregulation in this brain region (Singru et al., 2008). When examined in this current study there was significant upregulation of c-Fos expression displayed in the PVN after LPS administration at ZT4.

Another IEG –Egr-1- is a DNA binging transcription factor and it is also used as a marker of neuronal activation. Studies carried out in our laboratory showed the effect of long-lasting effects of sepsis on plasticity-related early gene expression. Post-septic mice displayed a significantly lower level of expression of EGR1 in the DG and CA1 (Anderson et al., 2015). The current study exhibited significant upregulation of immunoreactive EGR-1 in the vlSCN due to an LPS immune challenge in comparison to control, displayed 24 hours following an immune challenge at ZT4. There was also significant upregulation of Egr-1 expression displayed in the PVN after LPS administration at ZT4.

3.4.1.3 Expression of clock gene expression in the SCN and PVN post 24 hour LPS treatment.

Endotoxins such as LPS have been shown to modify clock gene expression in the SCN and PVN in rodents (Okada et al., 2008; Paladino et al., 2015). This change in clock gene expression was also displayed in peripheral tissues such as heart and liver, as a result of this immune challenge (Murphy et al., 2007; Okada et al., 2008; Yamamura et al, 2010). LPS administration in peripheral blood leukocytes of humans, results in alterations of clock gene expression (Haimovich et al., 2010). Studies carried out in rat have shown that LPS (1mg/kg) transiently suppresses the clock gene rPer2 in the SCN for 24 hours after treatment (Okada et al., 2008). The *Per2*$^{-/-}$ mouse is more resistant to endotoxic shock caused by LPS in comparison to the WT mouse. They also have reduced pro-inflammatory cytokines for example IFN-γ and Il-1β, therefore, implying a link between the circadian clock and the immune system (Liu et al., 2006).

Long term modulation of PER2 expression was witnessed in the SCN, and hippocampus areas including the DG and CA1 of post-septic mice (LPS 5mg/kg) one month after treatment (O'Callaghan, et al., 2012). Interestingly, when low dose LPS (100µg/kg) was administered in WT mice at ZT15, upregulation of PER2 expression could be seen in the SCN, however, this was not observed for the other clock gene, PER1. LPS treatment at ZT15, displayed no significant alterations for Per1 protein expression or *Per1* mRNA levels in the SCN (Paladino et al., 2015). Unlike Per2 expression, long term alterations in Per1 expression was not observed in the SCN one month post LPS treatment when compared to vehicle, this may be due to altered signalling mechanisms.

However, changes in Per1 expression did arise in the CA1 as a result of LPS (O'Callaghan et al., 2012). In this present study there was no significant modifications of expression of the clock gene PER1 displayed between LPS treated mice and controls in the SCN at ZT4. Therefore, from past and current studies, results may indicate that the expression of clock gene Per1 in the early subjective day and night is not affected by an immune challenge in the SCN.

When carrying out LPS treatment, Paladino and colleagues discovered that *Per1* mRNA was induced in the PVN in the hypothalamic region in the early subjective night in young adult mice (Paladino et al., 2010). The PVN is thought to play a role in mediating output information from the SCN (Kalsbeek et al., 2010), therefore, in this study it was examined whether there was alterations in clock gene PER1 expression within the PVN following LPS administration as this may affect rhythmic outputs of these animals. Studies exhibited an increase in Per1 expression at ZT15 (Paladino et al., 2015) and ZT22 (Takahashi et al., 2001) which suggested that the PVN responds to immune stimulation and is not time dependent. However, we do not find evidence for disruption of the molecular clockwork in young adult mice after an immune challenge during the early-mid subjective day. It was established that there was no significant alterations in relation to PER1 expression when analysing this clock gene in the PVN of LPS treated mice when compared to control mice, at ZT4. Hence, this finding suggests that the timing of the immune challenge may be crucial, as the subjective day does not seem to be affected by high dose LPS.

3.4.2 The direct effect of acute low dose LPS administration on circadian locomotor activity and the neurochemistry of the SCN and PVN.

As previously stated low dose LPS treatment at CT15 resulted in a photic like phase delay, with no significant phase shift displayed at any other CT as a result of LPS administration (Marpegán et al., 2005; Paladino et al., 2010). Cytokines such as Il-1β and TNF-α can cause similar phase delays seen with LPS at CT15. A 100µg/kg LPS treatment at CT15 undertaken in our lab also resulted in a significant phase delay of approximately 45 minutes when compared to saline controls. Marpégan and colleagues tested a variety of LPS dose from 25µg/kg to 250µg/kg which all induced phase delays of locomotor activity rhythms, 43 and 60 minutes respectively. Mice deficit in the TNFα receptor 1/p55

(Tnfr1 KO) still displayed comparable circadian behaviour with WT mice, however, they did not produced phase delays of locomotor activity rhythms after LPS administration which occurs in WT mice. Hence, there is a Tnfr1- dependent LPS circadian response at behavioural level (Paladino et al., 2015). In the current study there was a decrease but it was not significant, in rhythms amplitude and total wheel running activity following LPS treatment when compared to control. This was due to a temporary suppression of wheel running activity as a result of LPS for the few hours post treatment, which has being displayed in previous studies (Marpegán et al., 2005).

Administration of low dose LPS can cause an effect on the neurochemical responses in the SCN. A peripheral dose of 100 µg/kg LPS cannot cross the BBB, however, it can bind to specific receptors which results in it entering the endothelial cells, increasing BBB permeation, which triggers a series of signalling events resulting in the induction of pro-inflammatory responses in the brain (Singh & Jiang, 2004). One study investigated the effect of low dose (100µg/kg) LPS on hypothalamic mRNA transcription, 2 hours following treatment, using quantitative PCR. Findings resulted in a significant upregulation of IL-1β, TNF-α and IL-6 mRNA expression, as a result of a LPS challenge. Comparable findings were also displayed in the hippocampus (Skelly et al., 2013). Additionally, it is thought that that the SCN may express TLR4, since LPS applied directly to SCN tissue results in alterations to AVP production (Nava et al., 2000).

The IEG, c-Fos, displays oscillation throughout the day and night cycle under basal conditions (Aronin et al., 1990). Photic induction of c-Fos at CT15 results in expression throughout the whole SCN, whereas LPS treatment causes c-Fos expression in vlSCN at this time point (Marpegán et al., 2005). In this current study a similar result was demonstrated with increased c-Fos expression exhibited in the shell region of the SCN after low dose LPS administration at CT15. When the SCN was divided into sub regions, there was no significant alterations shown in the core between both treatment groups. The shell displayed significant alterations between LPS treated and control, similar to Marpegán and colleagues. There was significant upregulation of c-Fos displayed in the SCN, 4 hours after LPS treatment in contrast to controls. This was similar to other studies investigating low dose LPS treatment at both CT15, ZT14 and ZT15 in WT mice (Marpegán et al., 2005; Guerrero-Vargas et al., 2014; Paladino et al., 2015, 2010). The same LPS dose was administered at ZT2 and ZT3 with no alteration in c-Fos

expression displayed in the SCN, between both treatment groups, therefore, the timing of treatment is crucial (Guerrero-Vargas et al., 2014; Paladino et al., 2015). Studies suggest that Tnf, Tnfr1 and TLR4 are important in the variation of LPS-induced circadian effects on the SCN. Mice deficit in Tnfr1and TLR4 did not display LPS induced c-Fos expression in the SCN at ZT15 and CT15 respectively (Paladino et al., 2015, 2010).

Another area examined was the PVN in relation to modulation of IEG expression after LPS treatment. Paladino and colleagues recognised that low dose LPS administration to $TLR4^{-/-}$ mice at CT15 exhibited a significantly lower expression of c-Fos cells in the PVN in comparison to WT mice (Paladino et al., 2010). Also Paladino and colleagues also established that low dose LPS treatment at ZT15 resulted in a significant increase in c-Fos expression in the PVN (Paladino et al., 2015), with low dose LPS treatment at ZT14 exhibiting similar findings (Guerrero-Vargas et al., 2014). Using $Tnfr^{-/-}$ mice no alterations of c-Fos expression was recognised in the PVN as a result of LPS treatment when compared to control (Paladino et al., 2015). In this current study it was recognised that low dose LPS treatment at CT15 resulted in significant upregulation of c-Fos expression in the PVN.

A study was carried out, looking at the effect of low dose i.p LPS (100µg/kg) on C57BL/6 mice. Immunohistochemistry was utilised to investigate the expression of p65, two hours following this immune challenge. At this time point, differences were exhibited near blood vessels in the thalamus, hippocampus and the hypothalamus between treatment groups. In saline treated mice, NF-κB p65 expression, was contained to the cytoplasm. However, a LPS inflammatory challenge, resulted in nuclear localisation at the cerebral vasculature, with p65 staining evident in this area within two hours of an immune challenge (Skelly et al., 2013).

In this current study, the NF-κB component, p65, was investigated in relation to the effect of low dose i.p LPS administration at CT15. It was discovered that there was significant upregulation of the p65 expression in the SCN, in comparison to control. Significant upregulation of p65 expression was displayed in the shell as a consequence of low does LPS. However, this effect was not seen between both treatment groups in the PVN. In contrast, the component of the NF-κB, p-IκB, had no significant alterations of p-IκB expression in the SCN as a whole, or in either of the two sub regions of the SCN, nor was there significant change in the PVN as a result of low dose LPS. Other studies found that there was upregulation of IκBα mRNA expression in the PVN after a low dose

LPS treatment. Quan and colleagues also established that 100µg/kg of LPS along with 50mg/kg DEX increased the expression levels of IκBα mRNA further (Quan et al., 2000).

LPS has been shown to modulate clock gene expression in the SCN and PVN in WT mice, as it induces PER2 expression in the SCN and PER1 expression in the PVN (Paladino et al., 2015). There was no alterations when investing the role low dose LPS administration had on clock gene PER1 expression on the neurochemistry of the SCN at CT15 when examined 4 hours post treatment in this current experiment. Other studies also established similar findings with peripheral inoculation of low dose LPS at ZT15. The core and shell of the SCN, displayed comparable PER1 expression between both treatment groups, 90 minutes post treatment. Furthermore, there was diminutive difference between LPS and vehicle treatment groups when analysing *Per1* mRNA levels (Paladino et al., 2015). Additionally, it was discovered that low dose (50µg/kg) LPS administration at ZT22 displayed no alteration to *mPer1* mRNA in the SCN of CRF neurons, in comparison to controls (Takahashi et al., 2001).

In this current study, the PVN exhibited significant upregulation of PER1 expression after low dose LPS treatment. Comparable findings were visible in Paladino and colleagues work, where they found altered PER1 expression in the PVN at ZT15 after low dose LPS treatment (Paladino et al., 2015). Other studies displayed upregulation of *mPer1* mRNA in the PVN of CRF neurons, 1 hour after receiving low dose LPS treatment at ZT22. This alteration was not displayed 3 hours later, as *mPer1* expression returned to basal level. Therefore, there is indications that *mPer1* gene expression in the PVN is connected to stress-induced responses. (Takahashi et al., 2001).

3.4.3 Expression of IEG's and NF-κB components in the mid subjective day and night in the SCN and the PVN

When examining IEG's such as c-Fos expression, it is usually located in the ventrolateral region of the SCN (vlSCN), with peak c-Fos peptide expression between external time 8-11 under an LD cycle (Colwell & Menaker, 1992). There is change in c-Fos production in basal neuronal activity across the 24 hour cycle in the SCN, with studies reporting that c-Fos expression in the vlSCN tends to be high during the light cycle and low during the dark phase (Aronin et al., 1990; Colwell & Foster, 1992). In our findings it was discovered that during the mid-subjective day c-Fos expression was predominantly

in the dorsomedial region of the SCN (dmSCN), whereas during the mid-subjective night expression was located in the vlSCN. However, administration of LPS at ZT6, resulted in c-Fos expression occurring also in the vlSCN. There was upregulation of FOS expression in the SCN at this time point as a consequence of LPS, however, it was not a significant increase. When analysing c-Fos expression at ZT18, both treatment groups had significantly lower levels of expression during the dark phase in comparison to the light phase. LPS treatment did result in significant upregulation of c-Fos expression when compared to the control group in the mid subjective night. There was significant upregulation of FOS expression in the PVN after LPS treatment at ZT6 and ZT18.

The second IEG transcription factor examined in this experiment was Egr-1. There were higher levels of Egr-1 expression in the SCN exhibited during the mid-subjective day in comparison to the mid-subjective night. However, interestingly there was high levels of Egr-1 expression displayed in the SCN in control subjects which would not have been expected. At ZT6 there is an extremely high level of Egr-1 expression in the SCN, which is conflicting with other studies, which have minimal expression seen at this time point in WT vehicle subjects (O'Callaghan et al., 2012). Findings in this current experiment may be due to the fact that the mice utilized were B6.129S6-*Per2*^{tm1Jt}/J. These mice 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). It is thought that behaviour or neurochemistry should not be affected in B6.129S6 mice in comparison to WT mice (Yoo et al., 2004), however, when we were monitoring wheel running behaviour in these mice, they displayed a FRP that was advancing while other FRPs exhibited delays daily (n=16). Therefore, in that behavioural experiment, we reverted back to WT mice. This leads us to wonder if the high EGR-1 expression displayed in control mice obtained at ZT6, is caused by this mouse model. When the PVN was investigated there was significant upregulation of Egr-1 expression displayed as a result of LPS administration when compared to controls at both the mid-subjective day and night.

The NF-κB component, p-IκB, was also examined at ZT6 and ZT18 for variations of expression in the SCN and PVN after LPS administration. There was significant upregulation of p-IκB expression in the SCN at ZT6, but this was not displayed at ZT18, 24 hours post LPS treatment. A similar finding was discovered using the BALB/C-Tg

(IκBα::LUC) reporter mice. An immune challenge using the TLR5 agonist, CBLB502, was administered at ZT6, resulting in a significant level of NF-κB activation in the liver, which was not displayed at ZT18. This experiment was monitored using *in vivo* luciferase imaging, and Spengler also displayed that NF-κB activation peaked at ZT6, when analysing the effect of an immune challenge over a 24 hour period (Spengler et al., 2012). In this current study, LPS did not cause any significant alterations in p-IκB expression in the PVN in comparison to vehicle at either time point, although, upregulation was exhibited at both time points.

Finally, the NF-κB signalling component, p65, was also analysed in the middle of the day and night for alterations in relation to expression in the SCN and PVN after LPS treatment. Similarly to p-IκB, there was significant upregulation in the SCN at ZT6 as a result of LPS administration. However, there was no significant effect exhibited as a result of an immune challenge, administered in the mid subjective night. This again corresponds to the (Spengler, 2012) study, emphasising how an immune challenge seems to cause a higher NF-κB activation during the mid-subjective day in comparison to night, in the SCN. However, LPS did not cause any significant alterations of p65 expression in the PVN in comparison to vehicle at either time point.

3.4.4 The effects of acute LPS treatment on SCN expression in mPer2^{Luc} knock-in mice using double immunostaining

The microglial marker, IBA-1, was used to investigate the effect long lasting post-septic had on WT mice in our laboratory 3 months following LPS induced sepsis, in the mid subjective day (ZT5-8). Findings, discovered significant upregulation of microglial in the SCN and hippocampal subfields, such as DG, CA1 and CA3 in these post-sepsis mice (Anderson et al., 2015; O'Callaghan et al., 2012). Another studied revealed, that two months following LPS treatment, sustained microglial activation was exhibited in the frontal cortex (Weberpals et al., 2009). Furthermore, a study undertaken in 8 week old mice, also displays upregulation of IBA-1 expression in the following brain regions, the striatum, frontal cortex, medial septum, and the hippocampal subfields (DG, CA1 and CA3) after an acute LPS administration (Noh et al., 2014).

In this current study, a qualitative method was used to analysis the double immunostaining technique looking at IBA-1 and p65 expression in the SCN, 24 hours

following LPS (5mg/kg) treatment at ZT8. There was strong upregulation of IBA-1 and p65 post LPS treatment in comparison to vehicle, shown in the photomicrographs. Also displayed in this experiment when studying the IBA-1 expression post LPS treatment, was that the morphology was associated with activated microglia in SCN. This indicates that the SCN responds to a peripheral immune challenge within a short timescale.

LPS treatment has been found to cause upregulation of NF-κB activity in SCN astrocytes expression both *in vivo* and *in vitro*, with an increase in p65 expression displayed (Leone et al., 2006). In this study, the upregulation of p65 expression after an immune challenge appears to be in the form of glial cells in the SCN, which would correlate to previous findings by Listwak in which minimal NF-κB activity is found in neurons, with p65 expression displayed in the cytoplasm and nucleus in very low amounts (Listwak et al., 2013). In the current study, p65 expression in control mice exhibited morphology in what is thought to be microglial cells in their resting state (Kettenmann et al., 2011). Peripheral LPS treatment in this study caused the p65 microglia to become activated, displaying a hypertrophic appearance similar to phagocytic cells (Kreutzberg, 1996).

3.4.5 Conclusion

The activation of other transcription factors apart from NF-κB such as the MAPK as a result of LPS treatment, also play a role is driving an inflammatory response (Barton & Medzhitov, 2003). TLRs triggered by LPS can also trigger intracellular signalling through MAPKs which comprises of p38, ERK1/2 and JNK, leading to rapid transcriptional activation of IL-1β, IL-6, and TNF-α (Huang et al., 2009; Ji et al., 2013; Mogensen, 2009). This may account for the fact that some components of the NF-κB pathway, such as c-Rel and p-IκK, having minimal response in relation to acute LPS administration.

NF-κB seems to play a role in mediating LPS effects on circadian rhythmicity since the NF-κB inhibitor sulfasalazine can block this pathway. This highlights an association with immune suppressors that prevent NF-κB activity by blocking the immune-mediated LPS response (Marpegán et al., 2005). Another NF-κB inhibitor, PDTC, has also been shown to be involved in preventing NF-κB activity by blocking the immune-mediated LPS response. Recent work carried out in our laboratory discovered

that the NF-κB inhibitor, PDTC, significantly reduced the effects that LPS administration caused on behaviour and on microglial in the neuroimmune system. (Anderson et al., 2015). Future work that could be undertaken, is to assess whether the NF-κB inhibitors, sulfasalazine or PDTC, can block LPS effects in relation to the upregulation of NF-κB components -p65 and p-IκB- in the SCN, which we observed in our study.

As discussed in the previous chapter, extra care must be taken in interrupting results of neuronal NF-κB activity in the CNS, with numerous studies using antibodies that are not selective for p65 (Herkenham et al., 2011). Therefore, since antibodies can lack efficacy and specificity; experimental results can suffer as a consequence, in terms of perpetuating flawed findings. The more recent study (Listwak et al., 2013) found that there is minimal NF-κB activity in neurons even after LPS treatment, concluding that there is a lack of evidence that the NF-κB pathway plays any major role in the circadian clock, with a peripheral immune challenge only seen to cause an upregulation of p65 and p-IκB microglial cells in the SCN.

Chapter Four

Assessing how ageing and neuroinflammation impact on the roles of NF-κB signalling in the SCN clock and PVN.

4.1 Introduction

It has been recognised that ageing is concomitant with neuroinflammation which is thought to be involved in the decline of neuronal function (Godbout & Johnson, 2009). The NF-κB signalling pathway is implicated in ageing, (Tilstra et al, 2011) with ageing thought to be involved in increased brain NF-κB activity (Toliver-Kinsky et al., 1997). Ageing in humans and mammals may impact on NF-κB function in the SCN in the hypothalamus. NF-κB plays is vital in the body's immune system, therefore, targeting NF-κB by using inhibitors have been investigated as an approach to treating acute and chronic inflammatory conditions, and it is thought that this method may help in the treatment of neurodegenerative diseases such as AD and PD (Flood et al., 2011; Chen et al., 2005; Sung et al., 2004).

The aim of this chapter is to examine whether there are interactions in the circadian timekeeping system between aged and young mice, and also to assess whether ageing or neuroinflammation will alter the signalling properties of NF-κB in the SCN and PVN.

4.1.1 Neuroinflammation in the normal ageing brain and neurodegenerative brain

In the normal healthy ageing brain, a low-grade neuroinflammation is displayed (Godbout & Johnson, 2009), with astrocytes and microglial becoming more active or reactive as ageing progresses (Godbout & Johnson, 2006). Infection or a traumatic brain injury in an otherwise healthy ageing brain can result in a higher risk of memory deficiencies when compared to the younger adult brain (Barrientos et al., 2015). The normal healthy hippocampus of aged rats have primed microglial, so if an immune challenge does occur through infection (Godbout et al., 2005), surgery (Rosczyk et al.,

2008), or a stressor (Buchanan et al., 2008), the inflammatory response is exaggerated in comparison to younger adult rats (Barrientos et al., 2015).

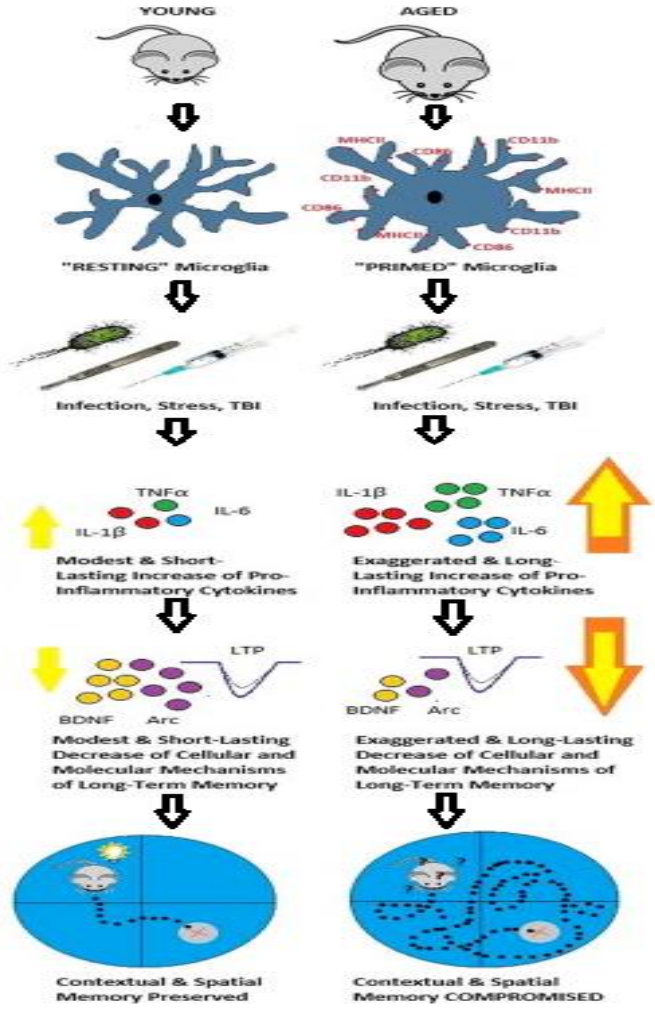


Figure 4.1: The effect an immune challenge has on microglial of young adult and aged animals, adapted from (Barrientos et al., 2015). Pro-inflammatory cytokines are released for roughly 24 hours to deal with the immune challenge. There is very little memory impairment in these young animals with LTP, BDNF and Arc attenuated slightly. However, aged microglia are primed displaying markers of activation including MHCII, CD86 and CD11b, ready for a subsequent challenge. When this challenge occurs, aged microglia have an increased neuroinflammatory response. In this instance, pro-inflammatory cytokines are released lasting over a week. LTP, BDNF and Arc are decreased significantly, and long term contextual and spatial memory is compromised.

A lot of emphasis has gone into the role neuronal loss plays in ageing. Equally, glial cells can also account for the development of senescence. Neuroinflammation in ageing is caused by microglial activation and the production of inflammatory cytokines leading to deficits in synaptic function which results in impaired learning and memory.

As discussed previously ageing results in cognitive decline with microglial activation playing a role. Even though microglia activation is vital in response to CNS injury there is also negative consequences resulting from over-activation of microglial, where releasing pro-inflammatory and cytotoxic factors for example Il-1 and nitric oxide can play a factor in neurodegenerative diseases (Lehnardt et al., 2003). Microglia can cause neuron damage and over production of microglia can result in uncontrolled neurotoxicity (Qin et al., 2007). Studies confirm a higher increase in pro-inflammatory cytokines and microglial cells in the brain after an immune challenge such as LPS administration in aged mice in comparison to young adult mice. Immunohistochemistry studies displayed a higher level of microglial cells in the hippocampus areas of aged mice including the dentate gyrus, CA1, CA2 and CA3 regions with an increase in IL-1β following LPS treatment. There was also disruption to the hippocampal processing in aged mice when compared to young adult mice (Chen et al., 2008).

The SCN's anatomy and physiology are altered in the normal healthy ageing brain and even more so in neurodegenerative brain due to molecular abnormalities (Biello, 2009; Harper et al., 2005). The responsible mechanisms for SCN discrepancies in ageing are not yet fully understood. The lifespan of *Bmal1*$^{-/-}$ KO mice is significantly decreased, and they also develop age-related complications when compared to WT mice (Kondratov et al., 2006). The consequences of ageing at a molecular level are, diminished expression of *Clock* and *Bmal1* in the SCN of rodents, which form part of the molecular machinery of the circadian clock (Kolker et al., 2003; Kolker et al., 2004). Kolker's laboratory discovered that although there were alterations of *Clock* and *Bmal1*expression over 24 hours in DD, between both aged groups, this was not presented in *Per* genes. However, there was a reduction in Per1 expression in the SCN following photic stimulation (Kolker et al., 2003). Asai and colleagues showed that circadian expression of rPer1, rPer2 and rCry1 mRNA were similar in the SCN and PVN of both young and aged rats. However, it was established that there was a decrease in Per1 and Per2 expression in the SCN of aged rats after photic induction. This may be due to impaired behavioural photic entrainment in aged rats (Asai et al., 2001).

When fetal SCN transplants were placed into aged rats it resulted in better circadian behavioural rhythms (Li & Satinoff, 1998; Van Reeth et al., 1994). This further emphasised the impact ageing has on the circadian clock. *In vitro* studies have shown that ageing also results in alterations to the phase shifting abilities of neurotransmitters in the

SCN of mice (Biello, 2009). The GABAergic network displays changes in the SCN in ageing animals in comparison to controls (Palomba et al., 2008). Biello proved that alterations at the level of the SCN in aged rodents plays a role in how it responses to certain photic stimuli and non-photic stimuli that are involved in coordinating the circadian clock. There is increased levels of pro-inflammatory cytokines for example IL-1β in normal healthy ageing (Godbout & Johnson, 2009) with the levels of cytokines dramatically increasing in neurodegenerative ageing brains for instance AD (Shaftel et al, 2008). In the normal healthy ageing brain, there is low-grade inflammation activity (Deng et al., 2006; Godbout & Johnson, 2009).

As discussed briefly, alterations in the ageing brain is thought to cause a rise in NF-κB activity. Kinsky and colleagues used electrophoretic mobility shift assays and western analysis to measure transcription factor NF-κB in aged and young rat brain tissues. They found that there was an increase in basal levels of NF-κB DNA-binding activity in the hippocampus and basal forebrain, with significant higher levels displayed at two and a half years of age (Toliver-Kinsky et al., 1997). Other studies have established that NF-κB becomes more active in the mediobasal hypothalamus (MBH) as mice age, with minimal hypothalamic NF-κB in young mice. NF-κB was labelled with GFP in young, middle-aged and aged mice, with proliferation of NF-κB exhibited as mice got older (Zhang et al., 2013). Immunohistochemistry was also carried out on the neuronal marker NeuN which showed noticeable NF-κB activation in neurons in aged mice. Whether this is due to NF-κB playing an important role in ageing or due to NF-κB being a side effect of some other aspect of ageing needs to be investigated further.

MBH-IKKβ mice had a shorter lifespan than controls and they had more of an age-related decline in terms of cognitive and physical health. In contrast, MBH-IκBα mice lived roughly 7 months longer, and had improved cognition and movement when compared to controls. When IKK-β, which activates NF-κB, was supressed in the hypothalamus in middle-old aged mice it prevented a surge of microglial cells in contrast to controls. There was a reduction in Iba-1 and TNF-α in middle-old and old aged mice where IKKβ was knockout. The IKKβ knockout mice also had cognitive and physical improvements in comparison to controls. In addition, the lifespan increased by 20-23% in ageing mice where IKK-β was suppressed throughout the brain. NF-κB counteracts the hormone gonadotropin releasing hormone (GnRH) resulting in a decrease in new brain cell growth and also halts the mice reproductive system. GnRH slowed down ageing when

injected into mice and also created the growth of new neurons (Zhang et al., 2013). For that reason, inflammation as a result of NF-κB can prompt the ageing process to begin. If GnRH can result in hippocampal neurogenesis which is important in terms of memory it may be worth investigating the role of NF-κB and GnRH in people with AD in relation to new possible treatments. The ageing process could be hastened or slowed down by either stimulating or inhibiting the immune pathway IKKβ/NF-κB in the hypothalamus in mice. These finding suggest that the hypothalamus plays a role in systemic ageing which is mediated by IKKβ/NF-κB pathway.

An increase in microglial cells occurred in the MBH as age increased. NF-κB was activated along with an over production of TNF-α in these microglial cells, suggesting that they are inflammatory. In young mice, over production of the cytokine TNF-α was generally localised to hypothalamic microglia. However, in aged mice TNF-α became widespread throughout the MBH and affected neurons. Since TNF-α can activate IKKβ/NF-κB, this may account for the IKKβ/NF-κB arbitrated microglia and neuron crosstalk which controls systemic ageing. Additionally, Iba-1 which is expressed in microglial cells was also upregulated in aged mice. Therefore, microglia in the hypothalamus use IKKβ/NF-κB in which they play a role in ageing in this brain region. (Zhang et al., 2013).

NFκB1$^{-/-}$ have a higher incidence of age related phenotypes when compared to controls at 12 months. *NF-κB1*$^{-/-}$ mice have increased brain GFAP staining in comparison to *NF-κB1*$^{+/+}$. Furthermore, the lifespan in *NF-κB1*$^{-/-}$ mice is attenuated in contrast to controls (Bernal et al., 2014). Lu and colleagues discovered that *NF-κB1*$^{-/-}$ mice aged between 6 and 10 months had significantly reduced body weight in comparison to control mice and they were beginning to die due to ageing (Lu et al., 2006).

Inflammation and microglia activation are observed in neurodegenerative diseases including PD, HD, MS and AD, and inflammation increases as each disease progresses (Boutajangout & Wisniewski, 2013; Xiao-Hua Deng et al., 2010; Ekdahl et al., 2003; Frank-Cannon et al., 2009). Furthermore, neuronal loss and tangles were observed in the SCN of AD patients (Stopa et al., 1999). NF-κB has been implicated in neurodegenerative diseases. Neurotoxicity in AD can occur due to excess NF-κB activity (Chen et al., 2005). In AD patients, activation of NF-κB has been displayed in early stage plaque formation and it plays a role in the expression of the APP gene. NF-κB is activated by Aβ, ROIs and

inflammatory cytokines in the brain which results in inducing a number of genes with relevance for AD. Therefore, NF-κB may be involved in the development of this disease. Future studies into a novel drug target may halt the progression of this disease (Kaltschmidt et al., 1997; Mushtaq et al., 2015). Using immunohistochemistry techniques the distribution of NF-κB in brain regions of AD and control post-mortem cases were examined using a polyclonal antibody against the NF-κB p65 subunit. The hippocampal formation and cerebral cortex region of AD patients displayed strong neuronal staining for NF-κB in neurons, neurofibrillary tangles and dystrophic neuritis. In the normal ageing brain there was only weak staining detected in some neurons, therefore, increased expression of NF-κB seems to occur in brain areas affected by AD (Terai et al., 1996).

4.1.2 Circadian rhythms perturbations in the normal ageing brain

Disruption of circadian rhythms in the normal ageing brain can cause increased wakefulness during the night and sleeping during the day in elderly patients. This fragmented sleep pattern attenuates their quality of life (Biello, 2009; Crowley, 2011). Perturbation of the circadian timing system as a result of ageing and neurodegeneration can impact on the ageing (Coogan et al., 2013; Wu & Swaab, 2007). The circadian timing system becomes progressively disturbed in the normal ageing human and mammal brain resulting in diminished amplitude and total activity (Hofman & Swaab, 2006; Nakamura et al., 2011; Swaab et al, 1996). There is also alterations in period length and altered phase-angle of entrainment in ageing compared to young (Hofman, 2000; Van Someren, 2000). There has been a variety of studies investigating chronic jet lag showing that dysfunction of the circadian timekeeping system can result in perturbed immune function (Castanon-Cervantes et al., 2010; Filipski et al., 2003; Filipski et al., 2006; Sukumaran et al., 2010). Chronic jet lag in aged mice increased the rate of mortality. Davidson and colleagues displayed that phase advancing the light schedule caused a higher mortality rate than when phase delaying the light schedule in aged mice (Davidson et al., 2006).

Alterations in the SCN due to ageing may cause circadian dysfunction which may result in reduced circadian neural activity. Nakamura and colleagues investigated one of the main neural outputs of the SCN, the subparaventricular zone (SPZ) in middle-aged mice compared to young mice. Findings established that there was a decrease in neural activity rhythms in the SCN and SPZ (Nakamura et al., 2011). Chronic low-grade

neuroinflammation can be seen to arise in the SCN (Deng et al., 2010). The ageing SCN has variations in the neural and temporal organisation, along with a reduced photic input to the clock (Biello, 2009). A reduced number of AVP expressing neurons in the SCN were displayed in ageing patients (Swaab et al., 1985) along with a decline of the sleep-wake cycle. Therefore, AVP may play a role in the output of the SCN (Kalsbeek et al., 2010). Ageing can affect the SCN by causing changes in serotonin rhythms (Jagota & Kalyani, 2010), altered neuropeptide content such as VIP (Kawakami et al., 1997) and AVP (Roozendaal et al., 1987), and GABAergic networks in the SCN (Palomba et al., 2008).

4.1.3 Circadian rhythms perturbations in neurodegenerative diseases

In neurodegenerative diseases such as HD, PD and AD, disruption to circadian rhythms and sleep is an early symptom (Harper et al., 2005; Hatfield et al., 2004; Naismith et al., 2011; Wulff et al., 2010). When comparing neurodegenerative diseases, such as AD, to normal ageing there is differences between their circadian rhythms. Hatfield and colleagues carried out studies using actograms to show that patients with severe AD have a more fragmented pattern of the rest/activity cycle and lower amplitude when compared to AD patients in their early stage of disease and the normal ageing brain (Hatfield et al., 2004). There is also perturbations in the phase and amplitude of the sleep rhythm (Satlin et al., 1991; Satlin et al., 1995; van Someren et al., 1996; Witting et al., 1990). As a result of these circadian alterations in AD patients, they display a greater cognitive decline, nocturnal insomnia, sleeping during the day, and confusion (Moe et al., 1995; Pollak & Perlick, 1991).

In AD patients, studies carried out showed that there is a decreased light input into the SCN. This is due to the degeneration of the retina and optic nerve which are involved in light input into the SCN (Hinton et al., 1986; Sadun & Bassi, 1990; Trick et al., 1989). This may result in the disruption to patient's circadian rhythms. In healthy aged subjects, male and female, there is a decline in AVP expressing neurons in the SCN when compared to young subjects as discussed previously (Hofman et al., 1996). However, there was a further AVP decrease reported in AD patients (Liu et al., 2000; Wu et al., 2007) and this decrease in the SCN also occurred at an earlier onset then normal ageing patients (Swaab et al., 1985). Furthermore, an attenuated amount of VIP was recognised

in the SCN in middle-aged males in comparison to younger males. In female AD patients, there was a decrease in VIP expressing neurons in the SCN when compared to controls. Therefore, Zhou established that VIP neuron expression in the SCN both was age related and AD related (Zhou, 1995). However, there has been conflicting results in relation to VIP and AVP immunoreactive neurons in the SCN of AD patients. Recent studies using immunohistochemical methods have shown that AD patients have a similar number of VIP and AVP neurons comparable to normal aged patients (Lim et al., 2014). Using other scientific methods such as *in situ* autoradiograms which count AVP mRNA expressing neurons (Liu et al., 2000) and immunohistochemical cell density expressed as a neuron:glia ratio (Harper et al., 2008), AD patients displayed attenuated levels of AVP expressing neurons in the SCN when compared to controls. The AD mouse model 3xTg also presented a decline in VIP and AVP expressing neurons in the SCN in comparison to control subjects (Sterniczuk et al., 2010). The decreases in VIP and AVP expression can change SCN neuronal function which might impact on AD patients (Hughes et al., 2004). All of these variations of the circadian system in AD patients may be due to underlying neuroinflammation.

Studies looking at circadian CLOCK gene polymorphisms linked to the fragmentation of the sleep-wake cycle in AD patients have been investigated. The 122 circadian-related polymorphisms resulted in largely negative outcomes (Yesavage et al., 2011). In other studies, research examining CLOCK gene expression was carried out across the day in the following brain areas: bed nucleus of the stria terminalis (BNST), cingulate cortex and the pineal gland in post-mortem brains of AD patients and aged subjects. There was significant diurnal variations in RNA expression levels of PER1, PER2, and BMAL1 between AD patients and aged controls proposing that the temporal synchronisation between oscillations is altered in the brain of AD patients (Cermakian et al., 2011). The alteration in clock gene coordination that was displayed in the tissues of Alzheimer's patients may play a factor in the fragmented sleep patterns that occur with AD. This study is of contrast to the work of Wu and colleagues who discovered rhythmic clock gene expression in the pineal glands of controls found no rhythmicity in AD patients (Wu et al., 2006). Investigating the role of CLOCK genes further is necessary to understand the role they may play in AD patients.

In PD patients between 60-90% reports sleep disturbances including fragmented sleep with a higher number of awakening and they experience rest tremors and dyskinesia

(Crowley, 2011; Stocchi et al., 1998; Thanvi et al., 2007). As AD and PD progresses, the altered sleep patterns worsen further, this can cause problems for families, as carers also will have disrupted sleep patterns which will impact on their own health (Wulff et al., 2010). Studies of neurological diseases such as HD, used R6/2 mice to display significant changes in the activity-rest cycle in comparison to WT. After 5 months, the HD mouse model exhibited patterns of arrhythmia throughout the 24 hour cycle (Reddy & O'Neill, 2010). An earlier study, also used the transgenic model of HD mice R6/2, Morton and colleagues established the fragmented sleep pattern which, in the end resulted in a total breakdown of circadian behaviour by week 13 (Morton et al., 2005). Sleep and circadian dysfunction does not only impact on the patient but also on many carers (Van Someren, 2000). Actiwatch-neurologica measured activity in HD patients and carers and they had similar, significantly increased nocturnal activity when compared to controls (Morton et al., 2005). Therefore, treatment to deal with sleep disturbances due to molecular abnormalities will hopefully be of benefit to neurodegenerative patients and carers. It is important to increase our knowledge of the process that causes the sleep-wake cycle disruption as this may result in improved treatments or therapies for AD, PD, and HD.

4.2 Materials and methods

4.2.1 Animals and housing

The animal models that were used in the following experiments were the male C57BL/6 strain of mouse and the B6.129S6-*Per2*^{tm1Jt}/J. The adult C57BL/6 male aged 6-8 weeks old (24-30g) were grouped house on arrival (3 per cage) from Charles Rivers, UK or Harlan, UK both designated breeding establishments. Homozygous B6.129S6-*Per2*^{tm1Jt}/J were also group housed, aged 6-8 weeks old (24-30g) and were obtained from a breeding colony that was established in Maynooth University. Behavioural analysis was undertaken by single housed mice as outlined in section 2.2.2.

4.2.2 Treatments

This chapter involves mice that have been treated with the endotoxin LPS from gram negative bacteria, Escherichia coli (Sigma, Ireland) or saline (NaCl).

4.2.2.1 Acute high grade inflammation induction

LPS was administrated into the abdominal cavity of mice. Mice were treated with a single (5mg/kg i.p dissolved in 0.9% sterile saline) injection of LPS or with an equal volume of sterile saline control (0.9% NaCl, 0.12ml). LPS treatment elicited the full spectrum of sickness behaviour evident 1 hour after treatment in these ageing mice. Roughly 10% of LPS 5mg/kg treated mice display a significant moribundity requiring euthanasia or mortality in the first 72 hours following treatment. Ageing mice display a comparable level of sickness behaviour to adult mice, though they do take longer to recover after LPS treatment (Chen et al., 2008; Godbout et al., 2005).

4.2.2.2 Chronic high grade inflammation induction

LPS was administrated into the abdominal cavity in this animal model of sepsis. Mice were treated with a single (5mg/kg i.p dissolved in 0.9% sterile saline) injection of LPS or with an equal volume of sterile saline control (0.9% NaCl, 0.12ml). LPS treatment elicited the full spectrum of sickness behaviour evident 1 hour after treatment in adult mice. Roughly 10% of LPS 5mg/kg treated mice display a significant moribundity requiring euthanasia or mortality in the first 72 hours following treatment.

4.2.3 Sepsis scoring

A sepsis score was calculated for LPS and control animals immediately after and 1 hour post treatment and subsequently at regular intervals for 24-48 hours post treatment pending on experiment. Scores were assigned on a five-point scale assessing alterations on the parameters of behaviour, appearance, dehydration and respiration. Each parameter was then graded on a scale of 0-4; 0 absent; 1, mild; 2, moderate; 3, severe; 4, very severe. The scores reverted back to zero roughly 48 hours post LPS treatment. If any animals displayed a high sepsis score or significant moribundity at this point they were humanely culled.

4.2.4 Analysis of circadian behavioural parameters in aged mice

Adult C57BL/6 male mice aged 16 months (n=6) and 3 months (n=6) were single housed in running wheels and allowed to habituate for two weeks in L: D 12:12 before experiment analysis took place. Both aged and young mice were then kept in L: D 12:12 conditions for a further two weeks to assess actogram data and circadian behavioural parameters such as rhythm amplitude and total wheel running activity undertaken. In order to assess the integrity of the retinohypothalamic tract or RHT transmission from it in ageing mice, the phase angles of entrainment were examined for each animal in both age groups under the 12:12 LD photoperiod, since alterations in RHT transmission impact upon the phase angle of entrainment. The average phase angles of entrainment for each animal was established and statistical analysis was carried out using independent t-tests.

The next step was that both groups were placed into DD for two weeks and allowed free run. Both aged and young mice were analysed for their FRP, rhythm amplitude and total wheel running activity. The Chronobiology Kit Chi Squared procedure was used to measure FRP and rhythm amplitude for all mice, and analysed using independent t-tests. To establish locomotor activity levels, the total daily wheel-running revolutions were calculated each day for all mice for 14 days in both LD and DD, using the Chronobiology Kit. The maximum wheel-running revolutions per minute observed per cycle was then averaged over the 14 day period for each animal. Statistical analysis for both groups were assessed using independent t-tests.

4.2.5 SCN neurochemistry in aged mice compared to young mice

B6.129S6-*Per2*^{tm1Jt}/J mice were group housed 3 per cage. Young mice at four months (n=8) and aged mice at 16 months (n=11) were placed in a 12:12 L: D cycle. In order to examine the effect ageing has on the SCN and PVN, both young and aged brains were perfused at ZT6 and ZT18. All brains were subsequently processed for immunohistochemistry staining (section 2.2.4) for components of NF-κB-p65, p-IκB, p-IKK and c-Rel. Quantitative analysis was carried out similar to (section 2.2.5). The mean of each group was confirmed and analysed by using a two way ANOVA. Results are stated by IOD ± standard error of mean, with $P<0.05$ accepted as statistical significant.

4.2.6 The effects of acute LPS treatment on SCN neurochemistry in aged mice

C57/BL6 aged mice (14-16 months) were group housed and allowed to habituate for two weeks in L: D 12:12 conditions prior to experiment. In order to examine the effects acute LPS treatment causes in the ageing SCN and PVN, expression of the clock gene protein PER1 and NF-κB components- p65, p-IκB- were analysed. C57BL/6 mice were treated with a single dose of LPS (n=5) (5mg/kg i.p, dissolved in 0.9% sterile saline) injection into the abdominal cavity or with an equal volume of sterile saline (n=5) control (0.9% NaCl, 0.12ml) at ZT4. Each brain was perfused 24 hours later. All brains were subsequently processed for immunohistochemistry staining (section 2.2.4). Quantitative analysis was carried out similar to (section 2.2.5). The mean of each group was confirmed and analysed by using an independent t-test. Results are stated by cell number mean value or IOD ± standard error of mean, $P<0.05$ is accepted as statistical significant.

4.2.7 The effects of chronic LPS treatment on SCN neurochemistry in aged mice

C57/BL6 mice (two months old) were group housed and administered a single dose of LPS injection (5mg/kg i.p dissolved in 0.9% sterile saline) into the abdominal cavity or with an equal volume of sterile saline control (0.9% NaCl, 0.12ml) at ZT4. After 16 months mice were perfused at ZT4 in order to assess the effects of chronic LPS treatment. All brains were subsequently processed for immunohistochemistry staining for p65 and p-IκB (section 2.2.4). Quantitative analysis was carried out similar to (section 2.2.5). Mean IOD were analysed using an independent t-test. Results are stated by IOD ± standard error of mean, with $P<0.05$ accepted as statistical significant.

4.3 Results

4.3.1 Analysis of circadian behavioural parameters in aged mice

The basic parameters were calculated in both aged and young mice under L: D 12:12 conditions. Comparison of mean amplitude between aged and young animals by independent t-test found no significant differences between both groups, with amplitude of 1640.22 ± 136.15 and 1938.35 ± 103.09 respectively ($P>0.05$). Independent t-test was

also carried out to analyse the total wheel running activity for a period of 14 days while placed under 12:12 L: D cycle. There were significant alterations seen in total wheel running activity in aged animals (11586.19 ± 1708.6) in contrast to young animals (20402.58 ± 2564.1, $P<0.05$) which is displayed in below actograms (Figure 4.2).

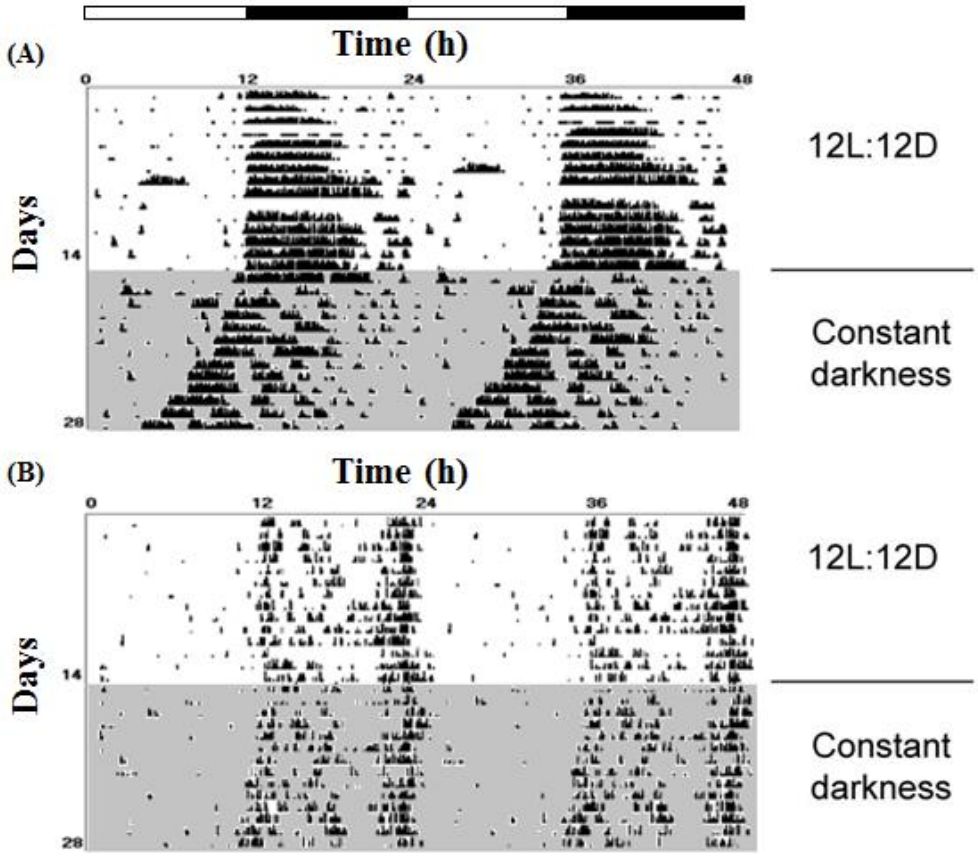


Figure 4.2: Locomotor activity rhythms under LD and DD. (A) and **(B)** are sample double plotted actograms from a young and aged animal respectively, showing their locomotor behaviour under 12L:12D for two weeks and constant darkness (DD) conditions for a further two weeks.

When placed in LD conditions for two weeks, the rhythm amplitude was attenuated in aged mice (1640.21 ± 136.15) when compared to young adult mice, (1938.35 ± 103.09). This however was not significant. There were significant differences seen in total wheel running activity in aged animals (11586.19 ± 1708.59) in contrast to young adult animals (20402.57 ± 2564.1, $P<0.05$) under LD conditions. These results are displayed in previous actograms (Figure 4.2). Furthermore, the phase angle of entrainment was significant when comparing both groups, aged (-33.86 ± 1.93) compared to young adult mice (-13.99 ± 1.65) (Figure 4.3).

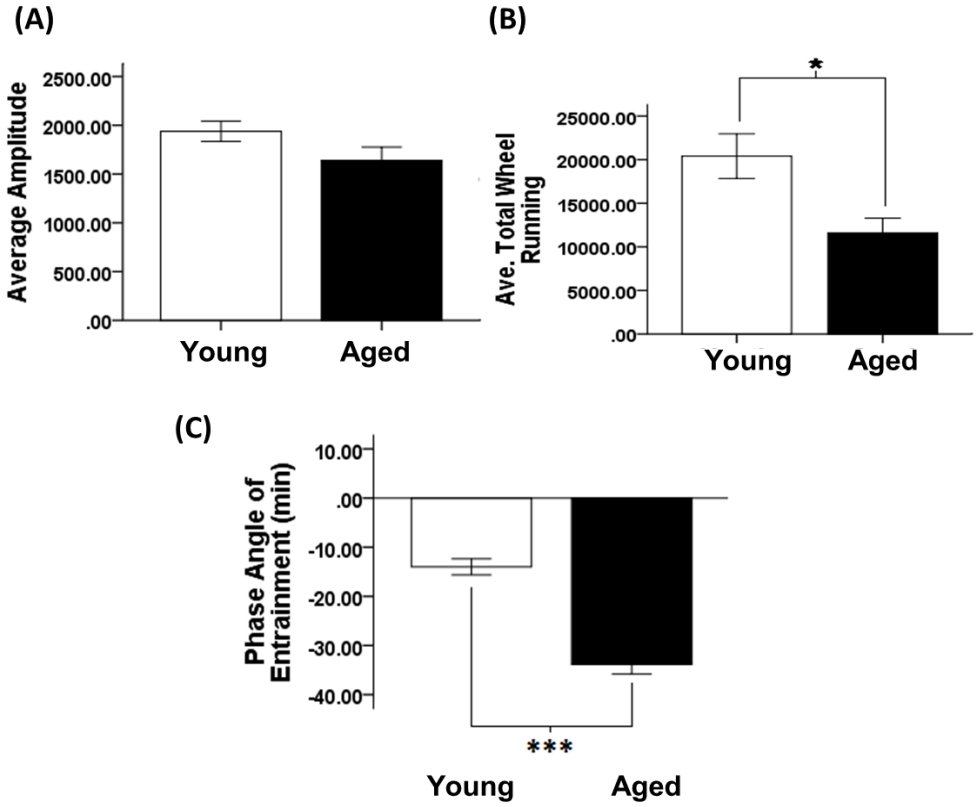


Figure 4.3: Bar graphs **s**howing **(A)** the average amplitude, **(B)** the average total wheel running activity and **(C)** the phase angle of entrainment of aged and young mice placed in LD for two weeks (n=6-7 per group). There was dampened total wheel running activity ($P<0.05$) and a delayed onset of wheel running activity ($P<0.001$) in aged mice.

When placed in DD conditions for two weeks, comparison of the mean free running period between aged and young mice was assessed using an independent t-test. Aged mice display a lengthened free running period, however, independent t-test found no statistically significant difference between aged and young, with free running periods of 23.96 ± 0.06 hrs and 23.73 ± 0.09 hrs respectively ($P>0.05$). Under housing in DD conditions, independent t-tests showed rhythm amplitude to be significantly different between aged (1113.68 ± 88.4) and young adult mice (1511.13 ± 110.1, $P<0.05$). In addition, there were significant differences also seen in total wheel running activity in aged animals (13036.19 ± 2321.2) in contrast to young adult animals (21522.57 ± 1503.8, $P<0.05$) when placed into DD conditions. These results are also displayed in previous actograms (Figure 4.4).

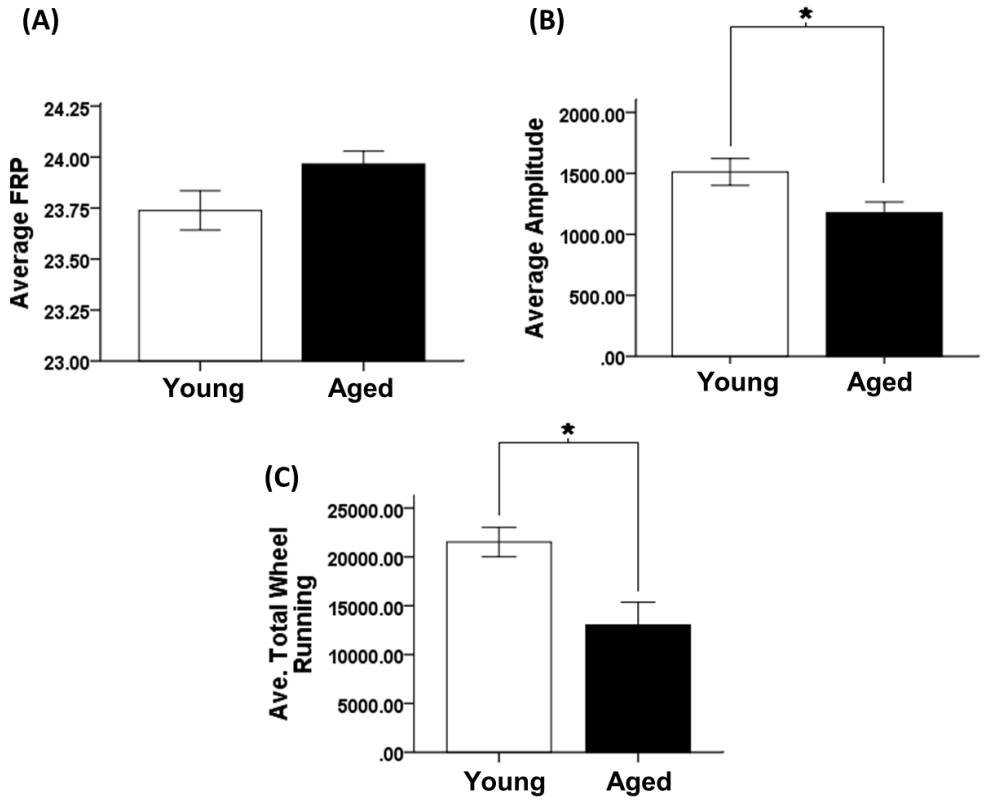


Figure 4.4: Bar graphs showing **(A)** the FRP **(B)** the average amplitude and **(C)** the average total wheel running activity of aged and young mice placed in DD for two weeks (n=6-7 per group). There was significant dampened average amplitude and reduced total wheel running activity in aged mice ($P<0.05$). There was a lengthened FRP in aged animals ($P>0.05$).

Table 5: Assessment of core circadian parameters under LD and DD conditions. Table displays rhythm amplitude, FRP and total wheel running activity for young and aged animals in LD and DD conditions. * denotes, $P<0.05$.

	Young	**Aged**
Rhythm amplitude in LD	1938.35 ± 103.09	1640.22 ± 136.15
Total wheel running activity in LD	20402.58 ± 2564.1	11586.19 ± 1708.6 *
Phase angle of entrainment in LD (min)	-13.99 ± 1.65	-33.86 ± 1.94 ***
FRP in DD (h)	23.73 ± 0.09	23.96 ± 0.06
Rhythm amplitude in DD	1511.13 ± 110.1	1113.68 ± 88.4 *
Total wheel running activity in DD	21522.57 ± 1503.8	13036.19 ± 2321.2 *

4.3.2 SCN and PVN neurochemistry in aged mice compared to young wild type mice

4.3.2.1 p65 expression in the SCN and PVN of aged and young mice at ZT6 or ZT18.

A two-way ANOVA was used to assess whether there was a main effect of either age or time on p65 expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,22}$=0.283, P=0.601) nor was there a main effect of age ($F_{1,22}$=5.65, P=0.029). There was no significant interaction effect between the two variables ($F_{1,22}$=3.619, P=0.073, Figure 4.5) for p65 expression levels in the SCN.

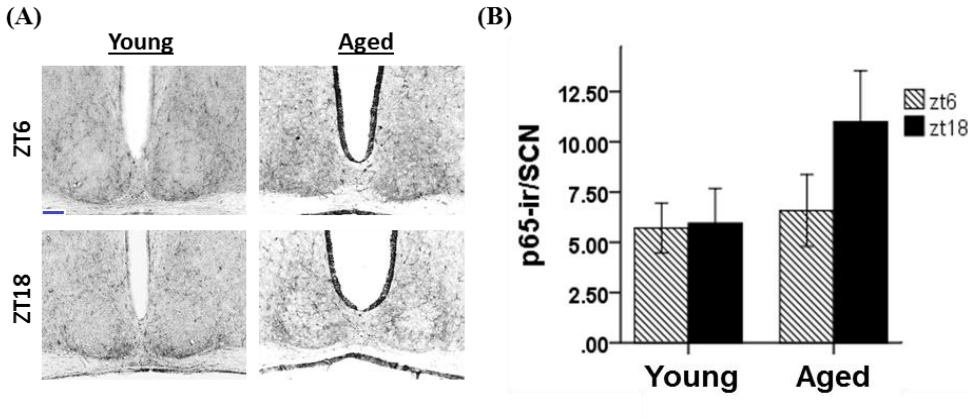


Figure 4.5: (A) Photomicrographs and **(B)** bar charts of the SCN illustrating the expression of the p65 in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is p65 upregulation in aged mice compared to control at ZT18. * denotes P<0.05.

When assessing the PVN it was revealed that there was no main effect of time ($F_{1,16}$=0.919, P=0.357). There was also no main effect of age ($F_{1,16}$=0.001, P=0.976). Finally, there was no significant interaction effect between the two variables ($F_{1,16}$=2.093, P=0.174, Figure 4.6) in relation to p65 expression in the PVN.

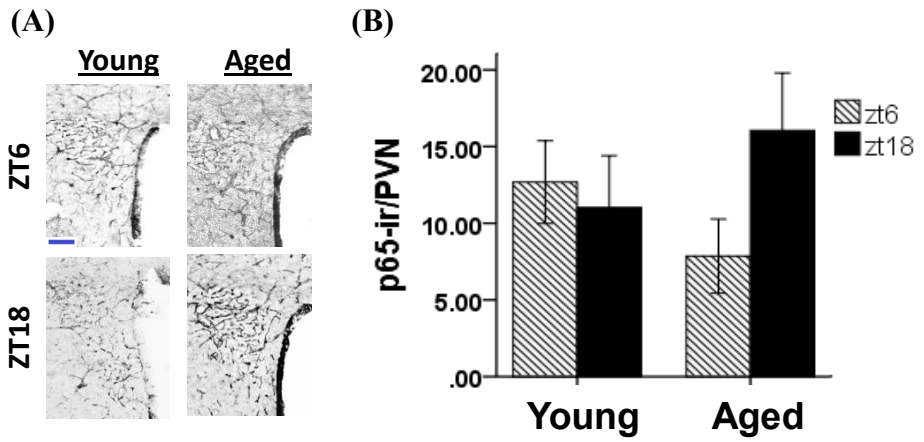


Figure 4.6: (A) Photomicrographs and **(B)** bar charts of the PVN- illustrating the expression of the p65 in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant p65 upregulation between age groups.

4.3.2.2 p-IκB expression in the SCN and PVN of aged and young mice at ZT6 or ZT18.

A two-way ANOVA was used to assess whether there was a main effect of either age or time on p-IκB expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,18}$=0.027, P=0.872). There was an overall main effect of age ($F_{1,18}$=5.98, P=0.028). An independent t-test revealed that young (10.51 ± 1.41) compared to aged (5.00 ± 1.38, P=0.017). There was no significant interaction effect between the two variables ($F_{1,18}$=0.004, P=0.949, Figure 4.7) for p-IκB expression levels in the SCN.

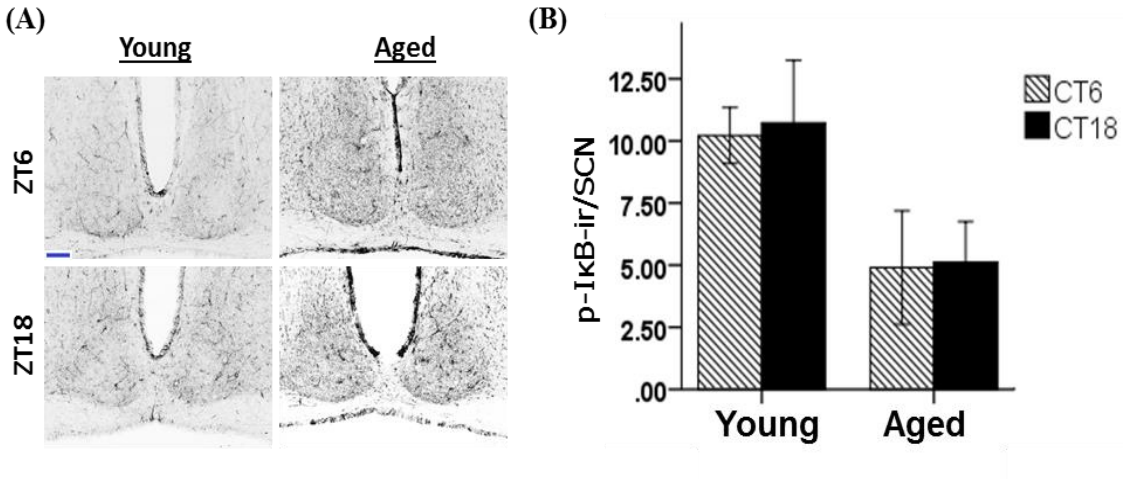


Figure 4.7: (A) Photomicrographs and **(B)** bar charts of the SCN, illustrating the expression of the p-IκB in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant p-IκB alterations between age groups.

Investigation of the PVN then took place, where there was found to be no main effect of time ($F_{1,17}$= 0.616, P=0.447). There was also no main effect of age ($F_{1,17}$= 1.150, P=0.303). A significant interaction effect was found between the two variables ($F_{1,17}$= 5.422, P=0.037, Figure 4.8). An independent t-test revealed that young (13.71 ± 4.36) compared to aged (29.18 ± 4.35, P=0.042).

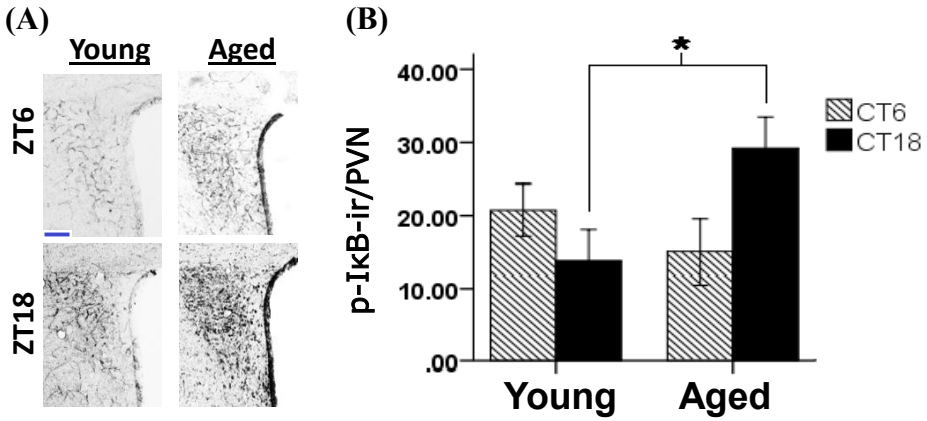


Figure 4.8: (A) Photomicrographs and **(B)** bar charts of the PVN, illustrating the expression of the p-IκB in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is significant upregulation of p-IκB between young compared to aged at ZT18.

4.3.2.3 p-IκK expression in the SCN and PVN of aged and young mice at ZT6 or ZT18.

A two way ANOVA was used to assess whether there was a main effect of either age or time on p-IκK expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,16}$=0.849, P=0.375). There also no main effect of age ($F_{1,16}$=2.88, P=0.115). There was no significant interaction effect between the two variables ($F_{1,16}$=0.126, P=0.728, Figure 4.9) for p-IκK expression levels in the SCN.

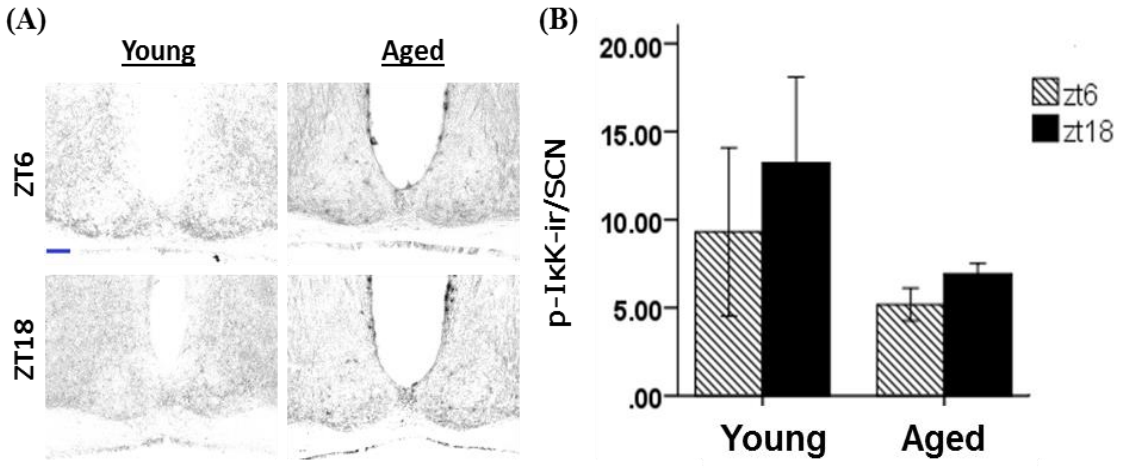


Figure 4.9: (A) Photomicrographs and **(B)** bar charts of the SCN, illustrating the expression of the p-IκK in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant alteration of p-IκK expression between both groups.

In addition, a two-way ANOVA was used to assess whether there was a main effect of either age or time on p-IκK expression in the PVN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,12}$=4.527, P=0.066). There also no main effect of age ($F_{1,12}$=0.521, P=0.491). There was no significant interaction effect between the two variables ($F_{1,12}$=1.169, P=0.311, Figure 4.10) for p-IκK expression levels in the PVN.

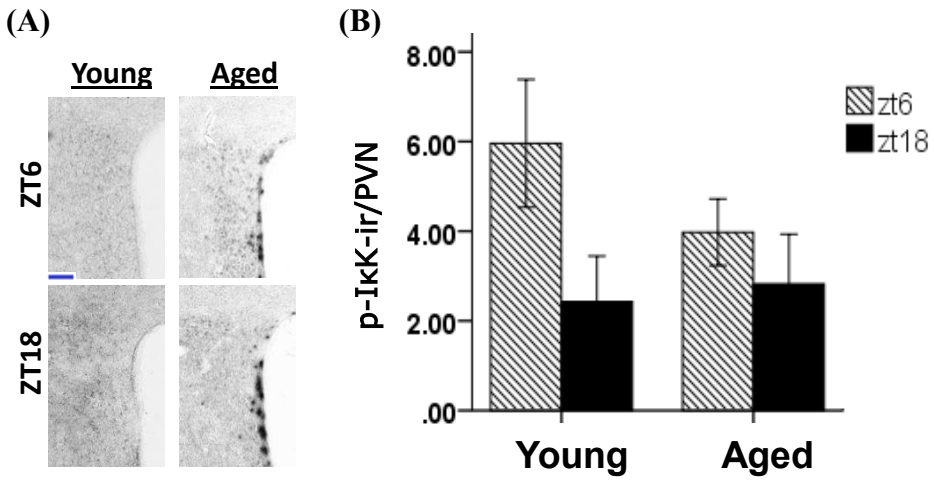


Figure 4.10: (A) Photomicrographs and **(B)** bar charts of the PVN, illustrating the expression of the p-IκK in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant alteration of p-IκK expression between both groups.

4.3.2.4 c-Rel expression in the SCN and PVN of aged and young mice at ZT6 or ZT18.

A two-way ANOVA was used to assess whether there was a main effect of either age or time on c-Rel expression in the SCN, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,18}$=2.261, P=0.155). There also no main effect of age ($F_{1,18}$=2.217, P=0.159). There was no significant interaction effect between the two variables ($F_{1,18}$=0.040, P=0.844, Figure 4.11) for c-Rel expression levels in the SCN.

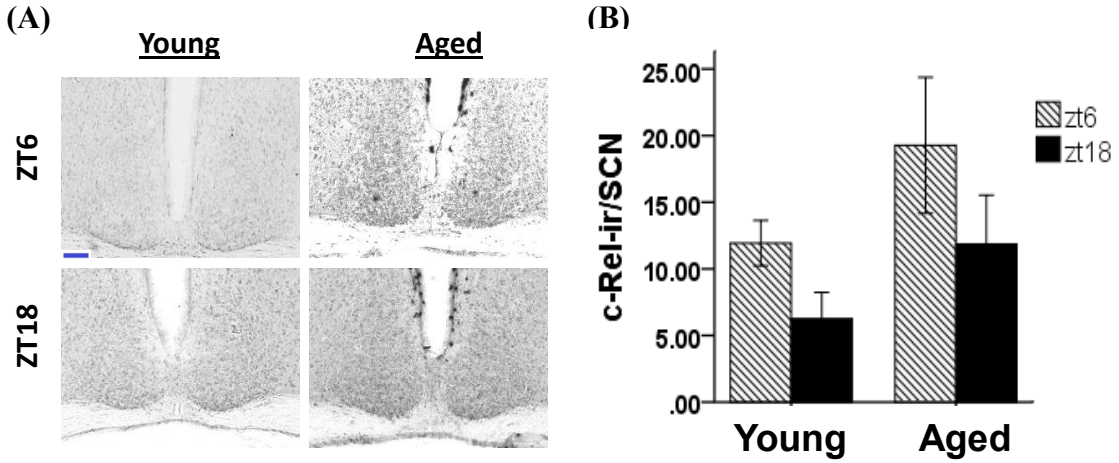


Figure 4.11: (A) Photomicrographs and **(B)** bar charts of the SCN, illustrating the expression of the c-Rel in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant alteration of c-Rel expression between both groups.

PVN analysis also used two-way ANOVA to assess whether there was a main effect of either age or time on c-Rel expression, or if there is an interaction effect between the two variables. There was no main effect of time ($F_{1,14}$=3.917, P=0.076). There was also no main effect of age ($F_{1,14}$=0.032, P=0.862). There was no significant interaction effect between the two variables ($F_{1,14}$=0.141, P=0.715, Figure 4.12) for c-Rel expression levels in the PVN.

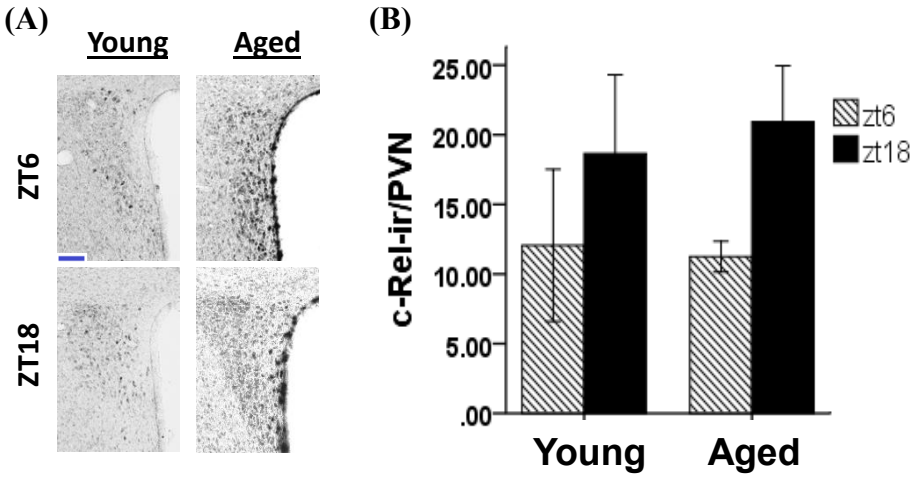


Figure 4.12: (A) Photomicrographs and **(B)** bar charts of the PVN, illustrating the expression of the c-Rel in young and aged mice at two different timepoints, ZT6 and ZT18 (scale bar 100µm). There is no significant alteration of c-Rel expression between both groups.

4.3.3 The effects of acute LPS treatment on SCN and PVN neurochemistry in aged mice

4.3.3.1 PER1 expression in the SCN and PVN following LPS 24 post treatment.

The SCN and PVN of aged mice were both examined for PER1 expression 24 hours post LPS or saline treatment. Analysing levels of PER1 immunoreactivity was carried out by manual count, followed by statistical analysis using an independent t-test to calculate whether there were alterations in the expression of PER1 in the SCN and PVN following treatment of either LPS or control at ZT4. Results indicate that there was no significant effect of treatment LPS (22.20 ± 5.46) versus saline (24.60 ± 3.94, P>0.05; Figure 4.13) on PER1 expression levels in the SCN. Further assessment was carried out of PER1 expression in the SCN subdivisions-core and shell. No significant differences were found in PER1 expression in the core of LPS mice (3.40± 1.53) when compared to controls (5.20 ± 0.86, P>0.05). There was no significant change of PER1 expression in the shell of LPS mice (18.8 ± 4.35) when compared to controls (19.4 ± 3.41, P>0.05). There was also no statistically significant effect of treatment of LPS (22.5 ± 4.11) and saline (15.75 ± 5.54, P>0.05; Figure 4.13) on the level of PER1 expression in the PVN.

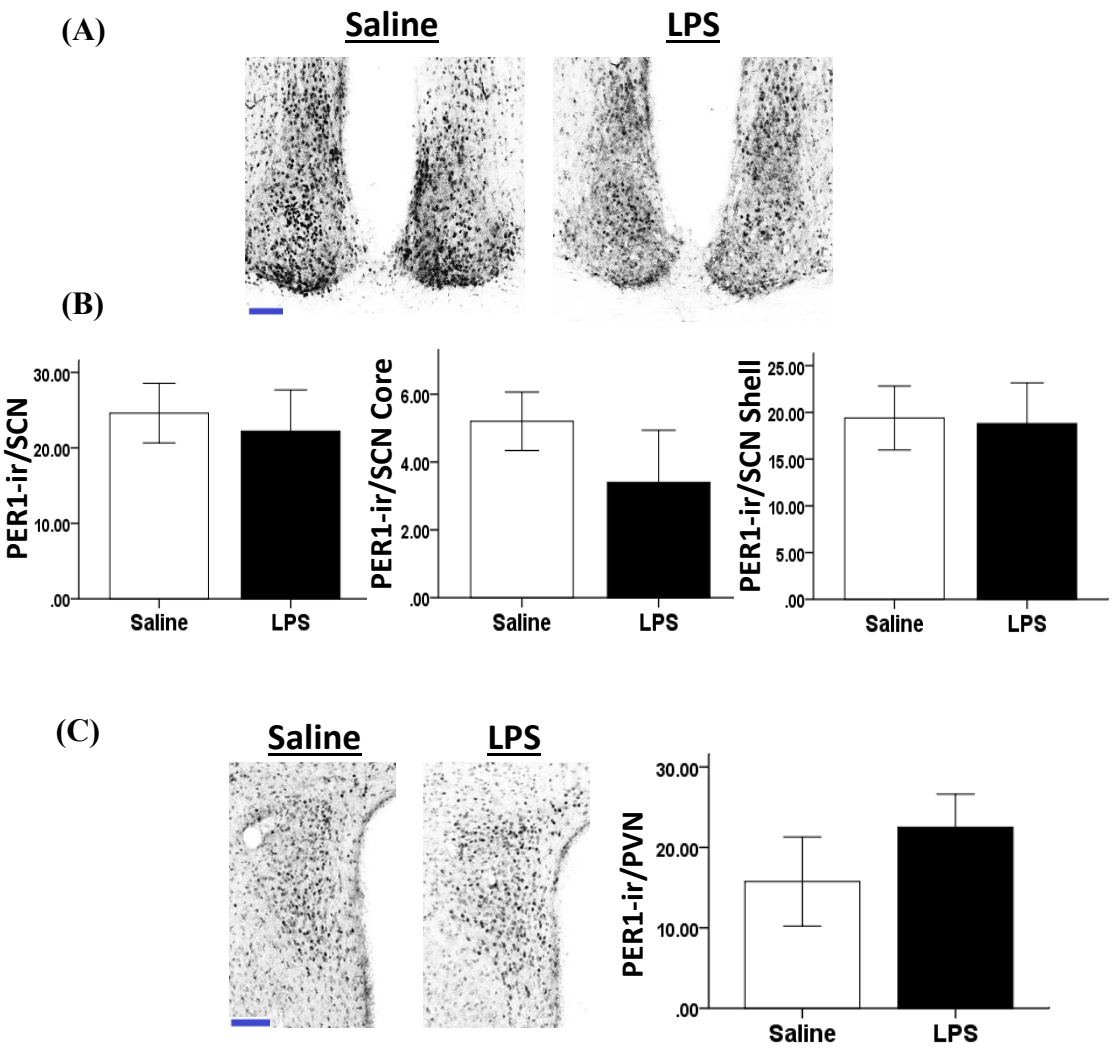


Figure 4.13: PER1 expression after 24 hours post LPS treatment in aged mice. (A) Representative photomicrographs illustrating of PER1 expression in the mid rostro-caudal level of the SCN (scale bar = 100µm) and (B) bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of the PER1 after treatment with either LPS or control. (C) Photomicrographs and bar graphs illustrating quantification of levels of PER1 immunostained cells in aged sections of the PVN of control and LPS treatment groups, 24 hours post treatment. There was no significant alterations of PER1 expression between treatment groups.

4.3.3.2 p65 expression in the SCN and PVN following LPS 24 post treatment.

The SCN and PVN of aged mice were both examined for p65 expression 24 hours following LPS or saline treatment. Analysing levels of p65 immunoreactivity was carried out by IOD. Statistical analysis using an independent t-test to calculate whether there

were differences in expression of p65 in the SCN and PVN following treatment of either LPS or control at ZT4 was carried out. Results indicate that there were significant alterations following treatment of LPS (14.37 ± 1.74) versus saline (7.06 ± 1.10, P<0.01; Figure 4.14) on p65 expression levels in the SCN. In addition, assessment of the core and shell for p65 expression was undertaken. There was no significant differences found in p65 expression in the core of LPS mice (14.96± 1.90) when compared to controls (11.04 ± 1.86, P>0.05). There was statically significant upregulation of p65 expression in the shell of LPS mice (14.14 ± 1.96) when compared to controls (5.87 ± 1.04, P<0.01). There was also a statistically significant effect of treatment of LPS (19.42 ± 3.31) and saline (10.00 ± 1.78, P<0.05; Figure 4.14) on the level of p65 expression in the PVN.

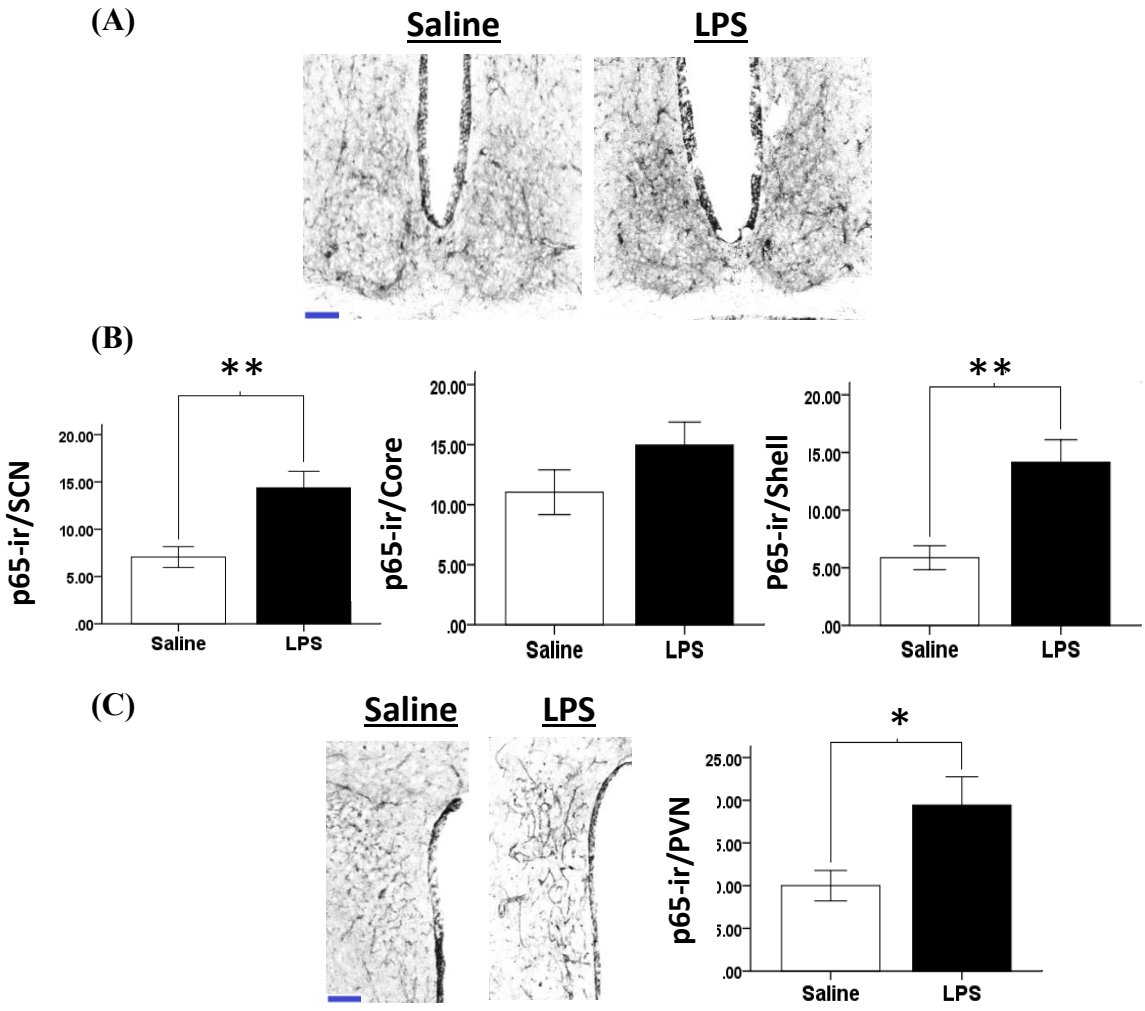
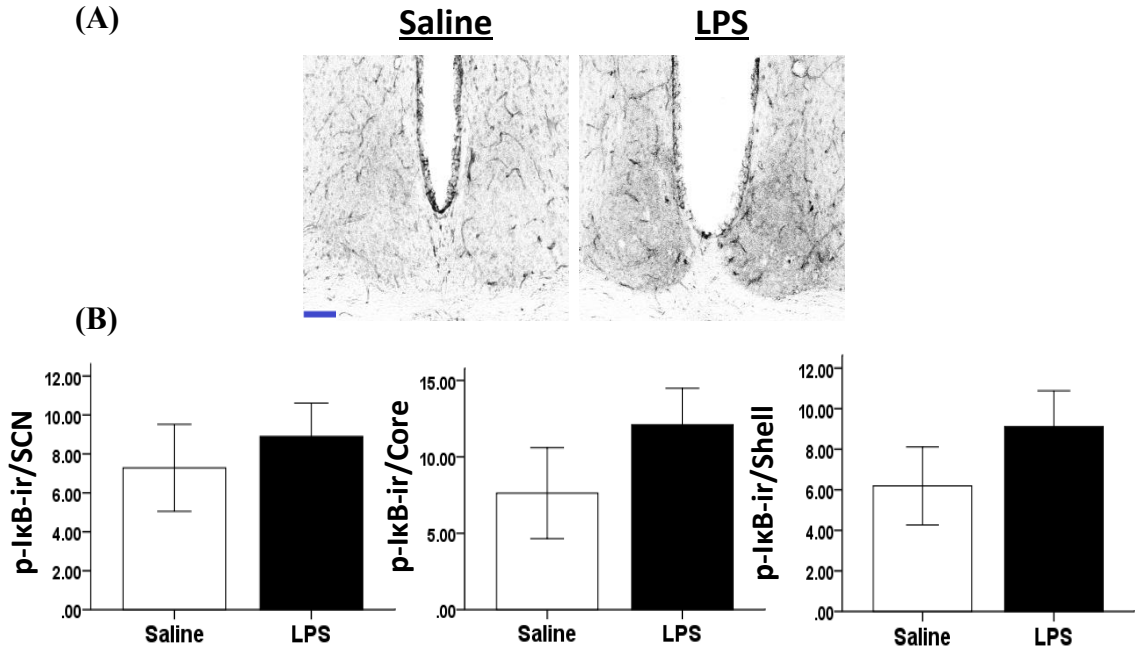


Figure 4.14: p65 expression after 24 hours post LPS treatment in aged mice. (A) Representative photomicrographs illustrating of p65 expression in the mid rostro-caudal level of the SCN (scale bar = 100µm) and (B) bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of p65

after treatment with either LPS or control. (C) Photomicrographs and bar graphs illustrating quantification of levels of p65 immunostained cells in aged sections of the PVN of control and LPS treatment groups, 24 hours post treatment (scale bar 100µm). There was significant upregulation of p65 in the mid rostro-caudal level of the SCN, the dorsomedial shell of the SCN and the PVN after LPS treatment.

4.3.3.3 p-IκB expression in the SCN and PVN following LPS 24 post treatment.

The SCN and PVN of aged mice were both examined for p-IκB expression 24 hours post LPS or saline treatment. Analysing levels of p-IκB immunoreactivity was carried out by IOD, followed by statistical analysis using an independent t-test to calculate whether there were alterations in the expression of p-IκB in the SCN and PVN following treatment of either LPS or control at ZT4. Results indicate that there was no significant effect of treatment LPS (8.90 ± 1.70) versus saline (7.28 ± 2.23, P>0.05; Figure 4.15) on p-IκB expression levels in the SCN. Further assessment was carried out of p65 expression in the SCN subdivisions-core and shell. No significant differences were found in p-IκB expression in the core of LPS mice (12.11± 2.37) when compared to controls (7.62 ± 2.97, P>0.05). There was no statistically significant upregulation of p65expression in the shell of LPS mice (9.11 ± 1.77) when compared to controls (6.19 ± 1.77, P>0.05). There was also no statistically significant effect of treatment of LPS (21.34 ± 2.46) and saline (26.06 ± 5.34, P>0.05; Figure 4.15) on the level of p-IκB expression in the PVN.



(C)

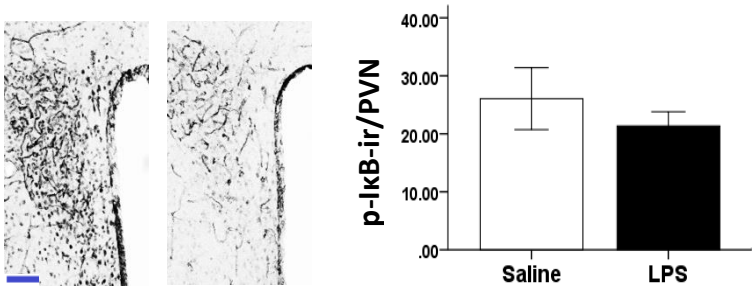


Figure 4.15: p-IκB expression after 24 hours post LPS treatment in aged mice. (A) Representative photomicrographs illustrating of p-IκB expression in the mid rostro-caudal level of the SCN (scale bar = 100μm) and (B) bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of p65 after treatment with either LPS or control. (C) Photomicrographs and bar graphs illustrating quantification of levels of p65 immunostained cells in aged sections of the PVN of control and LPS treatment groups, 24 hours post treatment.

4.3.4 The effects of chronic LPS treatment on SCN neurochemistry in aged mice

4.3.4.1 p65 expression in the SCN and PVN following LPS treatment post 16 months.

The SCN and PVN of aged mice (16 months) were both examined for p65 expression following an acute LPS or saline treatment administered at 3 months at ZT4. Analysing levels of p65 immunoreactivity was carried out by IOD. Statistical analysis using an independent t-test to calculate whether there was differences in expression of p65 in the SCN and PVN following chronic treatment of either LPS or control. Results indicate that there was no significant alterations following treatment of LPS (21.55 ± 5.25) versus saline (15.21 ± 2.45, P>0.05; Figure 4.16) on p65 expression levels in the SCN. In addition, assessment of the core and shell for p65 expression was undertaken. There was no significant difference found in p65 expression in the core of LPS mice (25.47± 4.09) when compared to controls (14.87 ± 4.31, P>0.05), nor was there significant alterations of p65 expression in the shell of LPS mice (22.44 ± 6.88) when compared to controls (15.52 ± 2.66, P>0.05). There was also no statistically significant effect of treatment of LPS (22.39 ± 4.57) and saline (11.86 ± 1.46, P>0.05; Figure 4.16) on the level of p65 expression in the PVN.

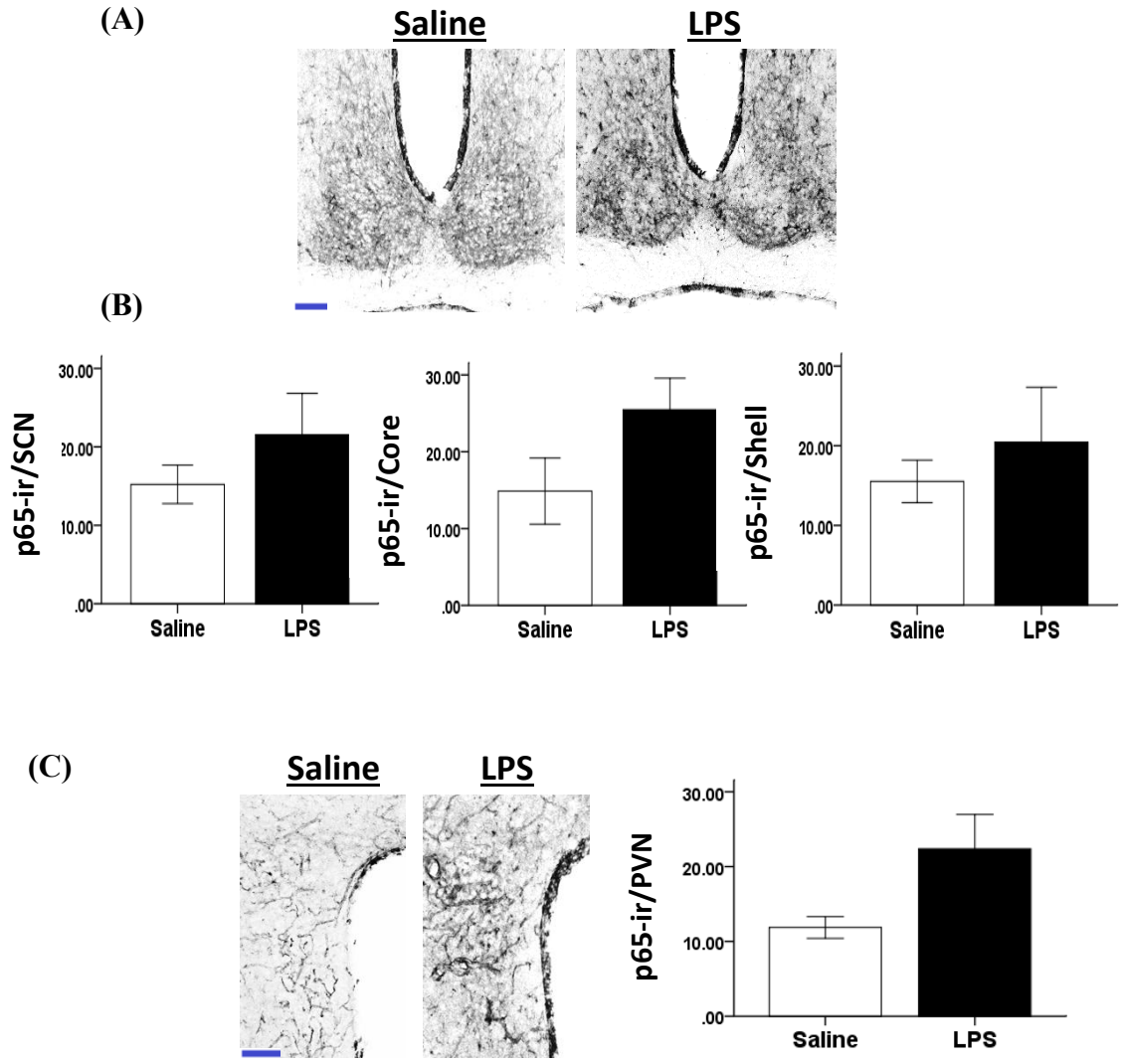


Figure 4.16: p65 expression 16 months post LPS treatment in aged mice. (A) Representative photomicrographs illustrating of p65 expression in the mid rostro-caudal level of the SCN (scale bar = 100µm) and (B) bar charts of the mid rostro-caudal level of the SCN, the SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of p65 after treatment with either LPS or control after 16 months. (C) Photomicrographs and bar graph illustrating quantification of levels of p65 immunostained cells in aged sections of the PVN of control and LPS treatment groups, 16 months post treatment.

4.3.4.2 p-IκB expression in the SCN and PVN following LPS treatment post treatment.

The SCN and PVN of aged mice (16 months) were both examined for p-IκB expression following an acute LPS or saline treatment administered at 3 months at ZT4. Analysis of levels of p-IκB immunoreactivity was carried out by IOD. Statistical analysis

using an independent t-test to calculate whether there were differences in expression of p-IκB in the SCN and PVN following chronic treatment of either LPS or control. Results indicate that there was no significant alterations following treatment of LPS (4.17 ± 1.93) versus saline (5.77 ± 0.80, P>0.05; Figure 4.17) on p-IκB expression levels in the SCN. In addition, assessment of the core and shell for p-IκB expression was undertaken. There were no significant differences found in p65 expression in the core of LPS mice (4.85 ± 1.98) when compared to controls (11.86 ± 4.47, P>0.05), nor was there significant alterations of p-IκB expression in the shell of LPS mice (3.40 ± 1.18) when compared to controls (3.18 ± 0.81, P>0.05). There was also no statistically significant effect of treatment of LPS (19.26 ± 4.66) and saline (21.61 ± 7.32, P>0.05; Figure 4.17) on the level of p-IκB expression in the PVN.

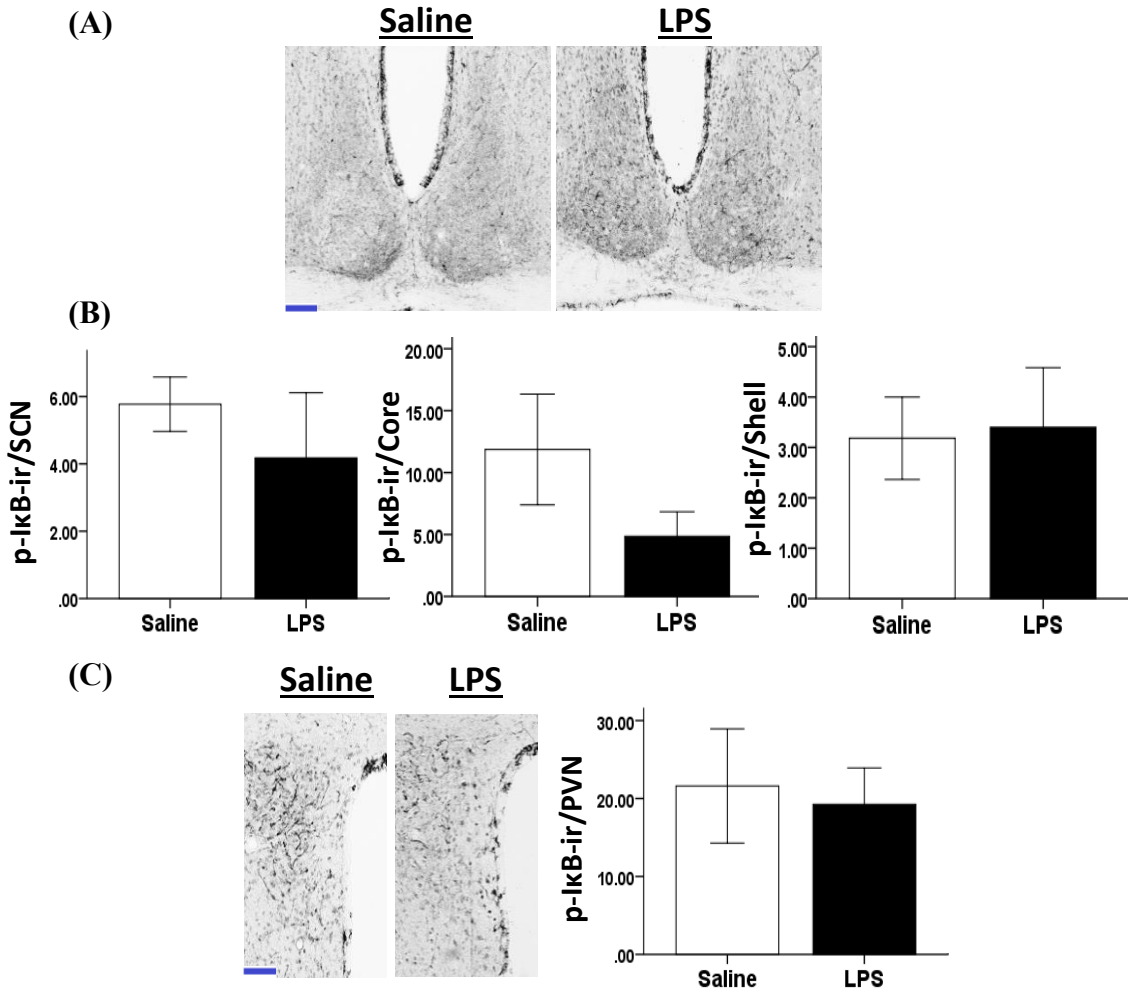


Figure 4.17: p-IκB expression after 16 months post LPS treatment in aged mice. (A) Representative photomicrographs illustrating of p-IκB expression in the mid rostro-caudal level of the SCN (scale bar = 100μm) and (B) bar charts of the mid rostro-caudal level of the SCN, the

SCN ventrolateral core and the dorsomedial shell of the SCN, illustrating the expression of p-IκB after treatment with either LPS or control after 16 months. (C) Photomicrographs and bar graph illustrating quantification of levels of p-IκB immunostained cells in aged sections of the PVN of control and LPS treatment groups, 16 months post treatment.

4.4 Discussion

The circadian clock plays a role in the synchronisation of physiology and metabolism in humans and animals, however, the circadian clock is not resilient to the ageing process which results in the decline of the circadian clocks synchronisation ability (Costa & Ripperger, 2015). Age related alterations in behavioural rhythms may be due to a disrupted central pacemaker, or may involve changes along the input pathway such as the RHT, IGL and the GHT. Deterioration in any of these input components as a result of ageing can cause circadian rhythm changes (Weinert, 2000). Finally, it may also involve reduced coupling between the SCN and its output pathways (Biello, 2009; Ruby et al., 1998). The PVN was also investigated due to the fact it is one of the main projection areas for the SCN and is implicated in circadian regulation of autonomic and endocrine function (Buijs et al., 2003; Mavroudis et al., 2013; Saper et al., 2005).

4.4.1 Examination of locomotor rhythm behaviour in the normal ageing and neurodegeneration

As discussed previously, ageing in humans and mammals can cause disruptions and deterioration in circadian rhythms, with a decrease in amplitude and activity of rhythmic behaviour. In the current study, findings showed a decrease in the total wheel running activity in aged mice when placed in LD. Similar finding were displayed in relation to activity in LD, when comparing mice aged 3 months to 18 months old (Farajnia et al., 2012). After transferring into DD, a decrease in amplitude and total wheel running activity were also discovered.

Fragmentation of sleep in ageing subjects also occurs, with difficulty staying awake during the daytime, and sleep loss during the night which results in diminished quality of life for subjects (Hofman & Swaab, 2006; Nakamura et al., 2011; Swaab et al., 1996). The fragmentation of sleep in increased further in neurodegenerative diseases such as AD (Bonanni et al., 2005; Vitiello & Borson, 2001), PD (Kudo et al., 2011) and HD,

(Morton, 2013; Morton et al., 2005; Reddy & O'Neill, 2010) and, as each disease progresses, evidently so does sleep disturbance with higher nocturnal awakening and diurnal naps (Bliwise et al., 1995). This fragmentation of activity-rest cycle sleep in neurodegenerative diseases usually leads to the institutionalisation of affected patients (Reddy & O'Neill, 2010). However, in the current study this was not recognised when analysing resting periods of normal ageing mice in comparison to young mice which can be seen in actograms (Figure 4.2). Locomotor activity is low in both groups when in their resting phase. Perhaps the low locomotor activity during the aged mice resting period was due to the fact that the mice used in this study were 16 months old. If older animals were used in this study, for example 24 months old mice, they may have had a more fragmented pattern in their resting phase, with recent findings displaying a higher incidence of 22-24 month old mice awake at the end of their resting period in comparison to controls (Farajnia et al., 2012; Wimmer et al., 2013). Furthermore, if the mice used in this experiment had been a neurodegenerative model there may also have been disturbances in their resting phase (Kuljis et al., 2012; Wulff et al., 2010). Another finding from the current study through observation from the actograms (Figure 4.2), was that there was an evident increase in fragmentation across the 12 hour active phase in ageing animals, with activity times being shorter than young adult mice. There was also longer periods of rest intervening between activity bouts, displayed in these ageing animals. Similar finding of fragmentation were demonstrated in 19-22 month old ageing mice (Valentinuzzi et al., 1997).

The double plotted actograms also demonstrated the reduction of total wheel running activity in ageing mice when compared to young mice in this current study whether in LD or DD. Other studies carried out also show a reduction in locomotor activity due to age in comparison to young mice (Farajnia et al., 2012; Godbout & Johnson, 2009). In addition, an immune challenge of LPS injections in ageing mice exacerbated locomotor activity further, causing an added significant decrease between both groups (Godbout & Johnson, 2009).

Evident change is displayed in aged subjects circadian rhythms locomotor activity when investigating the phase angle of entrainment to the LD cycle (Turek et al., 1995). Entrainment to the LD cycle is of vital importance to circadian clocks, and it can be affected by age dependent variations in period and photic sensitivity. Some studies have shown that aged hamsters exhibit earlier locomotor activity onset when light offset

occurred, when compared to young adult hamsters (Turek et al., 1995; Zee et al., 1992). Whereas, other findings suggest aged mice in LD displayed a delayed activity onset after lights turn off in comparison to controls (Possidente et al., 1995; Valentinuzzi et al., 1997). In this current study similar results were recognised when evaluating ageing mice entrained to a 12:12 LD cycle. There was a significant delay in wheel running activity onset when light offset arose in ageing mice. The significant delayed phase angle of entrainment in ageing mice is consistent with the lengthening of the FRP displayed in this current study.

Activity rhythms and circadian amplitude were studied to compare ageing in rodent subjects. The first study was undertaken in 1912 which found a decrease in amplitude as ageing progressed in rats (Slonaker, 1912.) There were also decreases found in the activity rhythm and amplitude in ageing mice and this reduction continued as ageing progressed (Valentinuzzi et al., 1997; Weinert & Weinert, 1998). Reduction in amplitude in aged animals was discovered when analysing the electrical activity rhythms in SCN slice cultures (Aujard et al., 2001; Li & Satinoff, 1998; Ruby et al., 1998; Watanabe et al., 1995). When analysing the circadian locomotor rhythms during wheel running activity in this current study, it was found that amplitude had significantly decreased in aged mice when compared to young adult mice when they were placed in DD. This result was comparable to findings by Nakamura and colleagues who also found an amplitude decline of circadian locomotor rhythms in similar ageing mice (Nakamura et al., 2011).

When examining the FRP in wheel running rhythms in the current study, there was a lengthening of the FRP in ageing mice. However, this was found not to be significant when compared to young mice in DD. This correlates to findings from previous studies. When investigating the period length in wheel running rhythms, there were no alterations between aged mice and control (Kolker et al., 2004; Wax, 1975; Weinert, 2000). There has been more inconsistent results found when investigating the FRP of wheel running in mice compared to other rodents, with some studies finding lengthened FRP's (Mayeda et al., 1997; Possidente et al., 1995; Valentinuzzi et al., 1997) and others discovering a shortening of FRP (Daan & Pittendrigh, 1976). In general, previous studies have reported that the period of the circadian locomotor activity rhythms is shortened in ageing rats and hamsters when compared to control subjects (Pittendrigh & Daan, 1974; Rosenberg et al., 1991; Witting et al, 1994). There is also variations

reported in human studies. Period lengthening was reported in one study even though it is thought that the period shortens during human ageing (Kendall et al., 2001). Whereas, other studies exhibit no significant difference of period between aged and young human subjects, with an average period of 24.8 hours (Czeisler et al., 1999).

The variations between findings when studying the affect ageing has on circadian rhythms is still unclear. Disparities between results may also be due to the fact different rodents are used in these studies. Another reason for these inconstancies may be due to that fact that studies used mice ageing from 16 months to 24 months which could be the reason for variations of FRP, re-entrainment and entrainment results. A variation in period length displayed from different studies, may also be due to the fact that some studies were not monitored for a long enough duration. Finally, different laboratories have used different experimental conditions such as altered LD cycles for example 12:12 (Valentinuzzi et al., 1997), 14/10 (Scarbrough et al., 1997; Turek et al., 1995) and 16/8 (Possidente et al., 1995) which could also impact on findings.

4.4.2 Examination of SCN and PVN neurochemistry in the normal healthy ageing mice

Reduced p50 DNA binding was displayed in aged mice when compared to young in wildtype mice (Bernal et al., 2014). Whereas other studies established that there is increased in nucleus-located NF-κB binding to DNA in the cerebellum and in the frontal cortex of aged rats. There were no alterations in the nuclear level of p50, p52 and p65 proteins components of the NF-κB displayed between the different age groups (Korhonen et al., 1997). Basal levels of NF-κB DNA-binding increased in the hippocampus and basal forebrain when comparing young and aged rats, whereas no significant difference was observed in the cortex and cerebellum between the two groups at 3 months and 30 months old respectively. When investigation took place of individual NF-κB proteins using supershift analysis by EMSA, only p65 and p50 were present in the brain areas studied. Using Western blot analysis, p65 and p50 were measured in nuclear and cytoplasmic extracts from 3 and 30 months old hippocampi. The NF-κB subunit, p65, levels increased in nuclear extracts with age, while levels attenuated in the cytoplasm. However, p50 nuclear extract levels were comparable between young and aged rats, with low cytoplasmic levels arising in both groups (Toliver-Kinsky et al., 1997).

Using immunohistochemistry in our studies we found that there was no significant difference of p65 expression between young mice and ageing mice in the SCN or PVN in either the subjective day and night. This may be due to the fact that the animals used in this study were ageing 16 months old mice, and perhaps we would have seen a significant upregulation of p65 expression had the mice been between 24-30 months old. Kinsky and colleagues had also examined NF-κB DNA binding levels of 18 month old rats and found no significant alterations compared to 3 months old rats. Therefore, the exact age may play a role in findings.

Other NF-κB proteins such as c-Rel, p52 and Rel B had no effect on oligonucleotide-binding intensity, therefore, not binding to NF-κB sequence mofit within the hippocampus and basal forebrain. Western analysis also confirmed similar results with no detection occurring for the 3 proteins (Toliver-Kinsky et al., 1997). In our study, p-IκB levels were not significantly different between the two age groups of mice in the SCN in either the subjective day or night. However, there was an upregulation of p-IκB in the PVN in the subjective night when comparing ageing and young animals. This may be due to the fact that there is an oscillation alteration at ZT18 in ageing animals when compared to controls. Furthermore, our analysis using immunohistochemistry found that the NF-κB protein c-Rel and p-IκK had low levels of nuclear staining observed in the SCN or PVN under basal conditions at both time points and age groups. To develop treatments to increase lifespan in healthy ageing, understanding the roles molecular ageing and anti-ageing mechanisms play is essential.

4.4.3 Examination of SCN and PVN neurochemistry 24 hours post LPS treatment in ageing mice

The levels of expression of NF-κB were also studied in the ageing SCN and PVN after administration of an acute peripheral immune challenge using LPS. As discussed in the previous chapter, LPS induces a systemic inflammatory response, which in turn increases systemic levels of pro-inflammatory cytokines such as TNF-α and IL-1β. NF-κB controls the transcription of genes involved in acute phase and inflammatory responses, for example TNF-α, IL-1, 2, and 6 and the immunoglobulin light chain κ gene (Toliver-Kinsky et al., 1997). Activating the peripheral innate immune system using LPS produces an exaggerated neuroinflammatory response and extends sickness behaviour

(Godbout et al., 2005a). The PVN contains the LPS receptor TLR4 (Laflamme & Rivest, 2001), therefore, the PVN may be directly stimulated by and respond to an LPS immune challenge in the ageing brain.

Studies have displayed that the use of α-tocopherol administered i.c.v for 3 consecutive days before a LPS i.p injection, resulted in reduced levels of NF-κB in the brain and also improved the recovery of sickness behaviour induced by LPS (Godbout et al., 2005b). Hence, antioxidants such as α-tocopherol can lessen NF-κB activity, and may play a vital role in attenuating inflammation in diseased aged brains.

The NF-κB component, p65, translocate to the nucleus from the cytoplasm when stimulated by LPS (Moynagh, 2005b). The SCN and PVN were sensitive to immune challenge in this study as the levels of p65 expression were significantly upregulated in the SCN and PVN in ageing mice compared to controls after 24 hours. Previous studies had established that there was an upregulation of p65 in the SCN and PVN post 24 hours treatment of LPS. A study carried out by Beynon, also discovered that there was upregulation of p65 in the PVN 6 hours post LPS treatment compared to controls, but this significant increase in p65 levels after 6 hours was not observed in the SCN. Therefore, the autonomic/endocrine response to LPS immune challenge seems to occur before the circadian clock (Beynon & Coogan, 2010). When investigating p-IκB levels after an acute peripheral immune challenge, there were no significant alterations displayed in the SCN nor was there any in the PVN in comparison to saline controls. In the previous chapter we established that p-IκB was significantly upregulated in the SCN after an LPS immune challenge in animals aged 3 months compared to saline controls, therefore, this result may be due to expression patterns changing as mice age.

The use of R6/2 mice, the transgenic model of HD, can be used to study pathophysiological alterations in the circadian clock. There was variation of expression of the circadian clock genes *mBmal1* and *mPer2* in the SCN. Firstly, the mRNA levels of *mBmal1* were reduced and lacked a significant circadian oscillation. Secondly, when monitoring the expression of *mPer2* the circadian nadir of this gene was attenuated in this mouse model compared to WT mice at week 16. There was also marked disruption of expression of the circadian clock gene *mPer2* in the striatum and motor cortex (Morton et al., 2005). IHC was used to investigate PER2 expression rhythms within the SCN, to determine if ageing affected the molecular machinery necessary to produce circadian oscillations. There was similar PER2 immunoreactivity displayed throughout the SCN at

four different time points in the 24 hour cycle in both the ageing mice and controls. Hence, ageing did not seem to cause disruption to the molecular clockwork when examining PER2 expression *in vivo*, (Nakamura et al., 2011) nor did it affect Per1 expression over a 24 hour period in hamsters (Kolker et al., 2003). Another study also established that the circadian clock gene *Per1* was elevated in the PVN following LPS administration, however, this was not seen in the SCN in young adult mice (Takahashi, 2001).

Since the PVN is thought to be involved in mediating output information communicated from the SCN (Kalsbeek et al., 2010), we examined whether there was a change in clock gene PER1 expression within the PVN following LPS administration, as this may affect rhythmic outputs of ageing animals. When analysing the SCN in our study of ageing mice compared to controls 24 hours post LPS administration, PER1 expression in both age groups were similar in the early subjective day. Comparable results were also obtained when analysing clock gene expression in the PVN, with no significant PER1 alterations displayed between aged and young adult mice. Therefore, we do not find evidence for disruption of the molecular clockwork in ageing mice after an immune challenge with this *in vivo* assay.

4.4.4 Examination of SCN and PVN neurochemistry 16 months following LPS treatment in ageing mice

Chronic inflammation is associated with normal and pathological ageing (Jurk et al., 2014). Inflammation's function is to protect the body's defence but can end up attaching itself leading to chronic inflammatory diseases (Neriah & Karin, 2011). Inflammation and microglial activation play a role in the pathogenesis of many chronic human diseases in the ageing including neurodegenerative diseases AD, HD, and PD (Nguyen et al., 2002). NF-κB activation is involved in these neurodegenerative diseases. In AD patients, activated NF-κB has been found in neurons and glial cells in Aβ plaque surrounding areas in the brain (Kaltschmidt et al., 1997; O'Neill & Kaltschmidt, 1997). Furthermore, NF-κB is activated in cultured neurons and glia by Aβ stimulation (Akama et al., 1998; Bales et al., 2000; Kaltschmidt et al., 1997). Studies have found that amyloid can be attenuated *in vitro* and in transgenic mice using NF-κB inhibitors (Chen et al., 2005; Eriksen et al., 2003; Sung et al., 2004), suggesting a role for NF-κB in the pathogenesis of AD. Targeting NF-κB has also been investigated in relation to treatment

for PD. Flood and colleagues discovered that inhibitors specific for IKKβ or IKKγ were able to inhibit neurodegeneration of TH+ DA- producing neurons in both the murine and primate models of PD. Therefore, it would suggest that targeting the NF-κB pathway to control chronic inflammation through the inhibition of the IKK complex, may be a vital therapeutic method to halt and reverse DA neuron loss in PD (Flood et al., 2011).

Chronic inflammation was administered by a peripheral i.p single dose of 5mg/kg LPS in 3 month old WT mice based on previous studies of endotoxin shock. Previous studies displayed that a single LPS treatment can cause sepsis shock resulting in long lasting neuroinflammation and progressive neurodegeneration, with upregulation of TNF-α and components of IL-1β system occurring, therefore, inducing a chronic effect as a result of acute treatment (Qin et al., 2007; Weberpals et al., 2009). Qin and colleagues injected LPS into both WT mice and mice lacking the TNFα receptor (TNF-α R1/R2$^{-/-}$). TNF-α mRNA and protein was raised for up to 10 months when examined in the brain when analysing WT mice. Furthermore, application of LPS activated microglial in WT mice. LPS administration did not activate microglial or increase pro-inflammatory cytokines in mice lacking the TNFα receptor. This proves the importance of the TNFα receptor in transferring peripheral inflammation in the form of LPS across the BBB to the brain to allow for activation of microglial and increased pro-inflammatory cytokines. When a systemic injection of TNFα was administered to WT mice it created TNFα mRNA and protein in the brain, along with pro-inflammatory factors such as IL-1β and NF-κB p65 subunit. Once again mice lacking TNFα receptors were not able to induce TNFα or other pro-inflammatory factors in the brain (Qin et al., 2007).

Our findings found that the NF-κB component, p65, was not statistically significant in the SCN and PVN, 13 months post LPS treatment in 16 month old mice. Another component of the NF-κB pathway, p-IκB, showed no significant difference in chronic inflammation in the SCN or PVN of aged mice. Similar results were obtained by O'Callaghan and colleagues when examining the chronic effects of sepsis on the SCN neurochemistry 3 months post LPS treatment. Two markers p65 and p-IκB did not show that NF-κB signalling is chronically upregulated in the SCN 3 months post LPS treatment (O'Callaghan et al., 2012). Studies carried out by the Qin group were comparable to ours, as they found no significant level of induced NF-κB p65 expression after administration of a single peripheral dose of LPS, at 5mg/kg post 10 months treatment in the brain (Qin et al., 2007). Chronic neuroinflammation caused by peripheral LPS application is

transferred from the peripheral to the brain by TNF-α (Qin et al., 2007) which may explain some upregulation of NF-κB p65 in the SCN and PVN in the above experiments. Qin and colleagues demonstrated that mice lacking TNF-α failed to display neuroinflammation in the brain following a single dose of peripheral LPS.

In conclusion, the findings from this chapter do not indicate any permutations in the signalling properties of NF-κB in either the SCN or PVN of healthy ageing mice, in either the subjective day or night. There were changes in the circadian timekeeping system between ageing and young adult mice when monitoring their wheel running activity, and this may be a result of disruption to the central pacemaker, or due to alterations along the input pathway such as the RHT, IGL and the GHT. When monitoring the effect following an acute LPS treatment on the neurochemistry of the SCN and PVN after 24 hours in ageing mice, the NF-κB subunit p65 was upregulated in ageing mice. Other components of NF-κB such as p-IκB and clock genes PER1 were not altered following an acute LPS treatment in ageing animals. Finally, when analysing acute LPS application, 16 months post treatment, there is no variations in the signalling properties of NF-κB displayed in this study in both the SCN and PVN. It would be of interest for future studies to investigate what effect older aged mice have on the normal healthy brain, as 24 month old mice may in fact have significant upregulation of NF-κB signalling components including p65, since NF-κB activity was seen to increase in the MBH the more mice aged (Zhang et al., 2013). Another future study of interest would be to see how circadian rhythms may be affected in NF-κB KO models with regard to ageing, whether their circadian parameters are diminished or stronger than WT ageing mice.

Chapter Five

Analysing PER2::LUC and NF-κB::LUC mice in the SCN using *in vitro* techniques

5.1 Introduction

Transgenic or knockout animals are commonly used tools to model inflammation and disease in the immune system using *in vivo* and *in vitro* techniques (Ngo et al., 2013; Picciotto & Wickman, 1998; Wang et al., 2013; Yoshida et al., 2014; Zhu et al., 2015). Knockout mice enable the study of a deleted or inactivated gene, by homologous recombination in certain tissues of the body (Capecchi, 2001; Sikorski & Peters, 1997; Vogel, 2007). There are multiple mutant mice available for research including various knockout and knockin mice. Humans have high levels of genetic homology with mice (Guénet, 2005; Pennacchio, 2003), therefore, these mutant mice are vital for investigating how a gene may be the cause or contributing factor to certain diseases in humans. Furthermore, some mutant mice are utilised to model neurodegenerative diseases (Aguzzi et al., 1996; Kudo et al., 2011; Lee et al., 1996; Nazem et al., 2015; Sterniczuk et al., 2010; Tilstra et al., 2011).

Alternatively, transgenic knockin reporter mice generally have a fluorescent tag, such as GFP (Kuhlman et al., 2000; LeSauter et al., 2003) or *luciferase* to produce bioluminescence (Yamazaki & Takahashi, 2005; Yoo et al., 2004), which can be excited with a bioluminescent substrate, and leads to co-expression of a light signal and a target gene, which has been inserted into their genome. Using PER1::GFP transgenic mice, it was established that GFP expression is rhythmic in the SCN, both *in vivo* and *in vitro* (LeSauter et al., 2003). Real-time luminescence monitoring of gene expression rhythms, using luciferase, was first carried in plants and cyanobacteria (Kondo et al., 1993; Millar et al, 1992). The enzyme luciferase, emits lights, by catalysing the oxidation of the substrate luciferin to oxyluciferin (Welsh et al., 2005). For this reaction to occur, it requires ATP, O_2 and Mg^{2+} (Aflalo, 1991; Baldwin, 1996; de Wet et al., 1987; McNabb et al., 2005; Thompson et al., 1991), and this reaction result in the release of a photon at 562 nm in 90% of catalytic series (Aflalo, 1991). Luciferase is known to have a moderately short half-life in mammalian cells, 3-4 hours (Leclerc et al., 2000). It has

become a standard method for both *in vivo* and *in vitro* reporting of transcriptional activity in mammalian cells (de Wet et al., 1987). Luminescence reporting is a useful system for analysing circadian studies. It is utilised in non-invasive assays of circadian oscillations, and it decreases the number of experimental animals necessary for each study. Cultured SCN explants have been reported to oscillate for up to nearly 2 years (Yamazaki & Takahashi, 2005). Several luminescent reporter lines using the firefly *Luc* gene, have been generated for the study of circadian rhythms *in vivo* and *in vitro*, including PER1::LUC rats, PER1::LUC mice and c-Fos::LUC mice (Asai et al., 2001; Geusz et al., 1997; Wilsbacher et al., 2002; Yamaguchi et al., 2000; Yamazaki et al., 2000).

PER2::LUC mice are generally utilised as reporter mice to investigate the circadian expression of the *PER2* gene. The luminescence can be measured by using media which contains luciferin substrate, which correlates with the expression of the *PER2* gene. Photomultiplier tubes (PMTs) are regularly used to measure the luminescence in either tissue or cell cultures (Yamazaki & Takahashi, 2005). In this chapter investigations took place using real-time luminescence reporting of circadian gene expression in two mice models, PER2::LUC knockin mice and NF-κB::LUC mice. Studies monitoring the effects of either an immune challenge, ageing or NF-κB inhibitors had on SCN slice cultures were carried out.

The PER2::LUC mice model is widely used in chronobiology in recent years to great effect. This model has a firefly luciferase (*luc*) gene is inserted in-frame into the 3' end of the endogenous *mPer2* gene, between exon 23 and the three primed untranslated region (3'UTR) of the B6.129S6-Per2^{tm1Jt}/J. Expression of mPER2::LUC fusion protein in real-time can be seen, with the SCN and peripheral tissues in explant cultures displaying robust and self-sustained circadian rhythms for up to 3 weeks. Mice that contain the PER2::LUC fusion gene displays similar circadian rhythms and also behavioural phase shifts, as a result of light, with WT mice, therefore, normal clock function is not altered in this knockin mouse model. The PER2::LUC knock-in mice are a valuable strain to show real-time reporting of circadian gene expression. Circadian patterns of Per2::Luc fusion protein expression is exhibited in the SCN along with peripheral tissues including cornea, liver, pituitary, kidney, and lung (Yoo et al., 2004).

Another useful mouse model was established for monitoring NF-κB activation *in vivo* (Carlsen et al., 2002). This transgenic NF-κB::LUC mice model contains the firefly (luc) gene which is driven by two NF-κB binding sites from the kappa light chain

enhancer in front of a minimal fos promotor (Voll et al., 2000). Therefore, this knockin mouse model expresses luciferase in all cells and tissues where NF-κB is present.

Real-time *in vivo* imaging can be carried out in a similar NF-κB knock-in mouse containing three NF-κB binding sites from the kappa light chain enhancer in front of a minimal fos promotor. This mouse model by i.v injection of luciferin, resulting in a bioluminescent reaction due to the presence of NF-κB transcriptional activity in tissues and cells (Mann, 2002). Carlsen and colleagues established that administration of LPS or TNF-α resulted in a rise in luminescence in organs including skin, lungs, spleen, and the small intestine. The liver, kidney, and heart also was affected however, exhibiting less NF-κB activity. Additionally, this study involving *in vivo* imaging displayed chronic inflammation in the form of NF-κB activity in joints, which was comparable with R.A (Carlsen et al., 2002).

Bioluminescence expression can be obtained also from peripheral mammalian oscillators including the liver, lung and pineal (Guenthner et al., 2014; Pezük et al., 2012; Yamazaki et al., 2002). When NF-κB activity was monitored in untreated living mice following i.v. injection of luciferin, luminescence was identified in the lymph nodes, the thymus and along the small intestine (Carlsen et al., 2002). Circadian rhythm can be observed using a luciferase reporter system, with bioluminescence remaining rhythmic for between 1 to 2 weeks in Rat-1 cells transfected with an *mBmal1::luc* reporter and primary fibroblasts cultured from PER2::LUC knockin mice. Circadian rhythm dampening was exhibited in these peripheral oscillators. This is due to the fact that peripheral cells need to be resynchronised following a few days in culture, to be able to retain circadian rhythmicity (Welsh et al., 2004).

Circadian expression of clock genes has been displayed in various peripheral tissues including the heart, liver, lung, kidney, pineal, pituitary, and cornea, even in the absence of the SCN (Abe et al., 2002; Carlsen et al., 2002; Gibbs et al., 2009; Gibbs et al., 2014; Li et al., 1999; Tahara et al., 2012; Yamazaki et al., 2002; Yoo et al., 2004). Studies have shown how isolated hippocampal microglia display rhythmic expression of circadian clock genes (Fonken et al., 2015). Further studies, using PER2::LUC mice, showed how hippocampus cultures isolated from the SCN displayed rhythmic *Per2* expression (Wang et al., 2009), therefore, this brain region is able to produce independent

circadian oscillations. However, it has been established that the overall hippocampal functioning still is dependent on SCN input (Phan et al., 2011).

Certain tissues, circadian rhythm expression does not continue under constant conditions without the input of the SCN (Balsalobre et al., 2000; Yamazaki et al., 2000). With regard to whether the liver can maintain circadian rhythms independently to the SCN, reports have been varied. Researchers analysing the liver in rodents *in vivo*, have acknowledged that non-photic stimuli, such as feeding, during their normal fasting time can entrain circadian rhythms in this organ. This is independent from the SCN as no phase shift was observed in the master circadian clock (Stokkan et al., 2001). Additional studies carried using *in vitro* techniques, established that liver explants from PER2::LUC transgenic mice expressed circadian rhythms for over 20 days *in vitro* (Yoo et al., 2004) while PER1::LUC rats maintained rhythmicity for approximately seven cycles in culture (Yamazaki et al., 2000). However, certain studies suggest that culture preparations and surgeries might result in the synchronising of liver explants (Guo et al., 2006; Saini et al., 2013; Tahara et al., 2012).

Aims of this chapter are to:

- Provide corroborative evidence for roles of NF-κB signalling in the circadian timekeeping system, using both PER2::LUC and NF-κB::LUC mice by:

1. Investigating how pharmacological perturbations of the NF-κB system as a result of an immune challenge may impact on *Per2* expression SCN slice cultures.

2. Examining whether NF-κB activity is required for rhythmic *Per2* expression, by administrating NF-κB inhibitors to SCN slice cultures.

3. Studying does age impact on the role of NF-κB in the circadian clock.

4. Investigating does SCN slice cultures from NF-κB::LUC mice display any evidence for circadian regulation of NF-κB mediated transcription.

5.2 Materials and methods

5.2.1 Animals and Housing

5.2.1.1 B6.129S6-Per2^{tm1Jt}/J strain

Homozygous B6.129S6-Per2^{tm1Jt}/J male and female mice were used in the following experiments. A mPer2^{Luc} knock-in homozygous breeding colony was maintained in Maynooth University, after been obtained from JAX mice (USA) via Charles River (U.K) as previous stated in section (3.2.1). Mice were ear punched and weaned at 21 days, animals were group housed (n=3) based on their sex. In addition, cages were equipped with appropriate environmental enrichment and were placed in a 12:12 L: D cycle. Mice were between 2 and 4 months old when used for experiment. However, in ageing *in vitro* experiments mice were between 14 and 16 months. The homozygous B6.129S6-*Per2*^{tm1Jt}/J mice display no alterations in their circadian behaviour or in entrainment parameters (Yoo et al., 2004).

5.2.1.2 B10.Cg-H2^{k}Tg(NFκB/Fos-luc)26Rinc/J strain

The B10.Cg-H2^{k}Tg(NFκB/Fos-luc)26Rinc/J male and female mice were also used in real-time experiments. This mouse model contains the firefly (luc) gene which is driven by two NF-κB binding sites from the kappa light chain enhancer in front of a minimal fos promotor (Voll et al., 2000). SCN slice cultures from NF-κB::LUC mice were examined for circadian regulation of NF-κB-mediated transcription. Hemizygous individuals were attained from JAX mice (USA) via Charles River (U.K), and a breeding colony was established in Maynooth University. Weaning and housing was carried out similar to section (5.2.1.1). Homozygous mice are not viable. Hemizygous were crossed with wild type mice, therefore, giving either hemizygous offspring with the transgene or wild type offspring. Genotyping was carried out in Maynooth University on offspring mice using ear punches to establish if they were hemizygous or wild type. Tissue was stored at -20°C in individual Eppendorf tubes until PCR was carried out. Male and female mice were between 2 and 4 months when used for experimental purposes.

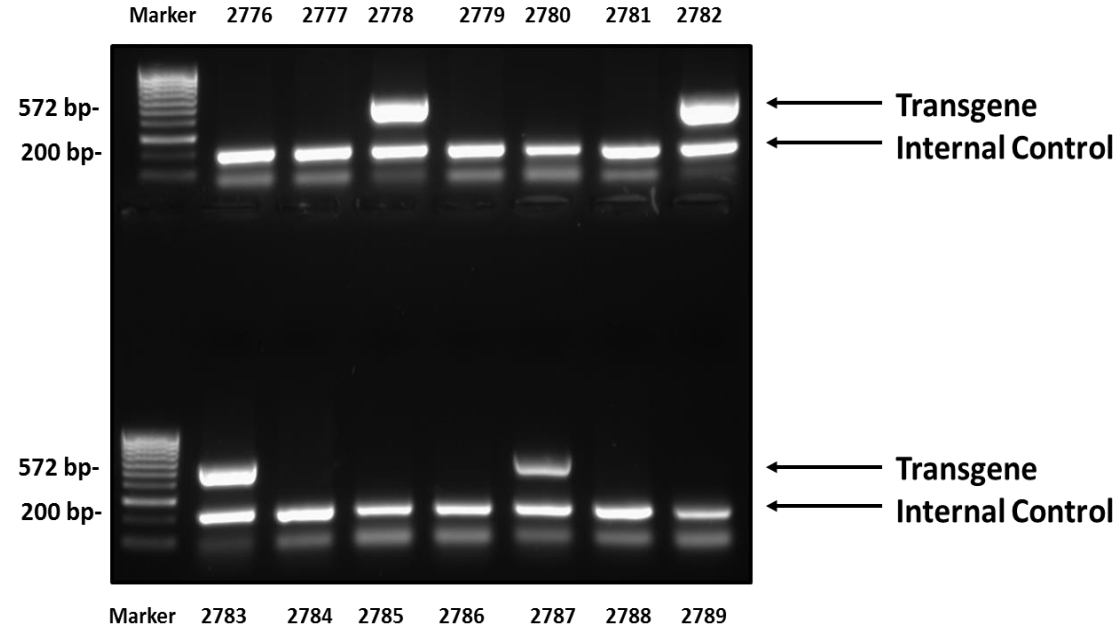


Figure 5.1: Genotyping using gel electrophoresis: PCR and gel electrophoresis techniques were used to distinguish between WT and hemizygous mice. From gel information we can see that there was 4 positive for the transgene (572 base pair).

5.2.2: Drug treatments

5.2.2.1 PDTC

Treatment of the NF-κB inhibitor, PDTC (Sigma Ireland) (n=21) or the control DMSO (n=23) was administered to PER2::LUC SCN slices for cell culturing to mice between 2 and 4 months. Application of PDTC (10μM) (Riera et al., 2015; Schreck et al., 1992) or DMSO (0.005%) took place at approximately ZT6, six hours after lights were turned on (ZT0 = 7am). The same dose of PDTC was administered to aged mice (n=6) in a separate experiment.

5.2.2.2 Sulfasalazine

Another NF-κB inhibitor, sulfasalazine (Abcam) (10μM) (n=7) was administered to PER2::LUC SCN cultures, aged between 2 and 4 months compared to DMSO control (Weber et al., 2000). Again application of treatment to SCN slices took place at roughly ZT6.

5.2.2.3 LPS

SCN slices from mPer2^{Luc}knock-in mice and NF-κB::LUC mice were treated with either LPS (100ng/ml) (n=8) or DMSO (0.005%) (n=8) (Gibbs et al., 2014; Guenthner et al., 2009; Wahl et al., 1998). In young mice aged between 2 and 4 months, application of treatment was administered at approximately ZT6. The same LPS dose was given to aged mice (n=7) and control (n=7) at similar times. NF-κB::LUC mice also received LPS (n=3) or DMSO (n=3) treatment, prior to incubation or 2 days post incubation in two separate experiments.

5.2.2.4 NMDA

NF-κB::LUC mice received NMDA (Sigma) (20μM) (n=3) or DMSO (n=3) treatment after 2 days of incubation (Asai et al., 2001; Pennartz et al., 2001; Shibata et al., 1994). Treatment to SCN cultures was carried out at ZT14.

5.2.3 Tissue preparation and cell culturing techniques

Prior to the experiment, distilled water was autoclaved at 120°C for 15 minutes. 70% ethanol was used to sterilize any equipment used in the procedure, followed by placing certain items under ultra violet (UV) light in a sterile tissue culture hood for 30 minutes to guarantee sterilization. The procedure needs to be done as quickly as possible to ensure that the SCN tissue remained living.

Mice were culled between ZT6 and ZT10 by cervical dislocation. This cell culture procedure required 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 10mM Hepes buffer (pH 7.2, Sigma), and 2.5ml of Penicillin-streptomycin (10,000 unit/ml- 10,00μg/ml, Gibco). These solutions were adjusted to a final volume of 1 litre using autoclaved Milli-Q water and mixed well. Glassware was covered in tinfoil as DMEM is light sensitive, and stored at 4°C pre-experiment. 0.1mM bettle luciferin potassium salt (Promega, U.S.A) was added to a certain volume of recording medium (for example: 6μl of luciferin in 6mls of recording medium) and placed

into a water bath at 37°C at the beginning of the experiment. Brains from PER2::LUC or NF-κB::LUC mice were carefully but promptly removed and placed on filter paper (Whatman, GE Healthcare UK Limited) which were placed in a petri dish. Drops of Hank's Balanced Salt Solution (HBSS) were placed on the brain to keep it cold. The HBSS was placed in glassware surrounded by ice to keep as cold as possible throughout the experiment. HBSS consists of (100ml Hank's balanced salt solution 10X, H1641, Sigma, U.S.A.; 10ml 1M HEPES buffer (Sigma), 4.7ml sodium bicarbonate solution (7.5%, Sigma), and 10ml of penicillin- streptomycin (10,000 unit/ml-10,000 µg/ml, Gibco, Invitrogen, U.S.A., which were all dissolved in autoclaved Milli-Q water and the total final volume of HBSS was adjusted to 1 litre and mixed thoroughly. Using a single edged razor blade brains were cut caudally and rostrally to remove the cerebellum and preoptic region respectively. The brain was placed with the caudal end attached using glue (Henkel) onto a mental block of a manual Vibratome (World Precision Instruments). The double edged blade (Camden Instruments Limited) was angled at 120^{o} with the ventral part of the brain facing it. The brain attached to block was placed into the vibroslice and immersed in ice cold HBSS to allow to cut for sections. The live brain was cut through till the optimum mid SCN region of 300µm in thickness was achieved. The paired SCN nuclei and retrochiasmatic area were identified and isolated from the slice using two single edged razor blades. This tissue was transferred to the sterile tissue culture hood and placed in a sterile petri dish with recording medium. A 35mm petri dish (Corning, U.S.A) was set up containing 1ml of (sterile recording medium and luciferin) + treatment or control. A Millicell culture membrane (Millipore, Ireland) was placed into petri dish, with the SCN tissue placed aseptically onto this membrane, prior to the luciferase activity assay. A 40mm coverslip (VWR) was then placed on the top of petri dish and sealed using autoclaved vacuum grease (Sigma) which was applied around the edge of dish. This ensured an airtight environment, stopping evaporation of the medium which resulted in appropriate nutrition, moisture and oxygen supply for the SCN tissue. The next step was to place each petri dish into a 37°c incubator (Memmert, Schwaback) which contains fitted PMT detector assemblies (Hamamtsu Photonic. U.K., LTD.) The incubator was waterproof and light tight. This system allowed for continuous monitoring of bioluminescence rhythms from individual SCN tissue culture explants for a period of up to two weeks.

5.2.4 Bioluminescence and statistical analysis

Evaluating the bioluminescence emitted from each SCN tissue culture was calculated by the programme Living Image 3.2 software (PMTmonTTL software by Dr. Shin Yamazaki). This allowed for the bioluminescence value to be recorded for one of every two minutes over a seven day period. Data sets were de-trended, with the 24hr running average been subtracted from the raw data. A 24 hour running average was then calculated from the de-trended data, from which a 3hr running average was then calculated. Using this 3 hr running average, it allowed for analysis using downloaded software called www.circadian.org which was developed by Dr. R. Refinetti. The Cosinor (Cosinor.exe, version 2.03) and LSP (Lsp.exe, version 2.07) software were both utilised to calculate the mean amplitude, mesor, acrosphase of rhythm (using Cosinor) and the circadian period of rhythm (using LSP). The mesor is a circadian rhythm-adjusted mean based on the parameters of a cosine function fitted to the raw data, while the acrophase represents the time at which the peak of a rhythm takes place.

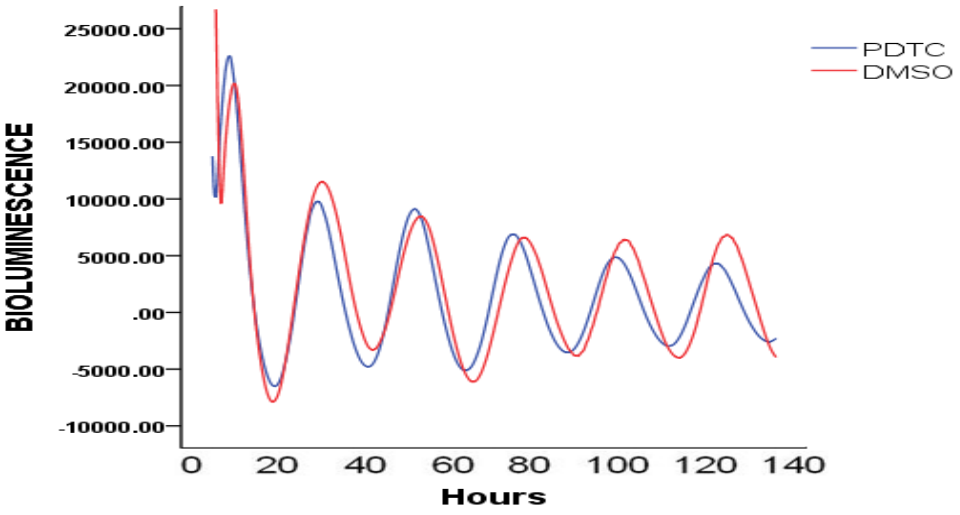
Each individual SCN tissue culture obtained a mean value for the period, amplitude, mesor and acrophase of the bioluminescence. For all the following cell tissue culture experiments, the mean value calculated for each tissue culture from both treatment groups were analysed, using an independent t-test. Results were stated as mean value ± standard error of the mean, with statistical significance been accepted at $P<0.05$.

5.3 Results

5.3.1 Application of NF-κB inhibitor PDTC to young SCN tissue cultures *in vitro* in PER2::LUC mice.

The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for PDTC and DMSO treated SCN tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. Bioluminescence was reported in relative light unit (RLU). Rhythm amplitude was found to be comparable between PDTC (3226.84 ± 316.58) and DMSO (3837.35 ± 528.56, P>0.05) SCN tissue cultures. Similar results also occurred when analysing the acrophase values with PDTC (16.55 ± 1.62) and DMSO (16.32 ± 1.96, P>0.05) for PER2::LUC rhythms. The mesor values for the PER2::LUC rhythms were found to be significant between PDTC (2489.01 ± 17.68) and DMSO (1120.68 ± 166.38, P<0.05) cultures. The Lomb-Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for PDTC and DMSO controls. When comparing the mean PER2::LUC period values there was no significant differences between the two treatment groups (PDTC, 24.10 ± 0.35 hrs vs. DMSO, 24.00 ± 0.22 hrs, P>0.05) (Figure 5.2).

(A)



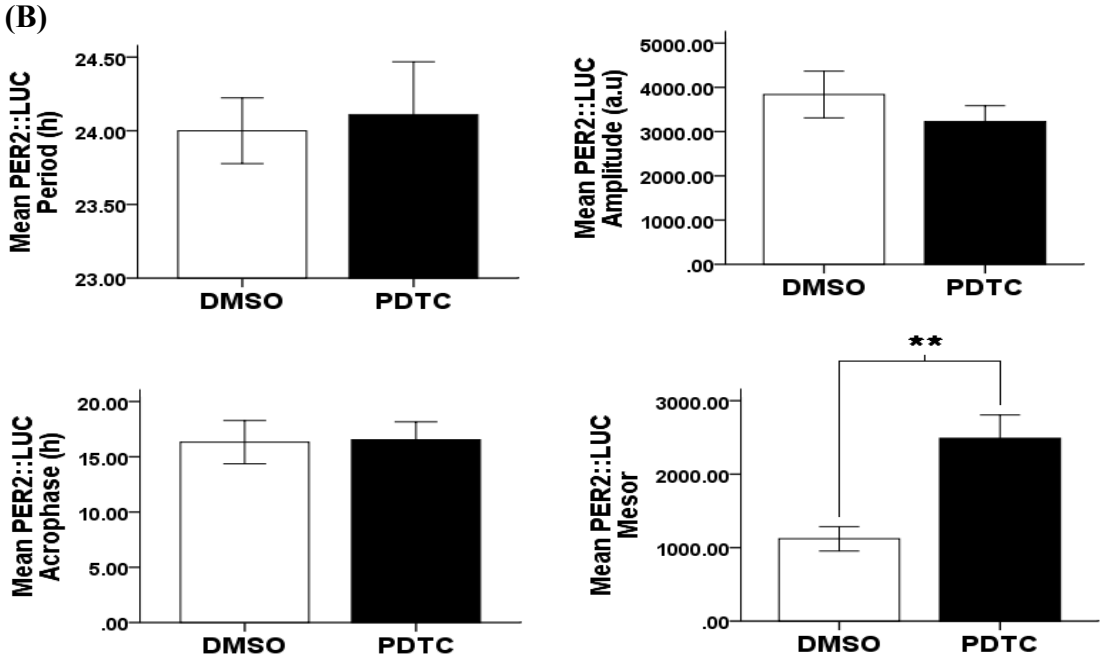


Figure 5.2: Circadian rhythms of PER2::LUC expression in PDTC and DMSO control in young SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue cultures treated with either PDTC (n=21) or DMSO (n=23). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. There was no differences displayed between the PER2::LUC rhythms of PDTC or DMSO treated cultures in any circadian parameter studied, apart from the mesor value. ** denotes P<0.01.

5.3.2 Application of NF-κB inhibitor sulfasalazine to young SCN tissue cultures *in vitro* in PER2::LUC mice.

A second NF-κB inhibitor sulfasalazine was utilised to examine if this inhibitor may have altered circadian parameters. The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for sulfasalazine and DMSO treated SCN tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. The mean rhythm amplitude was not significant between sulfasalazine (4880.37 ± 997.88) and DMSO (3741.21 ± 712.32, P>0.05), SCN tissue cultures. Similar results also occurred when analysing the acrophase values with sulfasalazine (15.46 ± 2.27) and DMSO (13.09 ± 2.97, P>0.05) for PER2::LUC rhythms. No significant alterations of mesor values for the PER2::LUC rhythms were found between sulfasalazine (3108.40 ±

1007.14) and DMSO (1053.43 ± 232.19, P>0.05) cultures. The Lomb-Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for sulfasalazine and DMSO controls. When comparing the mean PER2::LUC period values were no differences between the two treatment groups (sulfasalazine, 23.91 ± 0.57 hrs vs. DMSO, 23.87± 0.20 hrs, P>0.05) (Figure 5.3).

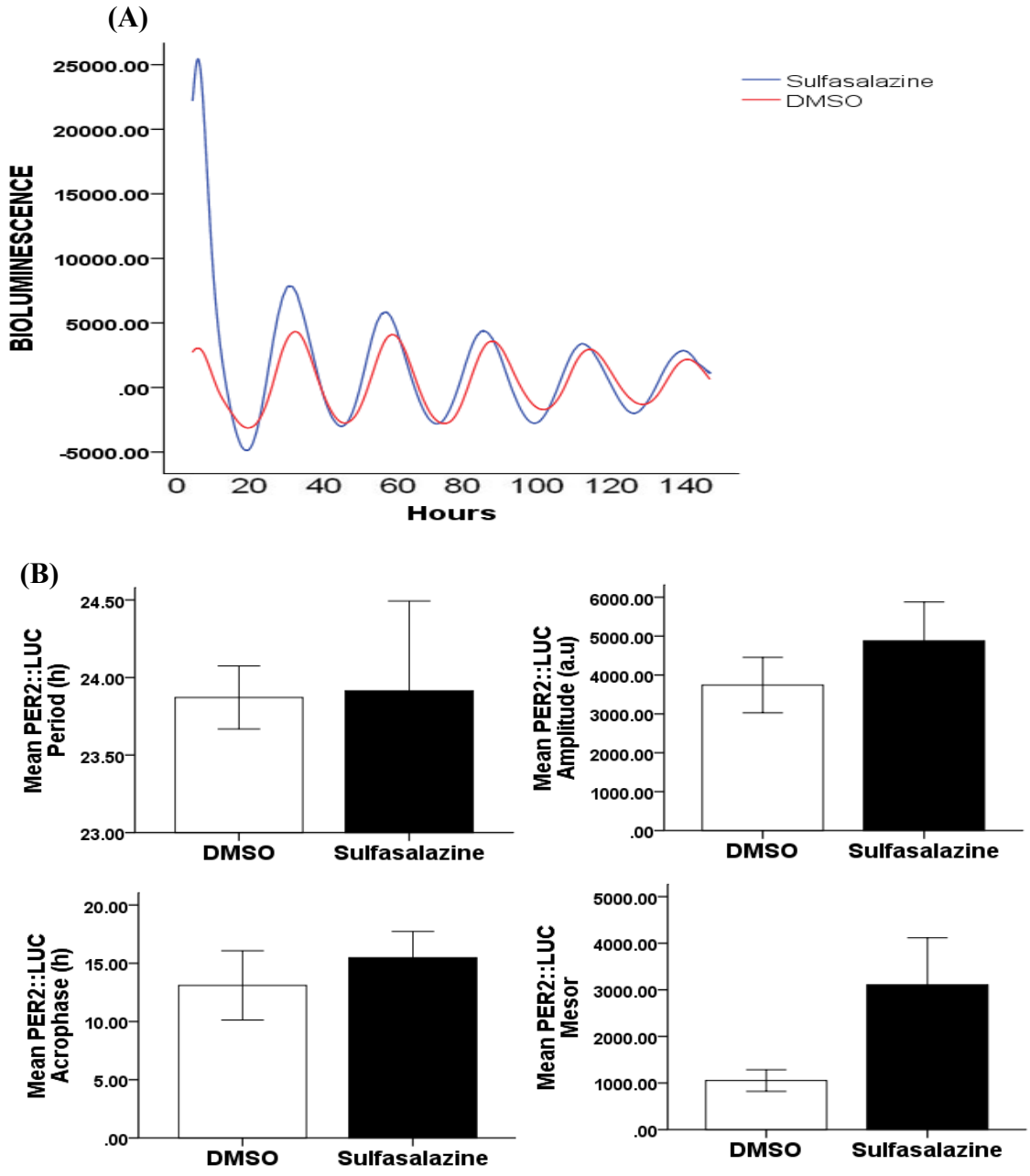


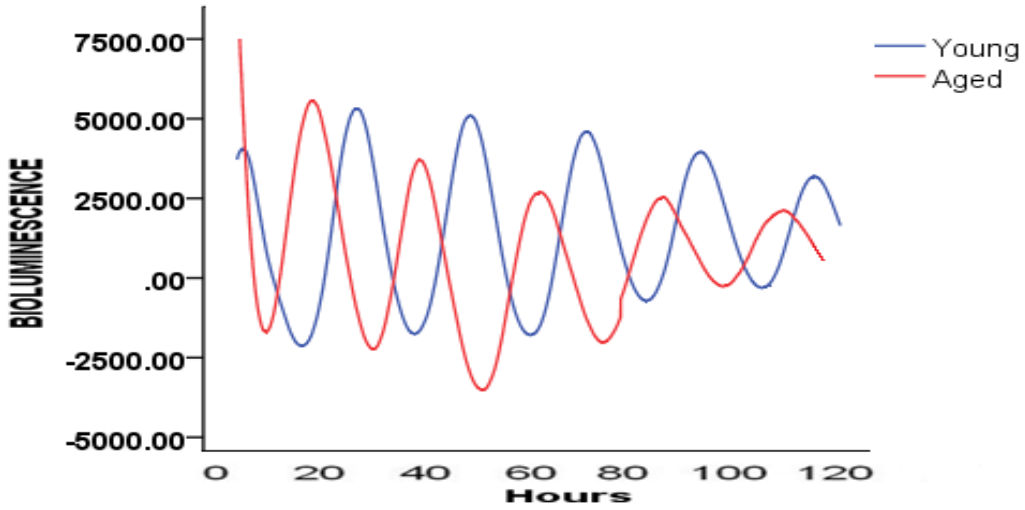
Figure 5.3: Circadian rhythms of PER2::LUC expression in sulfasalazine and DMSO control in young SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue cultures treated with either sulfasalazine

(n=7) or DMSO (n=7). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. There was no alterations displayed between the PER2::LUC rhythms of sulfasalazine or DMSO treated cultures in any circadian parameter studied.

5.3.3 Examining young adult PER2::LUC mice in comparison to ageing PER2::LUC mice SCN explants

The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for young adult and ageing SCN tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. There was a significant alteration of rhythm amplitude found between young adult (4260.65 ± 321.61) and ageing (2662.74 ± 496.74, $P<0.01$) SCN tissue cultures. Comparable results occurred when analysing the acrophase values of young adult (16.16 ± 3.91) and ageing (11.25 ± 1.62, $P>0.05$) for PER2::LUC rhythms. The mesor values for the PER2::LUC rhythms were found to be similar between young adult (1214.03 ± 175.35) and ageing (989.30 ± 230.39, $P>0.05$) cultures. The Lomb-Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for young adult and ageing SCN explants. When comparing the mean PER2::LUC period values there was no significant differences between the two treatment groups (young adult, 23.94 ± 0.09 hrs vs. ageing, 23.63 ± 0.21 hrs, $P>0.05$) (Figure 5.4).

(A)



(B)

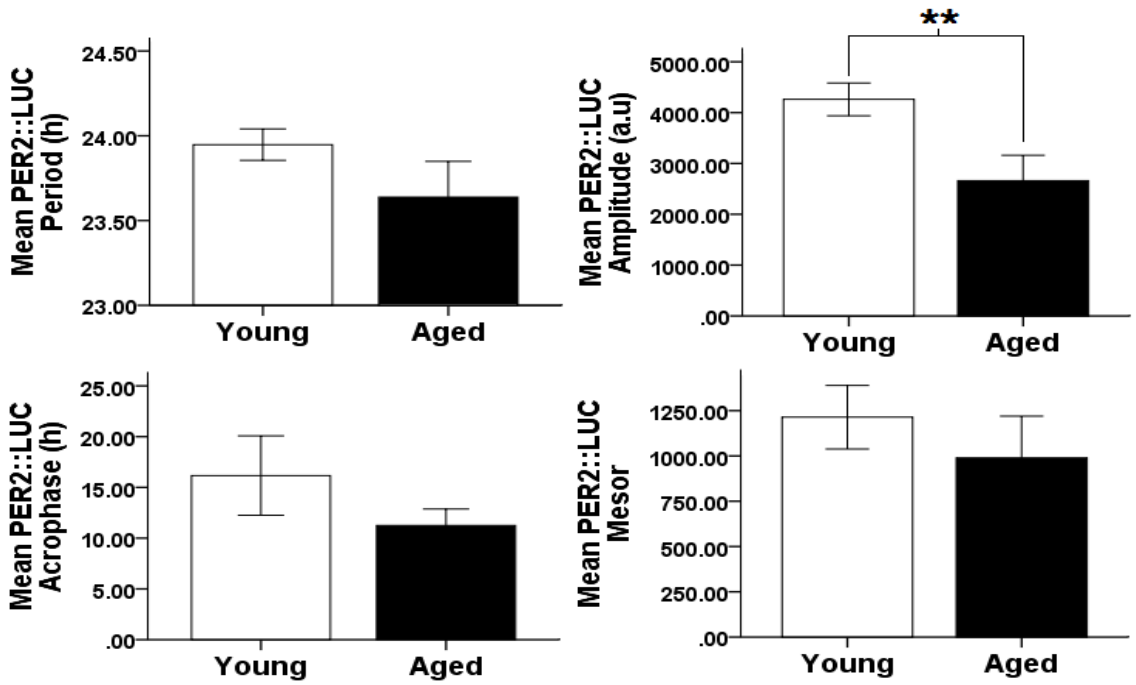


Figure 5.4: Circadian rhythms of PER2::LUC expression in young adult and aged PER2::LUC mice SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue of either young adult (n=19) or ageing SCN explants (n=13). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. There was no differences displayed between the PER2::LUC rhythms of young adult or ageing treated cultures in any circadian parameter studied, with the exception of amplitude. ** denotes P<0.01.

5.3.4 Application of NF-κB inhibitor PDTC to aged SCN tissue cultures *in vitro* in PER2::LUC mice.

The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for PDTC and DMSO treated SCN tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. Rhythm amplitude was not significant between PDTC (4435.95 ± 811.69) and DMSO (2400.90 ± 348.94, P>0.05) SCN tissue cultures. Similar results also occurred when analysing the acrophase values with PDTC (19.03 ± 3.68) and DMSO (17.83 ± 2.81, P>0.05) for PER2::LUC rhythms. The mesor values for the PER2::LUC rhythms were found to be significant between PDTC (5526.71 ± 100.36) and DMSO (727.66 ± 106.31, P<0.05) cultures. The Lomb-

Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for PDTC and DMSO controls. When comparing the mean PER2::LUC period values were no differences between the two treatment groups (PDTC, 23.82 ± 0.73 hrs vs. DMSO, 23.48± 0.28 hrs, P>0.05) (Figure 5.5).

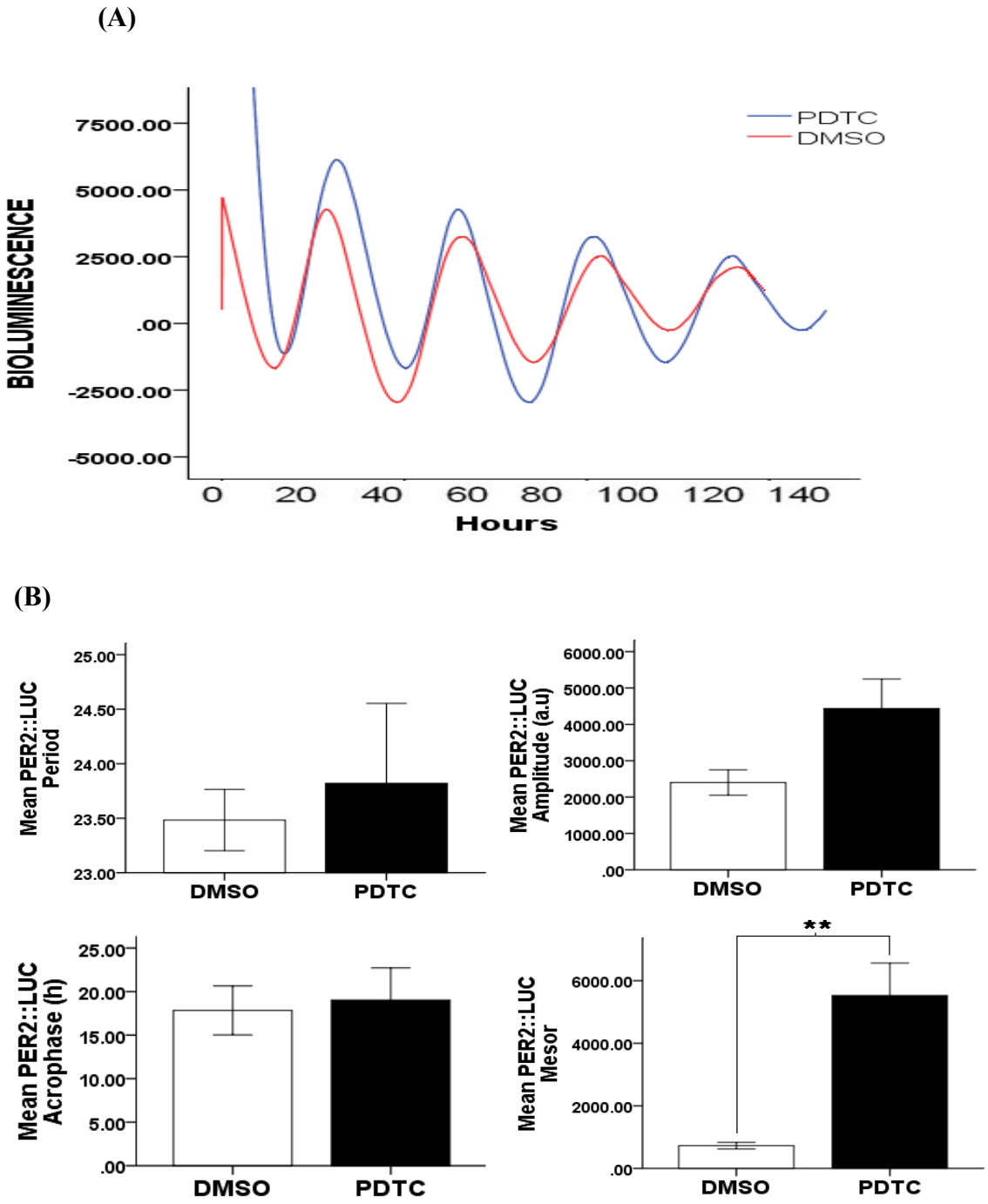


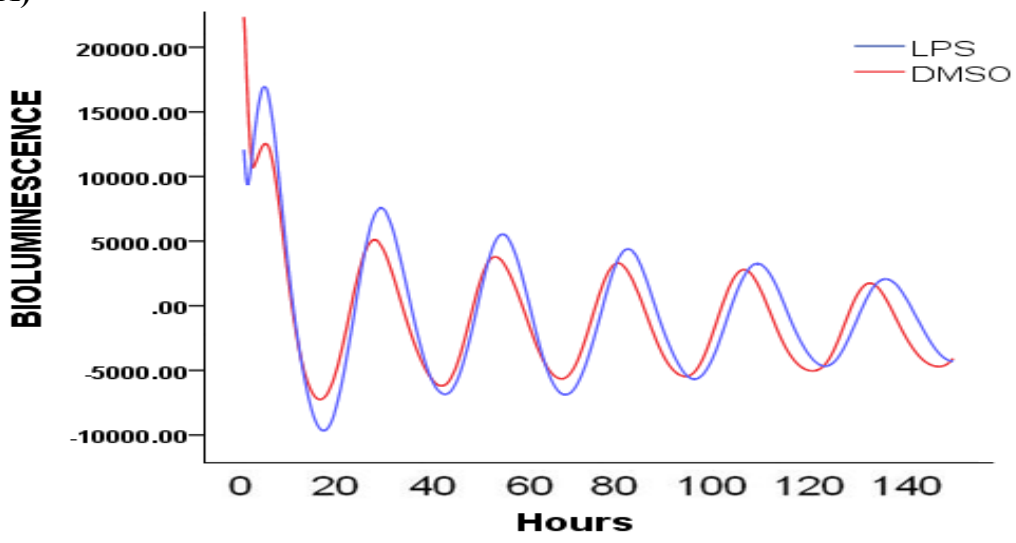
Figure 5.5: Circadian rhythms of PER2::LUC expression in PDTC and DMSO control in aged SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue cultures treated with either PDTC (n=10) or DMSO

(n=11). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. Treatment of SCN slice cultures from PER2::LUC mice with PDTC does not alter circadian rhythmicity of PER2::LUC expression compared to vehicle controls. There was however significant differences between treatment groups mesor value. ** denotes P<0.01.

5.3.5 Application of LPS to young SCN tissue cultures *in vitro* in PER2::LUC mice.

The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for LPS and DMSO treated SCN tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. The mean rhythm amplitude was upregulated as a result of LPS treatment (4389.33 ± 997.88) compared to DMSO (2611.47 ± 841.54, P>0.05) on SCN tissue cultures, but it was not statistically significant. No significant results occurred when analysing the mesor values with LPS (1302.64 ± 265.74) and DMSO (678.53 ± 265.21, P>0.05) for PER2::LUC rhythms. The acrophase values for the PER2::LUC rhythms were not found to be significant between LPS (8.84 ± 0.283) and DMSO (12.41 ± 2.18, P>0.05) cultures. The Lomb-Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for LPS and DMSO controls. When comparing the mean PER2::LUC period values were similar between the two treatment groups (LPS, 23.62 ± 0.21 hrs vs. DMSO, 23.82± 0.24 hrs, P>0.05) (Figure 5.6).

(A)



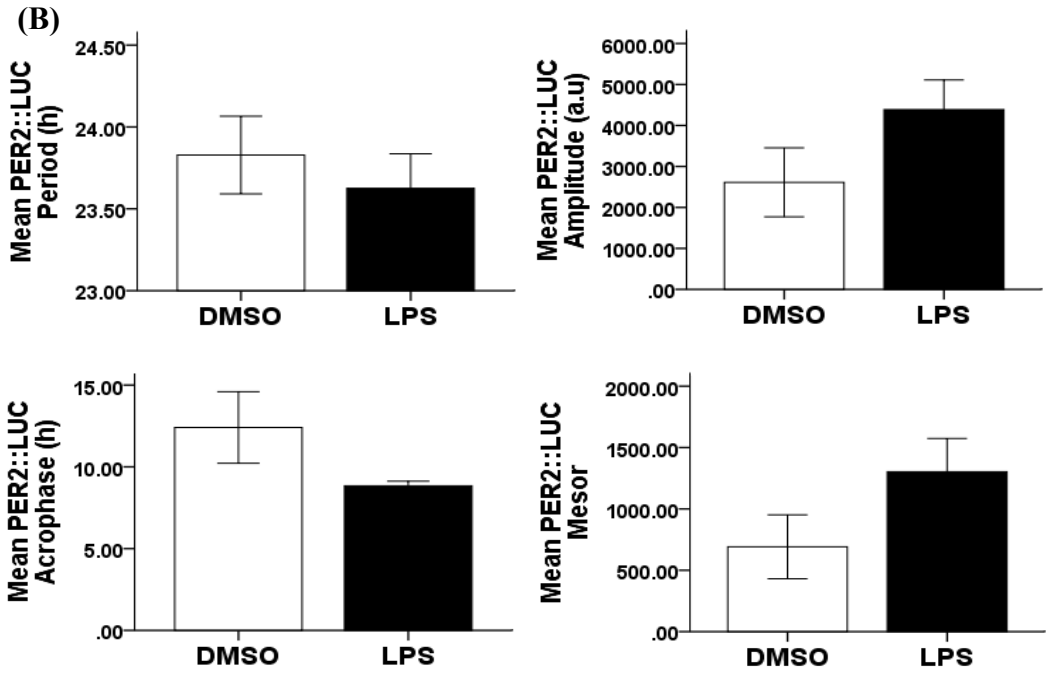


Figure 5.6: Circadian rhythms of PER2::LUC expression in LPS and DMSO control in young SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue cultures treated with either LPS (n=7) or DMSO (n=7). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. There was no alterations displayed between the PER2::LUC rhythms of LPS or DMSO treated cultures in any circadian parameter studied.

5.3.6 Application of LPS to aged SCN tissue cultures *in vitro* in PER2::LUC mice.

The cosinor method was used to calculate the mean amplitude, mesor and acrophase of PER2::LUC rhythms for LPS and DMSO treated SCN aged tissue cultures. The mean value for each individual SCN tissue culture in relation to their period, amplitude, mesor and acrophase of the bioluminescence rhythm was calculated, and evaluated between the two groups using an independent t-test. The mean rhythm amplitude was comparable between LPS (2818.88 ± 798.55) and DMSO (2887.18 ± 900.98, P>0.05) SCN tissue cultures. There was no significant difference when analysing the mesor values between LPS (910.11 ± 343.99) and DMSO (1213.55 ± 413.43, P>0.05) for PER2::LUC rhythms. The arcophase values for the PER2::LUC rhythms were found to be significant between LPS (10.37 ± 0.27) and DMSO (15.44 ± 2.88, P>0.05) cultures. The Lomb-Scargle periodogram procedure was utilised to measure the circadian period of the PER2::LUC rhythm for LPS and DMSO controls. The mean PER2::LUC period

values were similar between the two treatment groups (LPS, 23.51 ± 0.39 hrs vs. DMSO, 23.77± 0.32 hrs, P>0.05) (Figure 5.7).

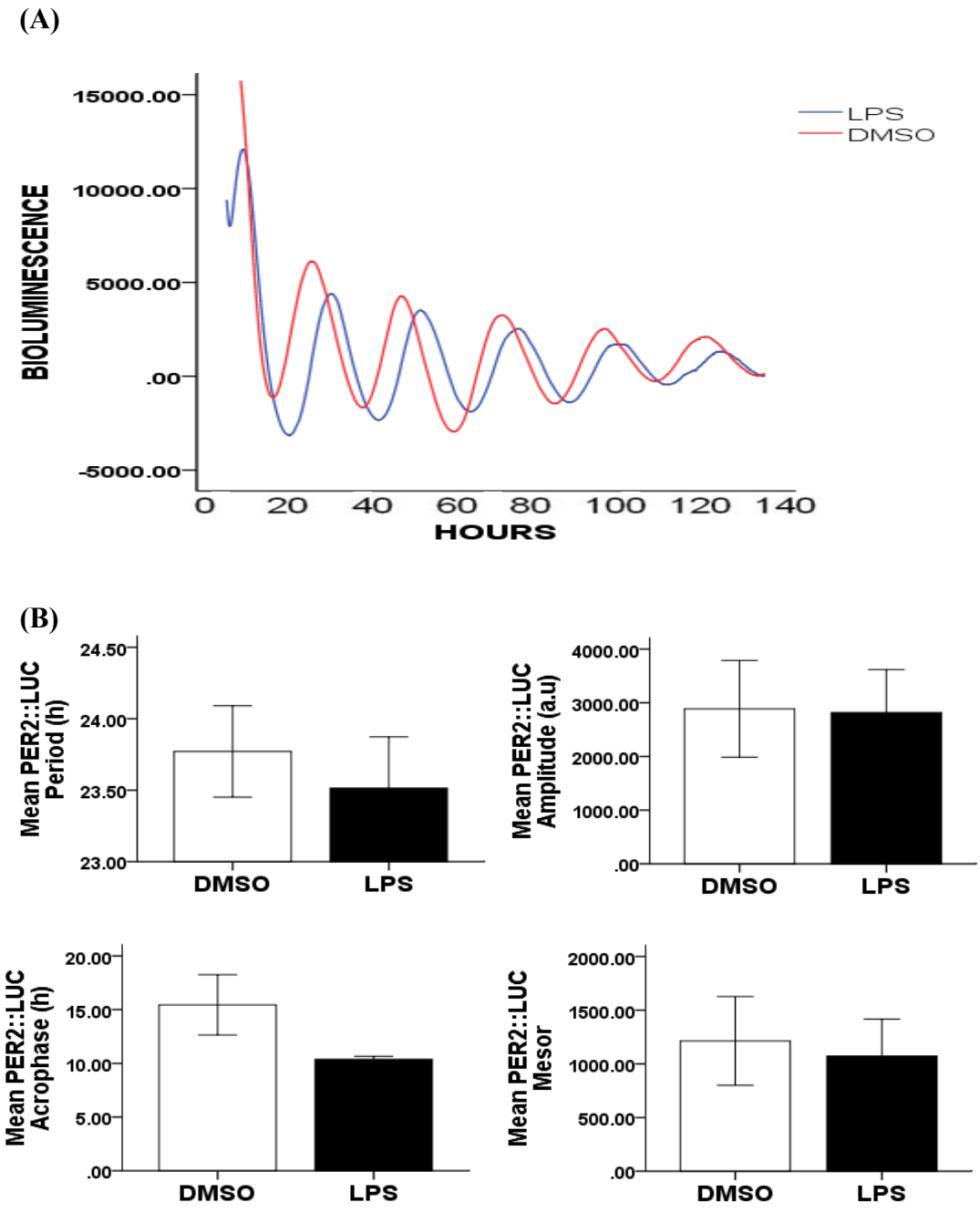


Figure 5.7: Circadian rhythms of PER2::LUC expression in LPS and DMSO control in aged SCN tissue cultures. (A) Representative multiple line plot of de-trended PER2::LUC bioluminescence rhythms from SCN tissue cultures treated with either LPS (n=7) or DMSO (n=7). The bioluminescence is plotted from the start time of culture preparation. **(B)** Bar graphs displaying PER2::LUC period, amplitude, acrophase and mesor values. There was no alterations displayed between the PER2::LUC rhythms of LPS or DMSO treated cultures in any circadian parameter studied.

5.3.7 Examining NF-κB::LUC SCN slice cultures *in vitro* for circadian regulation of NF-κB mediated transcription.

SCN slice cultures from NF-κB::LUC mice do not display any evidence for circadian regulation of NF-κB mediated transcription (Figure 5.8). Therefore, in the following experiments we looked at the affects of LPS and NMDA treatment on SCN cultures from NF-κB::LUC to establish if either of these can alter bioluminescence levels.

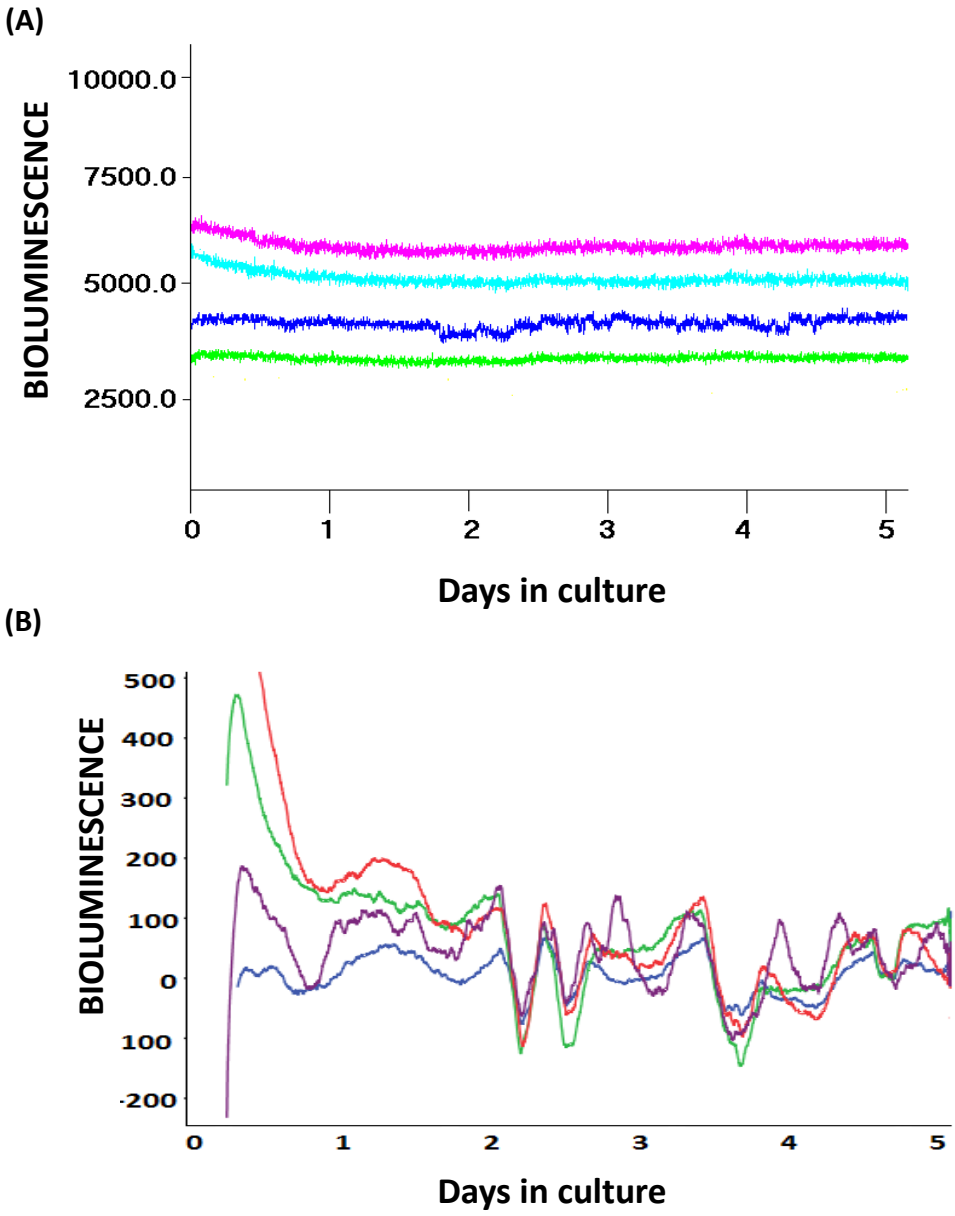


Figure 5.8: (A) Sample graph of raw traces from NF-κB::LUC SCN slices. **(B)** Sample graph of de-trended and smoothed data.

5.3.8 Examining NF-κB::LUC SCN slice cultures *in vitro* following LPS applications

LPS was administered to SCN cultures at the beginning of the experiment, and this resulted in an increased in bioluminescence when compared to DMSO controls (Figure 5.9). Previous studies have demonstrated that SCN explants are directly responsive to LPS treatment, including how LPS cause upregulation of p65 in SCN astrocytes *in vitro* (Leone et al., 2006), therefore, we expected to see some effect between treatment groups.

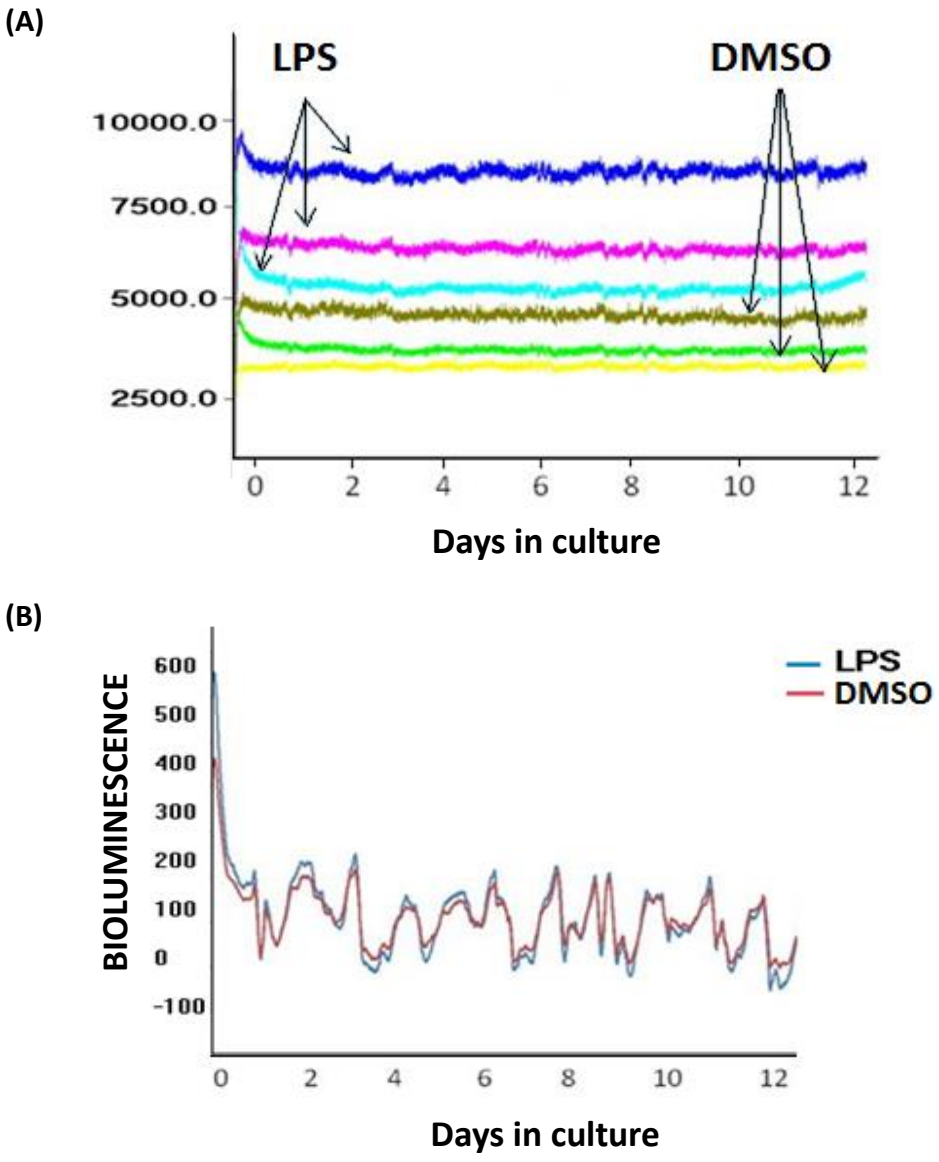


Figure 5.9: (A) Sample graph of raw traces from NF-κB::LUC SCN slices treated with LPS or control DMSO at the beginning of experiment. **(B)** Sample graph of de-trended and smoothed data from NF-κB::LUC slices after LPS or DMSO application. There is upregulation of bioluminescence in LPS treated slices compared to controls.

5.3.9 Examining NF-κB::LUC SCN slice cultures *in vitro* following LPS applications after two days in culture

LPS that was administered to SCN cultures 2 days after bioluminescence recordings had commenced, caused a slight upregulation in bioluminescence in comparison to DMSO controls (Figure 5.10).

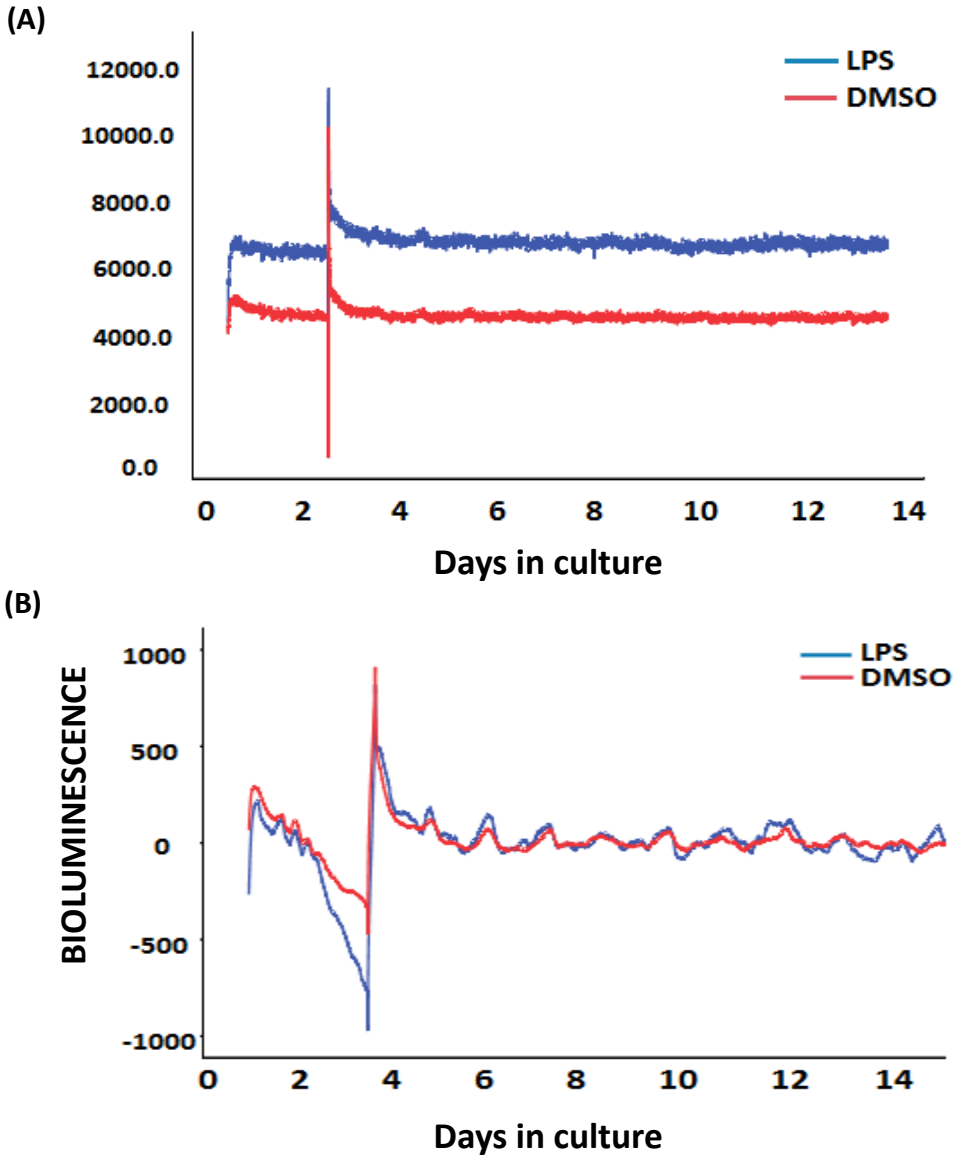


Figure 5.10: (A) Sample graph of raw traces from NF-κB::LUC SCN slices treated with LPS or control DMSO after two days in culture. **(B)** Sample graph of de-trended and smoothed data from NF-κB::LUC slices. LPS or DMSO control was administered two days into the experiment. Upregulation is displayed in graph (A) after LPS administration.

5.3.10 Examining NF-κB::LUC SCN slice cultures *in vitro* following NMDA applications after two days in culture

NMDA was administered to SCN cultures 2 days after bioluminescence recordings had begun, this resulted in a slight upregulation of NF::LUC bioluminescence which was not displayed in control cultures (Figure 5.11).

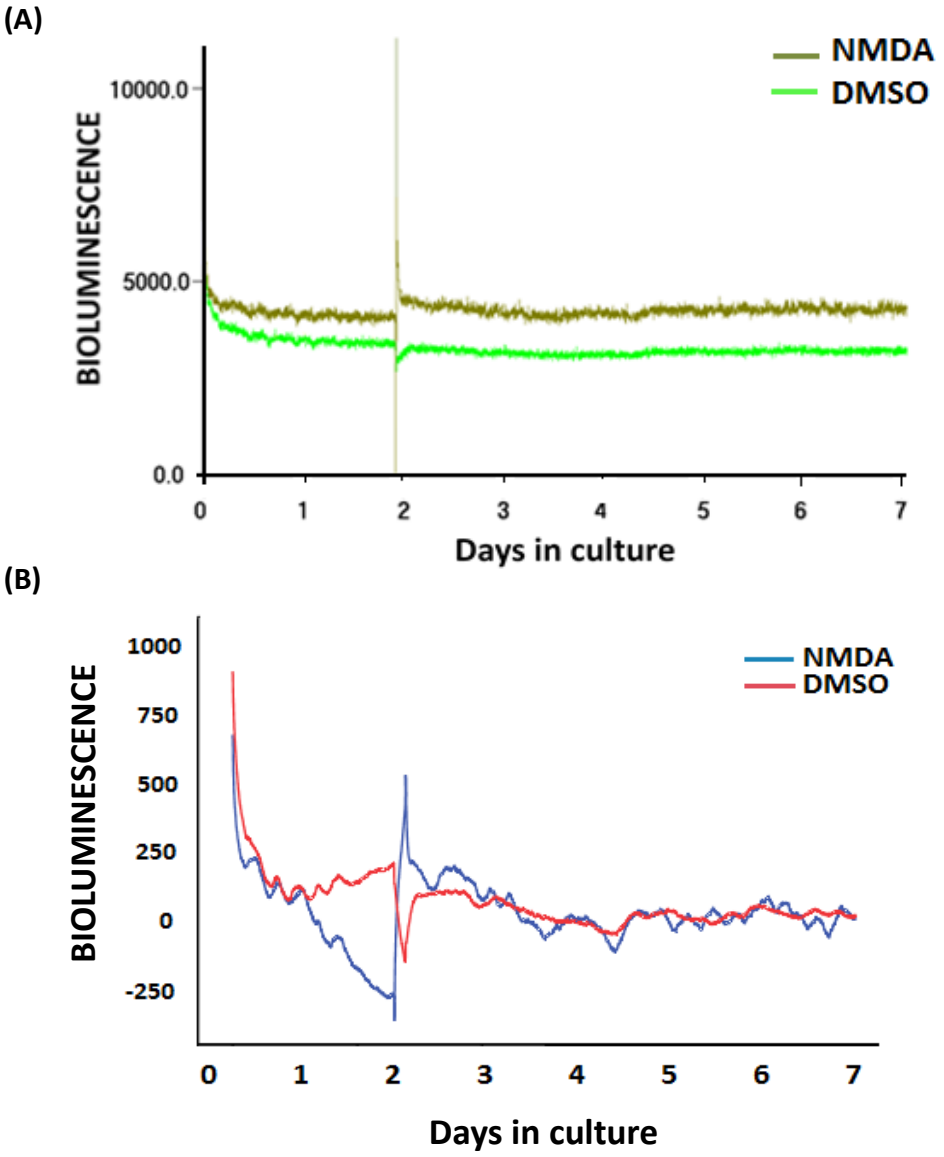


Figure 5.11: (A) Sample graph of raw traces from NF-κB::LUC SCN slices treated with NMDA or control DMSO after two days in culture. **(B)** Sample graph of detrended and smoothed data from NF-κB::LUC slices. NMDA or DMSO control was administered two days into the experiment. There was a small upregulation in bioluminescence after application of NMDA.

5.4 Discussion

Due to the presence of clock circadian machinery in most cells of the body, it has permitted circadian studies to be carried out in cell cultures. The use of cells isolated from humans or animal's tissue has allowed for advances *in vitro* studies. Isolated SCN explants are important to study to establish whether alterations in the circadian system are caused by changes within SCN properties, or whether it is a result of changes to input/output to and from the SCN. In this study we have investigated the bioluminescence of circadian rhythms using a luciferase reporter system in PER2::LUC and NF-κB knockin mice. Another important use of luciferase imagining, is that it can be carried out *in vivo* to analyse gene expression in the transgenic animal (Collaco et al., 2005; Hiler et al., 2006; Spengler et al., 2012).

Due to the close link between the molecular clock and the immune system, mice with a disrupted circadian clock often show disrupted immune responses also. The circadian and NF-κB pathways have been shown to contain a direct molecular link between each other, with both pathways coupled through the CLOCK protein, and this protein functions as a positive regulator of NF-κB–responsive promoters. Spengler and colleagues displayed that CLOCK causes an upregulation in NF-κB–mediated transcriptional activation, independent of BMAL1. *Clock-/-* and WT mice, expressing the κB-Luc reporter were compared, with *Clock-/-* mice exhibiting reduced immune responses in the form of the NF-κB subunit, p65, as a result of cultured hepatocytes and MEFs been administered CBLB502 and TNF-α respectively. Therefore, CLOCK upregulates NF-κB mediated transcription in various cells (Spengler et al., 2012). Therefore, it would be interesting to establish if an LPS immune challenge, *in vitro,* on SCN cultured, exhibits upregulation of NF-κB mediated transcription.

5.4.1 Does pharmacological perturbations of the NF-κB system, using *in vitro* techniques, impact on *per2* expression in young adult mice?

5.4.1.1 Circadian parameters in transgenic young adult knockin mice

SCN cultures from young adult mice were cut from the mid rostro-caudal for analysing. SCN cultures were normally prepared at ZT5, with studies displaying that the

peak phase was not reset at this time point, based on *in vitro* and *in vivo* studies, with regard to *Per1* expression (Asai et al., 2001; Miyake et al., 2000; Yoshikawa et al., 2005). Previous studies have described conflicting findings with regard to the behavioural FRP and the period of PER2::LUC, *Per1-Luc* and *Per1-Gfp* oscillations in SCN slices (Aton et al., 2004; Hughes et al., 2008; Mickman et al., 2008; Molyneux et al. , 2008).

In the current study, bioluminescence imaged from SCN control cultures displayed no signs PER2 expression rhythms damping during the first 5-7 days. When examining SCN control cultures in young adult mice during these experiments, the average period obtained was 23.9 ± 0.2 hrs. A similar period finding (23.6 ± 0.1 hrs) was established when monitoring circadian rhythms of PER2::LUC expression in the SCN (Guilding et al., 2009).

5.4.1.2 The effect blocking the NF-κB pathway has on young adult culture *in vitro*

Since excessive or deregulated NF-κB activation can lead to the development of pathological inflammation which can result in acute and chronic diseases occurring (Häcker & Karin, 2006; Karin & Greten, 2005; Wullaert et al., 2011), it is vital to target therapeutic repression of this pathway by using NF-κB inhibitors (Liu et al., 1999; Mariappan et al., 2010; Varghese et al., 2003; You et al., 2013). Previous studies *in vivo*, have documented that NF-κB inhibitors can block the effects of both an immune challenge (Marpegán et al., 2005) and photic induction (Marpegan et al., 2004). However, *in vivo* studies, undertaken in this project did not establish similar findings using the NF-κB inhibitor, PDTC. Therefore, it was necessary to investigate further the role the NF-κB pathway plays in the circadian timing system, using *in vitro* techniques.

In the current study, treatment of the NF-κB inhibitor, PDTC, to SCN slice cultures from Per2::LUC mice does not alter circadian rhythmicity of PER2::LUC expression compared to vehicle treated controls. Furthermore, a second NF-κB inhibitor, sulfasalazine, also exhibited no alterations in circadian parameters including amplitude, FRP, and acrophase. Both inhibitors did exhibit a significant alteration in mesor value between both treatment groups. However, it would seem that inhibiting the NF-κB pathway causes little effect on circadian processes *in vitro*.

5.4.1.3 An immune challenge on PER2::LUC young adult cultures

In vivo studies carried out using transgenic κB::LUC mice, demonstrated that NF-κB activation can be evaluated in living animals using luciferase as a reporter. Additionally, mice in this study received LPS i.v. (2mg/kg) to monitor luciferase activity in peripheral tissues following an immune challenge. LPS treatment caused high NF-κB-dependent luminescence in the skin, lungs, small intestines and spleen. Activity was also exhibited in the heart, kidney and liver (Carlsen et al., 2002). Another immune stimuli, TNF-α, is known to be involved in activating the JNK pathway or the NF-κB pathway (Deng et al., 2003; Pękalski et al., 2013). SCN astrocyte cultures of PER2::LUC mice were administered TNF-α, to allow for assessing how SCN astrocytes responded to an immune challenge using this stimuli. It was established that application of TNF-α results in alterations to the amplitude of PER2 expression rhythms and also causes phase shifts, which was time- dependent (Duhart et al., 2013). Furthermore, (Guenthner et al., 2009) also displayed that LPS has no effect on PER2 rhythms in the mouse SCN with regard to rhythm damping or circadian period.

In this current study, an assessment was carried out to monitor what effect LPS had on PER2::LUC knock-in mice. LPS was administered to PER2::LUC SCN cultures which resulted in no significant alterations of PER2 oscillation properties, between treatment group and control. There was an increase in the amplitude of PER2::LUC SCN explants after LPS treatment in comparison to control, though, overall there was no significant changes in amplitude. There was also no significant alterations in the mesor value. LPS treatment did however, reduce both the FRP and the acrophase, but again this was not significant. Therefore, even though LPS activates the NF-κB pathway, it does not seem to effect PER2 oscillation properties in young adult SCN cultures, with no evidence established that the NF-κB pathway may play a role in transducing immune events in the SCN using *in vitro* techniques. In chapter three, *in vivo* studies displayed that another clock gene, PER1, showed no alteration of expression following LPS administration at ZT4 in PER2::LUC mice.

Future studies could assess if there is any variations to PER2 oscillation properties *in vivo* after an immune challenge, since in this study the SCN is isolated, therefore, the culture is not influenced by signalling from peripheral or non- SCN oscillator site. Consequently, LPS treatment administered *in vivo*, may result in alterations in the

circadian system at levels downstream from the SCN or variations at the level of input to the SCN.

5.4.2 Does pharmacological perturbations of the NF-κB system impact on *per2* expression in ageing studies using *in vitro* techniques?

5.4.2.1 Circadian parameters in transgenic ageing knockin mice

Ageing affects the SCN as well as peripheral oscillators using PER1::LUC rats *in vitro* (Davidson et al., 2008). Previous studies *in vivo* have uncovered the effect ageing has on circadian function, with ageing resulting in amplitude reduction in aged animals when investigating the electrical activity rhythms in SCN slice cultures (Aujard et al., 2001; Biello, 2009; Li & Satinoff, 1998; Ruby et al., 1998; Watanabe et al., 1995). These age-related changes in electrical activity might be a result of variations in the amplitude and regularity of molecular components of the SCN clock such as *Per1* and *Per2* (Yamazaki et al., 2002). Individual SCN neurons explants from aged mice displayed a decline in amplitude of firing rate. They also had increased variability in period in comparison to young adult SCN cultures (Aujard et al., 2001). We hypothesised that ageing in PER2::LUC mice may also effect SCN properties *in vitro*.

We have already reproduced similar results to (Nakamura et al., 2011) and (Weinert & Weinert, 1998) in the previous chapter, with regard to wheel running activity patterns carried out in aged mice. It was established that aged mice had abnormal rhythms, in terms of diminished amplitude and a reduction in total wheel running activity. Therefore, to establish if circadian parameters were affected *in vitro*, a study was carried out to monitor basal levels of PER2::LUC SCN aged cultures in comparison to young adult SCN explants. A study was carried out by Yamazaki and colleagues using PER1::LUC rats, in which rhythmicity of SCN cultures lasted up to seven weeks. The FRP of the SCN was significantly reduced in aged PER1::LUC SCN cultures when compared to young adult SCN culture (Yamazaki et al., 2002). However, in this current study, it was discovered that ageing in SCN PER2::LUC explants did not lead to significant alterations in FRP, mesor or acrophase. Additionally, there was a significant decrease in amplitude exhibited in aged SCN PER2::LUC cultures when compared to young adult SCN cultures, similar to results obtained by Nakamura also using PER2::LUC mice (Nakamura et al., 2011).

Experiments carried out using *in vivo* techniques also established alterations in amplitude between the two age groups (Nakamura et al., 2011; Valentinuzzi et al., 1997; Weinert & Weinert, 2010).

This was not significantly found in the Yamazaki study (2002), when investigating the amplitude of PER1::LUC SCN cultures of rats, which is in contrast to *in vivo* locomotor behavioural studies. This contrast in amplitude reduction between the current study and Yamazaki may be due to different rodents been utilised or the fact that two different PER proteins are been investigated. Hence, the molecular component, PER2, may be more affected by ageing. Weinert found that the amplitude of night-day expression of *mPer2* in the SCN was reduced in aged mice, however, this was not found to be the case in the expression of *mPer1* (Weinert et al., 2001). However, contrasting *in vivo* results were provided by Asai, who found that rPer2 along with rPer1 had similar amplitude between young and aged rats (Asai et al., 2001).

5.4.2.2 The effect blocking the NF-κB pathway has on ageing cultures *in vitro*

The effect ageing has on the brain in terms of NF-κB function in the SCN clock was analysed *in vitro*. As discussed previously, the NF-κB signalling pathway is associated with ageing, (Tilstra et al, 2011) with ageing studies displaying increased brain NF-κB activity in certain brain regions including the hippocampus and basal forebrain (Toliver-Kinsky et al., 1997). Investigations were carried out to see what effect pharmacological inhibition of the NF-κB pathway had on circadian processes *in vitro* in aged PER2::LUC SCN explants. Administration of the NF-κB inhibitor, PDTC, to aged SCN cultures, exhibited no alterations of circadian parameters, apart from the mesor. There was no significant difference in FRP, amplitude and acrophase evident between the two groups, PDTC and vehicle. Therefore, similar to young adult SCN explants, it would appear that inhibition of the NF-κB pathway causes little effect on circadian processes *in vitro* in aged SCN explants.

5.4.2.3 An immune challenge on PER2::LUC aged cultures

An investigation took place to establish whether LPS treatment on aged SCN cultures, had a similar effect on PER2 expression rhythms as they do in young adult SCN

cultures. There was no alterations exhibited in the amplitude of PER2 expression rhythms in SCN explants after LPS treatment. Additionally no significant changes was displayed in the mesor value either. Similarly to young adult SCN cultures, LPS treatment in aged slices, caused a decrease in both the FRP and the acrophase, but again this effect was non-significant. Therefore, LPS does not seem to effect PER2 oscillation properties in aged SCN cultures either.

In chapter four, *in vivo* studies exhibited no alterations in PER1 expression following LPS administration in the aged SCN. A study using *in vivo* techniques such as IHC, to display PER2 expression, showed ageing did not appear to cause disruption to the molecular clockwork (Nakamura et al., 2011).

5.4.3 Is there circadian rhythms in NF-κB::LUC SCN explants?

In the current study, firstly we established that SCN cultures from NF-κB::LUC mice do not exhibit any evidence for circadian regulation of NF-κB mediated transcription. Additionally, an investigation took place to see how NF-κB::LUC SCN cultures would react to LPS treatment. A study carried out *in vivo* had previously shown how LPS administered to transgenic mice by i.v injection, caused a rise in luminescence in peripheral tissues, demonstrating NF-κB activation (Carlsen et al., 2002). Furthermore, previous *in vitro* studies that have administered LPS directly to SCN explants cause upregulation of the NF-κB component p65 in SCN astrocytes (Leone et al., 2006), therefore, we hypothesised that LPS would alter bioluminescence levels. We established that LPS treatment administered to SCN cultures at the beginning of the experiment resulted in a higher level of bioluminescence in comparison to SCN DMSO controls. Secondly, when LPS was administered two days into the experiment, this results in a spike in bioluminescence for SCN cultures for 12 days, when compared to controls.

NMDA is a glutamate receptor agonist and has been shown to induce phase shifts in circadian rhythms *in vitro* in the SCN of rats (Ding et al., 1994; Shibata et al., 1994). Ding and colleagues also displayed how application of NMDA antagonists inhibits the effect of glutamate *in vitro,* which is involved in photic entrainment (Ding et al., 1994). Furthermore, NMDA receptors are identified as playing a role in mediating the effect of light on circadian behaviour, since several NMDA antagonists are known to block photic phase shifts displayed in behavioural *in vivo* studies (Colwell et al., 1991; Colwell et al.,

1990). Application of NMDA was seen to increase the electrical firing rate in SCN WT mice cultures (vanderLeest et al., 2009).

Various studies have shown how NF-κB can be modulate by the glutamate receptor NMDA (Furukawa & Mattson, 1998; Zou & Crews, 2006), with glutamate neurotoxicity connected with an upregulation of NF-κB DNA binding. Neurotoxicity could be attenuated by agents that reduce NF-κB DNA binding (Zou & Crews, 2006). NF-κB can be activated in the hippocampus by glutamate (Kaltschmidt et al., 2005), with studies showing that this is a result of NMDA causing IκB-α degradation following ischemia (Shen et al, 2003; Nakai et al., 2000). *In vitro* studies displayed also how synaptic activation of NF-κB by NMDA can occur in cellbellar granule neurons (Guerrini et al., 1995). Since NMDA is known to modulate NF-κB, we tested what effect NMDA treatment would have on SCN NF-κB driven luc rhythms, two days after bioluminescence recording had begun. NMDA treatment resulted in upregulation of bioluminescence for the following 5 days. Another future study to carry out is to treat PER2::LUC young adult and aged explants with NMDA to demonstrate photic-like resetting of circadian rhythms.

5.4.4 Conclusion

The data collected in this chapter would suggest that while the NF-κB pathway may be involved in circadian function in peripheral clocks, it does not seem to play a key role in the SCN clock *in vitro,* adding to our previous findings established *in vivo*. Further studies could investigate if the NF-κB pathway plays a role *in vitro* in other brain regions, including the hippocampus and cortex, by investigating what effect LPS treatment has on the PER2::LUC cultures with regard to circadian oscillations, and also to establish if NF-κB inhibitors can block any effects that may occur due to this immune challenge. Since circadian control of hippocampal function seems to rely on SCN derived signals (Phan et al., 2011), one would not be surprised if no alterations, in circadian oscillations, from PER2::LUC cultures occurred from the hippocampus, due to the lack of evidence of NF-κB playing a role in the SCN.

In conclusion, even though studies have stated that there is basal expression of NF-κB in the CNS (Kaltschmidt et al., 1997; Kaltschmidt et al., 1994), other findings have

contradicted these previous studies, stating that immunohistochemistry and western blots used nonspecific antibodies for their studies, in relation to NF-κB expression. This resulted in certain antibodies which have been previously published, failing in specificity (Herkenham et al., 2011). A recent studies *in vitro* exhibits how IκB mRNA expression in microglia from the hippocampus was investigated, with findings displaying no temporal differences in young adult rats (Fonken et al., 2015). Other *in vivo* studies have shown how there is minimal NF-κB activity in neurons (Listwak et al., 2013) and in the MBH of young adult mice (Zhang et al., 2013). To further back up these results, this chapter using *in vitro* studies displays no evidence of the NF-κB pathway playing a role in the SCN. Bioluminescence expression from PER2::LUC cultures was not effected by blocking the NF-κB pathway using inhibitors, nor does an immune challenge result in any significant alterations in circadian oscillation properties. These results obtained were similar for both young adult and aged cultures. No circadian oscillations were exhibited when investigating the reporter NF-κB::LUC mice in the circadian timing system, further highlighting how this pathway has little or no effect in the SCN.

Chapter Six
General Discussion

The overall objective at the beginning of this thesis was to investigate if the NF-κB pathway played a role in the circadian clock, since there has been little investigation carried out previously on the regulation of SCN function by the NF-κB pathway. Chapter two conducted experiments to see if there was circadian regulation in NF-κB components in the SCN. Furthermore, photic regulation of the NF-κB component, p65, was investigated, along with pharmacological inhibition of the NF-κB pathway on circadian processes *in vivo* were also examined. Chapter three and four investigated the effects that an immune challenge had on young adult and ageing mice respectively, using behavioural techniques and IHC techniques. While lastly, chapter five, used *in vitro* techniques to further establish firstly, the effects of an immune challenge and secondly, pharmacological inhibition of the NF-κB pathway had on young adult and ageing SCN cultures.

Other signalling pathways have been identified as playing a key role in conveying events at the SCN neuronal membrane to core nuclear clockworks. These pathways include those involving MAPK (Coogan & Piggins, 2003) and protein kinase C (Bonsall & Lall, 2013; Lee et al., 2007). A molecular link between the NF-κB pathway and the clock-driven transcriptional pathway was uncovered (Bellet et al., 2012). Evidence from previous studies had suggested that there was constitutive NF-κB activity in neurons in various brain regions, including the hypothalamus (Bhakar et al., 2002; Joseph et al., 1996; Kaltschmidt & Kaltschmidt, 2009; Kaltschmidt et al., 2005; Kaltschmidt et al., 1994; Meffert & Baltimore, 2005; O'Neill & Kaltschmidt, 1997). However, recent evidence suggests that this may not be the case, with minimal NF-κB activity displayed in neurons (Listwak et al., 2013). Previous findings of constitutive NF-κB activity in neurons, are thought to be due to non-selective antibodies used in the previous studies, which was previously discussed in chapter two and three (Herkenham et al., 2011). Therefore, care needs to be taken when interpreting results in IHC with regard to NF-κB activity in neurons. In the CNS it has been established that astrocytes are the main cell type subject to NF-κB regulation (Lian et al., 2012), with findings from (Leone et al., 2006) showing that an immune challenge can cause upregulation of NF-κB activity in

SCN astrocytes. Our results seemed to represent an immune challenge causing NF-κB activity in the form of microglial cells demonstrated from their phagocytic morphology.

A considerable amount of evidence was ascertained in relation to the interaction between the circadian system and the immune system in recent years, with cytokine receptors of the immune system expressed rhythmically in the SCN (Beynon & Coogan, 2010), along with evidence for SCN regulation by TNF-α, LPS and interferon-γ (Kwak et al., 2008; Nygård et al., 2009; Paladino et al., 2010). Too further add to these findings, current literature has contributed that there is clear communication between the circadian system and the immune system, showing that TNF-α modulates mammalian circadian rhythms (Cermakian et al., 2013; Duhart et al., 2013; Fonken et al., 2015; Paladino et al., 2015).

Standard techniques to detect and analyse protein include Western blotting and enzyme-linked immunosorbent assay (ELISA) which allow for objective analysis with regard to quantitation (Aydin, 2015; Kurien & Scofield, 2006). Another major development over the past 20 years was the invention of Real-time PCR (RT PCR), which has revolutionised biological science (Deepak et al., 2007). This technique is extremely sensitive and allows for the quantification of the mRNA encoding the protein of interest (Bustin, 2000). There are also limitations involved with these three techniques (Guardigli et al., 2005). The majority of this project used immunohistochemistry (IHC) techniques to investigate if the NF-κB pathway played a role in the SCN. IHC technique is a widely used method of protein detection which is fast and sensitive when utilizing an appropriate detection antibody (Guardigli et al., 2005; Ramos-Vara, 2005). However, limitation do occur using this method, with regard to the fact that IHC only allows qualitative or semi-quantitative analysis of protein expression. Furthermore, the quantification of immunostaining is seen as subjective (Matos et al., 2010; Yaziji & Barry, 2006). Nonetheless, one main advantage that IHC has over its counterparts, is that it can demonstrate the location of the antigen within a cell or tissue (Ramos-Vara, 2011), which helped establish if LPS activated NF-κB to move from the cytoplasm into the nucleus, in our immune challenge studies.

The second technique used in this project was real-time luminescence reporting of circadian gene expression in mammals. The main advantage using this non-invasive assay, is that it allows for the measurement of real-time expression of circadian genes. In this study PER2::LUC mice permitted us to be able to monitor circadian rhythms of the

PER2 protein, to analyse immune challenge and ageing studies (Yamazaki & Takahashi, 2005; Yoo et al., 2004).

The administration of the NF-κB inhibitor PDTC by i.p injection, at both CT15 and CT22, could not block photic induction exhibited in our study. PDTC throughout this study resulted in conflicting findings when compared to other literature (Anderson et al., 2015; Marpegan et al., 2004), with both studies either attenuating the effects of LPS or photic induction in the SCN. The administration of oral PDTC in the drinking water of mice also not alter any behavioural circadian parameters, nor did it cause affect at a molecular level, with no alterations of diurnal rhythms in PER1, c-Fos and p65 expression in the SCN or PVN. This further emphasises that pharmacological inhibition of this pathway does not seem to affect circadian processes when carried out by *in vivo* techniques. The administration of PDTC and Sulfasalazine to PER2::LUC mice *in vitro* also highlights our previous findings, as both inhibitors did not affect any circadian parameters when administered to young adult SCN cultures, therefore establishing that blocking the NF-κB pathway is of little benefit to the SCN.

The effects of acute LPS treatment have been shown to impact upon the SCN (Leone et al., 2006; Marpegán et al., 2005; Paladino et al., 2010) and also effect the PVN (Laflamme & Rivest, 2001). In the current study, *in vivo* results showed that acute neuroinflammation was found to alter some signalling properties of NF-κB in the SCN and PVN. Components of the NF-kB pathway, p65 and p-IκB, may have a role in transducing immune events in the SCN. Other literature also found that peripheral LPS caused an upregulation of p65 in the SCN and PVN (Beynon & Coogan, 2010) and in SCN astrocytes (Leone et al., 2006). We confirmed similar behaviour findings to (Marpegán et al., 2005) with low dose LPS, causing phase delays at CT15, which is time-dependent. Recent behavioural findings have displayed the importance of the TNFα receptor 1/p55 and the TLR4 receptor, for transmitting the effects of LPS to the SCN by a series of signalling events, resulting in the induction of pro-inflammatory responses in the brain (Paladino et al., 2015, 2010).

At a molecular level it has been identified that the marker of neuronal activation, c-FOS, did not exhibit LPS induced FOS expression in the SCN, as a result of mice deficit in Tnfr1and TLR4 (Paladino et al., 2015, 2010). Therefore, highlighting the importance of these receptors at a behavioural and molecular level with regard to the master circadian clock, for example, if LPS cannot bind to TLR4, then NF-κB signalling cascade won't be

activated. An interesting finding with regard to low dose peripheral LPS administration was that it only caused upregulation in p65 expression, but not p-IκB, therefore the effects of LPS is dose dependent on the NF-κB pathway. Other findings witnessed in this study was that LPS is time dependent, with upregulation of p65 and p-IκB expression displayed through the subjective day (ZT4-8) in the SCN, this was not exhibited during the subjective night, hence displaying diurnal variation. Interestingly, *in vitro* studies using PER2::LUC young adult SCN cultures displayed no alterations to circadian parameters as a result of an immune challenge using LPS, but there was an increase in bioluminescence in LPS challenged NF-κB::LUC mice.

Ageing and chronic neuroinflammation was not found to alter the signalling properties of NF-κB in the SCN. We had hoped to find that ageing and chronic neuroinflammation might impact on the roles of NF-κB signalling in the SCN clock, since previous literature has already found that ageing can cause deterioration to the circadian timekeeping system (Weinert, 2000), and that the NF-κB signalling pathway is associated with ageing (Tilstra et al., 2011). Ageing has shown elevated levels in NF-κB/p65 DNA binding in peripheral tissues such as liver, kidney and cerebellum (Giardina & Hubbard, 2002; Kim et al., 2000; Korhonen et al., 1997). Recent studies have established that NF-κB increases as mice age in the mediobasal hypothalamus (MBH) when compared to young mice. NF-κB expression was investigated in young, middle-aged and aged mice, with a substantial increase of NF-κB expression exhibited as mice aged. The same study reveals that when IKK-β was inhibited in the hypothalamus in middle-old aged mice it reduced the number of microglial cells in comparison to controls. Additionally, Iba-1 and TNF-α decreased in middle aged and old aged mice where IKKβ was deficit. The IKKβ$^{-/-}$ mice also exhibited improvements with regard to their cognitive and physical wellbeing when compared to controls (Zhang et al., 2013). Another interesting finding from the Zhang study was that life expectancy increased up to 23% where IKK-β was inhibited, and MBH-IκBα mice had their life expectancy increased by approximately 7 months. In *Nfkb1*$^{-/-}$ mice have early onset ageing, with a decrease in their lifespan compared to *Nfkb1*$^{+/+}$ mice (Bernal et al., 2014). Therefore, findings from previous literature support that the NF-κB signalling pathway seems to be involved with ageing. Even though we did not find any significance in p65 expression between ageing and young adult mice in the SCN of healthy mice, this is likely to be due to the fact that the animals were only 16 months. Furthermore, other signalling properties of the NF-κB pathway including c-Rel,

p-IκB and p-IκK were not altered. Ageing mice did however display abnormalities in their behaviour locomotor rhythms *in vivo*, and circadian parameters were affected *in vitro*, when investigating basal levels of PER2::LUC SCN ageing cultures.

We established that an acute immune challenge caused by LPS increased p65 expression significantly in the SCN and the PVN, with similar results obtained from previous literature (Beynon & Coogan, 2010). LPS administration did not affect another signalling properties of the NF-κB pathway, p-IκB, in ageing mice. Additionally, chronic LPS administration was analysed to find out if NF-κB signalling is chronically upregulated. The data presented in this current study shows the two markers, p65 and p-IκB, do not show any alterations in expression between the two age groups in the SCN or PVN 16 months following treatment. This is comparable to findings found in other studies, regarding the SCN following chronic LPS dose after 3 and 10 months respectively (O'Callaghan et al., 2012; Qin et al., 2007). *In vitro* investigations using PER2::LUC mice, also added to the *in vivo* findings obtained, with ageing SCN culture explants not found to alter circadian parameters if challenged with LPS. *In vivo* results ascertained that there was no changes to PER1 expression after LPS treatment in ageing mice, therefore, we do not find evidence for disruption of the molecular clockwork in ageing mice after an immune challenge. The NF-κB inhibitor, PDTC, also did not significantly affect any circadian parameters when administered to ageing SCN explants when compared to controls with regard to period, amplitude and acrophase. This further emphasises that inhibiting the NF-κB pathway does not affect the circadian timing system *in vitro* with regards to ageing.

In conclusion, we have examined expression of constituents of the pathway across the circadian cycle in the SCN, have examined the effects of pharmacological inhibition of the pathway on circadian processes *in vivo* and *in vitro* and have examined whether there is evidence for circadian regulation of NF-kB-mediated transcription in the SCN. The findings gathered from these studies, suggests that there is lack of evidence that the NF-κB pathway is involved in the basal functioning of the SCN clock or in photic resetting. Some evidence obtained in this project suggests that the NF-κB pathway may have a role in transducing immune events in the SCN. To conclude, while the NF-kB pathway may be involved in circadian function in peripheral clocks, it does not appear to play a major role in the SCN clock.

Future investigations could look at the role NF-κB plays in non-photic clock resetting. Additionally, studies could be carried out in terms of the effect other NF-κB inhibitors have on the circadian clock, and whether they can block phase shifts as a result of photic induction in the early and late subjective night. Since NF-κB plays a vital role in regulating the immune and inflammatory response, it is essential to be careful in the study for new NF-κB inhibitor treatments aimed at various diseases, as blocking the NF-κB pathway for an extended period may result in severe side effects (Yamamoto & Gaynor, 2001). Certain inhibitors may also block other intracellular pathways causing dysfunction to other signalling cascades, therefore, caution needs to be taken (Gilmore & Herscovitch, 2006). Future studies into pharmacological treatment in identifying specific inhibitors of IKK activity may play a key role in alleviating inflammation, autoimmune diseases and cancer. However, from the data obtain in this study, we conclude that the NF-κB pathway does not play a significant role in the normal running or resetting of the SCN clock.

References

Abe, H., Honma, S., & Honma, K.-I. (2007). Daily restricted feeding resets the circadian clock in the suprachiasmatic nucleus of CS mice. The *American Journal of Physiology*, *292*(1), 607–15.

Abe, H., & Rusak, B. (1992). Stimulation of the hamster ventral lateral geniculate nucleus induces Fos-like immunoreactivity in suprachiasmatic nucleus cells. *Neuroscience Letters*, *148*(1-2), 185–189.

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., … Block, G. D. (2002). Circadian Rhythms in Isolated Brain Regions. *Journal of Neuroscience*, *22*(1), 350–356.

Abraham, C., & Cho, J. H. (2009). Inflammatory bowel disease. *The New England Journal of Medicine*, *361*(21), 2066–78.

Abrahamson, E. E., & Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Research*, *916*(1-2), 172–191.

Abrahamson, E. E., & Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain Research*, *916*(1-2), 172–91.

Aflalo, C. (1991). Biologically Localized Firefly Luciferase: A Tool to Study Cellular Processes. *International Review of Cytology*, *130*, 269–323.

Aguzzi, A., Brandner, S., Marino, S., & Steinbach, J. P. (1996). Transgenic and knockout mice in the study of neurodegenerative diseases. *Journal of Molecular Medicine*, *74*(3), 111–26.

Akama, K. T., Albanese, C., Pestell, R. G., & Van Eldik, L. J. (1998). Amyloid-peptide stimulates nitric oxide production in astrocytes through an NF-κB-dependent mechanism. *Proceedings of the National Academy of Sciences*, *95*(10), 5795–5800.

Akashi, M., Tsuchiya, Y., Yoshino, T., & Nishida, E. (2002). Control of Intracellular Dynamics of Mammalian Period Proteins by Casein Kinase I (CKI) and CKI in Cultured Cells. *Molecular and Cellular Biology*, *22*(6), 1693–1703.

Akira, S. (2009). Innate immunity to pathogens: diversity in receptors for microbial recognition. *Immunological Reviews*, *227*(1), 5–8.

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*, *124*(4), 783–801.

Albensi, B. C., & Mattson, M. P. (2000). Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. *Synapse*, *35*(2), 151–9.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). *Molecular Biology of the Cell*. Innate Immunity. 4th Edition. Garland Science, New York.

Albrecht, U., Sun, Z. S., Eichele, G., & Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell*, *91*(7), 1055–64.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S., & Lee, C. C. (2001). MPer1 and mper2 are essential for normal resetting of the circadian clock. *Journal of Biological Rhythms*, *16*(2), 100–4.

Albus, H., Vansteensel, M. J., Michel, S., Block, G. D., & Meijer, J. H. (2005). A GABAergic mechanism is necessary for coupling dissociable ventral and dorsal regional oscillators within the circadian clock. *Current Biology*, *15*(10), 886–93.

Ali, A. A. H., Schwarz-Herzke, B., Stahr, A., Prozorovski, T., Aktas, O., & von Gall, C. (2015). Premature aging of the hippocampal neurogenic niche in adult Bmal1-deficient mice. *Aging*, *7*(6), 435–49.

Anderson, S. T., Commins, S., Moynagh, P. N., & Coogan, A. N. (2015). Lipopolysaccharide-induced sepsis induces long-lasting affective changes in the mouse. *Brain, Behavior, and Immunity*, *43*, 98–109.

Anisman, H., Merali, Z., Poulter, M. O., & Hayley, S. (2005). Cytokines as a precipitant of depressive illness: animal and human studies. *Current Pharmaceutical Design*, *11*(8), 963–72.

Antle, M. C., Ogilvie, M. D., Pickard, G. E., & Mistlberger, R. E. (2003). Response of the mouse circadian system to serotonin 1A/2/7 agonists *in vivo*: surprisingly little. *Journal of Biological Rhythms*, *18*(2), 145–58.

Antle, M. C., & Silver, R. (2005). Orchestrating time: arrangements of the brain circadian clock. *Trends in Neurosciences*, *28*(3), 145–151.

Antoun, G., Bouchard-Cannon, P., Cannon, P. B., & Cheng, H.-Y. M. (2012). Regulation of MAPK/ERK signaling and photic entrainment of the suprachiasmatic nucleus circadian clock by Raf kinase inhibitor

protein. *The Journal of Neuroscience*, *32*(14), 4867–77.

Archer, S. N., Robilliard, D. L., Skene, D. J., Smits, M., Williams, A., Arendt, J., & von Schantz, M. (2003). A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. *Sleep*, *26*(4), 413–5.

Aronin, N., Sagar, S. M., Sharp, F. R., & Schwartz, W. J. (1990). Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(15), 5959–62.

Asai, M., Yoshinobu, Y., Kaneko, S., Mori, A., Nikaido, T., Moriya, T., … Shibata, S. (2001). Circadian profile of Per gene mRNA expression in the suprachiasmatic nucleus, paraventricular nucleus, and pineal body of aged rats. *Journal of Neuroscience Research*, *66*(6), 1133–9.

ASCHOFF, J. (1952). [Changes of frequency of periods of activity of mice in constant light and lasting darkness]. *Pflügers Archiv : European Journal of Physiology*, *255*(3), 197–203.

ASCHOFF, J. (1960). Exogenous and endogenous components in circadian rhythms. *Cold Spring Harbor Symposia on Quantitative Biology*, *25*, 11–28.

ASCHOFF, J. (1965). Circadian rhythms in man. *Science (New York, N.Y.)*, *148*(3676), 1427–32.

Asher, G., & Schibler, U. (2006). A CLOCK-less clock. *Trends in Cell Biology*, *16*(11), 547–9.

Aton, S. J., Block, G. D., Tei, H., Yamazaki, S., & Herzog, E. D. (2004). Plasticity of circadian behavior and the suprachiasmatic nucleus following exposure to non-24-hour light cycles. *Journal of Biological Rhythms*, *19*(3), 198–207.

Aton, S. J., Colwell, C. S., Harmar, A. J., Waschek, J., & Herzog, E. D. (2005). Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nature Neuroscience*, *8*(4), 476–83.

Atreya, I., Atreya, R., & Neurath, M. F. (2008). NF-kappaB in inflammatory bowel disease. *Journal of Internal Medicine*, *263*(6), 591–6.

Aujard, F., Herzog, E., & Block, G. (2001). Circadian rhythms in firing rate of individual suprachiasmatic nucleus neurons from adult and middle-aged mice. *Neuroscience*, *106*(2), 255–261.

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., & Karin, M. (1995). Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science, 270*(5234), 286–90.

Aupperle, K. R., Bennett, B. L., Boyle, D. L., Tak, P. P., Manning, A. M., & Firestein, G. S. (1999). NF-kappa B regulation by I kappa B kinase in primary fibroblast-like synoviocytes. *Journal of Immunology, 163*(1), 427–33.

Aupperle, K. R., Bennett, B. L., Han, Z., Boyle, D. L., Manning, A. M., & Firestein, G. S. (2001). NF- B Regulation by I B Kinase-2 in Rheumatoid Arthritis Synoviocytes. *The Journal of Immunology, 166*(4), 2705–2711.

Aydin, S. (2015). A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides,* 72, 4-15.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., & Weaver, D. R. (2001). Differential Functions of mPer1, mPer2, and mPer3 in the SCN Circadian Clock. *Neuron, 30*(2), 525–536.

Baeuerle, P. A., & Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. *Annual Review of Immunology, 12*, 141–79.

Bagchi, A., Herrup, E. A., Warren, H. S., Trigilio, J., Shin, H.-S., Valentine, C., & Hellman, J. (2007). MyD88-Dependent and MyD88-Independent Pathways in Synergy, Priming, and Tolerance between TLR Agonists. *The Journal of Immunology, 178*(2), 1164–1171.

Baldwin, T. O. (1996). Firefly luciferase: the structure is known, but the mystery remains. *Structure, 4*(3), 223–228.

Bales, K. R., Du, Y., Holtzman, D., Cordell, B., & Paul, S. M. Neuroinflammation and Alzheimer's disease: critical roles for cytokine/Abeta-induced glial activation, NF-kappaB, and apolipoprotein E. *Neurobiology of Aging, 21*(3), 427–32.

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., … Schibler, U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science, 289*(5488), 2344–7.

Balsalobre, A., Marcacci, L., & Schibler, U. (2000). Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Current Biology, 10*(20), 1291–4.

Banks, W. A., & Kastin, A. J. (1991). Blood to brain transport of interleukin links the immune and central nervous systems. *Life Sciences*, *48*(25), PL117–21.

Banks, W. A., Kastin, A. J., & Gutierrez, E. G. (1994). Penetration of interleukin-6 across the murine blood-brain barrier. *Neuroscience Letters*, *179*(1-2), 53–6.

Banks, W. A., & Robinson, S. M. (2010). Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. *Brain, Behavior, and Immunity*, *24*(1), 102–9.

Barnes, P. J., & Karin, M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *The New England Journal of Medicine*, *336*(15), 1066–71.

Barrientos, R. M., Kitt, M. M., Watkins, L. R., & Maier, S. F. (2015). Neuroinflammation in the normal aging hippocampus. *Neuroscience*.

Barton, G. M., & Medzhitov, R. (2003). Toll-like receptor signaling pathways. *Science*, *300*(5625), 1524–5.

Bass, J., & Takahashi, J. S. (2010). Circadian integration of metabolism and energetics. *Science (New York, N.Y.)*, *330*(6009), 1349–54.

Bechtold, D. a, Gibbs, J. E., & Loudon, A. S. I. (2010). Circadian dysfunction in disease. *Trends in Pharmacological Sciences*, *31*(5), 191–8.

Beg, A. A., & Baltimore, D. (1996). An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science (New York, N.Y.)*, *274*(5288), 782–4.

Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., & Baltimore, D. (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature*, *376*(6536), 167–70.

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

Benito, E., & Barco, A. (2015). The neuronal activity-driven transcriptome. *Molecular Neurobiology*, *51*(3), 1071–88.

Benloucif, S., & Dubocovich, M. L. (1996). Melatonin and light induce phase shifts of circadian activity rhythms in the C3H/HeN mouse. *Journal of Biological Rhythms*, *11*(2), 113–25.

Ben-Neriah, Y., & Karin, M. (2011). Inflammation meets cancer, with NF-κB as the matchmaker. *Nature Immunology*, *12*(8), 715–23.

Bennett, L. D., Beremand, P., Thomas, T. L., & Bell-Pedersen, D. (2013). Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in Neurospora crassa. *Eukaryotic Cell*, *12*(1), 59–69.

Bennett, M. R., Aronin, N., & Schwartz, W. J. (1996). *In vitro* stimulation of c-Fos protein expression in the suprachiasmatic nucleus of hypothalamic slices. *Brain Research. Molecular Brain Research*, *42*(1), 140–4.

Benson, S., Kattoor, J., Wegner, A., Hammes, F., Reidick, D., Grigoleit, J.-S., … Elsenbruch, S. (2012). Acute experimental endotoxemia induces visceral hypersensitivity and altered pain evaluation in healthy humans. *Pain*, *153*(4), 794–9.

Bernal, G. M., Wahlstrom, J. S., Crawley, C. D., Cahill, K. E., Pytel, P., Liang, H., Yamini, B. (2014). Loss of NF-κB1 leads to early onset aging. *Aging*, *6*(11), 931–43.

Berson, D. M., Dunn, F. A., & Takao, M. (2002). Phototransduction by retinal ganglion cells that set the circadian clock. *Science*, *295*(5557), 1070–3.

Berti, R., Williams, A. J., Moffett, J. R., Hale, S. L., Velarde, L. C., Elliott, P. J., Tortella, F. C. (2002). Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. *Journal of Cerebral Blood Flow and Metabolism*, *22*(9), 1068–79.

Bertolucci, C., Cavallari, N., Colognesi, I., Aguzzi, J., Chen, Z., Caruso, P., Pinotti, M. (2008). Evidence for an overlapping role of CLOCK and NPAS2 transcription factors in liver circadian oscillators. *Molecular and Cellular Biology*, *28*(9), 3070–5.

Beynon, A. L., & Coogan, A. N. (2010). Diurnal, age, and immune regulation of interleukin-1β and interleukin-1 type 1 receptor in the mouse suprachiasmatic nucleus. *Chronobiology International*, *27*(8), 1546–63.

Beynon, A. L., Thome, J., & Coogan, A. N. (2009). Age and time of day influences on the expression of transforming growth factor-beta and phosphorylated SMAD3 in the mouse suprachiasmatic and paraventricular nuclei. *Neuroimmunomodulation*, *16*(6), 392–9.

Bhakar, A. L., Tannis, L.-L., Zeindler, C., Russo, M. P., Jobin, C., Park, D. S., Barker, P. A. (2002). Constitutive Nuclear Factor-κB Activity Is Required for Central Neuron Survival. *The Journal of Neuroscience*, *22*(19), 8466–8475.

Biello, S. M. (2009). Circadian clock resetting in the mouse changes with age. *Age*, *31*(4), 293–303.

Biello, S. M., Harrington, M. E., & Mason, R. (1991). Geniculo-hypothalamic tract lesions block chlordiazepoxide-induced phase advances in Syrian hamsters. *Brain Research*, *552*(1), 47–52.

Bienkowski, M. S., & Rinaman, L. (2008). Noradrenergic inputs to the paraventricular hypothalamus contribute to hypothalamic-pituitary-adrenal axis and central Fos activation in rats after acute systemic endotoxin exposure. *Neuroscience*, *156*(4), 1093–102.

Biesmans, S., Meert, T. F., Bouwknecht, J. A., Acton, P. D., Davoodi, N., De Haes, P., Nuydens, R. (2013). Systemic immune activation leads to neuroinflammation and sickness behavior in mice. *Mediators of Inflammation*, *2013*, 271359.

Blasiak, T., & Lewandowski, M. H. (2013). Differential firing pattern and response to lighting conditions of rat intergeniculate leaflet neurons projecting to suprachiasmatic nucleus or contralateral intergeniculate leaflet. *Neuroscience*, *228*, 315–24.

Bliwise, D. L., Hughes, M., McMahon, P. M., & Kutner, N. (1995). Observed sleep/wakefulness and severity of dementia in an Alzheimer's disease special care unit. *The Journals of Gerontology.*, *50*(6), 303–6.

Block, M. L., & Hong, J.-S. (2005). Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Progress in Neurobiology*, *76*(2), 77–98.

Bluthé, R. M., Walter, V., Parnet, P., Layé, S., Lestage, J., Verrier, D., … Dantzer, R. (1994). Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. *Comptes Rendus de l'Académie Des Sciences. Série III, Sciences de La Vie*, *317*(6), 499–503.

Bohannon, J. K., Hernandez, A., Enkhbaatar, P., Adams, W. L., & Sherwood, E. R. (2013). The immunobiology of toll-like receptor 4 agonists: from endotoxin tolerance to immunoadjuvants. *Shock (Augusta, Ga.)*, *40*(6), 451–62.

Bonanni, E., Maestri, M., Tognoni, G., Fabbrini, M., Nucciarone, B.,

Manca, M. L., Murri, L. (2005). Daytime sleepiness in mild and moderate Alzheimer's disease and its relationship with cognitive impairment. *Journal of Sleep Research*, *14*(3), 311–7.

Bonetti, B., Stegagno, C., Cannella, B., Rizzuto, N., Moretto, G., & Raine, C. S. (1999). Activation of NF-kappaB and c-jun transcription factors in multiple sclerosis lesions. Implications for oligodendrocyte pathology. *The American Journal of Pathology*, *155*(5), 1433–8.

Bonizzi, G., Bebien, M., Otero, D. C., Johnson-Vroom, K. E., Cao, Y., Vu, D., Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *The EMBO Journal*, *23*(21), 4202–10.

Bonizzi, G., & Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends in Immunology*, *25*(6), 280–8.

Bonsall, D. R., & Lall, G. S. (2013). Protein kinase C differentially regulates entrainment of the mammalian circadian clock. *Chronobiology International*, *30*(4), 460–9.

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

Boulos, Z., & Rusak, B. (1982). Circadian phase response curves for dark pulses in the hamster. *Journal of Comparative Physiology*, *146*(4), 411–417.

Boutajangout, A., & Wisniewski, T. (2013). The innate immune system in Alzheimer's disease. *International Journal of Cell Biology*, *2013*, 576383.

Bozek, K., Kiełbasa, S. M., Kramer, A., & Herzel, H. (2007). Promoter analysis of Mammalian clock controlled genes. *Genome Informatics. International Conference on Genome Informatics*, *18*, 65–74.

Brandt, J. A., Churchill, L., Rehman, A., Ellis, G., Mémet, S., Israël, A., & Krueger, J. M. (2004). Sleep deprivation increases the activation of nuclear factor kappa B in lateral hypothalamic cells. *Brain Research*, *1004*(1-2), 91–7.

Brennan, P., & O'Neill, L. A. (1996). 2-mercaptoethanol restores the ability of nuclear factor kappa B (NF kappa B) to bind DNA in nuclear extracts from interleukin 1-treated cells incubated with pyrollidine

dithiocarbamate (PDTC). *The Biochemical Journal*, *320(3)*, 975–81.

Broad, A., Kirby, J. A., & Jones, D. E. J. (2007). Toll-like receptor interactions: tolerance of MyD88-dependent cytokines but enhancement of MyD88-independent interferon-beta production. *Immunology*, *120*(1), 103–11.

Brooks, E., & Canal, M. M. (2013). Development of circadian rhythms: role of postnatal light environment. *Neuroscience and Biobehavioral Reviews*, *37*(4), 551–60.

Brown, M. H., & Nunez, A. A. (1989). Vasopressin-deficient rats show a reduced amplitude of the circadian sleep rhythm. *Physiology & Behavior*, *46*(4), 759–62.

Brown, S. A., & Azzi, A. (2013). Peripheral circadian oscillators in mammals. *Handbook of Experimental Pharmacology*, (217), 45–66.

Bu, P., Gao, L., Zhuang, J., Feng, J., Yang, D., & Yan, X. (2006). Anti-CD146 monoclonal antibody AA98 inhibits angiogenesis via suppression of nuclear factor-kappaB activation. *Molecular Cancer Therapeutics*, *5*(11), 2872–8.

Buchanan, J. B., Sparkman, N. L., Chen, J., & Johnson, R. W. (2008). Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice. *Psychoneuroendocrinology*, *33*(6), 755–65.

Buhr, E. D., Yoo, S.-H., & Takahashi, J. S. (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. *Science, 330*(6002), 379–85. doi:10.1126/science.1195262

Buijs, R. M., & Kalsbeek, A. (2001). Hypothalamic integration of central and peripheral clocks. *Nature Reviews. Neuroscience*, *2*(7), 521–6.

Buijs, R. M., van Eden, C. G., Goncharuk, V. D., & Kalsbeek, A. (2003). The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. *The Journal of Endocrinology*, *177*(1), 17–26.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., … Bradfield, C. A. (2000). Mop3 Is an Essential Component of the Master Circadian Pacemaker in Mammals. *Cell*, *103*(7), 1009–1017.

Burkitt, M. J., Bishop, H. S., Milne, L., Tsang, S. Y., Provan, G. J., Nobel, C. S., … Slater, A. F. (1998). Dithiocarbamate toxicity toward

thymocytes involves their copper-catalyzed conversion to thiuram disulfides, which oxidize glutathione in a redox cycle without the release of reactive oxygen species. *Archives of Biochemistry and Biophysics*, *353*(1), 73–84.

Buschmann, T., Fuchs, S. Y., Lee, C. G., Pan, Z. Q., & Ronai, Z. (2000). SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell*, *101*(7), 753–62.

Bustin, S. A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology*, *25*(2), 169–93.

Butcher, G. Q., Doner, J., Dziema, H., Collamore, M., Burgoon, P. W., & Obrietan, K. (2002). The p42/44 mitogen-activated protein kinase pathway couples photic input to circadian clock entrainment. *The Journal of Biological Chemistry*, *277*(33), 29519–25.

Butler, M. P., & Silver, R. (2009). Basis of robustness and resilience in the suprachiasmatic nucleus: individual neurons form nodes in circuits that cycle daily. *Journal of Biological Rhythms*, *24*(5), 340–52.

Buttini, M., Sauter, A., & Boddeke, H. W. (1994). Induction of interleukin-1 beta mRNA after focal cerebral ischaemia in the rat. *Brain Research. Molecular Brain Research*, *23*(1-2), 126–34.

Byers, S. L., Wiles, M. V, Dunn, S. L., & Taft, R. A. (2012). Mouse estrous cycle identification tool and images. *PloS One*, *7*(4), e35538.

Canal, M. M., & Piggins, H. D. (2006). Resetting of the hamster circadian system by dark pulses. The *American Journal of Physiology*, *290*(3), R785–92.

Cao, J. P., Wang, H. J., Yu, J. K., Liu, H. M., & Gao, D. S. (2008). The involvement of NF-kappaB p65/p52 in the effects of GDNF on DA neurons in early PD rats. *Brain Research Bulletin*, *76*(5), 505–11.

Capecchi, M. R. (2001). Generating mice with targeted mutations. *Nature Medicine*, *7*(10), 1086–90.

Card, J. P., & Moore, R. Y. (1984). The suprachiasmatic nucleus of the golden hamster: immunohistochemical analysis of cell and fiber distribution. *Neuroscience*, *13*(2), 415–31.

Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J. J., & Sassone-Corsi, P. (2005). Circadian clock control by SUMOylation of BMAL1. *Science*, *309*(5739), 1390–4.

Carlsen, H., Moskaug, J. O., Fromm, S. H., & Blomhoff, R. (2002). *In Vivo* Imaging of NF- B Activity. *The Journal of Immunology*, *168*(3), 1441–1446.

Castanon-Cervantes, O., Wu, M., Ehlen, J. C., Paul, K., Gamble, K. L., Johnson, R. L., Davidson, A. J. (2010). Dysregulation of inflammatory responses by chronic circadian disruption. *Journal of Immunology*, *185*(10), 5796–805.

Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T., & Fontana, A. (2007). TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(31), 12843–8.

Cayetanot, F., Bentivoglio, M., & Aujard, F. (2005). Arginine-vasopressin and vasointestinal polypeptide rhythms in the suprachiasmatic nucleus of the mouse lemur reveal aging-related alterations of circadian pacemaker neurons in a non-human primate. *The European Journal of Neuroscience*, *22*(4), 902–10.

Cecon, E., Fernandes, P. A., Pinato, L., Ferreira, Z. S., & Markus, R. P. (2010). Daily variation of constitutively activated nuclear factor kappa B in rat pineal gland. *Chronobiology International*, *27*(1), 52–67.

Cermakian, N., Lamont, E. W., Boudreau, P., & Boivin, D. B. (2011). Circadian clock gene expression in brain regions of Alzheimer ’s disease patients and control subjects. *Journal of Biological Rhythms*, *26*(2), 160–70.

Cermakian, N., Lange, T., Golombek, D., Sarkar, D., Nakao, A., Shibata, S., & Mazzoccoli, G. (2013). Crosstalk between the circadian clock circuitry and the immune system. *Chronobiology International*, *30*(7), 870–88.

Cermakian, N., Monaco, L., Pando, M. P., Dierich, A., & Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. *The EMBO Journal*, *20*(15), 3967–74.

Chabicovsky, M., Prieschl-Grassauer, E., Seipelt, J., Muster, T., Szolar, O. H. J., Hebar, A., & Doblhoff-Dier, O. (2010). Pre-clinical safety evaluation of pyrrolidine dithiocarbamate. *Basic & Clinical Pharmacology & Toxicology*, *107*(3), 758–67.

Chakravarty, S., & Herkenham, M. (2005). Toll-like receptor 4 on

nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines. *The Journal of Neuroscience, 25*(7), 1788–96.

Challet, E. (2007). Minireview: Entrainment of the suprachiasmatic clockwork in diurnal and nocturnal mammals. *Endocrinology*, *148*(12), 5648–55.

Challet, E., Caldelas, I., Graff, C., & Pévet, P. (2003). Synchronization of the molecular clockwork by light- and food-related cues in mammals. *Biological Chemistry*, *384*(5), 711–9.

Challet, E., Scarbrough, K., Penev, P. D., & Turek, F. W. (1998). Roles of suprachiasmatic nuclei and intergeniculate leaflets in mediating the phase-shifting effects of a serotonergic agonist and their photic modulation during subjective day. *Journal of Biological Rhythms*, *13*(5), 410–21.

Chang, Y.-C., Tsai, M.-H., Sheu, W. H.-H., Hsieh, S.-C., & Chiang, A.-N. (2013). The therapeutic potential and mechanisms of action of quercetin in relation to lipopolysaccharide-induced sepsis *in vitro* and *in vivo*. *PloS One*, *8*(11), e80744.

Chaudhuri, A., Zangenehpour, S., Rahbar-Dehgan, F., & Ye, F. (2000). Molecular maps of neural activity and quiescence. *Acta Neurobiologiae Experimentalis*, *60*(3), 403–10.

Chen, D., Buchanan, G. F., Ding, J. M., Hannibal, J., & Gillette, M. U. (1999). Pituitary adenylyl cyclase-activating peptide: A pivotal modulator of glutamatergic regulation of the suprachiasmatic circadian clock. *Proceedings of the National Academy of Sciences*, *96*(23), 13468–13473.

Chen, J. (2005). SIRT1 Protects against Microglia-dependent Amyloid- Toxicity through Inhibiting NF- B Signaling. *Journal of Biological Chemistry*, *280*(48), 40364–40374.

Chen, J., Buchanan, J. B., Sparkman, N. L., Godbout, J. P., Freund, G. G., & Johnson, R. W. (2008). Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. *Brain, Behavior, and Immunity*, *22*(3), 301–11.

Chen, Z., Gardi, J., Kushikata, T., Fang, J., & Krueger, J. M. (1999). Nuclear factor-kappa B-like activity increases in murine cerebral cortex after sleep deprivation. The *American Journal of Physiology*, *276*(6), 1812–1818.

Chen, Z., Jalabi, W., Shpargel, K. B., Farabaugh, K. T., Dutta, R., Yin, X., … Trapp, B. D. (2012). Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. *The Journal of Neuroscience*, *32*(34), 11706–15.

Cheng, M. Y., Bullock, C. M., Li, C., Lee, A. G., Bermak, J. C., Belluzzi, J., … Zhou, Q.-Y. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature*, *417*(6887), 405–10.

Chou, T. C., Scammell, T. E., Gooley, J. J., Gaus, S. E., Saper, C. B., & Lu, J. (2003). Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. *The Journal of Neuroscience* , *23*(33), 10691–702.

Chourbaji, S., Urani, A., Inta, I., Sanchis-Segura, C., Brandwein, C., Zink, M., … Gass, P. (2006). IL-6 knockout mice exhibit resistance to stress-induced development of depression-like behaviors. *Neurobiology of Disease*, *23*(3), 587–94.

Chung, K. C., Park, J. H., Kim, C. H., Lee, H. W., Sato, N., Uchiyama, Y., & Ahn, Y. S. (2000). Novel biphasic effect of pyrrolidine dithiocarbamate on neuronal cell viability is mediated by the differential regulation of intracellular zinc and copper ion levels, NF-kappaB, and MAP kinases. *Journal of Neuroscience Research*, *59*(1), 117–25.

Claudio, E., Brown, K., Park, S., Wang, H., & Siebenlist, U. (2002). BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nature Immunology*, *3*(10), 958–65.

Clemens, J. A., Stephenson, D. T., Smalstig, E. B., Dixon, E. P., & Little, S. P. (1997). Global Ischemia Activates Nuclear Factor- B in Forebrain Neurons of Rats. *Stroke*, *28*(5), 1073–1081.

Collaco, A. M., Rahman, S., Dougherty, E. J., Williams, B. B., & Geusz, M. E. Circadian regulation of a viral gene promoter in live transgenic mice expressing firefly luciferase. *Molecular Imaging and Biology*, *7*(5), 342–50.

Colwell, C. S., & Foster, R. G. (1992). Photic regulation of Fos-like immunoreactivity in the suprachiasmatic nucleus of the mouse. *The Journal of Comparative Neurology*, *324*(2), 135–42.

Colwell, C. S., Foster, R. G., & Menaker, M. (1991). NMDA receptor

antagonists block the effects of light on circadian behavior in the mouse. *Brain Research*, *554*(1-2), 105–10.

Colwell, C. S., & Menaker, M. (1992). NMDA as Well as Non-NMDA Receptor Antagonists Can Prevent the Phase-Shifting Effects of Light on the Circadian System of the Golden Hamster. *Journal of Biological Rhythms*, *7*(2), 125–136.

Colwell, C. S., Ralph, M. R., & Menaker, M. (1990). Do NMDA receptors mediate the effects of light on circadian behavior? *Brain Research*, *523*(1), 117–20.

Coogan, A. N., & Piggins, H. D. (2003). Circadian and photic regulation of phosphorylation of ERK1/2 and Elk-1 in the suprachiasmatic nuclei of the Syrian hamster. *The Journal of Neuroscience*, *23*(7), 3085–93.

Coogan, A. N., & Piggins, H. D. (2005). Dark pulse suppression of P-ERK and c-Fos in the hamster suprachiasmatic nuclei. *The European Journal of Neuroscience*, *22*(1), 158–68.

Coogan, A. N., Schutová, B., Husung, S., Furczyk, K., Baune, B. T., Kropp, P., … Thome, J. (2013). The circadian system in Alzheimer's disease: disturbances, mechanisms, and opportunities. *Biological Psychiatry*, *74*(5), 333–9.

Coogan, A. N., & Thome, J. (2011). Chronotherapeutics and psychiatry: setting the clock to relieve the symptoms. *The World Journal of Biological Psychiatry*, *12(1)*, 40–3.

Coogan, A. N., & Wyse, C. a. (2008). Neuroimmunology of the circadian clock. *Brain Research*, *1232*, 104–12.

Coope, H. J., Atkinson, P. G. P., Huhse, B., Belich, M., Janzen, J., Holman, M. J., … Ley, S. C. (2002). CD40 regulates the processing of NF-kappaB2 p100 to p52. *The EMBO Journal*, *21*(20), 5375–85.

Cornélissen, G., Bakken, E., Delmore, P., Orth-Gomér, K., Akerstedt, T., Carandente, O., … Halberg, F. (1990). From various kinds of heart rate variability to chronocardiology. *The American Journal of Cardiology*, *66*(10), 863–8.

Courtois, G., & Gilmore, T. D. (2006). Mutations in the NF-kappaB signaling pathway: implications for human disease. *Oncogene*, *25*(51), 6831–43.

Crowley, K. (2011). Sleep and sleep disorders in older adults. *Neuropsychology Review*, *21*(1), 41–53.

Cruz, F. C., Koya, E., Guez-Barber, D. H., Bossert, J. M., Lupica, C. R., Shaham, Y., & Hope, B. T. (2013). New technologies for examining the role of neuronal ensembles in drug addiction and fear. *Nature Reviews. Neuroscience*, *14*(11), 743–54.

Cuesta, M., Aungier, J., & Morton, A. J. (2014). Behavioral therapy reverses circadian deficits in a transgenic mouse model of Huntington's disease. *Neurobiology of Disease*, *63*, 85–91.

Cuesta, M., Mendoza, J., Clesse, D., Pévet, P., & Challet, E. (2008). Serotonergic activation potentiates light resetting of the main circadian clock and alters clock gene expression in a diurnal rodent. *Experimental Neurology*, *210*(2), 501–13.

Curtis, A. M., Bellet, M. M., Sassone-Corsi, P., & O'Neill, L. A. J. (2014). Circadian clock proteins and immunity. *Immunity*, *40*(2), 178–86.

Curtis, A. M., Fagundes, C. T., Yang, G., Palsson-McDermott, E. M., Wochal, P., McGettrick, A. F., … O'Neill, L. A. J. (2015). Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(23), 7231–6.

Cutolo, M. (2012). Chronobiology and the treatment of rheumatoid arthritis. *Current Opinion in Rheumatology*, *24*(3), 312–8.

Cutrera, R. A., Ouarour, A., & Pévet, P. (1994). Effects of the 5-HT1a receptor agonist 8-OH-DPAT and other non-photic stimuli on the circadian rhythm of wheel-running activity in hamsters under different constant conditions. *Neuroscience Letters*, *172*(1-2), 27–30.

Cuzzocrea, S., Chatterjee, P. K., Mazzon, E., Dugo, L., Serraino, I., Britti, D., … Thiemermann, C. (2002). Pyrrolidine dithiocarbamate attenuates the development of acute and chronic inflammation. *British Journal of Pharmacology*, *135*(2), 496–510.

Cvek, B., & Dvorak, Z. (2007). Targeting of nuclear factor-kappaB and proteasome by dithiocarbamate complexes with metals. *Current Pharmaceutical Design*, *13*(30), 3155–67.

Czeisler, C. A., Duffy, J. F., Shanahan, T. L., Brown, E. N., Mitchell, J. F., Rimmer, D. W., … Kronauer, R. E. (1999). Stability, precision, and near-24-hour period of the human circadian pacemaker. *Science*, *284*(5423), 2177–81.

Daan, S., & Pittendrigh, C. S. (1976). A Functional analysis of circadian pacemakers in nocturnal rodents. *Journal of Comparative Physiology*,

106(3), 253–266.

Dai, J., Swaab, D. F., & Buijs, R. M. (1997). Distribution of vasopressin and vasoactive intestinal polypeptide (VIP) fibers in the human hypothalamus with special emphasis on suprachiasmatic nucleus efferent projections. *The Journal of Comparative Neurology*, *383*(4), 397–414.

Dallmann, R., DeBruyne, J. P., & Weaver, D. R. (2011). Photic resetting and entrainment in CLOCK-deficient mice. *Journal of Biological Rhythms*, *26*(5), 390–401.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes & Development*, *14*(23), 2950–61.

Dantzer, R. (2001). Cytokine-induced sickness behavior: where do we stand? *Brain, Behavior, and Immunity*, *15*(1), 7–24.

Dantzer, R. (2004). Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. *European Journal of Pharmacology*, *500*(1-3), 399–411.

Dantzer, R. (2009). Cytokine, sickness behavior, and depression. *Immunology and Allergy Clinics of North America*, *29*(2), 247–64.

Davidson, A. J., Sellix, M. T., Daniel, J., Yamazaki, S., Menaker, M., & Block, G. D. (2006). Chronic jet-lag increases mortality in aged mice. *Current Biology*, *16*(21), 914–6.

Davidson, A. J., Yamazaki, S., Arble, D. M., Menaker, M., & Block, G. D. (2008). Resetting of central and peripheral circadian oscillators in aged rats. *Neurobiology of Aging*, *29*(3), 471–7.

De Bosscher, K. (2000). Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. *Journal of Neuroimmunology*, *109*(1), 16–22.

De Jeu, M., & Pennartz, C. (2002). Circadian modulation of GABA function in the rat suprachiasmatic nucleus: excitatory effects during the night phase. *Journal of Neurophysiology*, *87*(2), 834–44.

de Paula, R. M., Lamb, T. M., Bennett, L., & Bell-Pedersen, D. (2008). A connection between MAPK pathways and circadian clocks. *Cell Cycle*, *7*(17), 2630–4.

de Wet, J. R., Wood, K. V, DeLuca, M., Helinski, D. R., & Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology*, *7*(2), 725–37.

Debruyne, J. P., Noton, E., Lambert, C. M., Maywood, E. S., Weaver, D. R., & Reppert, S. M. (2006). A clock shock: mouse CLOCK is not required for circadian oscillator function. *Neuron*, *50*(3), 465–77.

DeBruyne, J. P., Weaver, D. R., & Reppert, S. M. (2007a). CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. *Nature Neuroscience*, *10*(5), 543–5.

DeBruyne, J. P., Weaver, D. R., & Reppert, S. M. (2007b). Peripheral circadian oscillators require CLOCK. *Current Biology*, *17*(14), 538–9.

Deepak, S., Kottapalli, K., Rakwal, R., Oros, G., Rangappa, K., Iwahashi, H., … Agrawal, G. (2007). Real-Time PCR: Revolutionizing Detection and Expression Analysis of Genes. *Current Genomics*, *8*(4), 234–51.

Delerive, P., Monté, D., Dubois, G., Trottein, F., Fruchart-Najib, J., Mariani, J., … Staels, B. (2001). The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO Reports*, *2*(1), 42–8.

Deng, X.-H., Bertini, G., Palomba, M., Xu, Y.-Z., Bonaconsa, M., Nygård, M., & Bentivoglio, M. (2010). Glial transcripts and immune-challenged glia in the suprachiasmatic nucleus of young and aged mice. *Chronobiology International*, *27*(4), 742–67.

Deng, X.-H., Bertini, G., Xu, Y.-Z., Yan, Z., & Bentivoglio, M. (2006). Cytokine-induced activation of glial cells in the mouse brain is enhanced at an advanced age. *Neuroscience*, *141*(2), 645–61.

Deng, Y., Ren, X., Yang, L., Lin, Y., & Wu, X. (2003). A JNK-dependent pathway is required for TNFalpha-induced apoptosis. *Cell*, *115*(1), 61–70.

Desterro, J. M., Rodriguez, M. S., & Hay, R. T. (1998). SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Molecular Cell*, *2*(2), 233–9.

Deurveilher, S., & Semba, K. (2005). Indirect projections from the suprachiasmatic nucleus to major arousal-promoting cell groups in rat: implications for the circadian control of behavioural state. *Neuroscience*, *130*(1), 165–83.

Dibner, C., Schibler, U., & Albrecht, U. (2010). The mammalian circadian

timing system: organization and coordination of central and peripheral clocks. *Annual Review of Physiology*, *72*, 517–49.

Ding, J., Chen, D., Weber, E., Faiman, L., Rea, M., & Gillette, M. (1994). Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science*, *266*(5191), 1713–1717.

Ding, J. M., Chen, D., Weber, E. T., Faiman, L. E., Rea, M. A., & Gillette, M. U. (1994). Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. *Science*, *266*(5191), 1713–7.

Diz-Chaves, Y., Astiz, M., Bellini, M. J., & Garcia-Segura, L. M. (2013). Prenatal stress increases the expression of proinflammatory cytokines and exacerbates the inflammatory response to LPS in the hippocampal formation of adult male mice. *Brain, Behavior, and Immunity*, *28*, 196–206.

Dowling, G. A., Burr, R. L., Van Someren, E. J. W., Hubbard, E. M., Luxenberg, J. S., Mastick, J., & Cooper, B. A. (2008). Melatonin and bright-light treatment for rest-activity disruption in institutionalized patients with Alzheimer's disease. *Journal of the American Geriatrics Society*, *56*(2), 239–46.

Doyle, S. L., Shirey, K. A., McGettrick, A. F., Kenny, E. F., Carpenter, S., Caffrey, B. E., … O'Neill, L. A. (2013). Nuclear factor κB2 p52 protein has a role in antiviral immunity through IκB kinase epsilon-dependent induction of Sp1 protein and interleukin 15. *The Journal of Biological Chemistry*, *288*(35), 25066–75.

Dubrovsky, Y. V, Samsa, W. E., & Kondratov, R. V. (2010). Deficiency of circadian protein CLOCK reduces lifespan and increases age-related cataract development in mice. *Aging*, *2*(12), 936–44.

Duguay, D., & Cermakian, N. (2009). The crosstalk between physiology and circadian clock proteins. *Chronobiology International*, *26*(8), 1479–513.

Duhart, J. M., Leone, M. J., Paladino, N., Evans, J. A., Castanon-Cervantes, O., Davidson, A. J., & Golombek, D. A. (2013). Suprachiasmatic astrocytes modulate the circadian clock in response to TNF-α. *Journal of Immunology*, *191*(9), 4656–64.

Dziema, H., Oatis, B., Butcher, G. Q., Yates, R., Hoyt, K. R., & Obrietan, K. (2003). The ERK/MAP kinase pathway couples light to immediate-early gene expression in the suprachiasmatic nucleus. *The European Journal of Neuroscience*, *17*(8), 1617–27.

Earnest, D. J., & Olschowka, J. A. (1993). Circadian regulation of c-fos expression in the suprachiasmatic pacemaker by light. *Journal of Biological Rhythms*, *8*, 65–71.

Easton, A., Arbuzova, J., & Turek, F. W. (2003). The circadian Clock mutation increases exploratory activity and escape-seeking behavior. *Genes, Brain, and Behavior*, *2*(1), 11–9.

EBIHARA, S., TSUJI, K., & KONDO, K. (1978). Strain differences of the mouse's free-running circadian rhythm in continuous darkness☆. *Physiology & Behavior*, *20*(6), 795–799.

Ebisawa, T., Uchiyama, M., Kajimura, N., Mishima, K., Kamei, Y., Katoh, M., … Yamauchi, T. (2001). Association of structural polymorphisms in the human period3 gene with delayed sleep phase syndrome. *EMBO Reports*, *2*(4), 342–6.

EBLING, F. (1996). The role of glutamate in the photic regulation of the suprachiasmatic nucleus. *Progress in Neurobiology*, *50*(2-3), 109–132.

Ebling, F. J., Maywood, E. S., Staley, K., Humby, T., Hancock, D. C., Waters, C. M., … Hastings, M. H. (1991). The role of N-methyl-d-aspartate-type glutamatergic neurotransmission in the photic induction of immediate-early gene expression in the suprachiasmatic nuclei of the Syrian hamster. *Journal of Neuroendocrinology*, *3*(6), 641–52.

Edgar, D. M., Miller, J. D., Prosser, R. A., Dean, R. R., & Dement, W. C. (1993). Serotonin and the mammalian circadian system: II. Phase-shifting rat behavioral rhythms with serotonergic agonists. *Journal of Biological Rhythms*, *8*(1), 17–31.

Ehlen, J. C., Grossman, G. H., & Glass, J. D. (2001). *In Vivo* Resetting of the Hamster Circadian Clock by 5-HT7 Receptors in the Suprachiasmatic Nucleus. *The Journal of Neuroscience*, *21*(14), 5351–5357.

Ehlen, J. C., Novak, C. M., Karom, M. C., Gamble, K. L., Paul, K. N., & Albers, H. E. (2006). GABAA receptor activation suppresses Period 1 mRNA and Period 2 mRNA in the suprachiasmatic nucleus during the mid-subjective day. *The European Journal of Neuroscience*, *23*(12), 3328–36.

Ehrlich, L. C., Hu, S., Peterson, P. K., & Chao, C. C. (1998). IL-10 down-regulates human microglial IL-8 by inhibition of NF-kappaB activation. *Neuroreport*, *9*(8), 1723–6.

Eide, E. J., Vielhaber, E. L., Hinz, W. A., & Virshup, D. M. (2002). The

circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase Iepsilon. *The Journal of Biological Chemistry*, *277*(19), 17248–54.

Eide, E. J., Woolf, M. F., Kang, H., Woolf, P., Hurst, W., Camacho, F., … Virshup, D. M. (2005). Control of mammalian circadian rhythm by CKIepsilon-regulated proteasome-mediated PER2 degradation. *Molecular and Cellular Biology*, *25*(7), 2795–807.

Ekdahl, C. T., Claasen, J.-H., Bonde, S., Kokaia, Z., & Lindvall, O. (2003). Inflammation is detrimental for neurogenesis in adult brain. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(23), 13632–7.

Ekmekcioglu, C. (2006). Melatonin receptors in humans: biological role and clinical relevance. *Biomedicine & Pharmacotherapy*, *60*(3), 97–108.

Elmquist, J. K., Ackermann, M. R., Register, K. B., Rimler, R. B., Ross, L. R., & Jacobson, C. D. (1993). Induction of Fos-like immunoreactivity in the rat brain following Pasteurella multocida endotoxin administration. *Endocrinology*, *133*(6), 3054–7.

Emery, P. (2012). Optimizing outcomes in patients with rheumatoid arthritis and an inadequate response to anti-TNF treatment. *Rheumatology*, *51*(5), 22–30.

Eriksen, J. L., Sagi, S. A., Smith, T. E., Weggen, S., Das, P., McLendon, D. C., … Golde, T. E. (2003). NSAIDs and enantiomers of flurbiprofen target gamma-secretase and lower Abeta 42 *in vivo*. *The Journal of Clinical Investigation*, *112*(3), 440–9.

Evans, J. A., Carter, S. N., Freeman, D. A., & Gorman, M. R. (2012). Dim nighttime illumination alters photoperiodic responses of hamsters through the intergeniculate leaflet and other photic pathways. *Neuroscience*, *202*, 300–8.

Evans, J. A., & Davidson, A. J. (2013). Health consequences of circadian *disruption in humans and animal models. Progress in Molecular Biology and Translational Science*, *119, 283–323.*

Farajnia, S., Michel, S., Deboer, T., vanderLeest, H. T., Houben, T., Rohling, J. H. T., … Meijer, J. H. (2012). Evidence for neuronal desynchrony in the aged suprachiasmatic nucleus clock. *The Journal of Neuroscience*, *32*(17), 5891–9.

Farajnia, S., van Westering, T. L. E., Meijer, J. H., & Michel, S. (2014). Seasonal induction of GABAergic excitation in the central mammalian

clock. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(26), 9627–32.

Feldman, J. F., & Hoyle, M. N. (1976). Complementation analysis of linked circadian clock mutants of Neurospora crassa. *Genetics*, *82*(1), 9–17.

Feldmann, M., Brennan, F. M., & Maini, R. N. (1996). Role of cytokines in rheumatoid arthritis. *Annual Review of Immunology*, *14*, 397–440.

Ferguson, A. V, Latchford, K. J., & Samson, W. K. (2008). The paraventricular nucleus of the hypothalamus - a potential target for integrative treatment of autonomic dysfunction. *Expert Opinion on Therapeutic Targets*, *12*(6), 717–27.

Field, M. D., Maywood, E. S., O'Brien, J. A., Weaver, D. R., Reppert, S. M., & Hastings, M. H. (2000). Analysis of Clock Proteins in Mouse SCN Demonstrates Phylogenetic Divergence of the Circadian Clockwork and Resetting Mechanisms. *Neuron*, *25*(2), 437–447.

Filipski, E., King, V. M., Li, X., Granda, T. G., Mormont, M.-C., Claustrat, B., … Lévi, F. (2003). Disruption of circadian coordination accelerates malignant growth in mice. *Pathologie Biologie*, *51*(4), 216–219.

Filipski, E., Li, X. M., & Lévi, F. (2006). Disruption of circadian coordination and malignant growth. *Cancer Causes & Control*, *17*(4), 509–14.

Flood, P. M., Qian, L., Peterson, L. J., Zhang, F., Shi, J.-S., Gao, H.-M., & Hong, J.-S. (2011). Transcriptional Factor NF-κB as a Target for Therapy in Parkinson's Disease. *Parkinson's Disease*, 216298.

Fonken, L. K., Frank, M. G., Kitt, M. M., Barrientos, R. M., Watkins, L. R., & Maier, S. F. (2015). Microglia inflammatory responses are controlled by an intrinsic circadian clock. *Brain, Behavior, and Immunity*, *45*, 171–9.

Fonseca Costa, S. S., & Ripperger, J. A. (2015). Impact of the circadian clock on the aging process. *Frontiers in Neurology*, *6*, 43.

Foster, R. G., & Roenneberg, T. (2008). Human responses to the geophysical daily, annual and lunar cycles. *Current Biology*, *18*(17), 784–794.

Frank-Cannon, T. C., Alto, L. T., McAlpine, F. E., & Tansey, M. G. (2009). Does neuroinflammation fan the flame in neurodegenerative diseases? *Molecular Neurodegeneration*, *4*(1), 47.

Franzoso, G., Carlson, L., Poljak, L., Shores, E. W., Epstein, S., Leonardi,

A., … Siebenlist, U. (1998). Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *The Journal of Experimental Medicine*, *187*(2), 147–59.

Freeman, G. M., Krock, R. M., Aton, S. J., Thaben, P., & Herzog, E. D. (2013). GABA networks destabilize genetic oscillations in the circadian pacemaker. *Neuron*, *78*(5), 799–806.

Furukawa, K., & Mattson, M. P. (1998). The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. *Journal of Neurochemistry*, *70*(5), 1876–86.

Gallego, M., & Virshup, D. M. (2007). Post-translational modifications regulate the ticking of the circadian clock. *Nature Reviews. Molecular Cell Biology*, *8*(2), 139–48.

Gannon, R. L. (2003). Serotonergic serotonin (1A) mixed agonists/antagonists elicit large-magnitude phase shifts in hamster circadian wheel-running rhythms. *Neuroscience*, *119*(2), 567–76.

Gao, Y.-J., & Ji, R.-R. (2009). c-Fos and pERK, which is a better marker for neuronal activation and central sensitization after noxious stimulation and tissue injury? *The Open Pain Journal*, *2*, 11–17.

Gekakis, N. (1998). Role of the CLOCK Protein in the Mammalian Circadian Mechanism. *Science*, *280*(5369), 1564–1569.

Geusz, M. E., Fletcher, C., Block, G. D., Straume, M., Copeland, N. G., Jenkins, N. A., … Day, R. N. (1997). Long-term monitoring of circadian rhythms in c-fos gene expression from suprachiasmatic nucleus cultures. *Current Biology*, *7*(10), 758–66.

Ghosh, A., Roy, A., Liu, X., Kordower, J. H., Mufson, E. J., Hartley, D. M., … Pahan, K. (2007). Selective inhibition of NF-kappaB activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(47), 18754–9.

Ghosh, S., & Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell*, *109*, 81–96.

Ghosh, S., May, M. J., & Kopp, E. B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual Review of Immunology*, *16*, 225–60.

Giardina, C., & Hubbard, A. K. (2002). Growing old with nuclear factor-kappaB. *Cell Stress & Chaperones*, *7*(2), 207–12.

Gibbs, J. E., Beesley, S., Plumb, J., Singh, D., Farrow, S., Ray, D. W., & Loudon, A. S. I. (2009). Circadian timing in the lung; a specific role for bronchiolar epithelial cells. *Endocrinology*, *150*(1), 268–76.

Gibbs, J. E., Blaikley, J., Beesley, S., Matthews, L., Simpson, K. D., Boyce, S. H., … Loudon, A. S. I. (2012). The nuclear receptor REV-ERBα mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(2), 582–7.

Gibbs, J., Ince, L., Matthews, L., Mei, J., Bell, T., Yang, N., … Loudon, A. (2014). An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. *Nature Medicine*, *20*(8), 919–26.

Gillespie, C. F., Mintz, E. M., Marvel, C. L., Huhman, K. L., & Albers, H. E. (1997). GABA(A) and GABA(B) agonists and antagonists alter the phase-shifting effects of light when microinjected into the suprachiasmatic region. *Brain Research*, *759*(2), 181–9.

Gilmore, T. D., & Herscovitch, M. (2006). Inhibitors of NF-κB signaling: 785 and counting. *Oncogene*, *25*(51), 6887–6899.

Godbout, J. P., Berg, B. M., Krzyszton, C., & Johnson, R. W. (2005). Alpha-tocopherol attenuates NFkappaB activation and pro-inflammatory cytokine production in brain and improves recovery from lipopolysaccharide-induced sickness behavior. *Journal of Neuroimmunology*, *169*(1-2), 97–105.

Godbout, J. P., Chen, J., Abraham, J., Richwine, A. F., Berg, B. M., Kelley, K. W., & Johnson, R. W. (2005). Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. *Official Publication of the Federation of American Societies for Experimental Biology*, *19*(10), 1329–31.

Godbout, J. P., & Johnson, R. W. (2006). Age and neuroinflammation: a lifetime of psychoneuroimmune consequences. *Neurologic Clinics*, *24*(3), 521–38.

Godbout, J. P., & Johnson, R. W. (2009). Age and neuroinflammation: a lifetime of psychoneuroimmune consequences. *Immunology and Allergy Clinics of North America*, *29*(2), 321–37.

Goehler, L. E., Gaykema, R. P. A., Nguyen, K. T., Lee, J. E., Tilders, F. J. H., Maier, S. F., & Watkins, L. R. (1999). Interleukin-1beta in

Immune Cells of the Abdominal Vagus Nerve: a Link between the Immune and Nervous Systems? *J. Neuroscience.*, *19*(7), 2799–2806.

Goldsmith, C. S., & Bell-Pedersen, D. (2013). Diverse roles for MAPK signaling in circadian clocks. *Advances in Genetics*, *84*, 1–39.

Golombek, D. A., & Rosenstein, R. E. (2010). Physiology of circadian entrainment. *Physiological Reviews*, *90*(3), 1063–102.

Gonzalez, M. M. C., & Aston-Jones, G. (2008). Light deprivation damages monoamine neurons and produces a depressive behavioral phenotype in rats. *Proceedings of the National Academy of Sciences*, *105*(12),

Gooley, J. J., Lu, J., Chou, T. C., Scammell, T. E., & Saper, C. B. (2001). Melanopsin in cells of origin of the retinohypothalamic tract. *Nature Neuroscience*, *4*(12), 1165.

Greenberg, M. E., Thompson, M. A., & Sheng, M. (1992). Calcium regulation of immediate early gene transcription. *Journal of Physiology*, *86*(1-3), 99–108.

Greenberg, M. E., & Ziff, E. B. Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. *Nature*, *311*(5985), 433–8.

Grisham, M. B., Palombella, V. J., Elliott, P. J., Conner, E. M., Brand, S., Wong, H. L., … Adams, J. (1999). Inhibition of NF-kappa B activation *in vitro* and *in vivo*: role of 26S proteasome. *Methods in Enzymology*, *300*, 345–63.

Gu, J.-W., Young, E., Busby, B., Covington, J., & Johnson, J. W. (2009). Oral administration of pyrrolidine dithiocarbamate (PDTC) inhibits VEGF expression, tumor angiogenesis, and growth of breast cancer in female mice. *Cancer Biology & Therapy*, *8*(6), 514–21.

Guardigli, M., Marangi, M., Casanova, S., Grigioni, W. F., Roda, E., & Roda, A. (2005). Chemiluminescence quantitative immunohistochemical determination of MRP2 in liver biopsies. *J Histochem and Cytochem*, *53*(12), 1451–7.

Guénet, J. L. (2005). The mouse genome. *Genome Research*, *15*(12), 1729–40.

Guenthner, C. J., Bickar, D., & Harrington, M. E. (2009). Heme reversibly damps PERIOD2 rhythms in mouse suprachiasmatic nucleus explants. *Neuroscience*, *164*(2), 832–41.

Guenthner, C. J., Luitje, M. E., Pyle, L. A., Molyneux, P. C., Yu, J. K., Li, A. S., … Harrington, M. E. (2014). Circadian rhythms of Per2::Luc in

individual primary mouse hepatocytes and cultures. *PloS One*, *9*(2), e87573.

Guerrero-Vargas, N. N., Salgado-Delgado, R., Basualdo, M. D. C., García, J., Guzmán-Ruiz, M., Carrero, J. C., … Buijs, R. M. (2014). Reciprocal interaction between the suprachiasmatic nucleus and the immune system tunes down the inflammatory response to lipopolysaccharide. *Journal of Neuroimmunology*, *273*(1-2), 22–30.

Guerrini, L., Blasi, F., & Denis-Donini, S. (1995). Synaptic activation of NF-kappa B by glutamate in cerebellar granule neurons *in vitro*. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(20), 9077–81.

Guido, M. E., Goguen, D., De Guido, L., Robertson, H. A., & Rusak, B. (1999). Circadian and photic regulation of immediate-early gene expression in the hamster suprachiasmatic nucleus. *Neuroscience*, *90*(2), 555–71.

Guilding, C., Hughes, A. T. L., Brown, T. M., Namvar, S., & Piggins, H. D. (2009). A riot of rhythms: neuronal and glial circadian oscillators in the mediobasal hypothalamus. *Molecular Brain*, *2*(1), 28.

Guilding, C., & Piggins, H. D. (2007). Challenging the omnipotence of the suprachiasmatic timekeeper: are circadian oscillators present throughout the mammalian brain? *European Journal of Neuroscience*, *25*(11), 3195–216.

Guillaumond, F., Dardente, H., Giguère, V., & Cermakian, N. (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *Journal of Biological Rhythms*, *20*(5), 391–403.

Guo, H., Guo, H., Brewer, J. M., Lehman, M. N., & Bittman, E. L. (2006). Suprachiasmatic regulation of circadian rhythms of gene expression in hamster peripheral organs: effects of transplanting the pacemaker. *Journal of Neuroscience*, *26*(24), 6406–12.

Gupta, S. C., Sundaram, C., Reuter, S., & Aggarwal, B. B. (2010). Inhibiting NF-κB activation by small molecules as a therapeutic strategy. *Biochimica et Biophysica Acta*, *1799*(10-12), 775–87.

Gutierrez, E. G., Banks, W. A., & Kastin, A. J. (1993). Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. *Journal of Neuroimmunology*, *47*(2), 169–76. R

Guzowski, J. F., Lyford, G. L., Stevenson, G. D., Houston, F. P., McGaugh,

J. L., Worley, P. F., & Barnes, C. A. (2000). Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *The Journal of Neuroscience*, *20*(11), 3993–4001.

Guzowski, J. F., Timlin, J. A., Roysam, B., McNaughton, B. L., Worley, P. F., & Barnes, C. A. (2005). Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Current Opinion in Neurobiology*, *15*(5), 599–606.

Häcker, H., & Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. *Science's STKE*, *2006* (357), re13.

Haimovich, B., Calvano, J., Haimovich, A. D., Calvano, S. E., Coyle, S. M., & Lowry, S. F. (2010). *In vivo* endotoxin synchronizes and suppresses clock gene expression in human peripheral blood leukocytes. *Critical Care Medicine*, *38*(3), 751–8.

Halberg, F., Johnson, E. A., Brown, B. W., & Bittner, J. J. (1960). Susceptibility Rhythm to E. coli Endotoxin and Bioassay. *Experimental Biology and Medicine*, *103*(1), 142–144.

Hamada, T., LeSauter, J., Lokshin, M., Romero, M.-T., Yan, L., Venuti, J. M., & Silver, R. (2003). Calbindin Influences Response to Photic Input in Suprachiasmatic Nucleus. *Journal of Neuroscience*, *23*(26), 8820–8826.

Hamada, T., LeSauter, J., Venuti, J. M., & Silver, R. (2001). Expression of Period genes: rhythmic and nonrhythmic compartments of the suprachiasmatic nucleus pacemaker. *Journal of Neuroscience*, *21*(19), 7742–50.

Hang, C., Shi, J., Tian, J., Li, J., Wu, W., & Yin, H. (2004). Effect of systemic LPS injection on cortical NF-kappaB activity and inflammatory response following traumatic brain injury in rats. *Brain Research*, *1026*(1), 23–32.

Hannibal, J. (2002). Neurotransmitters of the retino-hypothalamic tract. *Cell and Tissue Research*, *309*(1), 73–88.

Hannibal, J. (2006). Roles of PACAP-containing retinal ganglion cells in circadian timing. *International Review of Cytology*, *251*, 1–39.

Hannibal, J., Ding, J. M., Chen, D., Fahrenkrug, J., Larsen, P. J., Gillette, M. U., & Mikkelsen, J. D. (1997). Pituitary adenylate cyclase-activating peptide (PACAP) in the retinohypothalamic tract: a potential daytime regulator of the biological clock. *The Journal of Neuroscience*,

17(7), 2637–44.

Hannibal, J., & Fahrenkrug, J. (2002). Immunoreactive substance P is not part of the retinohypothalamic tract in the rat. *Cell and Tissue Research*, *309*(2), 293–9.

Harms, E., Young, M. W., & Saez, L. (2003). CK1 and GSK3 in the Drosophila and mammalian circadian clock. *Novartis Foundation Symposium*, *253*, 267–84.

Harper, D. G., Stopa, E. G., Kuo-Leblanc, V., McKee, A. C., Asayama, K., Volicer, L., … Satlin, A. (2008). Dorsomedial SCN neuronal subpopulations subserve different functions in human dementia. *Brain*, *131*(6), 1609–17.

Harper, D. G., Volicer, L., Stopa, E. G., McKee, A. C., Nitta, M., & Satlin, A. (2005). Disturbance of Endogenous Circadian Rhythm in Aging and Alzheimer Disease. *The American Journal of Geriatric Psychiatry*, *13*(5), 359–368.

HARRINGTON, M. E. (1997). The Ventral Lateral Geniculate Nucleus and the Intergeniculate Leaflet: Interrelated Structures in the Visual and Circadian Systems. *Neuroscience & Biobehavioral Reviews*, *21*(5), 705–727.

Harrington, M. E., Hoque, S., Hall, A., Golombek, D., & Biello, S. (1999). Pituitary Adenylate Cyclase Activating Peptide Phase Shifts Circadian Rhythms in a Manner Similar to Light. *Journal of Neuroscience*, *19*(15), 6637–6642.

Harrington, M. E., & Rusak, B. (1986). Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. *Journal of Biological Rhythms*, *1*(4), 309–25.

Harrington, M., Molyneux, P., Soscia, S., Prabakar, C., McKinley-Brewer, J., & Lall, G. (2007). Behavioral and neurochemical sources of variability of circadian period and phase: studies of circadian rhythms of npy-/- mice. *American Journal of Physiology*, *292*(3), 1306–14.

Hastings, M. H., & Herzog, E. D. (2004). Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. *Journal of Biological Rhythms*, *19*(5), 400–13.

Hatfield, C. F., Herbert, J., van Someren, E. J. W., Hodges, J. R., & Hastings, M. H. (2004). Disrupted daily activity/rest cycles in relation to daily cortisol rhythms of home-dwelling patients with early Alzheimer's dementia. *Brain*, *127*(5), 1061–74.

Hattar, S., Kumar, M., Park, A., Tong, P., Tung, J., Yau, K.-W., & Berson, D. M. (2006). Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *The Journal of Comparative Neurology*, *497*(3), 326–49.

Hattar, S., Liao, H. W., Takao, M., Berson, D. M., & Yau, K. W. (2002). Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science*, *295*(5557), 1065–70.

Hayden, M. S., & Ghosh, S. (2004). Signaling to NF-kappaB. *Genes & Development*, *18*(18), 2195–224.

Hayden, M. S., & Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell*, *132*(3), 344–62.

Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., & Baeuerle, P. A. (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*, *365*(6442), 182–5.

Herdegen, T., Kovary, K., Buhl, A., Bravo, R., Zimmermann, M., & Gass, P. (1995). Basal expression of the inducible transcription factors c-Jun, JunB, JunD, c-Fos, FosB, and Krox-24 in the adult rat brain. *Journal of Comparative Neurology*, *354*(1), 39–56.

Herkenham, M., Rathore, P., Brown, P., & Listwak, S. J. (2011). Cautionary notes on the use of NF-κB p65 and p50 antibodies for CNS studies. *Journal of Neuroinflammation*, *8*(1), 141.

Hiler, D. J., Greenwald, M. L., & Geusz, M. E. (2006). Imaging gene expression in live transgenic mice after providing luciferin in drinking water. *Photochemical & Photobiological Sciences*, *5*(11), 1082–5.

Hinton, D. R., Sadun, A. A., Blanks, J. C., & Miller, C. A. (1986). Optic-nerve degeneration in Alzheimer's disease. *The New England Journal of Medicine*, *315*(8), 485–7.

Hirata, I., Yasumoto, S., Toshina, K., Inoue, T., Nishikawa, T., Murano, N., … Katsu, K. (2007). Evaluation of the effect of pyrrolidine dithiocarbamate in suppressing inflammation in mice with dextran sodium sulfate-induced colitis. *World Journal of Gastroenterology*, *13*(11), 1666–71.

Hirota, T., & Fukada, Y. (2004). Resetting mechanism of central and peripheral circadian clocks in mammals. *Zoological Science*, *21*(4), 359–68.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell*, *75*(2), 241–51.

Hofman, M. A. (2000). The human circadian clock and aging. *Chronobiology International*, *17*(3), 245–59.

Hofman, M. A., & Swaab, D. F. (1994). Alterations in circadian rhythmicity of the vasopressin-producing neurons of the human suprachiasmatic nucleus (SCN) with aging. *Brain Research*, *651*(1-2), 134–42.

Hofman, M. A., & Swaab, D. F. (2006). Living by the clock: the circadian pacemaker in older people. *Ageing Research Reviews*, *5*(1), 33–51.

Hofman, M. A., Zhou, J. N., & Swaab, D. F. (1996). Suprachiasmatic nucleus of the human brain: an immunocytochemical and morphometric analysis. *The Anatomical Record*, *244*(4), 552–62.

Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y. Z., Pray-Grant, M., … Bradfield, C. A. (1997). Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *Journal of Biological Chemistry*, *272*(13), 8581–93.

Hogenesch, J. B., Gu, Y. Z., Jain, S., & Bradfield, C. A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(10), 5474–9.

Honma, K. I., & Hiroshige, T. (1978). Endogenous ultradian rhythms in rats exposed to prolonged continuous light. *The American Journal of Physiology*, *235*(5), 250–6.

Honrado, G. I., Johnson, R. S., Golombek, D. A., Spiegelman, B. M., Papaioannou, V. E., & Ralph, M. R. (1996). The circadian system of c-fos deficient mice. *Journal of Comparative Physiology*, *178*(4), 563–70.

Horng, T., Barton, G. M., Flavell, R. A., & Medzhitov, R. (2002). The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature*, *420*(6913), 329–33.

Hrushesky, W. J., Langevin, T., Kim, Y. J., & Wood, P. A. (1994). Circadian dynamics of tumor necrosis factor alpha (cachectin) lethality. *The Journal of Experimental Medicine*, *180*(3), 1059–65.

Huang, G., Shi, L. Z., & Chi, H. (2009). Regulation of JNK and p38 MAPK in the immune system: signal integration, propagation and termination. *Cytokine*, *48*(3), 161–9.

Hughes, A. T., Fahey, B., Cutler, D. J., Coogan, A. N., & Piggins, H. D. (2004). Aberrant gating of photic input to the suprachiasmatic circadian pacemaker of mice lacking the VPAC2 receptor. *The Journal of Neuroscience*, *24*(14), 3522–6.

Hughes, A. T. L., Croft, C. L., Samuels, R. E., Myung, J., Takumi, T., & Piggins, H. D. (2015). Constant light enhances synchrony among circadian clock cells and promotes behavioral rhythms in VPAC2-signaling deficient mice. *Scientific Reports*, *5*, 14044.

Hughes, A. T. L., Guilding, C., Lennox, L., Samuels, R. E., McMahon, D. G., & Piggins, H. D. (2008). Live imaging of altered period1 expression in the suprachiasmatic nuclei of Vipr2-/- mice. *Journal of Neurochemistry*, *106*(4), 1646–57.

Hughes, P., & Dragunow, M. (1995). Induction of immediate-early genes and the control of neurotransmitter-regulated gene expression within the nervous system. *Pharmacological Reviews*, *47*(1), 133–78.

Hunot, S., Brugg, B., Ricard, D., Michel, P. P., Muriel, M. P., Ruberg, M., … Hirsch, E. C. (1997). Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(14), 7531–6.

Ingram, C. D., Ciobanu, R., Coculescu, I. L., Tanasescu, R., Coculescu, M., & Mihai, R. (1998). Vasopressin neurotransmission and the control of circadian rhythms in the suprachiasmatic nucleus. *Progress in Brain Research*, *119*, 351–64.

Ingram, C. D., Ciobanu, R., Coculescu, I. L., Tanasescu, R., Coculescu, M., & Mihai, R. (1999). *Advances in Brain Vasopressin*. *Progress in Brain Research* (Vol. 119). Elsevier.

Inouye, S. T., & Kawamura, H. (1979). Persistence of circadian rhythmicity in a mammalian hypothalamic “island” containing the suprachiasmatic nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(11), 5962–6.

Ivan, A. L. M., Campanini, M. Z., Martinez, R. M., Ferreira, V. S., Steffen, V. S., Vicentini, F. T. M. C., … Casagrande, R. (2014). Pyrrolidine dithiocarbamate inhibits UVB-induced skin inflammation and

oxidative stress in hairless mice and exhibits antioxidant activity *in vitro*. *Journal of Photochemistry and Photobiology*, *138*, 124–133.

Ivanenkov, Y. A., Balakin, K. V, & Lavrovsky, Y. (2011). Small molecule inhibitors of NF-kB and JAK/STAT signal transduction pathways as promising anti-inflammatory therapeutics. *Mini Reviews in Medicinal Chemistry*, *11*(1), 55–78.

Jacob, A., Hensley, L. K., Safratowich, B. D., Quigg, R. J., & Alexander, J. J. (2007). The role of the complement cascade in endotoxin-induced septic encephalopathy. *Laboratory Investigation*, *87*(12), 1186–1194.

Jagannath, A., Butler, R., Godinho, S. I. H., Couch, Y., Brown, L. A., Vasudevan, S. R., … Peirson, S. N. (2013). The CRTC1-SIK1 pathway regulates entrainment of the circadian clock. *Cell*, *154*(5), 1100–11.

Jagota, A., & Kalyani, D. (2010). Effect of melatonin on age induced changes in daily serotonin rhythms in suprachiasmatic nucleus of male Wistar rat. *Biogerontology*, *11*(3), 299–308.

Jellinger, K. A. (2002). Recent developments in the pathology of Parkinson's disease. *Journal of Neural Transmission*, (62), 347–76.

Jhaveri, K. A., Ramkumar, V., Trammell, R. A., & Toth, L. A. (2006). Spontaneous, homeostatic, and inflammation-induced sleep in NF-kappaB p50 knockout mice. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *291*(5), 1516–26.

Ji, R., Tian, S., Lu, H. J., Lu, Q., Zheng, Y., Wang, X., … Lu, Q. (2013). TAM receptors affect adult brain neurogenesis by negative regulation of microglial cell activation. *Journal of Immunology*, *191*(12), 6165–77.

Joans, J., Tackenberg, M., & McMahon, D. (2015). Manipulating circadian clock neuron firing rate resets molecular circadian rhythms and behavior. *Nature Neuroscience*, *18*, 373-375.

Johnson, C. H., Elliott, J. A., & Foster, R. (2003). Entrainment of circadian programs. *Chronobiology International*, *20*(5), 741–74.

Johnson, R. . (2002). The concept of sickness behavior: a brief chronological account of four key discoveries. *Veterinary Immunology and Immunopathology*, *87*(3-4), 443–450.

Johnson, R. F., Moore, R. Y., & Morin, L. P. (1988). Loss of entrainment and anatomical plasticity after lesions of the hamster retinohypothalamic tract. *Brain Research*, *460*(2), 297–313.

Joseph, S. A., Tassorelli, C., Prasad, A. V, & Lynd-Balta, E. (1996). NF-kappa B transcription factor subunits in rat brain: colocalization of p65 and alpha-MSH. *Peptides*, *17*(4), 655–64.

Juan, T. S.-C., Kelley, M. J., Johnson, D. A., Busse, L. A., Hailman, E., Wright, S. D., & Lichenstein, H. S. (1995). Soluble CD14 Truncated at Amino Acid 152 Binds Lipopolysaccharide (LPS) and Enables Cellular Response to LPS. *Journal of Biological Chemistry*, *270*(3), 1382–1387.

Jud, C., Schmutz, I., Hampp, G., Oster, H., & Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biological Procedures Online*, *7*, 101–16.

Jurk, D., Wilson, C., Passos, J. F., Oakley, F., Correia-Melo, C., Greaves, L., … von Zglinicki, T. (2014). Chronic inflammation induces telomere dysfunction and accelerates ageing in mice. *Nature Communications*, *2*, 4172.

Kaczmarek, L., & Chaudhuri, A. (1997). Sensory regulation of immediate-early gene expression in mammalian visual cortex: implications for functional mapping and neural plasticity. *Brain Research*, *23*(3), 237–56.

Kakizaki, Y., Watanobe, H., Kohsaka, A., & Suda, T. (1999). Temporal profiles of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in the plasma and hypothalamic paraventricular nucleus after intravenous or intraperitoneal administration of lipopolysaccharide in the rat: estimation by push-pull per. *Endocrine Journal*, *46*(4), 487–96.

Kalsbeek, A., & Buijs, R. M. (2002). Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. *Cell and Tissue Research*, *309*(1), 109–18.

Kalsbeek, A., Fliers, E., Hofman, M. A., Swaab, D. F., & Buijs, R. M. (2010). Vasopressin and the output of the hypothalamic biological clock. *Journal of Neuroendocrinology*, *22*(5), 362–72.

Kalsbeek, A., Palm, I. F., La Fleur, S. E., Scheer, F. A. J. L., Perreau-Lenz, S., Ruiter, M., … Buijs, R. M. (2006). SCN outputs and the hypothalamic balance of life. *Journal of Biological Rhythms*, *21*(6),

Kaltschmidt, B., & Kaltschmidt, C. (2009). NF-kappaB in the nervous system. *Cold Spring Harbor Perspectives in Biology*, *1*(3), a001271.

Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P. A., & Kaltschmidt, C. (1997). Transcription factor NF-kappaB is activated in primary neurons

by amyloid beta peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(6), 2642–7.

Kaltschmidt, B., Widera, D., & Kaltschmidt, C. (2005). Signaling via NF-kappaB in the nervous system. *Biochimica et Biophysica Acta*, *1745*(3), 287–99.

Kaltschmidt, C., Kaltschmidt, B., & Baeuerle, P. A. (1995). Stimulation of ionotropic glutamate receptors activates transcription factor NF-kappa B in primary neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(21), 9618–22.

Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H., & Baeuerle, P. A. (1994a). Constitutive NF-kappa B activity in neurons. *Molecular and Cellular Biology*, *14*(6), 3981–3992.

Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., … Bredesen, D. E. (1993). Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species. *Science*, *262*(5137), 1274–7.

Karin, M., & Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annual Review of Immunology*, *18*, 621–63.

Karin, M., & Greten, F. R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nature Immunology*, *5*(10), 749–59.

Kassed, C. A., & Herkenham, M. (2004). NF-kappaB p50-deficient mice show reduced anxiety-like behaviors in tests of exploratory drive and anxiety. *Behavioural Brain Research*, *154*(2), 577–84.

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, *11*(5), 373–84.

Kawai, T., & Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*, *34*(5), 637–50.

Kawakami, F., Okamura, H., Tamada, Y., Maebayashi, Y., Fukui, K., & Ibata, Y. (1997). Loss of day-night differences in VIP mRNA levels in the suprachiasmatic nucleus of aged rats. *Neuroscience Letters*, *222*(2), 99–102.

Keating, S. E., Maloney, G. M., Moran, E. M., & Bowie, A. G. (2007). IRAK-2 participates in multiple toll-like receptor signaling pathways to NFkappaB via activation of TRAF6 ubiquitination. *Journal of Biological Chemistry*, *282*(46), 33435–43.

Kendall, A. R., Lewy, A. J., & Sack, R. L. (2001). Effects of aging on the intrinsic circadian period of totally blind humans. *Journal of Biological Rhythms*, *16*(1), 87–95.

Kettenmann, H., Hanisch, U.-K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. *Physiological Reviews*, *91*(2), 461–553.

Khoshnan, A., Ko, J., Watkin, E. E., Paige, L. A., Reinhart, P. H., & Patterson, P. H. (2004). Activation of the IkappaB kinase complex and nuclear factor-kappaB contributes to mutant huntingtin neurotoxicity. *Journal of Neuroscience*, *24*(37), 7999–8008.

Kikusui, T., Isaka, Y., & Mori, Y. (2005). Early weaning deprives mouse pups of maternal care and decreases their maternal behavior in adulthood. *Behavioural Brain Research*, *162*(2), 200–6.

Kim, C. H., Kim, J. H., Xu, J., Hsu, C. Y., & Ahn, Y. S. (1999). Pyrrolidine dithiocarbamate induces bovine cerebral endothelial cell death by increasing the intracellular zinc level. *Journal of Neurochemistry*, *72*(4), 1586–92.

Kim, H. J., & Harrington, M. E. (2008). Neuropeptide Y-deficient mice show altered circadian response to simulated natural photoperiod. *Brain Research*, *1246*, 96–100.

Kim, H. J., Kim, K. W., Yu, B. P., & Chung, H. Y. (2000). The effect of age on cyclooxygenase-2 gene expression: NF-kappaB activation and IkappaBalpha degradation. *Free Radical Biology & Medicine*, *28*(5), 683–92.

Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Human Molecular Genetics*, *15*(2), 271–7.

Kolker, D. E., Fukuyama, H., Huang, D. S., Takahashi, J. S., Horton, T. H., & Turek, F. W. (2003). Aging alters circadian and light-induced expression of clock genes in golden hamsters. *Journal of Biological Rhythms*, *18*(2), 159–69.

Kolker, D. E., Vitaterna, M. H., Fruechte, E. M., Takahashi, J. S., & Turek, F. W. (2004). Effects of age on circadian rhythms are similar in wild-type and heterozygous Clock mutant mice. *Neurobiology of Aging*, *25*(4), 517–23.

Kondo, T., Strayer, C. A., Kulkarni, R. D., Taylor, W., Ishiura, M., Golden, S. S., & Johnson, C. H. (1993). Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(12), 5672–6.

Kondratov, R. V, Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V, & Antoch, M. P. (2006). Early aging and age-related pathologies in mice deficient in BMAL1, the core componentof the circadian clock. *Genes & Development*, *20*(14), 1868–73.

Kondratova, A. A., Dubrovsky, Y. V, Antoch, M. P., & Kondratov, R. V. (2010). Circadian clock proteins control adaptation to novel environment and memory formation. *Aging*, *2*(5), 285–97.

Kondratova, A. A., & Kondratov, R. V. (2012). The circadian clock and pathology of the ageing brain. *Nature Reviews Neuroscience*, *13*(5), 325–35.

Konopka, R. J., & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *68*(9), 2112–6.

Konsman, J. P., Kelley, K., & Dantzer, R. (1999). Temporal and spatial relationships between lipopolysaccharide-induced expression of Fos, interleukin-1beta and inducible nitric oxide synthase in rat brain. *Neuroscience*, *89*(2), 535–48.

Konsman, J. P., Parnet, P., & Dantzer, R. (2002). Cytokine-induced sickness behaviour: mechanisms and implications. *Trends in Neurosciences*, *25*(3), 154–9.

Köntgen, F., Grumont, R. J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., & Gerondakis, S. (1995). Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. *Genes & Development*, *9*(16), 1965–77.

Kopp, E., & Ghosh, S. (1994). Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science*, *265*(5174), 956–9.

Korb, E., & Finkbeiner, S. (2011). Arc in synaptic plasticity: from gene to behavior. *Trends in Neurosciences*, *34*(11), 591–8.

Korhonen, P., Helenius, M., & Salminen, A. (1997). Age-related changes in the regulation of transcription factor NF-kappa B in rat brain. *Neuroscience Letters*, *225*(1), 61–4.

Kornhauser, J. M., Nelson, D. E., Mayo, K. E., & Takahashi, J. S. (1990). Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. *Neuron*, *5*(2), 127–34.

Kovács, K. J. (2008). Measurement of immediate-early gene activation- c-fos and beyond. *Journal of Neuroendocrinology*, *20*(6), 665–72.

Koyanagi, S. (2002). Alteration of Intrinsic Biological Rhythms during Interferon Treatment and Its Possible Mechanism. *Molecular Pharmacology*, *62*(6), 1393–1399.

Kraves, S., & Weitz, C. J. (2006). A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. *Nature Neuroscience*, *9*(2), 212–9.

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends in Neurosciences*, *19*(8), 312–318.

Krueger, J. M. (2008). The role of cytokines in sleep regulation. *Current Pharmaceutical Design*, *14*(32), 3408–16.

Krueger, J. M., Obál, F. J., Fang, J., Kubota, T., & Taishi, P. (2001). The role of cytokines in physiological sleep regulation. *Annals of the New York Academy of Sciences*, *933*, 211–21.

Kubo, T., Ozasa, K., Mikami, K., Wakai, K., Fujino, Y., Watanabe, Y., … Tamakoshi, A. (2006). Prospective cohort study of the risk of prostate cancer among rotating-shift workers: findings from the Japan collaborative cohort study. *American Journal of Epidemiology*, *164*(6), 549–55.

Kudo, T., Loh, D. H., Truong, D., Wu, Y., & Colwell, C. S. (2011). Circadian dysfunction in a mouse model of Parkinson's disease. *Experimental Neurology*, *232*(1), 66–75.

Kudo, T., Schroeder, A., Loh, D. H., Kuljis, D., Jordan, M. C., Roos, K. P., & Colwell, C. S. (2011). Dysfunctions in circadian behavior and physiology in mouse models of Huntington's disease. *Experimental Neurology*, *228*(1), 80–90.

Kuhlman, S. J., Quintero, J. E., & McMahon, D. G. (2000). GFP fluorescence reports Period 1 circadian gene regulation in the mammalian biological clock. *Neuroreport*, *11*(7), 1479–82.

Kuljis, D., Schroeder, A. M., Kudo, T., Loh, D. H., Willison, D. L., & Colwell, C. S. (2012). Sleep and circadian dysfunction in neurodegenerative disorders: insights from a mouse model of

Huntington's disease. *Minerva Pneumologica*, *51*(3), 93–106.

Kumar, A., Takada, Y., Boriek, A. M., & Aggarwal, B. B. (2004). Nuclear factor-kappaB: its role in health and disease. *Journal of Molecular Medicine*, *82*(7), 434–48.

Kumar, D., Singla, S. K., Puri, V., & Puri, S. (2015). The restrained expression of NF-kB in renal tissue ameliorates folic acid induced acute kidney injury in mice. *PloS One*, *10*(1), e115947.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., … Reppert, S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell*, *98*(2), 193–205.

Kurabayashi, N., Hirota, T., Harada, Y., Sakai, M., & Fukada, Y. (2006). Phosphorylation of mCRY2 at Ser557 in the hypothalamic suprachiasmatic nucleus of the mouse. *Chronobiology International*, *23*(1-2), 129–34.

Kurien, B. T., & Scofield, R. H. (2006). Western blotting. *Methods*, *38*(4), 283–93.

Kwak, Y., Lundkvist, G. B., Brask, J., Davidson, A., Menaker, M., Kristensson, K., & Block, G. D. (2008). Interferon-gamma alters electrical activity and clock gene expression in suprachiasmatic nucleus neurons. *Journal of Biological Rhythms*, *23*(2), 150–9.

Laflamme, N., & Rivest, S. (2001). Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *Official Publication of the Federation of American Societies for Experimental Biology*, *15*(1), 155–163.

Lall, G. S., & Biello, S. M. (2002). Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study. *Brain Research*, *957*(1), 109–116.

Lall, G. S., & Biello, S. M. (2003a). Attenuation of circadian light induced phase advances and delays by neuropeptide Y and a neuropeptide Y Y1/Y5 receptor agonist. *Neuroscience*, *119*(2), 611–8.

Lall, G. S., & Biello, S. M. (2003b). Neuropeptide Y, GABA and circadian phase shifts to photic stimuli. *Neuroscience*, *120*(4), 915–21.

Lamia, K. A., Storch, K.-F., & Weitz, C. J. (2008). Physiological significance of a peripheral tissue circadian clock. *Proceedings of the*

National Academy of Sciences of the United States of America, *105*(39), 15172–7.

Laposky, A. D., Bass, J., Kohsaka, A., & Turek, F. W. (2008). Sleep and circadian rhythms: key components in the regulation of energy metabolism. *FEBS Letters*, *582*(1), 142–51.

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*, *1*(6), a001651.

Laye, S., Bluthe, R. M., Kent, S., Combe, C., Medina, C., Parnet, P., … Dantzer, R. (1995). Subdiaphragmatic vagotomy blocks induction of IL-1 beta mRNA in mice brain in response to peripheral LPS. *The American Journal of Physiology*, *268*(5), 1327–1331.

Leclerc, G. M., Boockfor, F. R., Faught, W. J., & Frawley, L. S. (2000). Development of a destabilized firefly luciferase enzyme for measurement of gene expression. *BioTechniques*, *29*(3), 590–98.

Lee, B., Almad, A., Butcher, G. Q., & Obrietan, K. (2007). Protein kinase C modulates the phase-delaying effects of light in the mammalian circadian clock. *The European Journal of Neuroscience*, *26*(2), 451–62.

Lee, H., Chen, R., Lee, Y., Yoo, S., & Lee, C. (2009). Essential roles of CKIdelta and CKIepsilon in the mammalian circadian clock. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(50), 21359–64.

Lee, J. H., & Sancar, A. (2011). Regulation of apoptosis by the circadian clock through NF-kappaB signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(29), 12036–41.

Lee, M. K., Borchelt, D. R., Wong, P. C., Sisodia, S. S., & Price, D. L. (1996). Transgenic models of neurodegenerative diseases. *Current Opinion in Neurobiology*, *6*(5), 651–60.

Lentsch, A. B., Shanley, T. P., Sarma, V., & Ward, P. A. (1997). *In vivo* suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. *The Journal of Clinical Investigation*, *100*(10), 2443–8.

Leone, M. J., Marpegan, L., Bekinschtein, T. A., Costas, M. A., & Golombek, D. A. (2006). Suprachiasmatic astrocytes as an interface for immune-circadian signalling. *Journal of Neuroscience Research*, *84*(7), 1521–7.

Leone, M. J., Marpegan, L., Duhart, J. M., & Golombek, D. A. (2012). Role of proinflammatory cytokines on lipopolysaccharide-induced phase shifts in locomotor activity circadian rhythm. *Chronobiology International*, *29*(6), 715–23.

LeSauter, J., Yan, L., Vishnubhotla, B., Quintero, J. E., Kuhlman, S. J., McMahon, D. G., & Silver, R. (2003). A short half-life GFP mouse model for analysis of suprachiasmatic nucleus organization. *Brain Research*, *964*(2), 279–87.

Levi, F., & Schibler, U. (2007). Circadian rhythms: mechanisms and therapeutic implications. *Annual Review of Pharmacology and Toxicology*, *47*, 593–628.

Li, H., & Satinoff, E. (1998). Fetal tissue containing the suprachiasmatic nucleus restores multiple circadian rhythms in old rats. *The American Journal of Physiology*, *275*(6), 1735–1744.

Li, P., Sur, S. H., Mistlberger, R. E., & Morris, M. (1999). Circadian blood pressure and heart rate rhythms in mice. *The American Journal of Physiology*, *276*(2), 500–504.

Lian, H., Shim, D. J., Gaddam, S. S. K., Rodriguez-Rivera, J., Bitner, B. R., Pautler, R. G., … Zheng, H. (2012). IκBα deficiency in brain leads to elevated basal neuroinflammation and attenuated response following traumatic brain injury: implications for functional recovery. *Molecular Neurodegeneration*, *7*(1), 47.

Lim, A. S. P., Ellison, B. A., Wang, J. L., Yu, L., Schneider, J. A., Buchman, A. S., … Saper, C. B. (2014). Sleep is related to neuron numbers in the ventrolateral preoptic/intermediate nucleus in older adults with and without Alzheimer's disease. *Brain*, *137*(10), 2847–61.

Ling, J., & Kumar, R. (2012). Crosstalk between NFkB and glucocorticoid signaling: a potential target of breast cancer therapy. *Cancer Letters*, *322*(2), 119–26.

Listwak, S. J., Rathore, P., & Herkenham, M. (2013). Minimal NF-κB activity in neurons. *Neuroscience*, *250*, 282–99.

Liu, A. C., Tran, H. G., Zhang, E. E., Priest, A. A., Welsh, D. K., & Kay, S. A. (2008). Redundant function of REV-ERBalpha and beta and non-essential role for Bmal1 cycling in transcriptional regulation of intracellular circadian rhythms. *PLoS Genetics*, *4*(2), e1000023.

Liu, A. C., Welsh, D. K., Ko, C. H., Tran, H. G., Zhang, E. E., Priest, A. A., … Kay, S. A. (2007). Intercellular coupling confers robustness against

mutations in the SCN circadian clock network. *Cell*, *129*(3), 605–16.

Liu, C., & Reppert, S. M. (2000). GABA synchronizes clock cells within the suprachiasmatic circadian clock. *Neuron*, *25*(1), 123–8.

Liu, J., Malkani, G., Mankani, G., Shi, X., Meyer, M., Cunningham-Runddles, S., … Sun, Z. S. (2006). The circadian clock Period 2 gene regulates gamma interferon production of NK cells in host response to lipopolysaccharide-induced endotoxic shock. *Infection and Immunity*, *74*(8), 4750–6.

Liu, R. Y., Zhou, J. N., Hoogendijk, W. J., van Heerikhuize, J., Kamphorst, W., Unmehopa, U. A., … Swaab, D. F. (2000). Decreased vasopressin gene expression in the biological clock of Alzheimer disease patients with and without depression. *Journal of Neuropathology and Experimental Neurology*, *59*(4), 314–22.

Liu, S. F., Ye, X., & Malik, A. B. (1999). Inhibition of NF- B Activation by Pyrrolidine Dithiocarbamate Prevents *In Vivo* Expression of Proinflammatory Genes. *Circulation*, *100*(12), 1330–1337.

Liu, S. F., Ye, X., & Malik, A. B. (1999). Pyrrolidine Dithiocarbamate Prevents I-kappa B Degradation and Reduces Microvascular Injury Induced by Lipopolysaccharide in Multiple Organs. *Molecular Pharmacology*, *55*(4), 658–667.

Lockley, S. W., Skene, D. J., James, K., Thapan, K., Wright, J., & Arendt, J. (2000). Melatonin administration can entrain the free-running circadian system of blind subjects. *The Journal of Endocrinology*, *164*(1), 1–6.

Logan, R. W., & Sarkar, D. K. (2012). Circadian nature of immune function. *Molecular and Cellular Endocrinology*, *349*(1), 82–90.

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., … Takahashi, J. S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science*, *288*(5465), 483–92.

Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annual Review of Genomics and Human Genetics*, *5*, 407–41.

Lu, J., Zhang, Y. H., Chou, T. C., Gaus, S. E., Elmquist, J. K., Shiromani, P., & Saper, C. B. (2001). Contrasting effects of ibotenate lesions of the paraventricular nucleus and subparaventricular zone on sleep-wake cycle and temperature regulation. *The Journal of Neuroscience* , *21*(13), 4864–74.

Lu, Y.-C., Yeh, W.-C., & Ohashi, P. S. (2008). LPS/TLR4 signal transduction pathway. *Cytokine*, *42*(2), 145–51.

Lu, Z.-Y., Yu, S. P., Wei, J.-F., & Wei, L. (2006). Age-related neural degeneration in nuclear-factor kappaB p50 knockout mice. *Neuroscience*, *139*(3), 965–78.

Luna-Moreno, D., Aguilar-Roblero, R., & Díaz-Muñoz, M. (2009). Restricted feeding entrains rhythms of inflammation-related factors without promoting an acute-phase response. *Chronobiology International*, *26*(7), 1409–29.

Lundkvist, G. B., Andersson, A., Robertson, B., Rottenberg, M. E., & Kristensson, K. (1999). Light-dependent regulation and postnatal development of the interferon-gamma receptor in the rat suprachiasmatic nuclei. *Brain Research*, *849*(1-2), 231–4.

Luo, H., Wang, X., Wang, J., Chen, C., Wang, N., Xu, Z., … Zeng, C. (2015). Chronic NF-κB blockade improves renal angiotensin II type 1 receptor functions and reduces blood pressure in Zucker diabetic rats. *Cardiovascular Diabetology*, *14*(1), 76.

Lv, R., Xu, X., Luo, Z., Shen, N., Wang, F., & Zhao, Y. (2014). Pyrrolidine dithiocarbamate (PDTC) inhibits the overexpression of MCP-1 and attenuates microglial activation in the hippocampus of a pilocarpine-induced status epilepticus rat model. *Experimental and Therapeutic Medicine*, *7*(1), 39–45.

Lyford, G. L., Yamagata, K., Kaufmann, W. E., Barnes, C. A., Sanders, L. K., Copeland, N. G., … Worley, P. F. (1995). Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron*, *14*(2), 433–445.

Maes, M., Meltzer, H. Y., Bosmans, E., Bergmans, R., Vandoolaeghe, E., Ranjan, R., & Desnyder, R. (1995). Increased plasma concentrations of interleukin-6, soluble interleukin-6, soluble interleukin-2 and transferrin receptor in major depression. *Journal of Affective Disorders*, *34*(4), 301–309.

Mahoney, M., Bult, A., & Smale, L. (2001). Phase response curve and light-induced fos expression in the suprachiasmatic nucleus and adjacent hypothalamus of Arvicanthis niloticus. *Journal of Biological Rhythms*, *16*(2), 149–62.

Malm, T. M., Iivonen, H., Goldsteins, G., Keksa-Goldsteine, V., Ahtoniemi, T., Kanninen, K., … Koistinaho, J. (2007). Pyrrolidine dithiocarbamate

activates Akt and improves spatial learning in APP/PS1 mice without affecting beta-amyloid burden. *The Journal of Neuroscience*, *27*(14), 3712–21.

Mann, D. A. (2002). The NFkappaB luciferase mouse: a new tool for real time measurement of NFkappaB activation in the whole animal. *Gut*, *51*(6), 769–770.

Manna, S. K., Bueso-Ramos, C., Alvarado, F., & Aggarwal, B. B. (2003). Calagualine inhibits nuclear transcription factors-kappaB activated by various inflammatory and tumor promoting agents. *Cancer Letters*, *190*(2), 171–82.

Marcheva, B., Ramsey, K. M., Affinati, A., & Bass, J. (2009). Clock genes and metabolic disease. *Journal of Applied Physiology*, *107*(5), 1638–46.

Mariappan, N., Elks, C. M., Sriramula, S., Guggilam, A., Liu, Z., Borkhsenious, O., & Francis, J. (2010). NF-kappaB-induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes. *Cardiovascular Research*, *85*(3), 473–83.

Marpegán, L., Bekinschtein, T. A., Costas, M. A., & Golombek, D. A. (2005). Circadian responses to endotoxin treatment in mice. *Journal of Neuroimmunology*, *160*(1-2), 102–9.

Marpegan, L., Bekinschtein, T. A., Freudenthal, R., Rubio, M. F., Ferreyra, G. A., Romano, A., & Golombek, D. A. (2004). Participation of transcription factors from the Rel/NF-kappa B family in the circadian system in hamsters. *Neuroscience Letters*, *358*(1), 9–12.

Marpegan, L., Leone, M. J., Katz, M. E., Sobrero, P. M., Bekinstein, T. A., & Golombek, D. A. (2009). Diurnal variation in endotoxin-induced mortality in mice: correlation with proinflammatory factors. *Chronobiology International*, *26*(7), 1430–42.

Martich, G. D., Boujoukos, A. J., & Suffredini, A. F. (1993). Response of man to endotoxin. *Immunobiology*, *187*(3-5), 403–16.

Martin, J. B., & Gusella, J. F. (1986). Huntington's disease. Pathogenesis and management. *The New England Journal of Medicine*, *315*(20), 1267–76.

Marvel, F. A., Chen, C.-C., Badr, N., Gaykema, R. P. A., & Goehler, L. E. (2004). Reversible inactivation of the dorsal vagal complex blocks lipopolysaccharide-induced social withdrawal and c-Fos expression in central autonomic nuclei. *Brain, Behavior, and Immunity*, *18*(2), 123–

34.

Marzolo, M. P., von Bernhardi, R., Bu, G., & Inestrosa, N. C. (2000). Expression of alpha(2)-macroglobulin receptor/low density lipoprotein receptor-related protein (LRP) in rat microglial cells. *Journal of Neuroscience Research*, *60*(3), 401–11.

Mathias, S., Schiffelholz, T., Linthorst, A. C., Pollmächer, T., & Lancel, M. (2000). Diurnal variations in lipopolysaccharide-induced sleep, sickness behavior and changes in corticosterone levels in the rat. *Neuroendocrinology*, *71*(6), 375–85.

Matos, L. L. de, Trufelli, D. C., de Matos, M. G. L., & da Silva Pinhal, M. A. (2010). Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomarker Insights*, *5*, 9–20.

Mattson, M. P., & Camandola, S. (2001). NF-kappaB in neuronal plasticity and neurodegenerative disorders. *The Journal of Clinical Investigation*, *107*(3), 247–54.

Mattson, M. P., & Meffert, M. K. (2006). Roles for NF-kappaB in nerve cell survival, plasticity, and disease. *Cell Death and Differentiation*, *13*(5), 852–60.

Mavroudis, P. D., Scheff, J. D., Calvano, S. E., & Androulakis, I. P. (2013). Systems biology of circadian-immune interactions. *Journal of Innate Immunity*, *5*(2), 153–62.

Mayeda, A. R., Hofstetter, J. R., & Possidente, B. (1997). Aging lengthens TauDD in C57BL/6J, DBA/2J, and outbred SWR male mice (Mus musculus). *Chronobiology International*, *14*(1), 19–23.

McClung, C. A. (2011). Circadian rhythms and mood regulation: insights from pre-clinical models. *European Neuropsychopharmacology*, *21(4)*, 683–93.

McClung, C. A., Sidiropoulou, K., Vitaterna, M., Takahashi, J. S., White, F. J., Cooper, D. C., & Nestler, E. J. (2005). Regulation of dopaminergic transmission and cocaine reward by the Clock gene. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(26), 9377–81.

McNabb, D. S., Reed, R., & Marciniak, R. A. (2005). Dual luciferase assay system for rapid assessment of gene expression in Saccharomyces cerevisiae. *Eukaryotic Cell*, *4*(9), 1539–49.

Meberg, P. J., Kinney, W. R., Valcourt, E. G., & Routtenberg, A. (1996).

Gene expression of the transcription factor NF-kappa B in hippocampus: regulation by synaptic activity. *Molecular Brain Research*, *38*(2), 179–90.

Medanic, M., & Gillette, M. U. (1992). Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker *in vitro* only during the subjective day. *The Journal of Physiology*, *450*, 629–42.

Medzhitov, R., & Janeway, C. J. (2000). Innate immune recognition: mechanisms and pathways. *Immunological Reviews*, *173*(1), 89–97.

Meffert, M. K., & Baltimore, D. (2005). Physiological functions for brain NF-kappaB. *Trends in Neurosciences*, *28*(1), 37–43.

Meffert, M. K., Chang, J. M., Wiltgen, B. J., Fanselow, M. S., & Baltimore, D. (2003). NF-kappa B functions in synaptic signaling and behavior. *Nature Neuroscience*, *6*(10), 1072–8.

Meijer, J. H., & Schwartz, W. J. (2003). In search of the pathways for light-induced pacemaker resetting in the suprachiasmatic nucleus. *Journal of Biological Rhythms*, *18*(3), 235–49.

Mendoza, J., Graff, C., Dardente, H., Pevet, P., & Challet, E. (2005). Feeding cues alter clock gene oscillations and photic responses in the suprachiasmatic nuclei of mice exposed to a light/dark cycle. *The Journal of Neuroscience*, *25*(6), 1514–22.

Mendoza, J., Revel, F. G., Pévet, P., & Challet, E. (2007). Shedding light on circadian clock resetting by dark exposure: differential effects between diurnal and nocturnal rodents. *The European Journal of Neuroscience*, *25*(10), 3080–90.

Mendoza, J. Y., Dardente, H., Escobar, C., Pevet, P., & Challet, E. (2004). Dark pulse resetting of the suprachiasmatic clock in Syrian hamsters: behavioral phase-shifts and clock gene expression. *Neuroscience*, *127*(2), 529–37.

Meyer-Bernstein, E. L., & Morin, L. P. (1996). Differential serotonergic innervation of the suprachiasmatic nucleus and the intergeniculate leaflet and its role in circadian rhythm modulation. *The Journal of Neuroscience*, *16*(6), 2097–111.

Miagkov, A. V, Kovalenko, D. V, Brown, C. E., Didsbury, J. R., Cogswell, J. P., Stimpson, S. A., … Makarov, S. S. (1998). NF-kappaB activation provides the potential link between inflammation and hyperplasia in the arthritic joint. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(23), 13859–64.

Mickman, C. T., Stubblefield, J. J., Stubblefield, J. S., Harrington, M. E., & Nelson, D. E. (2008). Photoperiod alters phase difference between activity onset *in vivo* and mPer2::luc peak *in vitro*. *The American Journal of Physiology, 295*(5), 1688–94.

Mihai, R., Coculescu, M., Wakerley, J. B., & Ingram, C. D. (1994). The effects of [Arg8]vasopressin and [Arg8]vasotocin on the firing rate of suprachiasmatic neurons *in vitro*. *Neuroscience*, *62*(3), 783–92.

Mikkelsen, J. D., Larsen, P. J., Mick, G., Vrang, N., Ebling, F. J., Maywood, E. S., … Møller, M. (1995). Gating of retinal inputs through the suprachiasmatic nucleus: role of excitatory neurotransmission. *Neurochemistry International*, *27*(3), 263–72.

Millar, A. J., Short, S. R., Chua, N. H., & Kay, S. A. (1992). A novel circadian phenotype based on firefly luciferase expression in transgenic plants. *The Plant Cell*, *4*(9), 1075–87.

Min, Y. K., Park, J. H., Chong, S. A., Kim, Y. S., Ahn, Y. S., Seo, J. T., … Chung, K. C. (2003). Pyrrolidine dithiocarbamate-induced neuronal cell death is mediated by Akt, casein kinase 2, c-Jun N-terminal kinase, and IkappaB kinase in embryonic hippocampal progenitor cells. *Journal of Neuroscience Research*, *71*(5), 689–700.

Mintz, E. M., Gillespie, C. F., Marvel, C. L., Huhman, K. L., & Albers, H. E. (1997). Serotonergic regulation of circadian rhythms in Syrian hamsters. *Neuroscience*, *79*(2), 563–9.

Mintz, E. M., Marvel, C. L., Gillespie, C. F., Price, K. M., & Albers, H. E. (1999). Activation of NMDA receptors in the suprachiasmatic nucleus produces light-like phase shifts of the circadian clock *in vivo*. *The Journal of Neuroscience*, *19*(12), 5124–30.

Mishima, K., Okawa, M., Hishikawa, Y., Hozumi, S., Hori, H., & Takahashi, K. (1994). Morning bright light therapy for sleep and behavior disorders in elderly patients with dementia. *Acta Psychiatrica Scandinavica*, *89*(1), 1–7.

Mistlberger, R. E., & Skene, D. J. (2005). Nonphotic entrainment in humans? *Journal of Biological Rhythms*, *20*(4), 339–52.

Miyake, S., Sumi, Y., Yan, L., Takekida, S., Fukuyama, T., Ishida, Y., … Okamura, H. (2000). Phase-dependent responses of Per1 and Per2 genes to a light-stimulus in the suprachiasmatic nucleus of the rat. *Neuroscience Letters*, *294*(1), 41–4.

Moe, K., Vitiello, M., Larsen, L., & Prinz, P. (1995). Symposium: Cognitive

processes and sleep disturbances: Sleep/wake patterns in Alzheimer's disease: relationships with cognition and function. *Journal of Sleep Research*, *4*(1), 15–20.

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews*, *22*(2), 240–73.

Mogi, M., Kondo, T., Mizuno, Y., & Nagatsu, T. (2007). p53 protein, interferon-gamma, and NF-kappaB levels are elevated in the parkinsonian brain. *Neuroscience Letters*, *414*(1), 94–7.

Molyneux, P. C., Dahlgren, M. K., & Harrington, M. E. (2008). Circadian entrainment aftereffects in suprachiasmatic nuclei and peripheral tissues *in vitro*. *Brain Research*, *1228*, 127–34.

Monje, F. J., Cabatic, M., Divisch, I., Kim, E.-J., Herkner, K. R., Binder, B. R., & Pollak, D. D. (2011). Constant darkness induces IL-6-dependent depression-like behavior through the NF-κB signaling pathway. *The Journal of Neuroscience*, *31*(25), 9075–83.

Monk, T. H., & Welsh, D. K. (2003). The role of chronobiology in sleep disorders medicine. *Sleep Medicine Reviews*, *7*(6), 455–473.

Moore, R. Y. (1983). Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. *Federation Proceedings*, *42*(11), 2783–9.

Moore, R. Y., & Card, J. P. (1994). Intergeniculate leaflet: an anatomically and functionally distinct subdivision of the lateral geniculate complex. *The Journal of Comparative Neurology*, *344*(3), 403–30.

Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Research*, *42*(1), 201–206.

Moore, R. Y., & Speh, J. C. (1993). GABA is the principal neurotransmitter of the circadian system. *Neuroscience Letters*, *150*(1), 112–116.

Moore, R. Y., Speh, J. C., & Leak, R. K. (2002). Suprachiasmatic nucleus organization. *Cell and Tissue Research*, *309*(1), 89–98.

Morgan, J. I., & Curran, T. (1991). Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. *Annual Review of Neuroscience*, *14*, 421–51.

Morin, L. P. (1999). Serotonin and the regulation of mammalian circadian rhythmicity. *Annals of Medicine*, *31*(1), 12–33.

Morin, L. P. (2007). SCN organization reconsidered. *Journal of Biological Rhythms*, *22*(1), 3–13.

Morin, L. P., & Allen, C. N. (2006). The circadian visual system, 2005. *Brain Research Reviews*, *51*(1), 1–60.

Morin, L. P., Blanchard, J. H., & Provencio, I. (2003). Retinal ganglion cell projections to the hamster suprachiasmatic nucleus, intergeniculate leaflet, and visual midbrain: bifurcation and melanopsin immunoreactivity. *The Journal of Comparative Neurology*, *465*(3), 401–16.

Morin, L. P., Blanchard, J., & Moore, R. Y. (1992). Intergeniculate leaflet and suprachiasmatic nucleus organization and connections in the golden hamster. *Visual Neuroscience*, *8*(03), 219–230.

Morton, A. J. (2013). Circadian and sleep disorder in Huntington's disease. *Experimental Neurology*, *243*, 34–44.

Morton, A. J., Wood, N. I., Hastings, M. H., Hurelbrink, C., Barker, R. A., & Maywood, E. S. (2005). Disintegration of the sleep-wake cycle and circadian timing in Huntington's disease. *The Journal of Neuroscience*, *25*(1), 157–63.

Motzkus, D., Albrecht, U., & Maronde, E. (2002). The Human PER1 Gene is Inducible by Interleukin-6. *Journal of Molecular Neuroscience*, *18*(1-2), 105–110.

Moynagh, P. N. (2005a). The NF-kappaB pathway. *Journal of Cell Science*, *118*(20), 4589–92.

Moynagh, P. N. (2005b). TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. *Trends in Immunology*, *26*(9), 469–76.

Mrosovsky, N. (1989). Nonphotic enhancement of adjustment to new light-dark cycles: masking interpretation discounted. *Journal of Biological Rhythms*, *4*(3), 365–70.

Mrosovsky, N. (1996). Locomotor activity and non-photic influences on circadian clocks. *Biological Reviews of the Cambridge Philosophical Society*, *71*(3), 343–72.

Müller, R., Bravo, R., Burckhardt, J., & Curran, T. (1984). Induction of c-fos gene and protein by growth factors precedes activation of c-myc. *Nature*, *312*(5996), 716–720.

Murphy, B. A., Vick, M. M., Sessions, D. R., Cook, R. F., & Fitzgerald, B.

P. (2007). Acute systemic inflammation transiently synchronizes clock gene expression in equine peripheral blood. *Brain, Behavior, and Immunity*, *21*(4), 467–76.

Mushtaq, G., Khan, J. A., Kumosani, T. A., & Kamal, M. A. (2015). Alzheimer's disease and type 2 diabetes via chronic inflammatory mechanisms. *Saudi Journal of Biological Sciences*, *22*(1), 4–13.

Musiek, E. S., Lim, M. M., Yang, G., Bauer, A. Q., Qi, L., Lee, Y., … Fitzgerald, G. A. (2013). Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *The Journal of Clinical Investigation*, *123*(12), 5389–400.

Mutsuga, N., Shahar, T., Verbalis, J. G., Brownstein, M. J., Xiang, C. C., Bonner, R. F., & Gainer, H. (2004). Selective gene expression in magnocellular neurons in rat supraoptic nucleus. *The Journal of Neuroscience*, *24*(32), 7174–85.

Nadjar, A., Bluthé, R.-M., May, M. J., Dantzer, R., & Parnet, P. (2005). Inactivation of the cerebral NFkappaB pathway inhibits interleukin-1beta-induced sickness behavior and c-Fos expression in various brain nuclei. *Neuropsychopharmacology*, *30*(8), 1492–9.

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., … Miyake, K. (2002). Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature Immunology*, *3*(7), 667–72.

Naismith, S. L., Lewis, S. J. G., & Rogers, N. L. (2011). Sleep-wake changes and cognition in neurodegenerative disease. *Progress in Brain Research*, *190*, 21–52.

Nakamura, T. J., Nakamura, W., Yamazaki, S., Kudo, T., Cutler, T., Colwell, C. S., & Block, G. D. (2011). Age-related decline in circadian output. *The Journal of Neuroscience*, *31*(28), 10201–5.

Narasimamurthy, R., Hatori, M., Nayak, S. K., Liu, F., Panda, S., & Verma, I. M. (2012). Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(31), 12662–7.

Nava, F., Carta, G., & Haynes, L. W. (2000). Lipopolysaccharide increases arginine-vasopressin release from rat suprachiasmatic nucleus slice cultures. *Neuroscience Letters*, *288*(3), 228–230.

Nazem, A., Sankowski, R., Bacher, M., & Al-Abed, Y. (2015). Rodent models of neuroinflammation for Alzheimer's disease. *Journal of*

Neuroinflammation, *12*(1), 74.

Nelson, W., Tong, Y. L., Lee, J. K., & Halberg, F. Methods for cosinor-rhythmometry. *Chronobiologia*, *6*(4), 305–23.

Nemzek, J. A., Hugunin, K. M. S., & Opp, M. R. (2008). Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comparative Medicine*, *58*(2), 120–8.

NEURATH, M. F. (1998). Pathogenesis of inflammatory bowel disease: transcription factors in the spotlight. *Gut*, *42*(4), 458–459.

Ngo, D., Beaulieu, E., Gu, R., Leaney, A., Santos, L., Fan, H., … Morand, E. F. (2013). Divergent effects of endogenous and exogenous glucocorticoid-induced leucine zipper in animal models of inflammation and arthritis. *Arthritis and Rheumatism*, *65*(5), 1203–12.

Nguyen, M. D., Julien, J.-P., & Rivest, S. (2002). Innate immunity: the missing link in neuroprotection and neurodegeneration? *Nature Reviews. Neuroscience*, *3*(3), 216–27.

Nielsen, H. S., Hannibal, J., Knudsen, S. M., & Fahrenkrug, J. (2001). Pituitary adenylate cyclase-activating polypeptide induces period1 and period2 gene expression in the rat suprachiasmatic nucleus during late night. *Neuroscience*, *103*(2), 433–41.

Nishimura, M., Yamagata, K., Sugiura, H., & Okamura, H. (2003). The activity-regulated cytoskeleton-associated (Arc) gene is a new light-inducible early gene in the mouse suprachiasmatic nucleus. *Neuroscience*, *116*(4), 1141–7.

Noh, H., Jeon, J., & Seo, H. (2014). Systemic injection of LPS induces region-specific neuroinflammation and mitochondrial dysfunction in normal mouse brain. *Neurochemistry International*, *69*, 35–40.

Nonaka, M., Chen, X. H., Pierce, J. E., Leoni, M. J., McIntosh, T. K., Wolf, J. A., & Smith, D. H. (1999). Prolonged activation of NF-kappaB following traumatic brain injury in rats. *Journal of Neurotrauma*, *16*(11), 1023–34.

Novak, C. M., & Albers, H. E. (2004). Circadian phase alteration by GABA and light differs in diurnal and nocturnal rodents during the day. *Behavioral Neuroscience*, *118*(3), 498–504.

Novak, C. M., & Albers, H. E. (2004). Novel phase-shifting effects of GABAA receptor activation in the suprachiasmatic nucleus of a diurnal rodent. *The American Journal of Physiology*, *286*(5), 820–5.

Nurmi, A., Vartiainen, N., Pihlaja, R., Goldsteins, G., Yrjänheikki, J., & Koistinaho, J. (2004). Pyrrolidine dithiocarbamate inhibits translocation of nuclear factor kappa-B in neurons and protects against brain ischaemia with a wide therapeutic time window. *Journal of Neurochemistry*, *91*(3), 755–65.

Nygård, M., Lundkvist, G. B., Hill, R. H., & Kristensson, K. (2009). Rapid nitric oxide-dependent effects of tumor necrosis factor-alpha on suprachiasmatic nuclei neuronal activity. *Neuroreport*, *20*(2), 213–7.

O'Callaghan, E. K., Anderson, S. T., Moynagh, P. N., & Coogan, A. N. (2012). Long-lasting effects of sepsis on circadian rhythms in the mouse. *PloS One*, *7*(10), e47087.

O'Neill, L. A. ., & Kaltschmidt, C. (1997). NF-kB: a crucial transcription factor for glial and neuronal cell function. *Trends in Neurosciences*, *20*(6), 252–258.

Oakeshott, S., Balci, F., Filippov, I., Murphy, C., Port, R., Connor, D., … Brunner, D. (2011). Circadian Abnormalities in Motor Activity in a BAC Transgenic Mouse Model of Huntington's Disease. *PLoS Currents*, *3*, RRN1225.

Obrietan, K., Impey, S., Smith, D., Athos, J., & Storm, D. R. (1999). Circadian Regulation of cAMP Response Element-mediated Gene Expression in the Suprachiasmatic Nuclei. *Journal of Biological Chemistry*, *274*(25), 17748–17756.

Obrietan, K., Impey, S., & Storm, D. R. (1998). Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nature Neuroscience*, *1*(8), 693–700.

Ogilvie, K. M., Lee, S., & Rivier, C. (1998). Divergence in the Expression of Molecular Markers of Neuronal Activation in the Parvocellular Paraventricular Nucleus of the Hypothalamus Evoked by Alcohol Administration via Different Routes. *Journal of Neuroscience*, *18*(11), 4344–4352.

Ohta, H., Yamazaki, S., & McMahon, D. G. (2005). Constant light desynchronizes mammalian clock neurons. *Nature Neuroscience*, *8*(3), 267–9.

Ohta, K., Nakayama, K., Kurokawa, T., Kikuchi, T., & Yoshimura, N. (2002). Inhibitory effects of pyrrolidine dithiocarbamate on endotoxin-induced uveitis in Lewis rats. *Investigative Ophthalmology & Visual Science*, *43*(3), 744–50.

Okada, K., Yano, M., Doki, Y., Azama, T., Iwanaga, H., Miki, H., … Monden, M. (2008). Injection of LPS causes transient suppression of biological clock genes in rats. *The Journal of Surgical Research*, *145*(1), 5–12.

Okayasu, I., Hatakeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y., & Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology*, *98*(3), 694–702.

Olanow, C. W., & Tatton, W. G. (1999). Etiology and pathogenesis of Parkinson's disease. *Annual Review of Neuroscience*, *22*, 123–44.

Pacifico, F., & Leonardi, A. (2006). NF-kappaB in solid tumors. *Biochemical Pharmacology*, *72*(9), 1142–52.

Paladino, N., Fedele, M. L. M., Duhart, J. M., Marpegan, L., & Golombek, D. A. (2014). Modulation of mammalian circadian rhythms by tumor necrosis factor-α, *Chronobiology International*, 31(5), 668-79.

Paladino, N., Leone, M. J., Plano, S. a, & Golombek, D. a. (2010). Paying the circadian toll: the circadian response to LPS injection is dependent on the Toll-like receptor 4. *Journal of Neuroimmunology*, *225*(1-2), 62–7.

Pallier, P. N., Maywood, E. S., Zheng, Z., Chesham, J. E., Inyushkin, A. N., Dyball, R., … Morton, A. J. (2007). Pharmacological imposition of sleep slows cognitive decline and reverses dysregulation of circadian gene expression in a transgenic mouse model of Huntington's disease. *The Journal of Neuroscience*, *27*(29), 7869–78.

Palomba, M., & Bentivoglio, M. (2008). Chronic inflammation affects the photic response of the suprachiasmatic nucleus. *Journal of Neuroimmunology*, *193*(1-2), 24–7.

Palomba, M., Nygård, M., Florenzano, F., Bertini, G., Kristensson, K., & Bentivoglio, M. (2008). Decline of the presynaptic network, including GABAergic terminals, in the aging suprachiasmatic nucleus of the mouse. *Journal of Biological Rhythms*, *23*(3), 220–31.

Pålsson-McDermott, E. M., & O'Neill, L. A. J. (2004). Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*, *113*(2), 153–62.

Pan, W., Ding, Y., Yu, Y., Ohtaki, H., Nakamachi, T., & Kastin, A. J. (2006). Stroke upregulates TNFalpha transport across the blood-brain barrier. *Experimental Neurology*, *198*(1), 222–33.

Partonen, T., Treutlein, J., Alpman, A., Frank, J., Johansson, C., Depner, M., … Schumann, G. (2007). Three circadian clock genes Per2, Arntl, and Npas2 contribute to winter depression. *Annals of Medicine*, *39*(3), 229–38.

Paschos, G. K., Ibrahim, S., Song, W.-L., Kunieda, T., Grant, G., Reyes, T. M., … Fitzgerald, G. A. (2012). Obesity in mice with adipocyte-specific deletion of clock component Arntl. *Nature Medicine*, *18*(12), 1768–77.

Pękalski, J., Zuk, P. J., Kochańczyk, M., Junkin, M., Kellogg, R., Tay, S., & Lipniacki, T. (2013). Spontaneous NF-κB activation by autocrine TNFα signaling: a computational analysis. *PloS One*, *8*(11), e78887.

Pendergast, J. S., Friday, R. C., & Yamazaki, S. (2009). Endogenous rhythms in Period1 mutant suprachiasmatic nuclei *in vitro* do not represent circadian behavior. *The Journal of Neuroscience*, *29*(46), 14681–6.

Pendergast, J. S., Friday, R. C., & Yamazaki, S. (2010). Distinct functions of Period2 and Period3 in the mouse circadian system revealed by *in vitro* analysis. *PloS One*, *5*(1), e8552.

Pendergast, J. S., Niswender, K. D., & Yamazaki, S. (2012). Tissue-specific function of Period3 in circadian rhythmicity. *PloS One*, *7*(1), e30254.

Pennacchio, L. A. (2003). Insights from human/mouse genome comparisons. *Mammalian Genome*, *14*(7), 429–36.

Pennartz, C. M., Hamstra, R., & Geurtsen, A. M. (2001). Enhanced NMDA receptor activity in retinal inputs to the rat suprachiasmatic nucleus during the subjective night. *The Journal of Physiology*, *532*(1), 181–94.

Pereira, D. S., Kushner, D. B., Ricciardi, R. P., & Graham, F. L. (1996). Testing NF-kappa B1-p50 antibody specificity using knockout mice. *Oncogene*, *13*(2), 445–6.

Perkins, N. D. (2006). Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene*, *25*(51), 6717–30.

Perkins, N. D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nature Reviews. Molecular Cell Biology*, *8*(1), 49–62.

Petrovsky, N., McNair, P., & Harrison, L. C. (1998). Diurnal rhythms of pro-inflammatory cytokines: regulation by plasma cortisol and

therapeutic implications. *Cytokine*, *10*(4), 307–12.

Pevet, P., & Challet, E. (2011). Melatonin: both master clock output and internal time-giver in the circadian clocks network. *Journal of Physiology*, *105*(4-6), 170–82.

Pezük, P., Mohawk, J. A., Wang, L. A., & Menaker, M. (2012). Glucocorticoids as entraining signals for peripheral circadian oscillators. *Endocrinology*, *153*(10), 4775–83.

Phan, T. X., Phan, T. H., Chan, G. C.-K., Sindreu, C. B., Eckel-Mahan, K. L., & Storm, D. R. (2011). The diurnal oscillation of MAP (mitogen-activated protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus. *The Journal of Neuroscience*, *31*(29), 10640–7.

Phifer-Rixey, M., & Nachman, M. W. (2015). Insights into mammalian biology from the wild house mouse Mus musculus. *eLife*, *4*.

PICCIOTTO, M. R., & WICKMAN, K. (1998). Using Knockout and Transgenic Mice to Study Neurophysiology and Behavior. *Physiol Rev*, *78*(4), 1131–1163.

Pickard, G. E. (1989). Entrainment of the circadian rhythm of wheel-running activity is phase shifted by ablation of the intergeniculate leaflet. *Brain Research*, *494*(1), 151–4.

Pickard, G. E., Ralph, M. R., & Menaker, M. (1987). The intergeniculate leaflet partially mediates effects of light on circadian rhythms. *Journal of Biological Rhythms*, *2*(1), 35–56.

Piggins, H. D., Antle, M. C., & Rusak, B. (1995). Neuropeptides phase shift the mammalian circadian pacemaker. *The Journal of Neuroscience*, *15*(8), 5612–22.

Piggins, H. D., & Cutler, D. J. (2003). The roles of vasoactive intestinal polypeptide in the mammalian circadian clock. *The Journal of Endocrinology*, *177*(1), 7–15.

Pitrosky, B., Kirsch, R., Malan, A., Mocaer, E., & Pevet, P. (1999). Organization of rat circadian rhythms during daily infusion of melatonin or S20098, a melatonin agonist. *The American Journal of Physiology*, *277*(2), 812–28.

Pittendrigh, C. S., & Daan, S. (1974). Circadian oscillations in rodents: a systematic increase of their frequency with age. *Science*, *186*(4163), 548–50.

Pittendrigh, C. S., & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents. *Journal of Comparative Physiology*, *106*(3), 291–331.

Pizzi, M., & Spano, P. (2006). Distinct roles of diverse nuclear factor-kappaB complexes in neuropathological mechanisms. *European Journal of Pharmacology*, *545*(1), 22–8.

Plath, N., Ohana, O., Dammermann, B., Errington, M. L., Schmitz, D., Gross, C., … Kuhl, D. (2006). Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron*, *52*(3), 437–44.

Pollak, C. P., & Perlick, D. Sleep problems and institutionalization of the elderly. *Journal of Geriatric Psychiatry and Neurology*, *4*(4), 204–10.

Pomerantz, J. L., & Baltimore, D. (2002). Two pathways to NF-kappaB. *Molecular Cell*, *10*(4), 693–5.

Popa-Wagner, A., Buga, A.-M., Dumitrascu, D. I., Uzoni, A., Thome, J., & Coogan, A. N. (2015). How does healthy aging impact on the circadian clock? *Journal of Neural Transmission*.

Possidente, B., McEldowney, S., & Pabon, A. (1995). Aging lengthens circadian period for wheel-running activity in C57BL mice. *Physiology & Behavior*, *57*(3), 575–9.

Prasad, A. V, Pilcher, W. H., & Joseph, S. A. (1994). Nuclear factor-kappa B in rat brain: enhanced DNA-binding activity following convulsant-induced seizures. *Neuroscience Letters*, *170*(1), 145–8.

Preitner, N., Damiola, F., Zakany, J., Duboule, D., Albrecht, U., & Schibler, U. (2002). The Orphan Nuclear Receptor REV-ERBα Controls Circadian Transcription within the Positive Limb of the Mammalian Circadian Oscillator. *Cell*, *110*(2), 251–260.

Prosser, R. A. (2000). Serotonergic Actions and Interactions on the SCN Circadian Pacemaker: *In Vitro* Investigations. *Biological Rhythm Research*, *31*(3), 315–339.

Prosser, R. A. (2003). Serotonin phase-shifts the mouse suprachiasmatic circadian clock *in vitro*. *Brain Research*, *966*(1), 110–5.

Prosser, R. A., Lee, H.-M., & Wehner, A. (2006). Serotonergic pre-treatments block *in vitro* serotonergic phase shifts of the mouse suprachiasmatic nucleus circadian clock. *Neuroscience*, *142*(2), 547–55.

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J.-S., … Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*, *55*(5), 453–62.

Quan, N., He, L., & Lai, W. (2003). Endothelial activation is an intermediate step for peripheral lipopolysaccharide induced activation of paraventricular nucleus. *Brain Research Bulletin*, *59*(6), 447–452.

Quan, N., He, L., Lai, W., Shen, T., & Herkenham, M. (2000). Induction of Ikappa Balpha mRNA Expression in the Brain by Glucocorticoids: A Negative Feedback Mechanism for Immune-to-Brain Signaling. *The Journal of Neuroscience*, *20*(17), 6473–6477.

Quan, N., Whiteside, M., Kim, L., & Herkenham, M. (1997). Induction of inhibitory factor kappaBalpha mRNA in the central nervous system after peripheral lipopolysaccharide administration: an in situ hybridization histochemistry study in the rat. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(20), 10985–90.

Raetz, C. R. H., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*, *71*, 635–700.

Ralph, M., & Menaker, M. (1988). A mutation of the circadian system in golden hamsters. *Science*, *241*(4870), 1225–1227.

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science*, *247*(4945), 975–8.

Ramkisoensing, A., & Meijer, J. H. (2015). Synchronization of Biological Clock Neurons by Light and Peripheral Feedback Systems Promotes Circadian Rhythms and Health. *Frontiers in Neurology*, *6*, 128.

Ramos-Vara, J. A. (2005). Technical aspects of immunohistochemistry. *Veterinary Pathology*, *42*(4), 405–26.

Ramos-Vara, J. A. (2011). Principles and methods of Immunohistochemistry. Gautier (Vol. 691). *Drug Safety Evaluation*, 83-96.

Rangan, G. K., Wang, Y., Tay, Y. C., & Harris, D. C. (1999). Inhibition of nuclear factor-kappaB activation reduces cortical tubulointerstitial injury in proteinuric rats. *Kidney International*, *56*(1), 118–34.

Rayet, B., & Gélinas, C. (1999). Aberrant rel/nfkb genes and activity in human cancer. *Oncogene*, *18*(49), 6938–47.

Rea, M. A. (1989). Light increases Fos-related protein immunoreactivity in the rat suprachiasmatic nuclei. *Brain Research Bulletin*, *23*(6), 577–81.

Rea, M. A. (1998). Photic entrainment of circadian rhythms in rodents. *Chronobiology International*, *15*(5), 395–423.

Rea, M. A., & Pickard, G. G. E. (2010). Serotonergic Modulation of Photic Entrainment in the Syrian Hamster. *Biological Rhythm Research*, 31(3), 284-314.

Reddy, A. B., & O'Neill, J. S. (2010). Healthy clocks, healthy body, healthy mind. *Trends in Cell Biology*, *20*(1), 36–44.

Redman, J., Armstrong, S., & Ng, K. T. (1983). Free-running activity rhythms in the rat: entrainment by melatonin. *Science*, *219*(4588), 1089–91.

Reed, H. E., Meyer-Spasche, A., Cutler, D. J., Coen, C. W., & Piggins, H. D. (2001). Vasoactive intestinal polypeptide (VIP) phase-shifts the rat suprachiasmatic nucleus clock *in vitro*. *The European Journal of Neuroscience*, *13*(4), 839–43.

Reghunandanan, V., Badgaiyan, R. D., Marya, R. K., & Maini, B. K. (1987). Suprachiasmatic injection of a vasopressin antagonist modifies the circadian rhythm of food intake. *Behavioral and Neural Biology*, *48*(3), 344–51.

Reghunandanan, V., & Reghunandanan, R. (2006). Neurotransmitters of the suprachiasmatic nuclei. *Journal of Circadian Rhythms*, *4*, 2.

Reghunandanan, V., Reghunandanan, R., Marya, R. K., & Singh, P. I. (1992). Vasopressin antagonist disrupts the circadian rhythm of water intake on suprachiasmatic injection. *Chronobiology International*, *9*(5), 356–61.

Reick, M., Garcia, J. A., Dudley, C., & McKnight, S. L. (2001). NPAS2: an analog of clock operative in the mammalian forebrain. *Science*, *293*(5529), 506–9.

Reppert, S. M., Perlow, M. J., Ungerleider, L. G., Mishkin, M., Tamarkin, L., Orloff, D. G., … Klein, D. C. (1981). Effects of damage to the suprachiasmatic area of the anterior hypothalamus on the daily melatonin and cortisol rhythms in the rhesus monkey. *The Journal of Neuroscience*, *1*(12), 1414–25.

Reppert, S. M., & Weaver, D. R. (2001). Molecular analysis of mammalian circadian rhythms. *Annual Review of Physiology*, *63*, 647–76.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature*, *418*(6901), 935–41.

Reppert, S. M., Weaver, D. R., Rivkees, S. A., & Stopa, E. G. (1988). Putative melatonin receptors in a human biological clock. *Science*, *242*(4875), 78–81.

Revell, V. L., Burgess, H. J., Gazda, C. J., Smith, M. R., Fogg, L. F., & Eastman, C. I. (2006). Advancing human circadian rhythms with afternoon melatonin and morning intermittent bright light. *The Journal of Clinical Endocrinology and Metabolism*, *91*(1), 54–9.

Riera, H., Afonso, V., Collin, P., & Lomri, A. (2015). A Central Role for JNK/AP-1 Pathway in the Pro-Oxidant Effect of Pyrrolidine Dithiocarbamate through Superoxide Dismutase 1 Gene Repression and Reactive Oxygen Species Generation in Hematopoietic Human Cancer Cell Line U937. *PloS One*, *10*(5), e0127571.

Ripperger, J. A., Jud, C., & Albrecht, U. (2011). The daily rhythm of mice. *FEBS Letters*, *585*(10), 1384–92.

Rivest, S. (2003). Molecular insights on the cerebral innate immune system. *Brain, Behavior, and Immunity*, *17*(1), 13–9.

Rivest, S., & Laflamme, N. (1995). Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. *Journal of Neuroendocrinology*, *7*(7), 501–25.

Rogler, G., Brand, K., Vogl, D., Page, S., Hofmeister, R., Andus, T., … Gross, V. (1998). Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology*, *115*(2), 357–69.

Rollag, M. D., Berson, D. M., & Provencio, I. (2003). Melanopsin, ganglion-cell photoreceptors, and mammalian photoentrainment. *Journal of Biological Rhythms*, *18*(3), 227–34.

Roozendaal, B., van Gool, W. A., Swaab, D. F., Hoogendijk, J. E., & Mirmiran, M. (1987). Changes in vasopressin cells of the rat suprachiasmatic nucleus with aging. *Brain Research*, *409*(2), 259–64.

Rosczyk, H. A., Sparkman, N. L., & Johnson, R. W. (2008). Neuroinflammation and cognitive function in aged mice following minor surgery. *Experimental Gerontology*, *43*(9), 840–6.

Rosenberg, R. S., Zee, P. C., & Turek, F. W. (1991). Phase response curves to light in young and old hamsters. *The American Journal of*

Physiology, *261*(2), 491–495.

Rosenwasser, A. M. (2009). Functional neuroanatomy of sleep and circadian rhythms. *Brain Research Reviews*, *61*(2), 281–306.

Rosenwasser, A. M., & Dwyer, S. M. (2002). Phase shifting the hamster circadian clock by 15-minute dark pulses. *Journal of Biological Rhythms*, *17*(3), 238–47.

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., & Hauschildt, S. (2011). LPS-induced cytokine production in human monocytes and macrophages. *Critical Reviews in Immunology*, *31*(5), 379–446.

Rothstein, J. D., Bristol, L. A., Hosler, B., Brown, R. H., & Kuncl, R. W. (1994). Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(10), 4155–9.

Ruby, N. F., Brennan, T. J., Xie, X., Cao, V., Franken, P., Heller, H. C., & O'Hara, B. F. (2002). Role of melanopsin in circadian responses to light. *Science*, *298*(5601), 2211–3.

Ruby, N. F., Edgar, D. M., Heller, H. C., & Miller, J. D. (1998). The aged suprachiasmatic nucleus is phase-shifted by cAMP *in vitro*. *Brain Research*, *779*(1-2), 338–341.

Rudic, R. D., McNamara, P., Curtis, A.-M., Boston, R. C., Panda, S., Hogenesch, J. B., & Fitzgerald, G. A. (2004). BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biology*, *2*(11), e377.

Rusak, B., McNaughton, L., Robertson, H. A., & Hunt, S. P. (1992). Circadian variation in photic regulation of immediate-early gene mRNAs in rat suprachiasmatic nucleus cells. *Brain Research. Molecular Brain Research*, *14*(1-2), 124–30.

Rusak, B., Robertson, H. A., Wisden, W., & Hunt, S. P. (1990). Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. *Science*, *248*(4960), 1237–40.

Sack, R. L., Brandes, R. W., Kendall, A. R., & Lewy, A. J. (2000). Entrainment of free-running circadian rhythms by melatonin in blind people. *The New England Journal of Medicine*, *343*(15), 1070–7.

Sack, R. L., & Lewy, A. J. (1997). Melatonin as a chronobiotic: treatment of circadian desynchrony in night workers and the blind. *Journal of*

Biological Rhythms, *12*(6), 595–603.

Sadki, A., Bentivoglio, M., Kristensson, K., & Nygård, M. (2007). Suppressors, receptors and effects of cytokines on the aging mouse biological clock. *Neurobiology of Aging*, *28*(2), 296–305.

Sadun, A. A., & Bassi, C. J. (1990). Optic nerve damage in Alzheimer's disease. *Ophthalmology*, *97*(1), 9–17.

Saeb-Parsy, K., Lombardelli, S., Khan, F. Z., McDowall, K., Au-Yong, I. T., & Dyball, R. E. (2000). Neural connections of hypothalamic neuroendocrine nuclei in the rat. *Journal of Neuroendocrinology*, *12*(7), 635–48.

Sagar, S. M., Sharp, F. R., & Curran, T. (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. *Science*, *240*(4857), 1328–31.

Saini, C., Liani, A., Curie, T., Gos, P., Kreppel, F., Emmenegger, Y., … Schibler, U. (2013). Real-time recording of circadian liver gene expression in freely moving mice reveals the phase-setting behavior of hepatocyte clocks. *Genes & Development*, *27*(13), 1526–36.

Sam, S., Corbridge, T. C., Mokhlesi, B., Comellas, A. P., & Molitch, M. E. (2004). Cortisol levels and mortality in severe sepsis. *Clinical Endocrinology*, *60*(1), 29–35.

Saper, C. B., Lu, J., Chou, T. C., & Gooley, J. (2005). The hypothalamic integrator for circadian rhythms. *Trends in Neurosciences*, *28*(3), 152–7.

Satlin, A., Teicher, M. H., Lieberman, H. R., Baldessarini, R. J., Volicer, L., & Rheaume, Y. (1991). Circadian locomotor activity rhythms in Alzheimer's disease. *Neuropsychopharmacology*, *5*(2), 115–26.

Satlin, A., Volicer, L., Stopa, E. G., & Harper, D. (1995). Circadian locomotor activity and core-body temperature rhythms in Alzheimer's disease. *Neurobiology of Aging*, *16*(5), 765–771.

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., … Hogenesch, J. B. (2004). A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron*, *43*(4), 527–37.

Sato, T. K., Yamada, R. G., Ukai, H., Baggs, J. E., Miraglia, L. J., Kobayashi, T. J., … Hogenesch, J. B. (2006). Feedback repression is required for mammalian circadian clock function. *Nature Genetics*,

38(3), 312–9.

Scarbrough, K., Losee-Olson, S., Wallen, E. P., & Turek, F. W. (1997). Aging and photoperiod affect entrainment and quantitative aspects of locomotor behavior in Syrian hamsters. *The American Journal of Physiology*, *272*(4), 1219–1225.

Scheidereit, C. (2006). IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene*, *25*(51), 6685–705.

Scheiermann, C., Kunisaki, Y., & Frenette, P. S. (2013). Circadian control of the immune system. *Nature Review Immunology*, *13*(3), 190–198.

Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., & Baldwin, A. S. (1995). Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science*, *270*(5234), 283–6.

Schernhammer, E. S., Kroenke, C. H., Laden, F., & Hankinson, S. E. (2006). Night work and risk of breast cancer. *Epidemiology*, *17*(1), 108–11.

Schmidt, T. M., Chen, S.-K., & Hattar, S. (2011). Intrinsically photosensitive retinal ganglion cells: many subtypes, diverse functions. *Trends in Neurosciences*, *34*(11), 572–80.

Schmidt-Ullrich, R., Mémet, S., Lilienbaum, A., Feuillard, J., Raphaël, M., & Israel, A. (1996). NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity. *Development*, *122*(7), 2117–28.

Schmitz, M. L., Mattioli, I., Buss, H., & Kracht, M. (2004). NF-kappaB: a multifaceted transcription factor regulated at several levels. *Chembiochem*, *5*(10), 1348–58.

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675.

Schreck, R., Meier, B., Männel, D. N., Dröge, W., & Baeuerle, P. A. (1992). Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *The Journal of Experimental Medicine*, *175*(5), 1181–94. t

Schreiber, S., Nikolaus, S., & Hampe, J. (1998). Activation of nuclear factor kappa B inflammatory bowel disease. *Gut*, *42*(4), 477–84.

Schulze-Luehrmann, J., & Ghosh, S. (2006). Antigen-receptor signaling to nuclear factor kappa B. *Immunity*, *25*(5), 701–15.

Schwartz, W. J., Aronin, N., Takeuchi, J., Bennett, M. R., & Peters, R. V. (1995). Towards a molecular biology of the suprachiasmatic nucleus: photic and temporal regulation of c-fosgene expression. *Seminars in Neuroscience*, *7*(1), 53–60.

Schwartz, W. J., & Zimmerman, P. (1990). Circadian timekeeping in BALB/c and C57BL/6 inbred mouse strains. *The Journal of Neuroscience*, *10*(11), 3685–94.

Selim, M., Glass, J. D., Hauser, U. E., & Rea, M. A. (1993). Serotonergic inhibition of light-induced fos protein expression and extracellular glutamate in the suprachiasmatic nuclei. *Brain Research*, *621*(2), 181–188.

Sellix, M. T., Evans, J. A., Leise, T. L., Castanon-Cervantes, O., Hill, D. D., DeLisser, P., … Davidson, A. J. (2012). Aging differentially affects the re-entrainment response of central and peripheral circadian oscillators. *The Journal of Neuroscience*, *32*(46), 16193–202.

Semmler, A., Okulla, T., Sastre, M., Dumitrescu-Ozimek, L., & Heneka, M. T. (2005). Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *Journal of Chemical Neuroanatomy*, *30*(2-3), 144–57.

Sen, R., & Baltimore, D. (1986). Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. *Cell*, *47*(6), 921–928.

Sha, W. C., Liou, H. C., Tuomanen, E. I., & Baltimore, D. (1995). Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. *Cell*, *80*(2), 321–30.

Shaftel, S. S., Griffin, W. S. T., & O'Banion, M. K. (2008). The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. *Journal of Neuroinflammation*, *5*(1), 7.

Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., … Reppert, S. M. (2000). Interacting molecular loops in the mammalian circadian clock. *Science*, *288*(5468), 1013–9.

Shen, W.-H., Zhang, C.-Y., & Zhang, G.-Y. (2003). Modulation of IkappaB kinase autophosphorylation and activity following brain ischemia. *Acta Pharmacologica Sinica*, *24*(4), 311–5.

Sheng, M., & Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron*, *4*(4), 477–485.

Shibata, S., & Moore, R. Y. (1993). Neuropeptide Y and optic chiasm stimulation of affect suprachiasmatic nucleus circadian function *in vitro*. *Brain Research*, *615*(1), 95–100.

Shibata, S., Tsuneyoshi, A., Hamada, T., Tominaga, K., & Watanabe, S. (1992). Phase-resetting effect of 8-OH-DPAT, a serotonin1A receptor agonist, on the circadian rhythm of firing rate in the rat suprachiasmatic nuclei *in vitro*. *Brain Research*, *582*(2), 353–6.

Shibata, S., Watanabe, A., Hamada, T., Ono, M., & Watanabe, S. (1994). N-methyl-D-aspartate induces phase shifts in circadian rhythm of neuronal activity of rat SCN *in vitro*. *The American Journal of Physiology*, *267*(2), 360–4.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., … Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell*, *91*(7), 1043–53.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., & Kimoto, M. (1999). MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like Receptor 4. *Journal of Experimental Medicine*, *189*(11), 1777–1782.

Shimomura, K., Low-Zeddies, S. S., King, D. P., Steeves, T. D., Whiteley, A., Kushla, J., … Takahashi, J. S. (2001). Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. *Genome Research*, *11*(6), 959–80.

Shinohara, K., Hiruma, H., Funabashi, T., & Kimura, F. (2000). GABAergic modulation of gap junction communication in slice cultures of the rat suprachiasmatic nucleus. *Neuroscience*, *96*(3), 591–596.

Shirakawa, T., Honma, S., Katsuno, Y., Oguchi, H., & Honma, K. I. (2000). Synchronization of circadian firing rhythms in cultured rat suprachiasmatic neurons. *The European Journal of Neuroscience*, *12*(8), 2833–8.

Shirogane, T., Jin, J., Ang, X. L., & Harper, J. W. (2005). SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. *The Journal of Biological Chemistry*, *280*(29), 26863–72.

Siegel, A. L., Bledsoe, C., Lavin, J., Gatti, F., Berge, J., Millman, G., … Carlson, C. G. (2009). Treatment with inhibitors of the NF-kappaB pathway improves whole body tension development in the mdx mouse.

Neuromuscular Disorders : NMD, *19*(2), 131–9. doi:10.1016/j.nmd.2008.10.006

Sikorski, R., & Peters, R. (1997). Transgenics on the Internet. *Nature Biotechnology*, *15*(3), 289.

Silver, A. C., Arjona, A., Walker, W. E., & Fikrig, E. (2012). The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity*, *36*(2), 251–61.

Silver, R., LeSauter, J., Tresco, P. A., & Lehman, M. N. (1996). A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. *Nature*, *382*(6594), 810–3.

Singh, A. K., & Jiang, Y. (2004). How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology*, *201*(1-3), 197–207.

Singru, P. S., Sánchez, E., Acharya, R., Fekete, C., & Lechan, R. M. (2008). Mitogen-activated protein kinase contributes to lipopolysaccharide-induced activation of corticotropin-releasing hormone synthesizing neurons in the hypothalamic paraventricular nucleus. *Endocrinology*, *149*(5), 2283–92.

Skelly, D. T., Hennessy, E., Dansereau, M.-A., & Cunningham, C. (2013). A systematic analysis of the peripheral and CNS effects of systemic LPS, IL-1β, [corrected] TNF-α and IL-6 challenges in C57BL/6 mice. *PloS One*, *8*(7), e69123.

Slonaker, J. R. 1970. The normal activity of the albino rat from birth to natural death, its rate of growth and the duration of life. Journal of Animal Behavior, 2(1), 20-42.

Slotten, H. A., Krekling, S., Sicard, B., & Pévet, P. (2002). Daily infusion of melatonin entrains circadian activity rhythms in the diurnal rodent Arvicanthis ansorgei. *Behavioural Brain Research*, *133*(1), 11–9.

Slotten, H. A., Pitrosky, B., & Pévet, P. (1999). Influence of the mode of daily melatonin administration on entrainment of rat circadian rhythms. *Journal of Biological Rhythms*, *14*(5), 347–53.

Smith, R. D., Inouye, S., & Turek, F. W. (1989). Central administration of muscimol phase-shifts the mammalian circadian clock. *Journal of Comparative Physiology*, *164*(6), 805–14.

Smolen, J., & Keystone, E. C. (2012). Rheumatoid arthritis: where are we now? *Rheumatology*, *51*(5), 1–2.

Son, G. H., Chung, S., Choe, H. K., Kim, H.-D., Baik, S.-M., Lee, H., … Kim, K. (2008). Adrenal peripheral clock controls the autonomous circadian rhythm of glucocorticoid by causing rhythmic steroid production. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(52), 20970–5.

Son, G. H., Chung, S., & Kim, K. (2011). The adrenal peripheral clock: glucocorticoid and the circadian timing system. *Frontiers in Neuroendocrinology*, *32*(4), 451–65.

Song, X. R., Torphy, T. J., Griswold, D. E., & Shealy, D. (2002). Coming of age: anti-cytokine therapies. *Molecular Interventions*, *2*(1), 36–46.

Spengler, M. L., Kuropatwinski, K. K., Comas, M., Gasparian, A. V, Fedtsova, N., Gleiberman, A. S., … Antoch, M. P. (2012). Core circadian protein CLOCK is a positive regulator of NF-κB-mediated transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(37), E2457–65.

Sprouse, J., Li, X., Stock, J., McNeish, J., & Reynolds, L. (2005). Circadian rhythm phenotype of 5-HT7 receptor knockout mice: 5-HT and 8-OH-DPAT-induced phase advances of SCN neuronal firing. *Journal of Biological Rhythms*, *20*(2), 122–31.

Steinlechner, S., Jacobmeier, B., Scherbarth, F., Dernbach, H., Kruse, F., & Albrecht, U. (2002). Robust circadian rhythmicity of Per1 and Per2 mutant mice in constant light, and dynamics of Per1 and Per2 gene expression under long and short photoperiods. *Journal of Biological Rhythms*, *17*(3), 202–9.

Stephan, F. K., & Zucker, I. (1972). Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proceedings of the National Academy of Sciences of the United States of America*, *69*(6), 1583–6.

Sterniczuk, R., Dyck, R. H., Laferla, F. M., & Antle, M. C. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 1. Circadian changes. *Brain Research*, *1348*, 139–48.

Sterniczuk, R., Stepkowski, A., Jones, M., & Antle, M. C. (2008). Enhancement of photic shifts with the 5-HT1A mixed agonist/antagonist NAN-190: intra-suprachiasmatic nucleus pathway. *Neuroscience*, *153*(3), 571–80.

Steward, O., & Worley, P. F. (2001). Selective Targeting of Newly Synthesized Arc mRNA to Active Synapses Requires NMDA Receptor

Activation. *Neuron*, *30*(1), 227–240.

Stocchi, F., Barbato, L., Nordera, G., Berardelli, A., & Ruggieri, S. (1998). Sleep disorders in Parkinson's disease. *Journal of Neurology*, *245(1)*, 15–18.

Stojkovic, K., Wing, S. S., & Cermakian, N. (2014). A central role for ubiquitination within a circadian clock protein modification code. *Frontiers in Molecular Neuroscience*, *7*, 69.

Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y., & Menaker, M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science*, *291*(5503), 490–3.

Stopa, E. G., Volicer, L., Kuo-Leblanc, V., Harper, D., Lathi, D., Tate, B., & Satlin, A. (1999). Pathologic evaluation of the human suprachiasmatic nucleus in severe dementia. *Journal of Neuropathology and Experimental Neurology*, *58*(1), 29–39.

Sudo, M., Sasahara, K., Moriya, T., Akiyama, M., Hamada, T., & Shibata, S. (2003). Constant light housing attenuates circadian rhythms of mPer2 mRNA and mPER2 protein expression in the suprachiasmatic nucleus of mice. *Neuroscience*, *121*(2), 493–9.

Sukumaran, S., Almon, R. R., DuBois, D. C., & Jusko, W. J. (2010). Circadian rhythms in gene expression: Relationship to physiology, disease, drug disposition and drug action. *Advanced Drug Delivery Reviews*, *62*(9-10), 904–17.

Sumová, A., & Illnerová, H. (2005). Effect of photic stimuli disturbing overt circadian rhythms on the dorsomedial and ventrolateral SCN rhythmicity. *Brain Research*, *1048*(1-2), 161–9.

Sumová, A., Trávníčková, Z., Mikkelsen, J. D., & Illnerová, H. (1998). Spontaneous rhythm in c-Fos immunoreactivity in the dorsomedial part of the rat suprachiasmatic nucleus. *Brain Research*, *801*(1-2), 254–258.

Sun, S.-C. (2011). Non-canonical NF-κB signaling pathway. *Cell Research*, *21*(1), 71–85.

Sung, S., Yang, H., Uryu, K., Lee, E. B., Zhao, L., Shineman, D., … Praticò, D. (2004). Modulation of nuclear factor-kappa B activity by indomethacin influences A beta levels but not A beta precursor protein metabolism in a model of Alzheimer's disease. *The American Journal of Pathology*, *165*(6), 2197–206.

Swaab, D. F., Fliers, E., & Partiman, T. S. (1985). The suprachiasmatic

nucleus of the human brain in relation to sex, age and senile dementia. *Brain Research*, *342*(1), 37–44.

Swaab, D. F., Van Someren, E. J., Zhou, J. N., & Hofman, M. A. (1996). Biological rhythms in the human life cycle and their relationship to functional changes in the suprachiasmatic nucleus. *Progress in Brain Research*, *111*, 349–68.

Swanson, L. W., & Sawchenko, P. E. (1980). Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology*, *31*(6), 410–7.

Swinney, D. C., Xu, Y.-Z., Scarafia, L. E., Lee, I., Mak, A. Y., Gan, Q.-F., … Huber, M. (2002). A small molecule ubiquitination inhibitor blocks NF-kappa B-dependent cytokine expression in cells and rats. *The Journal of Biological Chemistry*, *277*(26), 23573–81.

Tafani, M., Pucci, B., Russo, A., Schito, L., Pellegrini, L., Perrone, G. A., … Russo, M. A. (2013). Modulators of HIF1α and NFkB in Cancer Treatment: Is it a Rational Approach for Controlling Malignant Progression? *Frontiers in Pharmacology*, *4*, 13.

Tahara, Y., Kuroda, H., Saito, K., Nakajima, Y., Kubo, Y., Ohnishi, N., … Shibata, S. (2012). *In vivo* monitoring of peripheral circadian clocks in the mouse. *Current Biology*, *22*(11), 1029–34.

Taishi, P., Bredow, S., Guha-Thakurta, N., Obál, F., & Krueger, J. M. (1997). Diurnal variations of interleukin-1 beta mRNA and beta-actin mRNA in rat brain. *Journal of Neuroimmunology*, *75*(1-2), 69–74.

Takahashi, J. S. (2004). Finding new clock components: past and future. *Journal of Biological Rhythms*, *19*(5), 339–47.

Takahashi, J. S., DeCoursey, P. J., Bauman, L., & Menaker, M. (n.d.). Spectral sensitivity of a novel photoreceptive system mediating entrainment of mammalian circadian rhythms. *Nature*, *308*(5955), 186–8.

Takahashi, S. (2001). Physical and Inflammatory Stressors Elevate Circadian Clock Gene mPer1 mRNA Levels in the Paraventricular Nucleus of the Mouse. *Endocrinology*, *142*(11), 4910–4917.

Takahashi, S., Yokota, S., Hara, R., Kobayashi, T., Akiyama, M., Moriya, T., & Shibata, S. (2001). Physical and inflammatory stressors elevate circadian clock gene mPer1 mRNA levels in the paraventricular nucleus of the mouse. *Endocrinology*, *142*(11), 4910–7.

Takasu, N. N., Pendergast, J. S., Olivas, C. S., Yamazaki, S., & Nakamura, W. (2013). *In vivo* monitoring of multi-unit neural activity in the suprachiasmatic nucleus reveals robust circadian rhythms in Period1$^{-/-}$ mice. *PloS One*, *8*(5), e64333.

Takeda, K., & Akira, S. (2004). TLR signaling pathways. *Seminars in Immunology*, *16*(1), 3–9.

Terai, K., Matsuo, A., & McGeer, P. L. (1996). Enhancement of immunoreactivity for NF-kappa B in the hippocampal formation and cerebral cortex of Alzheimer's disease. *Brain Research*, *735*(1), 159–68.

Thanvi, B., Lo, N., & Robinson, T. (2007). Levodopa-induced dyskinesia in Parkinson's disease: clinical features, pathogenesis, prevention and treatment. *Postgraduate Medical Journal*, *83*(980), 384–8.

Thome, J., Coogan, A. N., Woods, A. G., Darie, C. C., & Häßler, F. (2011). CLOCK Genes and Circadian Rhythmicity in Alzheimer Disease. *Journal of Aging Research*, *2011*, 383091, 4.

Thompson, J. F., Hayes, L. S., & Lloyd, D. B. (1991). Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene*, *103*(2), 171–177.

Tilstra, J. S., Clauson, C. L., Niedernhofer, L. J., & Robbins, P. D. (2011). NF-κB in Aging and Disease. *Aging and Disease*, *2*(6), 449–65.

Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., … Fu, Y. H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science*, *291*(5506), 1040–3.

Toliver-Kinsky, T., Papaconstantinou, J., & Perez-Polo, J. R. (1997). Age-associated alterations in hippocampal and basal forebrain nuclear factor kappa B activity. *Journal of Neuroscience Research*, *48*(6), 580–7.

Tominaga, K., Shibata, S., Ueki, S., & Watanabe, S. (1992). Effects of 5-HT1A receptor agonists on the circadian rhythm of wheel-running activity in hamsters. *European Journal of Pharmacology*, *214*(1), 79–84.

Trick, G. L., Barris, M. C., & Bickler-Bluth, M. (1989). Abnormal pattern electroretinograms in patients with senile dementia of the Alzheimer type. *Annals of Neurology*, *26*(2), 226–31.

Tu, Y.-C., Huang, D.-Y., Shiah, S.-G., Wang, J.-S., & Lin, W.-W. (2013). Regulation of c-Fos gene expression by NF-κB: a p65 homodimer

binding site in mouse embryonic fibroblasts but not human HEK293 cells. *PloS One*, *8*(12), e84062.

Turek, F. W. (2005). Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. *Science*, *308*(5724), 1043–1045.

Turek, F. W., Penev, P., Zhang, Y., van Reeth, O., & Zee, P. (1995). Effects of age on the circadian system. *Neuroscience & Biobehavioral Reviews*, *19*(1), 53–58.

Turrigiano, G. G. (2008). The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell*, *135*(3), 422–35.

Ulevitch, R. J., & Tobias, P. S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annual Review of Immunology*, *13*, 437–57.

Unlap, M. T., & Jope, R. S. (1997). Dexamethasone attenuates NF-kappa B DNA binding activity without inducing I kappa B levels in rat brain *in vivo*. *Brain Research*, *45*(1), 83–9.

Unlap, T., & Jope, R. S. (1995). Inhibition of NFkB DNA binding activity by glucocorticoids in rat brain. *Neuroscience Letters*, *198*(1), 41–4.

Valentinuzzi, V. S., Scarbrough, K., Takahashi, J. S., & Turek, F. W. (1997). Effects of aging on the circadian rhythm of wheel-running activity in C57BL/6 mice. *The American Journal of Physiology*, *273*(6), 1957–1964.

Van Amersfoort, E. S., Van Berkel, T. J. C., & Kuiper, J. (2003). Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clinical Microbiology Reviews*, *16*(3), 379–414.

van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., … Yasui, A. (1999). Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. *Nature*, *398*(6728), 627–30.

Van Reeth, O., Zhang, Y., Zee, P. C., & Turek, F. W. (1994). Grafting fetal suprachiasmatic nuclei in the hypothalamus of old hamsters restores responsiveness of the circadian clock to a phase shifting stimulus. *Brain Research*, *643*(1-2), 338–42.

Van Someren, E. J. (2000). Circadian rhythms and sleep in human aging. *Chronobiology International*, *17*(3), 233–43.

van Someren, E. J., Hagebeuk, E. E., Lijzenga, C., Scheltens, P., de Rooij, S. E., Jonker, C., … Swaab, D. F. (1996). Circadian rest-activity

rhythm disturbances in Alzheimer's disease. *Biological Psychiatry*, *40*(4), 259–70.

van Vollenhoven, R. F., Ernestam, S., Geborek, P., Petersson, I. F., Cöster, L., Waltbrand, E., … Bratt, J. (2009). Addition of infliximab compared with addition of sulfasalazine and hydroxychloroquine to methotrexate in patients with early rheumatoid arthritis (Swefot trial): 1-year results of a randomised trial. *Lancet*, *374*(9688), 459–66.

vanderLeest, H. T., Rohling, J. H. T., Michel, S., & Meijer, J. H. (2009). Phase shifting capacity of the circadian pacemaker determined by the SCN neuronal network organization. *PloS One*, *4*(3), e4976.

Varghese, T. K., Yan, S., Hummel, M., & Abecassis, M. (2003). PDTC prevents reactivation of latent cytomegalovirus both *in vivo* and *in vitro*. *Journal of Surgical Research*, *114*(2), 280.

Vindlacheruvu, R. R., Ebling, F. J. P., Maywood, E. S., & Hastings, M. H. (1992). Blockade of Glutamatergic Neurotransmission in the Suprachiasmatic Nucleus Prevents Cellular and Behavioural Responses of the Circadian System to Light. *The European Journal of Neuroscience*, *4*(7), 673–679.

Vitaterna, M. H., King, D. P., Chang, A. M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., … Takahashi, J. S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. *Science*, *264*(5159), 719–25.

Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., … Sancar, A. (1999). Differential regulation of mammalian Period genes and circadian rhythmicity by cryptochromes 1 and 2. *Proceedings of the National Academy of Sciences*, *96*(21), 12114–12119.

Vitiello, M. V, & Borson, S. (2001). Sleep disturbances in patients with Alzheimer's disease: epidemiology, pathophysiology and treatment. *CNS Drugs*, *15*(10), 777–96.

Vogel, G. (2007). Nobel Prizes. A knockout award in medicine. *Science*, *318*(5848), 178–9.

Voll, R. E., Jimi, E., Phillips, R. J., Barber, D. F., Rincon, M., Hayday, A. C., … Ghosh, S. (2000). NF-κB Activation by the Pre-T Cell Receptor Serves as a Selective Survival Signal in T Lymphocyte Development. *Immunity*, *13*(5), 677–689.

Wager-Smith, K., & Kay, S. A. (2000). Circadian rhythm genetics: from

flies to mice to humans. *Nature Genetics*, *26*(1), 23–7.

Wagner, S., Castel, M., Gainer, H., & Yarom, Y. (1997). GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity. *Nature*, *387*(6633), 598–603.

Wahl, C., Liptay, S., Adler, G., & Schmid, R. M. (1998). Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. *The Journal of Clinical Investigation*, *101*(5), 1163–74.

Waltereit, R., Dammermann, B., Wulff, P., Scafidi, J., Staubli, U., Kauselmann, G., … Kuhl, D. (2001). Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. *The Journal of Neuroscience*, *21*(15), 5484–93.

Wang, G. (2002). Signaling components that drive circadian rhythms. *Current Opinion in Neurobiology*, *12*(3), 331–338.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., & Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*, *153*(4), 910–8.

Wang, L. M.-C., Dragich, J. M., Kudo, T., Odom, I. H., Welsh, D. K., O'Dell, T. J., & Colwell, C. S. (2009). Expression of the circadian clock gene Period2 in the hippocampus: possible implications for synaptic plasticity and learned behaviour. *ASN Neuro*, *1*(3).

Watanabe, A., Shibata, S., & Watanabe, S. (1995). Circadian rhythm of spontaneous neuronal activity in the suprachiasmatic nucleus of old hamster *in vitro*. *Brain Research*, *695*(2), 237–9.

Wax, T. M. (1975). Runwheel activity patterns of mature-young and senescent mice: the effect of constant lighting conditions. *Journal of Gerontology*, *30*(1), 22–7.

Weaver, D. R., & Emery, P. (2013). Ciracadian Timekeeping. Elsevier (4^{th}), *Fundamental Neuroscience*, 819-845.

Webb, I. C., Coolen, L. M., & Lehman, M. N. (2013). NMDA and PACAP receptor signaling interact to mediate retinal-induced scn cellular rhythmicity in the absence of light. *PloS One*, *8*(10), e76365.

Weber, C. K., Liptay, S., Wirth, T., Adler, G., & Schmid, R. M. (2000). Suppression of NF-κB activity by sulfasalazine is mediated by direct inhibition of IκB kinases α and β. *Gastroenterology*, *119*(5), 1209–

1218.

Weber, E. T., Gannon, R. L., & Rea, M. A. (1998). Local administration of serotonin agonists blocks light-induced phase advances of the circadian activity rhythm in the hamster. *Journal of Biological Rhythms*, *13*(3), 209–18.

Weberpals, M., Hermes, M., Hermann, S., Kummer, M. P., Terwel, D., Semmler, A., … Heneka, M. T. (2009). NOS2 gene deficiency protects from sepsis-induced long-term cognitive deficits. *The Journal of Neuroscience*, *29*(45), 14177–84.

Weinert, D. (2000). Age-dependent changes of the circadian system. *Chronobiology International*, *17*(3), 261–83.

Weinert, H., & Weinert, D. (1998). Circadian activity rhythms of laboratory mice during the last weeks of their life. *Biological Rhythm Research*, 29(2),159-178.

Weinert, H., Weinert, D., Schurov, I., Maywood, E. S., & Hastings, M. H. (2001). Impaired expression of the mPer2 circadian clock gene in the suprachiasmatic nuclei of aging mice. *Chronobiology International*, *18*(3), 559–65.

Welsh, D. K. (2009). Limits of single-cell autonomy in the suprachiasmatic nucleus. *Sleep and Biological Rhythms*, *7*(4), 252–259.

Welsh, D. K., Imaizumi, T., & Kay, S. A. (2005). Real-time reporting of circadian-regulated gene expression by luciferase imaging in plants and mammalian cells. *Methods in Enzymology*, *393*, 269–88.

Welsh, D. K., Logothetis, D. E., Meister, M., & Reppert, S. M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron*, *14*(4), 697–706.

Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annual Review of Physiology*, *72*, 551–77.

Welsh, D. K., Yoo, S.-H., Liu, A. C., Takahashi, J. S., & Kay, S. A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Current Biology*, *14*(24), 2289–95.

Westrich, L., Sprouse, J., & Sánchez, C. (2013). The effects of combining serotonin reuptake inhibition and 5-HT7 receptor blockade on

circadian rhythm regulation in rodents. *Physiology & Behavior*, *110-111*, 42–50.

Wetterberg, L. (1994). Light and biological rhythms. *Journal of Internal Medicine*, *235*(1), 5–19.

Wickland, C., & Turek, F. W. (1994). Lesions of the thalamic intergeniculate leaflet block activity-induced phase shifts in the circadian activity rhythm of the golden hamster. *Brain Research*, *660*(2), 293–300.

Wilsbacher, L. D., Yamazaki, S., Herzog, E. D., Song, E.-J., Radcliffe, L. A., Abe, M., … Takahashi, J. S. (2002). Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice invivo. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(1), 489–94.

Wimmer, M. E., Rising, J., Galante, R. J., Wyner, A., Pack, A. I., & Abel, T. (2013). Aging in mice reduces the ability to sustain sleep/wake states. *PloS One*, *8*(12), e81880.

Witting, W., Kwa, I. H., Eikelenboom, P., Mirmiran, M., & Swaab, D. F. (1990). Alterations in the circadian rest-activity rhythm in aging and Alzheimer's disease. *Biological Psychiatry*, *27*(6), 563–72.

Witting, W., Mirmiran, M., Bos, N. P., & Swaab, D. F. (1994). The effect of old age on the free-running period of circadian rhythms in rat. *Chronobiology International*, *11*(2), 103–12.

Wright, S. D. (1999). Toll, A New Piece in the Puzzle of Innate Immunity. *Journal of Experimental Medicine*, *189*(4), 605–609.

Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., & Mathison, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, *249*(4975), 1431–3.

Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Arsura, M., Katz, D., … Sonenshein, G. E. (1996). Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells. *The EMBO Journal*, *15*(17), 4682–90.

Wu, Y.-H., Fischer, D. F., Kalsbeek, A., Garidou-Boof, M.-L., van der Vliet, J., van Heijningen, C., … Swaab, D. F. (2006). Pineal clock gene oscillation is disturbed in Alzheimer's disease, due to functional disconnection from the "master clock". *Official Publication of the Federation of American Societies for Experimental Biology*, *20*(11), 1874–6.

Wu, Y.-H., & Swaab, D. F. (2007). Disturbance and strategies for reactivation of the circadian rhythm system in aging and Alzheimer's disease. *Sleep Medicine*, *8*(6), 623–36.

Wu, Y.-H., Zhou, J.-N., Van Heerikhuize, J., Jockers, R., & Swaab, D. F. (2007). Decreased MT1 melatonin receptor expression in the suprachiasmatic nucleus in aging and Alzheimer's disease. *Neurobiology of Aging*, *28*(8), 1239–1247.

Wulff, K., Gatti, S., Wettstein, J. G., & Foster, R. G. (2010). Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nature Reviews. Neuroscience*, *11*(8), 589–99.

Wullaert, A., Bonnet, M. C., & Pasparakis, M. (2011). NF-κB in the regulation of epithelial homeostasis and inflammation. *Cell Research*, *21*(1), 146–58. doi:10.1038/cr.2010.175

Xiao, G., Harhaj, E. W., & Sun, S.-C. (2001). NF-κB-Inducing Kinase Regulates the Processing of NF-κB2 p100. *Molecular Cell*, *7*(2), 401–409.

Xu, Y., Padiath, Q. S., Shapiro, R. E., Jones, C. R., Wu, S. C., Saigoh, N., … Fu, Y.-H. (2005). Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. *Nature*, *434*(7033), 640–4.

Yadav, U. C. S., Kalariya, N. M., & Ramana, K. V. (2011). Emerging role of antioxidants in the protection of uveitis complications. *Current Medicinal Chemistry*, *18*(6), 931–42.

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A., & Worley, P. F. (1993). Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. *Neuron*, *11*(2), 371–386.

Yamaguchi, S., Mitsui, S., Miyake, S., Yan, L., Onishi, H., Yagita, K., … Okamura, H. (2000). The 5' upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. *Current Biology*, *10*(14), 873–6.

Yamaguchi, Y., Suzuki, T., Mizoro, Y., Kori, H., Okada, K., Chen, Y., … Okamura, H. (2013). Mice genetically deficient in vasopressin V1a and V1b receptors are resistant to jet lag. *Science*, *342*(6154), 85–90.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., … Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science*, *301*(5633), 640–3.

Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., … Akira, S. (2003). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature Immunology*, *4*(11), 1144–50.

Yamamoto, T., Nakahata, Y., Soma, H., Akashi, M., Mamine, T., & Takumi, T. (2004). Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Molecular Biology*, *5*, 18.

Yamamoto, Y., & Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *The Journal of Clinical Investigation*, *107*(2), 135–42.

Yamamura, Y., Yano, I., Kudo, T., & Shibata, S. (2010). Time-dependent inhibitory effect of lipopolysaccharide injection on Per1 and Per2 gene expression in the mouse heart and liver. *Chronobiology International*, *27*(2), 213–32.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., … Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, *288*(5466), 682–5.

Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., & Block, G. D. (2002). Effects of aging on central and peripheral mammalian clocks. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10801–6.

Yamazaki, S., & Takahashi, J. S. (2005). Real-time luminescence reporting of circadian gene expression in mammals. *Methods in Enzymology*, *393*, 288–301.

Yan, L. (2009). Expression of clock genes in the suprachiasmatic nucleus: effect of environmental lighting conditions. *Reviews in Endocrine & Metabolic Disorders*, *10*(4), 301–10.

Yan, L., Karatsoreos, I., Lesauter, J., Welsh, D. K., Kay, S., Foley, D., & Silver, R. (2007). Exploring spatiotemporal organization of SCN circuits. *Cold Spring Harbor Symposia on Quantitative Biology*, *72*, 527–41.

Yan, L., & Silver, R. (2004). Resetting the brain clock: time course and localization of mPER1 and mPER2 protein expression in suprachiasmatic nuclei during phase shifts. *The European Journal of Neuroscience*, *19*(4), 1105–9.

Yaziji, H., & Barry, T. (2006). Diagnostic Immunohistochemistry: what can go wrong? *Advances in Anatomic Pathology*, *13*(5), 238–46.

Yesavage, J. A., Noda, A., Hernandez, B., Friedman, L., Cheng, J. J., Tinklenberg, J. R., … Zeitzer, J. M. (2011). Circadian clock gene polymorphisms and sleep-wake disturbance in Alzheimer disease. *The American Journal of Geriatric Psychiatry*, *19*(7), 635–43.

Yoo, S.-H., Yamazaki, S., Lowrey, P. L., Shimomura, K., Ko, C. H., Buhr, E. D., … Takahashi, J. S. (2004). PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(15), 5339–46. doi:10.1073/pnas.0308709101

Yoshida, Y., Yoshimi, R., Yoshii, H., Kim, D., Dey, A., Xiong, H., … Ozato, K. (2014). The transcription factor IRF8 activates integrin-mediated TGF-β signaling and promotes neuroinflammation. *Immunity*, *40*(2), 187–98.

Yoshikawa, T., Yamazaki, S., & Menaker, M. (2005). Effects of preparation time on phase of cultured tissues reveal complexity of circadian organization. *Journal of Biological Rhythms*, *20*(6), 500–12.

You, W.-C., Wang, C.-X., Pan, Y.-X., Zhang, X., Zhou, X.-M., Zhang, X.-S., … Zhou, M.-L. (2013). Activation of nuclear factor-κB in the brain after experimental subarachnoid hemorrhage and its potential role in delayed brain injury. *PloS One*, *8*(3), e60290.

Young, M. W., & Kay, S. A. (2001). Time zones: a comparative genetics of circadian clocks. *Nature Reviews. Genetics*, *2*(9), 702–15.

Yu, Z., Zhou, D., Cheng, G., & Mattson, M. P. (2000). Neuroprotective role for the p50 subunit of NF-kappaB in an experimental model of Huntington's disease. *Journal of Molecular Neuroscience*, *15*(1), 31–44.

Zee, P. C., Rosenberg, R. S., & Turek, F. W. (1992). Effects of aging on entrainment and rate of resynchronization of circadian locomotor activity. *The American Journal of Physiology*, *263*(5), 1099–1103.

Zhang, C., Truong, K. K., & Zhou, Q.-Y. (2009). Efferent projections of prokineticin 2 expressing neurons in the mouse suprachiasmatic nucleus. *PloS One*, *4*(9), e7151.

Zhang, G., Li, J., Purkayastha, S., Tang, Y., Zhang, H., Yin, Y., … Cai, D. (2013). Hypothalamic programming of systemic ageing involving IKK-β, NF-κB and GnRH. *Nature*, *497*(7448), 211–6.

Zhang, J., Jiang, W., & Zuo, Z. (2014). Pyrrolidine dithiocarbamate

attenuates surgery-induced neuroinflammation and cognitive dysfunction possibly via inhibition of nuclear factor κB. *Neuroscience*, *261*, 1–10.

Zhao, Z., Huang, G., Wang, B., & Zhong, Y. (2013). Inhibition of NF-kappaB activation by Pyrrolidine dithiocarbamate partially attenuates hippocampal MMP-9 activation and improves cognitive deficits in streptozotocin-induced diabetic rats. *Behavioural Brain Research*, *238*, 44–7.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., … Lee, C. C. (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. *Cell*, *105*(5), 683–94.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., … Bradley, A. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature*, *400*(6740), 169–73.

Zhou, H., Monack, D. M., Kayagaki, N., Wertz, I., Yin, J., Wolf, B., & Dixit, V. M. (2005). Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation. *The Journal of Experimental Medicine*, *202*(10), 1327–32.

Zhou, J. (1995). VIP neurons in the human SCN in relation to sex, age, and Alzheimer's disease. *Neurobiology of Aging*, *16*(4), 571–576.

Zhou, Y. D., Barnard, M., Tian, H., Li, X., Ring, H. Z., Francke, U., … McKnight, S. L. (1997). Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(2), 713–8.

Zhu, X., Messer, J. S., Wang, Y., Lin, F., Cham, C. M., Chang, J., … Chang, E. B. (2015). Cytosolic HMGB1 controls the cellular autophagy/apoptosis checkpoint during inflammation. *The Journal of Clinical Investigation*, *125*(3), 1098–110.

Zou, J., & Crews, F. CREB and NF-kappaB transcription factors regulate sensitivity to excitotoxic and oxidative stress induced neuronal cell death. *Cellular and Molecular Neurobiology*, *26*(4-6), 385–405.

Zylka, M. J., Shearman, L. P., Levine, J. D., Jin, X., Weaver, D. R., & Reppert, S. M. (1998). Molecular Analysis of Mammalian Timeless. *Neuron*, *21*(5), 1115–1122.