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Circadian desynchrony in the mouse: how does chronic exposure to rotating shift work-like patterns of light/dark affect circadian rhythms and neurobehavioural outcomes.

Thesis submitted to the Department of Psychology, Faculty of Science and Engineering, in fulfilment of the requirements for the degree of Master of Science

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Contents

Organic functions themselves were regulated by it: one ate, not upon feeling hungry, but when prompted by the clock: one slept, not when one was tired, but when the clock sanctioned it…

The gain in mechanical efficiency through co-ordination and through the closer articulation of the day's events cannot be overestimated: while this increase cannot be measured in mere horsepower, one has only to imagine its absence today to foresee the speedy disruption and eventual collapse of our entire society. The modern industrial régime could do without coal and iron and steam easier than it could do without the clock.

--- Mumford (1934)

Acknowledgments

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Abstract

Disruption of circadian rhythms is associated with several deleterious health consequences, increased prevalence of obesity, maladaptive changes in affect, and cognitive impairment. Still it is estimated that as much as 20% of the work force are exposed to this risk factor experiencing some degree of chronodisruption by way of recurring weekly patterns of shift work. It is not presently clear therefore how efficiently the mammalian circadian system entrains to alternative sleep/wake cycles such as those found in shift work schedules or what the resulting physiological and neurobehavioural changes as a result of a *round-the-clock* activity regime might be.

The present study examines male CD-1 mice treated with three different paradigms of rapidly rotating shift work-like light/dark manipulation compared to two control groups maintained on a standard 12:12 h light/dark cycle. Animal actigraphy used to assess locomotor rhythm entrainment and circadian parameter plasticity revealed phenotypically distinct entrainment patterns for different work paradigms. In contrast to previous studies circadian desynchrony did not produce changes in animal body-weight. Behavioural testing suggests possible anxiogenic and hyperactive outcomes dependent on rotation speed as animals displayed open field thigmotaxis and hyperlocomotion. To test the hypothesis that rotating light/dark cycle weakens the circadian pacemaker examination of the SCN was carried out with results indicating no aberrant upregulation of inflammatory markers and normal rhythmic expression of core molecular clock proteins PER1 and PER2.

Together these observations suggest that major alterations in circadian rhythm components are induced by light/dark cycles which resemble shift work and are predictive of long term changes in behaviour and learning which persist after photoperiod has been remedied. Despite this, master pacemaker functioning appears to maintain coherency and is not weakened possibly pointing to desynchrony in peripheral clocks or a role of other mechanisms, such as stress or sleep disturbance, in mediating the effects described.

1. Introduction

1.1 Circadian rhythms

Circadian rhythms (*lat: circa-,* approximately; *diem-,* a day) are endogenous rhythmic processes which orchestrate several biological and behavioural parameters about a day – hence the name *circadian*. In nature these rhythms are present in almost all organisms ranging from single-celled eurkaryota, to plant and fungal life, on to higher order mammalian species such as man (Merrow, Spoelstra, & Roenneberg, 2005). In mammals the circadian pacemaker oscillates with a period of close to but not quite 24 h, allowing the system to manoeuvre entrainment to external time cues, or zeitgebers. While several zeitgebers of the system such as feeding time, temperature, exercise, and social interaction, have been found to produce phase changes in the physiology, the most potent stimulus which circadian rhythms respond to is the recurring light/dark cycle (Vitaterna, Takahashi, & Turek, 2001). In effect circadian rhythms enable the organism to organise its physiology and behaviour in anticipation of a changing environment.

Perhaps the most obvious example of the biological clock is that of the sleep-wake cycle; however it has also been demonstrated that these rhythms are present in regulating body temperature, hormone fluctuation, metabolism, immune activity, attention, mood, and cognition (Reppert & Weaver, 2001; Kohsaka & Bass, 2006; Bollinger *et al.,* 2010). The ability to pre-empt environmental changes and to regulate these processes in a pro-active manner affords the organism with an important evolutionary advantage, the ubiquity of which across all forms of life illustrates aptly the importance of adaption to the recurring cycle of day and night in our shared planetary environment influencing strategic timing of feeding, reproduction, and rest ensuring survivability of the organism. Moreover due to the system's ability to economise various organic functional parameters to be primed during the animals normal active phase, when desynchrony arises in the

system between internal and external factors it can lead to profound detriments across several biological and behavioural faculties. This principle holds true for man also and is particularly relevant for shift-workers who engage in non-conventional or inconsistent work patterns. Evidence suggests that disparity between internal circadian rhythm and the external pattern of light/dark can promote negative health and psychological consequences (Karatsoreos *et al.,* 2011; Fritschi *et al.,* 2010).

1.1.1 Site of the mammalian pacemaker

The central pacemaker in mammals is situated within the suprachiasmatic nucleus (SCN), a bilateral complex of approximately 20,000 phenotypically distinct cells located in the anterior hypothalamus superior to the optic chiasma (Morin & Allen, 2005). The significance of the SCN in regulating mammalian timekeeping was realised in the 1970s through a series of studies which established that ablation of the region through electrolytic or chemical lesions resulted in loss of circadian rhythmicity in a number of biological and behavioural rhythms (Moore & Eichler, 1972; Stephan & Zucker, 1972; Raisman & Brown-Grant, 1977; Ibuka *et al.,* 1977). This finding was reinforced by fetal transplant experiments which revealed that grafted tissue implants into the third ventricle ameliorated arrhythmicity with a period that corresponded to the genotype of the donor animal (Ralph *et al.,* 1990; Sollars *et al.,* 1995).The utilisation of donor grafts from *tau* mutant hamsters expedited this argument as the homozygotic free-running period of 20 h transplanted to a free-running wild-type host with a period of 24.1 h (both in constant darkness: DD) removed any error in delineating between the rhythm of the donor versus that of the residual host rhythm (Ralph *et al.,* 1990). It has also previously been demonstrated by *in vitro* recording that SCN tissue sections display ongoing circadian rhythmicity which suggests that the SCN can maintainan endogenous pacemaker function in absence of its organic environment (Green & Gillette, 1982). Since its early discovery

in the field of chronobiology, the SCN has been extensively investigated and as a result the functional anatomy of the master pacemaker has been well characterised from a neurophysiological and neurochemical perspective (see Rosenwasser & Turek, 2011; Rosenwasser, 2009; Morin & Allen, 2005; Reppert & Weaver, 2002; and Moore, Speh, & Leak, 2002 for reviews). A brief review highlighting the current understanding of circadian pacemaker functioning will be discussed here.

The first anatomical characteristic of the SCN is the compartmentalisation of each nucleus into two anatomically and functionally distinct regions (Figure 1.1) comprising of the ventrolateral *'core'* and the dorsomedial *'shell'* subnuclei (Moore *et al.,* 2002). Different neurotransmitter and neuropeptide profiles of each region serve to delineate between the two with the core region characterised by the presence of neurons containing vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP), while the shell SCN is characterised by arginine vasopressin (AVP) rich neurons (Rosenwasser, 2009). Colocalisted in each region with the respective aforementioned neuropeptides is γ-aminobutyric acid (GABA). The prevailing hypothesis explaining the division between subnuclei is that the core receives and organises pacemaker inputs and relays its signal to the shell which is responsible for outgoing circadian signal. This is evidenced by the finding that several major SCN afferent pathways project directly to the core region while several SCN efferents project outwards from the shell to the thalamus and other extra-SCN targets (Rosenwasser, 2009). Another supportive finding is that within the SCN core neurons project to neurons in the shell while the shell does not reciprocally project to the core. Indeed it is also found that GABA and core neuropeptide VIP are implicated in intercellular synchronisation as application or disruption of such is found to respectively promote synchrony or compromise behavioural rhythm coherence (Liu & Reppert, 2000; Colwell *et al,* 2003). A caveat to this core-afferent/shell-efferent model however is that neither the core nor shell regions are precluded from driving output or receiving input

signals as there are also outward projections from the core to extra-SCN regions and minor neurotransmitter inputs to the shell from acetylcholine, epinephrine, and histamine neurons (Rosenwasser & Turek, 2011).

The three major well characterised afferent projections to the SCN stem from the retina, the thalamic intergeniculate leaflet (IGL), and the raphe nuclei, and converge in the SCN core (Rosenwasser, 2009; see Figure 1.2). Retinal projections deliver photic input to the SCN through a system distinct from the primary visual pathway known as the retinohypothalamic tract (RHT). This dedicated circuit facilitates normal photic circadian entrainment through retinal ganglion cells specific from those involved in sensory vision (Moore *et al.* 1995; Rosenwasser, 2009). Early investigation revealed that the RHT was necessary for photic entrainment of the clock through selective ablation of retinal pathways (Johnson, Moore, & Morin, 1988). Freedman *et al.* (1999) more recently reported that genetically engineered mice without cone and rod photoreceptors sufficiently entrained to light suggesting that a novel, non-*'classical'* photoreceptor may be involved in the photic entrainment of circadian rhythms. Present understanding implicates the photopigment melanopsin, which is found within a subset of retinal ganglion cells in the RHT, as a circadian photoreceptor molecule involved in the photosensitivity of the RHT (Gooley *et al.,* 2001; Provencio *et al.,* 2000). Immunoreactive investigation into the neurotransmitters and neuropeptides active in the RHT has revealed glutamate, substance-P (SP), and pituitary adenyl cyclise-activating polypeptide (PACAP) present where the projection terminates in the SCN (Moore, Speh, & Leak, 2002). Species differences have also been noted pertaining to RHT content of SP and its significance in the pacemaker in rodents (Piggins, Samuels, Coogan, & Cutler, 2001).

The IGL too receives retinal input via a separate limb of the RHT as well as transmitting its own non-photic entrainment signals both of which are transmitted to the

central pacemaker via the geniculohypothalamic tract (GHT). The phase shifting capabilities of non-photic zeitgebers such as novel wheel running and benzodiazepine treatment have been compromised following IGL lesioning (Janik & Mrolovsky, 1994; Wickland & Turek, 1994; Meyer *et al.*, 1993; Schuhler *et al.*, 1999). The functional significance of such findings suggests that the IGL is likely involved in phase shifting in response to behaviourally arousing stimuli and reward circuit interaction. In addition to the arousal related pacemaker cues which seem to be signalled by the IGL, the RHT-IGL pathway has been identified as a possible *'secondary',* indirect avenue for photic entrainment to the SCN (Rosenwasser, 2009).

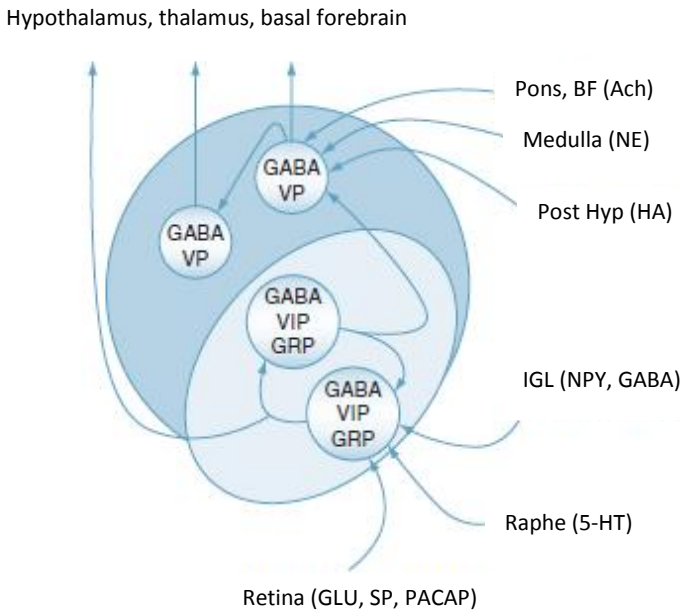


Figure 1.1. Core and shell organisation of SCN. The SCN core subnucleus (light blue) is rich in GABA neurons which are commonly colocalised with both VIP and/or GRP. The shell subnucleus (dark blue) is also rich in GABA which is colocalised with VP neurons. The three major afferent pathways converge in the SCN core which receives projections from the retina, IGL, and raphe nuclei. Several less well characterised afferents project to the SCN shell from extra-SCN targets which deliver clock entrainable signals via cholinergic (Ach), noradrenergic (NE), and histaminergic (HA) projections. Intra-SCN information flows outward from core to shell. Shell efferent projections relay pacemaker signal to extra-SCN targets. Figure source: Rosenwasser and Turek (2011).

Ach, acetylcholine; BF, basal forebrain; GABA, γ-aminobutyric acid; GLU, glutamate; GRP, gastrin-releasing peptide; 5-HT, 5-hydroxytryptamine (serotonin); IGL, intergeniculate leaflet; NE, norepinephrine; NPY, neuropeptide Y; OX, optic chiasma; PACAP, pituitary adenyl cyclise-activating polypeptide; SP, substance-P; VIP, vasoactive intestinal peptide; VP, arginine vasopressin; 3V, third ventricle.

It has been demonstrated experimentally for example that IGL lesions may augment or suppress the phase shifting effects of light throughout different circadian phases (Harrington & Rusak, 1986). It is thought that neuropeptide Y (NPY) and GABA are the

The final major afferent pathway to the pacemaker to be discussed here is the serotonergic (5-HT) projection from the raphe nuclei. It is known also that this pathway also innervates the IGL providing an alternative route by which 5-hydroxytryptamine (5-HT; serotonin) neurotransmitter could alter SCN function (Rosenwasser & Turek, 2011). It has been shown previously that serotonin can interfere with photic entrainment of the circadian clock causing both attenuated light induced phase shifting after treatment with serotonin agonists or reuptake inhibitors (Glass *et al.*, 1995; Rea & Pickard, 2000; Gannon & Millan, 2007) and, conversely, potentiating photic signalling in the presence of serotonergic neurotoxic lesioning (Bradbury, Dement, & Edgar, 1997).

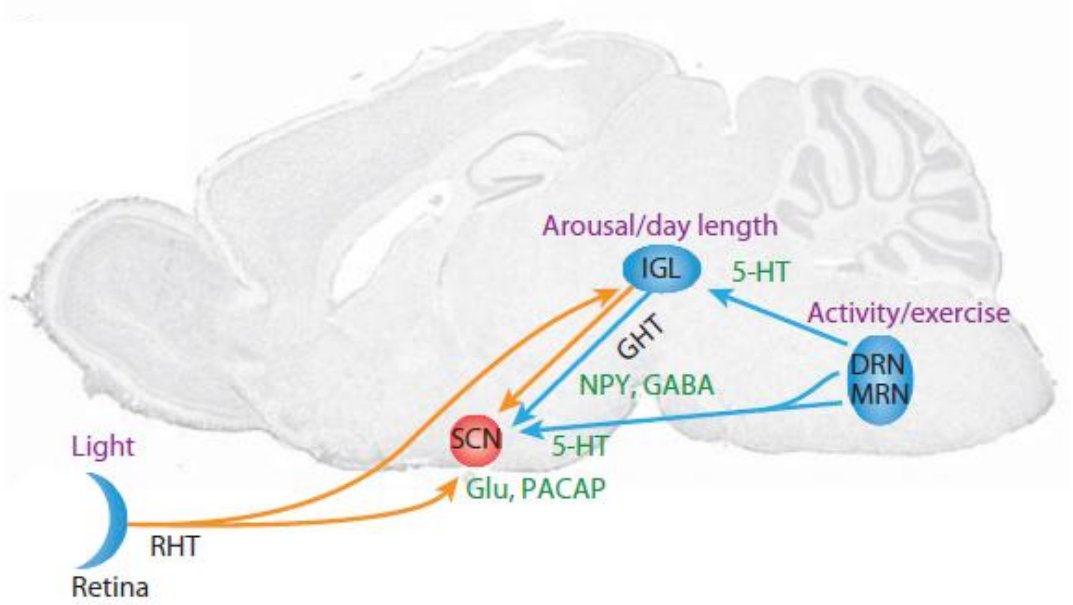


Figure 1.2. Major SCN afferents in the murine brain. Afferent projections from the retina and dorsal raphe nuclei (DRN) target the intergeniculate leaflet of the thalamus (IGL) which in turn projects to the SCN via the geniculohypothalamic tract (GHT). Retinal projections directly target the SCN via the retinohypothalamic tract (RHT). Serotonergic afferents also converge in the SCN projecting from the median raphe nuclei (MRN). Orange arrows = photic input, blue arrows = non-photic input. Figure source: Dibner, Schibler, and Albrecht (2010).

GABA, γ-aminobutyric acid; GLU, glutamate; 5-HT, 5-hydroxytryptamine (serotonin); NPY, neuropeptide Y; PACAP, pituitary adenyl cyclise-activating polypeptide.

There is reasonable evidence to also suggest that 5-HT is involved with arousal mediated entrainment of the clock as entrainment via restricted wheel running access and scheduled treadmill activity have shown state-dependent variation of the neurotransmitter (Edgar *et al.,* 1997; Marchant *et al.,* 1997). In one review on SCN neurotransmitter function it is mentioned that the raphe projection may be of significance in relating mood disorders with circadian desynchrony, specifically seasonal affective disorder (SAD) as 5-HT is a well known mediator of affective change in those with mood disorders (Reghunandanan & Reghunandanan, 2006).

1.1.2 The hierarchical multioscillatory system

Aside from potentiating circadian rhythms themselves components of the circadian clock are involved in transcription of several genes not involved with the circadian system – it is estimated that up to 10% of all mammalian genes rely on some element of these circadian factors (Duffield *et al.,* 2002). Therefore elucidating the mechanisms by which the master pacemaker orchestrates these other functions is of prime importance in understanding how uncoupling of rhythms within the organism can result in internal desynchrony. An important heuristic of the circiadan system is the manner in which biological timekeeping is regulated through a hierarchy of multioscillatory cell populations spread throughout the organism.

A number of extra SCN targets scattered across the periphery (Figure 1.3) are also known to possess rhythmic properties which are circadian in nature (Balsalobre, Damiola, & Schibler, 1998; Zylka *et al.,* 1998). It has been demonstrated that *in vivo* circumstances however that peripheral cells can only maintain 24 h oscillations for no more than a few days whereas SCN explants can maintain coherent rhythmicity for much longer (Yamazaki *et al.,* 2000). As part of the circadian system these subordinate peripheral oscillators rely on synchronising signals from the master pacemaker in order to orchestrate oscillations which are precise in phase and stable in amplitude (Pando *et al.,*

2002). Thus communication filters down the hierarchy from the master pacemaker to 'slave' oscillators the function of which is to promote global synchrony. The prevailing hypothesis of how these signals are transmitted implicates several pathways originating in the SCN for calibrating biological rhythms.

Innervation from the autonomic nervous system supports direct neural synaptic transmission pathways which project from the SCN to peripheral oscillators (Bartness, Song, & Demas, 2001; Dibner *et al.,* 2010). Endocrine axis stimulation is also an important mechanism for time signalling to peripheral oscillators as hormonal factors have been shown to express entraining properties. Glucocortical agonists for example can phase shifts circadian rhythms of peripheral oscillators (Balsalobre *et al.,* 2000) and melatonin, the circadian hormone associated with darkness, is known to be a powerful synchroniser of circadian rhythms at various levels of the circadian hieracrchy (Pévet *et al.,* 2006).

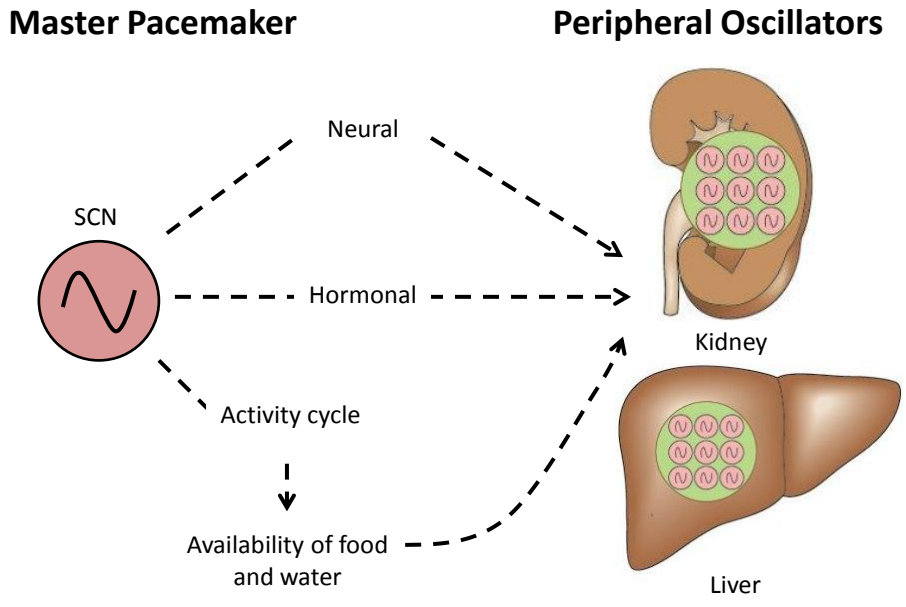


Figure 1.3 Communication between master and slave circadian clocks. Emerging evidence suggests that the SCN synchronisies biological timekeeping through neural, endocrine, and behavioural signalling. Figure adapted from Reppert and Weaver (2002).

Finally the master pacemaker can synchronise peripheral components of the system in an indirect manner by orchestrating organism sleep/wake cycle and thereby controlling exposure to external zeitgebers such as food availability which themselves entrain peripheral oscillators to appropriate physical responses in a time dependent manner (Reppert & Weaver, 2002).

1.1.3 How molecular clocks regulate circadian rhythms

Since the 1970s exploration of the circadian clock mechanisms in *Drosophila* and *Neurospora* has lead to the current molecular circadian models which are validated today (Young & Kay, 2001). Mammalian homologues of insect genes have been cloned in mice which in the contemporary literature have aided the formulation of the intracellular molecular circadian clock model we understand today as being responsible for governing circadian oscillations from a molecular to system wide level (Reppert & Weaver, 2002).

In mammals the molecular circadian clock mechanism consists of a cell-autonomous transcription-translation feedback loop (Figure 1.4) which oscillates with a periodicy of about a day (Reppert & Weaver, 2001; Ko & Takahashi, 2006). Current understanding implicates eight canonical '*clock'* genes and their protein products with the rudimentary functioning of the molecular pacemaker. These are: Circadian Locomotor Output Cycles Kaput protein (CLOCK), Brain and Muscle ARNT-like protein 1 (BMAL1), the *Period* genes (*Per 1, 2, 3*), the *Cryptochrome* genes (*Cry 1, 2*), and Casein Kinase 1 epsilon (CK1 ε). The transcriptional activator consists of a heterodimer between CLOCK and BMAL1 which translocates to the cytoplasm to bind to E-box sequences in the promoter regions of the *Per* and *Cry* genes. In turn PER and CRY translocate to the cytoplasm and heterodimerise and are phosphorylated by CK1ε leading to changes in stability and aided nuclear entry (McClung, 2007; Kurabayashi *et al.,* 2006). After translocating to the nucleus the PER-CRY complex inhibits the potentiating drive of the CLOCK-BMAL1

heterodimer, and thus *Per* and *Cry* activation, thereby forming the negative limb of the feedback loop.

In peripheral cells and forebrain regions where CLOCK is absent in the cell nucleus a homologous protein called Neuronal PAS Domain Protein 2 (NPAS2) is functionally identical to CLOCK's role and binds with BMAL1 to activate the transcription-translation feedback loop (Reick et al., 2001). Finally a second negative feedback loop which the CLOCK-BMAL1 dimer activates consists of the genes *Rev-erbα* and RAR-related orphan receptor alpha (*Rorα*) which, once translated, their protein products may positively or negatively drive *BMAL1* transcription (Sato *et al.*, 2004; Guillaumond *et al.*, 2005).

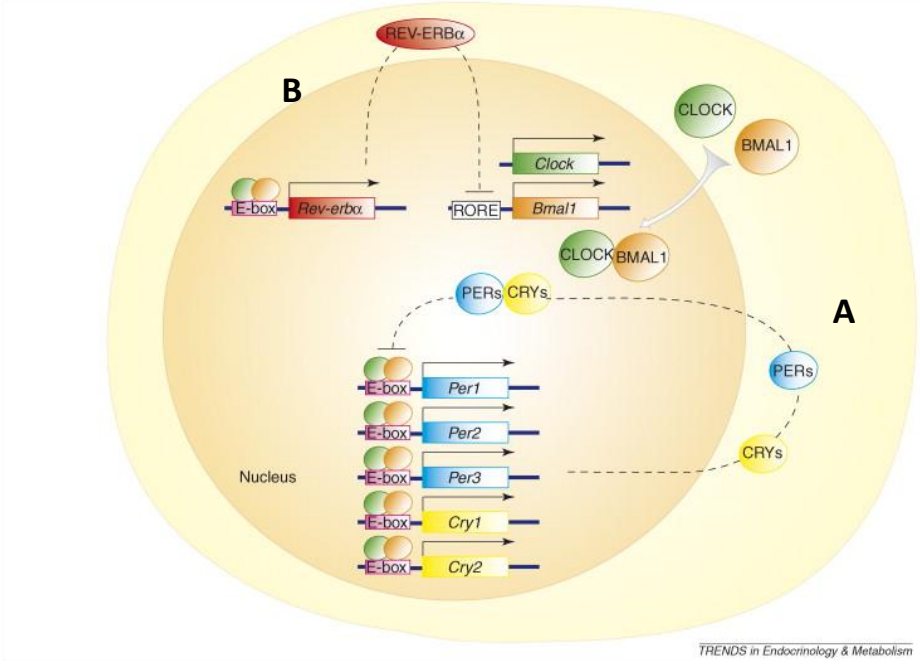


Figure 1.4 Autonomous molecular circadian feedback machinery. The above schematic represents **(A)** The action of CLOCK and BMAL1 proteins which dimerise to produce a complex which promotes the transcription of the *Per* and *Cry* families of genes. PER and CRY products in turn dimerise in the cytoplasm and inhibit CLOCK-BMAL1 thereby indirectly regulating their own transcription. This loop occurs over a period of 24 h and generates mammalian circadian rhythms **(B)** A secondary transcription mechanism exists between CLOCK-BMAL1 and the genes *Rev-erbα* and *Rorα* (not shown above), the proteins of which respectively negatively or positively drive *BMAL1* transcription. Figure adapted from Kohsaka and Bass (2007).

1.2 Shift-work and the health consequences of working around the clock

It is estimated that up to 20% of the workforce in Europe and the United States engage in some type of shift-work as part of their recurring weekly work schedules (Kanterman *et al.,* 2010). The necessity of 24 h operation is apparent across a broad range professions and occupations such as healthcare, transportation, factory production, the emergency services and telecommunications. Despite the productive and practical advantages afforded to society by operating around the clock, the detrimental consequences of working at odds against the circadian system have only begun to be reflected in the epidemiology. Over the past two decades accumulating evidence has implicated shift-work as a risk factor in the development of a number of chronic medical conditions. Studies from the epidemiological literature suggest that those who have been exposed to chronic shift-work are particularly susceptible to negative health outcomes. In many cases these studies are suggestive of a dose-response relationship between shift-work and poor health outcome – the prevailing trend being that the amount of years involved in a shift-work type regime and the degree of chronodisruption incurred the more severe the proportional impact on worker health

Studies examining worker mortality have yielded mixed results. While studies have implicated excess mortality rates in those exposed to long-term shift-work (Pati & Achari, 2007) other reports have found no remarkable differences in shift-worker and day-worker longevity (Bøggild *et al.,* 1999; Åkerstedt, Kecklund, & Johansson, 2004). Despite no unchallenged claim that shift-work adversely effects mortality, there are numerous studies which do associate shift-work with potentially fatal chronic illnesses. Among the associated risk factors are several types of cancer, cardiovascular problems, and diabetes. Studies have also revealed acute detriments to executive functioning and mental health and an increased propensity of developing sleep disorders in shift-workers (Folkard & Tucker, 2003; Drake *et al.,* 2004). The following is a brief review of such findings:

1.2.1 Chronic illness and shift-work

In 2007 the International Agency for Research on Cancer (IARC) designated shift-work as a class 2A carcinogen concluding that *'shift-work that involved circadian disruption is probably carcinogenic to humans'* based on *'sufficient'* animal evidence but *'limited evidence in humans'* (Straif *et al.,* 2007). Epidemiological findings investigating the carcinogenicity of shift-work have implicated risk of cancer among several sites with long term exposure to shift-work. Previously studies have revealed a significant association between shift-work and prostate cancer, colorectal cancer, and non-Hodgkin lymphoma. Breast cancer however remains the distinguished focus in the majority of findings (Brudnowska & Pepłońska, 2011).

Numerous studies suggest that there is a positive significant relationship between breast cancer and exposure to shift-work across various occupations including nursing, air transport services, and radio and telegraph operations (Lie, Roessink, & Kjaerhiem, 2006; Erren *et al.,* 2010; Tynes *et al.,* 1996). Schernhammer *et al.* (2006) reported that, among the 155,000 nurses recruited in the second Nurses' Health Study, those engaged in rotating shift-work for a period of ≥20 years had an almost 80% elevated risk of developing breast cancer (RR=1.79; 95% CI: 1.06-3.01). In each study confounding risk factors such as age, alcohol consumption, use of oral contraception, menarcheal age, and familial history of breast cancer were adjusted for. Among male shift-workers prostate cancer has been identified as a potential hazard related to shift-work. Kubo *et al.* (2006) first reported a significantly increased risk of prostate cancer in men working rotating-shift schedules compared to those who did not (RR=3.0; 95% CI: 1.2-7.7). Similar findings have been reported by Conlon and colleagues (2007) who observed an association between full-time rotating shift-work and an increased incidence of prostate cancer (OR=1.2, 95% CI: 1.0-1.4). The most recent study on the topic reported some

increased risk of prostate cancer in shift-workers however the increase was not statistically meaningful (Kubo *et al.*, 2011).

A number of studies have also examined increased risk of cardiovascular disease (CVD) and shift-work. A review on the topic that examined longitudinal, cohort, and case-referent studies concluded that those who engaged in shift-work had a 40% excess risk of CVD compared to those who did not (Bøggild & Knutsson, 1999). Studies examining coronary heart disease have suggested that shift-workers have an increased susceptibility to developing the disorder adjusting for other factors such as smoking, BMI, hypertension, hypercholesterolaemia, diabetes, alcohol intake, and physical activity (Ellingsen, Bener, & Gehani, 2007; Ha & Park, 2005). Furthermore it has been shown by Kawachi and colleagues (1995) that risk increases over time stating that the excess risk of the disease was 21% among women reporting less than 6 years of shift-work (RR=1.21; 95% CI: 0.92-1.59) increasing to 51% in those who engaged in more than 6 years of rotating shifts (RR=1.51; 95% CI: 1.12-2.03).

Tüchsen (1993) examined the relative risk of ischaemic heart disease related hospital admittance in a cohort of over 1,200,000 men revealing men belonging to occupational groups which involved frequent night work had a higher rate of admittance. A Japanese study on the subject revealed that compared to day workers rotating shift-workers had a significantly higher risk of death due to ischaemic heart disease (RR=2.32; 95% CI: 1.37-3.95). The relative risk for selected coronary risk factors in a multivariate model of the disease in the same study revealed that those with hypertension, high BMI, and high habitual alcohol consumption, were at increased susceptibility to the effects of rotating shift-work and risk of death (Fujino *et al.,* 2006).

Studies examining myocardial infarction (MI) among shift-workers suggest that shift-work may be a contributory factor to the illness. A Swedish study investigating risk of MI and work environment revealed that occupations with a *'continuously changing day and*

night work schedule' represented a higher risk of developing MI in a male sample of 334 cases and 882 controls (Alfredsson, Karasek, & Theorell, 1982). Another more recent Swedish study demonstrated that MI was associated with shift-work in both men (OR=1.3; 95% CI: 1.1-1.6) and women (OR=1.3; 95% CI: 0.9-1.8) in a sample of 2006 cases and 2642 controls (Knutsson *et al.,* 1999). Haupt *et al.* (2008) published findings which support the above; investigating the links between shift-work and atherosclerosis and MI the authors report that shift-work is associated with increased risk of MI adjusting for confounders such as age, sex, and pack years in smokers (HR=1.53; 95% CI: 1.06-2.22).

There is evidence also which suggests an adverse association between shift-work and risk of metabolic syndrome. Metabolic syndrome can be defined as a clustering of risk factors including central obesity, raised triglycerides, lowered high-density lipoprotein (HDL) cholesterol, raised fasting glucose, and hypertension, which are often found to occur comorbidly (Alberti, Zimmet, & Shaw, 2005). In a study by Karlsson *et al.* (2001) both men and women who worked shifts were found to have an increased risk of obesity, hypertension, and elevated triglycerides. A Japanese study on the topic implicated shift-work with hypercholesterolemia in a cohort of male workers (Dochi *et al.,* 2008). Another Japanese study found a significant effect of shift-work and risk of excess weight however found no significant difference in biological markers of metabolic syndrome versus day workers (Morikawa *et al.,* 2007). A Belgian study reported the excess risk of metabolic syndrome was 46% in shift-workers compared to those that only worked during the daytime (OR=1.46; 95% CI: 1.04-2.07; De Bacquer *et al.,* 2009). Considering the associated risk factors of metabolic syndrome previously outlined, studies have also examined the risk of diabetes among shift-workers. Nagaya *et al.* (2002) found that markers of insulin resistance were more common in shift-workers compared to day workers under the age of 50. To date only one study reports a significant difference in

prevalence of diabetes between shift-workers (2.9%) and day workers (0.9%) despite statistically elevated risk in a number of metabolic risk factors outlined above (Mikuni, Ohoshi, Hayashi, & Miyamura, 1983).

A recent study examining risk of multiple sclerosis (MS) in those that began shift-work at a young age revealed that there may be an association between the inflammatory disorder and shift-work (Hedström *et al.,* 2011). The report was based on two case-control studies examining incident cases and prevalence cases. In each study a significant association between working shifts and MS was observed. The increased risk was 60% (OR=1.6; 95% CI: 1.2-2.1) and 30% (OR=1.3; 95% CI: 1.0-1.6) for each respective study. In addition to this finding those that had worked shifts for 3 years or more before the age of 20 had a significantly increased odds ratio of developing the disorder compared to reference (OR=2.1; 95% CI: 1.3-3.4).

1.2.2 Cognitive deficits, affective change, and sleep disorders

Aside from the longer-term health consequences, there are acute neurobehavioural outcomes associated with shift-work. Diminished executive functioning and maladaptive affective changes are among the psychological detriments associated with typical shift-work regimes. Indeed several of the widely publicised work-related catastrophes such as the Chernobyl and Three-Mile-Island disasters are related to stress and fatigue arising from rotational shift-work schedules.

Folkard and Tucker (2003) reported increased risk of accidents associated with shift-work with relative risk of workplace accidents increased by 18% in the afternoon shift compared to the morning, and by 30% for the night shift compared to the morning. Furthermore the researchers reported that risk of accident increased to 36% after a period of four successive night shifts. Worryingly studies targeting safety sensitive professions specifically have revealed that those employed physicians, policemen, nurses, and pilots

that are exposed to chronic shift-lag perform significantly worse on measures of cognition and work related fatigue (Machi *et al.,* 2012; Rajaratnam *et al.,* 2011; Chang *et al.,* 2011; Cho *et al.,* 2000). Neurological imaging suggests that chronic shift-lag may also induce temporal lobe atrophy dependant on the time of recovery periods permitted by work schedules. Cho (2001) found that in a sample of female flight crew attendants exposed to chronic shift-lag for ≥5 years mean normalised right temporal lobe volume was significantly reduced in those that had a short-recovery time compared to those with a long-recovery time between shifts. The MRI data were supported by the finding that short-recovery sample had a longer mean reaction time and more incorrect responses when tested with a visual spatial cognitive task.

In addition to the cognitive effects experienced by shift-workers there is evidence to suggest that shift-lag may increase the risk of morbid affective change. Depression is one of the most common disorders found in working populations (Sanderson & Andrews, 2006). Several lines of evidence implicate recurring schedules of shift-work in one's working arrangement with symptoms of depression and anxiety. A number of studies have examined shift-work as a potential risk factor for depression. A British longitudinal study revealed that men that had worked shifts for ≥ 4 years had an increased risk of reporting anxiety (OR=2.58, 95% CI: 1.22-5.48) and depression (OR=6.08, 95% CI: 2.06-17.92; Bora & Arber, 2009). Findings from the Maastricht Cohort Study also report that men involved in shift-work are at increased risk of reporting depressed mood (OR=2.05, 95% CI: 1.52-2.77; Driesen *et al.,* 2010). An Iranian study on the topic revealed that shift-work was significantly associated with anxiety measured by the GHQ-28 among nurses that engaged in shift-work with incidence of other mental illnesses being no different from the referent population (Ardekani *et al.,* 2008).

Finally, and perhaps unsurprisingly, sleep complaints are a common concern among shift-workers. In many individuals engaged in non-conventional patterns of work,

mismatch between work roster and normally active hours on non-working days can interfere with productivity, social activity, and domestic responsibility. It is estimated up to 30% of shift-workers develop a similar collection of disturbances (Gumenyuk, Roth, & Drake, 2010) which is severe enough to enter the clinical range in about 10% of shift-workers (Drake *et al.,* 2004), in which case a distinct circadian rhythm sleep disorder known as shift-work disorder (SWD) is diagnosed.

Characterised by excessive day-time sleepiness, insomnia during normal resting phase, and sleep which is unrefreshing (Åkerstedt, 2003; Knuatch *et al.,* 1980), SWD produces a maladaptive syndrome which can put shift-workers' health and safety at risk. Obstacles to recognition exist also due to the symptoms comorbidity with a host of other physiological and psychological complaints leading SWD to be misdiagnosed (Dagan, 2002). Many of the identifiable symptoms of the disorder for example are present in previously discussed affective disorders. The mainstays of treatment for SWD focus on re-aligning the circadian drive to sleep through bright-light therapy and pharmacological treatment with melatonin (Barion & Zee, 2007). Aside from these interventional targets research has demonstrated promising preventative measures which may be undertaken to strategise which individuals are selected to work different roster types. Recently Gamble *et al.* (2011) found that chronotype, or the behavioural measure of sleep onset and offset, and different genotype variations (i.e. mutations or polymorphisms in circadian genes) are factors of interest in contributing to shift pattern adaptation. Self reported diurnal preference and genotype of single nucleotide polymorphisms (SNPs) in a number of circadian clock related genes were predictive of napping propensity and use of stimulants as a strategy for coping with shift-work. These findings may be applied in a clinical manner to provide better outcome for those affected or perhaps even be implemented in occupational environments as a preventative measure in shift-workers susceptible to sleep disorder.

1.2.3 Mechanisms behind poor health outcomes

Several theories have been put forward to account for the detrimental health consequences of shift-work. Evidence from animal models and epidemiological studies have aided in indentifying several supposed biological mechanisms which may put shift-workers at risk. Indeed the basis for the poor health outcomes experienced by shift-workers likely consists of a multitude of factors, the contributory sum of which interact to produce maladaptive changes that may precipitate illness.

To begin with, segregating a single mechanism for experimental analysis is difficult due to the increased propensity of shift-workers to engage in a number of other personal habits which themselves produce unfavourable health outcomes. It is reported for example that shift-workers are more likely to smoke compared to the general population (Puttonen, Härmä, & Hublin, 2010). Also due to mismatch between normally sociable hours and work schedule it may be difficult for shift-workers to gain access to healthy foods, which are generally available during the day, attributing to poor diet (Stewart & Wahlqvist, 1985). The former points are often coupled with a lack of physical exercise as a result of being sedentary during normal recreational hours which may further contribute to an unhealthy lifestyle (Atkinson & Davenne, 2007). These habits have all been identified in the medical literature as associated risk factors for chronic illnesses such as heart disease and cancer.

The *'light at night'* hypothesis first purported by Stevens (1987) suggests that attenuated melatonin production as a result of exposure to bright light during working hours may have a facilitating role for certain chronic illnesses in shift-workers. The hormone melatonin, which is produced in darkness, has long been characterised as a potent free radical scavenger and is known for its endogenous anti-oxidative capabilities (Reiter, Manchester, & Tan, 2010; Tan *et al.*, 2002; Tan *et al.*, 1993). Functionally reducing the generation of carcinogenic cells and stimulating the release of enzymes that

detoxify oxygen radicals, the hormone may have a protective role against a broad spectrum of malignancies. Several *in vitro* experiments using MCF-7 breast cancer cells exposed to melatonin have demonstrated the anti-proliferative and anti-metastatic properties of the hormone. (Cos *et al.,* 1996; Cos *et al.,*1998). Animal models have also supported oncostatic evidence, finding an inhibitory effect of melatonin on tumour growth in animals with intestinal and hepatocellular carcinomas (Anisimove, Popovich, & Zabezhinski, 1997; Cini, Coronnello, Mini, & Neri, 1998).

Additionally the modulatory effect of melatonin on estrogenic hormones is of potential interest to shift-worker health. It is suggested that attenuated melatonin production may disrupt the neuroendocrine reproductive axis and at a cellular level regulate estrogenic aromatisation (Cos *et al.,* 2006). As the epidemiological evidence reveals breast and prostate cancer among the highest risks associated with shift-work, and both illnesses are associated with deviations in sex hormone profiles, disrupted melatonin induced hormonal changes may be of particular relevance among the pathogenesis of these cancers in shift-workers. In addition to the reproductive function of the hormone, melatonin is known as an important immune signaller. Numerous findings have reported a reduction in endogenous melatonin production with a decrease in the production of lymphocytes and circulating immune cells such as NK cells, IL-2, IL-12, IFN-γ, and TNF-α (Blask *et al.,* 2009). Several of these immune factors demonstrate particular protective function against viruses and are involved with suppression of tumour growth. The discovery of binding sites for melatonin in immunocompetent cells further suggests an important role of the hormone in immunomodulation.

Despite a sound etiological foundation, studies examining shift-work disturbance in the biological rhythm profiles of melatonin in workers have reported contrasting results. Some studies exploring urinary excretion of 6-sulfatoxymelatonin, a primary metabolite of melatonin, in shift-workers have demonstrated lower excretions in nurses that work

nights (Marie Hansen, Helene Garde, & Hansen, 2006; Pepłońska *et al.,* 2012). Such evidence is not uncontested however as other studies report no significant differences in metabolite excretion (Dumont *et al.,* 2012) and salivary measurement of melatonin is found to yield similar results regardless of shift type (Grundy *et al.,* 2011). It is not clear therefore that attenuated melatonin production is a universal consequence of prolonged exposure to shift-work; however the potential intermediate effect of the hormone remains of relevance in some chronic illnesses and as the evidence suggests may be implicated with risk of breast cancer.

Another putative hypothesis for disease in shift-workers involves lack of sufficient exposure to sunlight. It has been suggested that shift-workers may have lower circulating vitamin D as a result of nocturnal work schedules (Fritschi *et al.,* 2010; Kimlin & Tenkate, 2007). Indeed limited sun exposure is known to have negative health outcomes involving musculoskeletal and oesteopathic disorders.

Given the recent evidence linking multiple sclerosis (MS) and shift-work the current theories regarding the etiology of vitamin D in MS are of particular mechanistic relevance for the apparent shift-work related morbidity of the disease. It is known that vitamin D is important in the immune regulation of several factors involved with MS and supplementation of the vitamin has been suggested as a potential protective therapy against the disease (Niino *et al.,* 2008). Animal models of MS have also revealed that the hormone 1, 25-dihydroxyvitamin D_3 has an ameliorative role against the disease (Hayes, Cantorna, & DeLuca, 1997). It is reasonable then to suggest then that low-sunlight conditions which may arise by working at an inappropriate circadian time can inhibit endogenous vitamin D production resulting in increased susceptibility for developing MS.

Cancer has also been implicated with a lack of the vitamin. A Czech pilot study found that incidence of serum hypovitaminosis D was significantly higher in patients diagnosed with various types of cancer such as breast, colorectal, and prostate, compared to a

healthy control group (Pazdiora *et al.,* 2011). As demonstrated in the epidemiological studies several of these cancers have been associated with shift-work. It is thought that vitamin D is involved with many cellular processes such as apoptosis, differentiation, and proliferation, disruption of which may facilitate cancer development (Kang *et al.,* 2011). Evidence that shift-workers' vitamin D levels are affected remains scarce however. To date only one study has examined the relationship between nocturnal shift-work and serum 25-hydroxyvitamin D, a circulating metabolic marker of vitamin D, finding no significant difference between work groups (Itoh *et al.,* 2011). The power of the study is limited by its small sample size however and further investigation is required before the vitamin D hypothesis of shift-work can be excluded or verified.

Despite the multitude of putative mechanisms by which shift-work may produce negative health outcomes, elucidating a definitive causal pathway and verifying it experimentally has proven elusive. The difficulty persists in part due to the previously noted fact that usually the suggested causes are present concurrently in a shift-worker sample and thereby unequivocally quantifying a single risk factor is arguably impossible. Furthermore, where physiological changes are suspected studies which report diminution in melatonin or vitamin D function in shift-workers are contested by other findings which find no effect (Dumont *et al.,* 2012). Finally, the majority of suggested hypotheses are either directly or indirectly mediated by an organisms' circadian machinery. There is an accummilating body of evidence which suggest that circadian desynchrony in and of itself likely exerts a pivotal influence on the etiology of shift-work related illnesses.

Several invertebrate studies have shown that longevity is negatively impacted by a challenging circadian period. Specifically insects maintained on a 24 h light/dark cycle lived longer than cohorts maintained on a schedule shorter in period length than their endogenous period (*tau;* Pittendrigh & Minis, 1972; von Saint Paul & Aschoff, 1978). It has been observed also in mammals that proximity of *tau* to 24 is predictive of greater

longevity among several strains of primate and laboratory mouse (Wyse *et al.,* 2010) suggesting better survival outcomes for animals in which circadian physiology more precisely adapts to planetary rotation.

Circadian desynchrony too is a relevant causal factor in the pathophysiology precursive of obesity. Numerous animal studies indicate that disruptive photoperiods result in increased body-weight and deleterious physiological changes in metabolism (Karatsoreos *et al.,* 2011; Oishi, 2009). In agreement with animal reports, in human participants the profound effect of disturbed circadian rhythms on metabolism is readily demonstrated in a chronodisruptive model which forces 28 h sleep-wake cycles (8 h sleep, 20 h wake; Scheer *et al.,* 2009). After 4 cycles this activity paradigm produces attenuated leptin rhythms, spikes in glucose and insulin levels and mismatched cortisol rhythms, all of which were concomitant with circadian misalignment by up to 12 h, additionally almost half the cohort exhibited a pre-diabetic state during the paradigm.

An intriguing hypothesis dealing with the mechanisms underlying circadian desynchrony and obesity purports that photoperiod mediated adaptation of feeding and metabolism in seasonal animals may provide insight into the maladaptive changes experienced in humans. Wyse *et al.* (2011) suggests that many of the changes in feeding habits and metabolism observed in animal studies are analogous to the arrangement of animal physiology in adaptive mechanisms such as hibernation and torpor. Such adaptive phenotypes have evolved as a survival mechanism in animals whose environment presents inhospitable climatory change of which daylight availability is a predictor. Thus is it suggested that in other mammals, for example the human shift-worker, irregular or rotating photoperiods may activate an ancient mammalian mechanism inducing conservational changes in metabolism and homeostasis, the modern manifestation of which is no longer protective but rather may contribute to the prevalence of obesity.

It is also known that interference of circadian rhythms can have a meaningful impact on animal survival when presented with acute immune challenge (references). Epidemiological data reveals a circadian trend in the severity of number chronic inflammatory conditions (Cutolo, 2012; Manfredini *et al.,* 2012). Despite a well known association between immune system and the neuroendocrine system, which itself is subjected to circadian influence, the manner in which circadian timing orchestrates immune function remains an enigma. It is known also that circadian insult through acute sleep deprivation is known to increase circulating levels of pro-inflammatory cytokines (Prather *et al.,* 2009). Interestingly at a molecular level the expression of numerous immune factors are found to be circadian in nature. Circulating T-lymphocytes CD3, CD4, and CD8, have been shown to be rhythmic expressing one to two peaks throughout the day (Lévi *et al.,* 1988). Circadian patterns are also evidenced in circulating cytokines IL-6, IL-2, IL-10, TNF-α, and GM-CSF (Sothern *et al.,* 1994; Young *et al,* 1995). Importantly IL-6 secretion was found to be most elevated during the night. Of particular interest here with respect to shift-work related disease is the effects of mismatch between behavioural cycle and the underlying cycle of immune effectors. In the long term shift-work which forces environmental desynchrony could give rise to chronic adaption of circulating immune factors which may result in impaired immune functioning. Modulators of the immune response IL-6 and TNF-α are of particular relevance here as they induce secretion of other factors responsible for host deference (Shields, 2002). Thus if environmental factors such as shift-work extinct the natural rhythms of circulating cytokines responsible for modulating systemic host defence the repercussive effect may be a less resilient defence in dealing with chronic inflammatory conditions.

1.3. Designing work schedules to facilitate the circadian system

Due to the significant operational advantages afforded by round-the-clock work schedules and the ubiquity of shift-work in modern industry, removing non-conventional working hours in the interest of circumventing associated health risks is unlikely. Instead research has focused on better roster design of shift-work schedules in an effort to reach a compromise which may prevent against deleterious impact to physical and mental health (Arendt, 2010). In the shift-work literature for example, debate has arisen regarding whether rotating or fixed-shift schedules produce better health outcomes for shift-worker health, sleep quality, and performance (Wedderburn, 1992; Folkard, 1992).

Recommendations from studies undertaken by Czeisler and colleagues suggest that fixed-shift rosters or those that are slow rotating are more favourable than rapidly rotating schedules arguing that more permanent shift schedules encourage circadian adjustment and thus minimise biological rhythm inertia (Czeisler, Moore-Ede, & Coleman, 1982). A review of 6 separate studies examining melatonin rhythms of workers on permanent night-shifts however challenges the assumption of better circadian adjustment being facilitated by such fixed rosters. The researchers reported only a small minority (<3%) of permanent night workers completely adjusted their endogenous rhythms and less than 25% of permanent night workers adjusted their rhythm in such a fashion that they would derive any benefit from adaptation (Folkard, 2008).

Studies in shift-workers which have demonstrated circadian adjustment have taken place in unusual circumstances. Research into shift-workers at the Halley Base Antarctic research station found that the majority of members who worked rotating weekly schedules of night-work (2000-0800 h) were found to align urinary aMT6 rhythms with work schedule (Midwinter & Arendt, 1991; Ross *et al.,* 1995). Similar studies involving off-shore oil rigs in the North Sea are reminiscent of the Halley Base findings indicating that schedules of night-work which persist for greater than one week without change

produce full circadian adaption, also measured by aMT6 concentrations, in the majority of cases (Gibbs *et al.,* 2002; Barnes *et al.,* 1998). Further, in shift-worker samples from an onshore Australian mining operation, time of onset of melatonin secretion changed significantly across both weeks of day and night shifts albeit the researchers noted that rhythm entrainment was not as substantial as previously described reports (Ferguson *et al.,* 2012).

The predictive efficacy of such studies outside of non-conventional work environments is somewhat limited however. In such unusual occupational environments which the aforementioned studies have explored, circadian entrainment is seen without the interplay of natural factors such as social obligations, change in photic environment during home/workplace commute, or, in the case of Antarctic studies, circadian adaption may be greater facilitated by abnormal longitudinal daylight length (Ng, Morgan, & Arendt, 2003). Thus a significant challenge arises in interpreting the effect of shift pattern independent of outside factors.

To date the majority of studies which have demonstrated circadian rhythm adaption in fixed-shift work schedules have haven place in abnormal environments however circadian entrainment in offshore shift-workers is contested. The finding that circadian rhythms entrain better in unique circumstances is contested also however by results from a study which examined submarine crews that worked 18 h days and found that melatonin rhythms began to free-run (Kelly *et al.,* 1999).

An alternative suggestion for better shift roster design proposed by other studies (e.g. Bambra, 2008) maintains the opposite to that of Czeisler favouring fast rotations in shift-work schedule (i.e. 2-3 days between pattern changes). The justification here being that if the body can no longer resynchronise to rapidly changing shift schedules the jet-lag effects of constant stress of having to resynchronise will be minimised. Additionally, in shift rosters that do rotate the consensus among the literature appears to be that forward-

shifting patterns are more beneficial to shift-workers than reverse-shifting patterns (Barton & Folkard, 1993; Knuath, 1993). The reason for this being that phase delays caused by repeatedly rotating the sleep/wake in a forward fashion are thought to be better tolerated by the circadian physiology than phase advances produced by reverse-rotating schedules (Barton & Folkard, 1993).

Despite advising against reverse rotating shift-work patterns there is little empirical evidence to support this view. Instead there is evidence to suggest that in air traffic control cohorts, which commonly work rapidly rotating shifts, the direction of shift rotation does not play a significant role on sleep quality, sleep onset, and subjective measures of affect (Cruz *et al.,* 2003). Furthermore the actigraphic results of the study indicate that both rotation groups are equally sensitive to sleep problems. The study represented the first inquiry into the role of rotational direction on subjective shift-worker outcomes and since its publication there exists a paucity of experimentally controlled investigations regarding rapidly rotating shift schedules and rotation direction.

1.4 The use of animal models to investigate circadian desynchrony

To date designing and implementing a human experimental model of shift-work which lends itself to clinical manipulation to facilitate scientific inquiry has proven difficult. In the majority of cases comparison of shift-work patterns is retrospective and precise control over work hours is largely inaccessible due to the productive interests of the services which utilise shift-work schedules. Additionally, as previously noted, repetitive shift-work cycles which have previously been investigated may be confounded by environmental factors or be experimentally underpowered (i.e. some of the studies from the Folkard review). There may also be ethical conflicts in placing an individual on a potentially deleterious schedule compared to one which might yield better outcomes for the purpose of comparison between the two groups. Therefore there may be an

opportunity to use animal models to compare differential patterns of shift-work for the purpose of translational research and achieving better roster design for workers.

Prior animal models of shift-work have been proposed to aid this investigation each with their own ethnographically different approach. One such model proposed by Murphy *et al.* (2003) involved restricting food and exercise access to the rats' inactive phase. Though the rats did entrain activity in the cohort appeared to focus around LD transitions rather than appropriating in a continuously throughout the light cycle as would be representative of shift-worker behaviour.

Another approach involves enforced locomotion in rotating activity drums to define periods of work with weekends or days off modelled by return to home cage (Salgado-Delgado *et al.,* 2008; Salgado-Delgado *et al.,* 2010). Similarly a model introduced by Leenaars *et al.*(2011) involves a variably rotating upright chamber which is sporadically rotated in such a manner which would organise wake and rest hours to rotate in non-rotating night-shifts. In such models care is necessary to prevent against enforced locomotor activity resulting in increased corticosterone concentrations which may then exert an endocrine mediated influence on neurobehavioural parameters and metabolism (Leenaars *et al.,* 2011). A significant drawback of such models however, is that on-line locomotor rhythms cannot be determined with ease due to the masking effect of forced locomotion as a means of perturbing the sleep/wake cycle. Such parameters are necessary in order to assess the underlying changes in the system.

In other animal models of shift-work repetitive phase shifting of the LD cycle has been used to represent rotating shift patterns (Bartol-Munier *et al.,* 2006; Tsai *et al.,* 2004). This method affords the advantage of actigraphic measurement important for elucidating how the system entrains. Previously authors have criticised this model arguing that this type of resembles jet-lag as a result of transmeridian travel rather than shift-work (Salgado-Delgado *et al.,* 2010). It is believed that light-dark exposure is an important

factor in facilitating circadian entrainment however and is a relevant factor in predicting how shift-workers might entrain to occupational patterns. Ultimately however the type of intervention used to model shift-work in animals is dependent on the individual aims of the experiment and the questions being asked. The following reviews important findings in the animal shift-work literature:

1.4.1 Metabolic factors and circadian desynchrony

A complex relationship exists between the circadian time-keeping mechanism and mammalian metabolism. An important finding in the animal literature suggests that the maladaptive effect of circadian insult which result in metabolic syndrome and obesity in shift-workers is also transferrable to animal models of circadian desynchrony.

To begin with many of the genes involved with metabolism and metabolic processes possess circadian components (Panda *et al.,* 2002; Kohsaka & Bass). Furthermore it has been demonstrated that that genetic disruption of circadian clock genes in rodents produces deviant changes in metabolism and body-weight. *Per2* mutant mice develop enhanced growth curves and are obese (Yang *et al.,* 2009) while *Bmal1* knockout mice have impaired glucose and triglyceride rhythms leading to increased body-weight (Lamia, Storch, & Weitz, 2008; Rudic *et al.,* 2004). Mutant *Clock* mice also display hyperphagic and obesity phenotypes (Turek *et al.,* 2005; Rudic *et al.,* 2004).

While the relationship between circadian timing and metabolism may only partially be understood several lines of evidence suggest a reciprocal relationship between the two. Peripheral clocks for example display entrainment to feeding time in a uniquely independent manner than that observed in the SCN (Damiola *et al.,* 2000; Mendoza *et al.,* 2005). Feeding behaviours can even uncouple behavioural and metabolic rhythms form the master pacemaker (Baez-Ruiz *et al.,* 2005; Stephan, 2002).This is of particular relevance for shift-workers as food intake during the normal resting phase may produce

internal desynchrony leading to detrimental consequences for human health (Salgado-Delgado, 2008).

In a rat model of shift-work Salgado-Delgado and colleagues (2008) forced animals to be active during their normal inactive phase via forced locomotion in rotating drums while the LD schedule remained unaltered. Comparison of body-weight between experimental and control groups revealed that rats forced to be active during the phase when lights were on showed significant weight gain versus rats forced to be active in their normal active phase. Moreover 'shift-worker' rats exhibited a loss of glucose rhythmicity and reversed triglyceride rhythms. Another observation of interest reported in the study was that total daily food intake remained the same between groups suggesting that circadian desynchronisation alone was sufficient to result in weight gain. More recently Salgado-Delgado *et al.* (2010) found that rats' propensity to obesity was rescued when food intake was limited to the normally active phase in shift-worker rats undergoing a similar model of forced locomotion. In addition previously perturbed metabolic rhythms were reverted to control ranges when diet was restricted to the dark phase (Salgado-Delgado, 2010).

In a model manipulating the photic zeitgeber Tsai and colleagues (2005) demonstrated that repeated 12 h light-dark phase shifts twice weekly could also hasten body-weight gain in male rats. Greater body weight was a feature which persisted even after the LD cycle was remedied.While previous animal studies point to a shift-work releated obese phenotype characterised by enhanced weight-gain and disrupted metabolic rhythms these findings are not homogenous among all experimental animals.

In the Leenaars *et al.* (2011) model exposure to non-rotating shift-patterns results in attenuated growth curves in intervention rats relative to age-related controls. Interestingly other models have found that female Brown Norway rats (Kort *et al.,* 1986) and female CD2F1 mice (Nelson & Halberg, 1986) also gain less weight in relative to controls under

shift-work paradigms suggesting possible sex differences and strain differences on metabolic sensitivity to chronodisruption.

1.4.2 Animal models of shift-work and cognitive impairment

In agreement with studies which demonstrate cognitive impairments in human shift-workers animal models of chronic circadian disruption have demonstrated acute negative outcomes on learning and memory.

Gibson *et al.* (2010) found that performance on a hippocampal memory task was compromised in a cohort of female Syrian hamsters undergoing a chronic phase advance jet-lag paradigm. The procedure involved conditioned place preference (CPP) in which control animals successfully learned the associated between context (white/black chamber) and rewarding appetitive stimulus (running wheel). Remarkably the researchers demonstrated that differences in CPP learning persisted between control and shifted animals when tested four weeks after cessation of the chronic jet-lag protocol suggesting that repeated circadian desynchrony negatively impacts learning and memory well past the point of readjustment to fixed LD cycle. The researchers also demonstrated in separate cohorts that jet-lag produced a marked suppression of hippocampal cell proliferation and neurogenesis, reducing the number of cells by approximately fifty per cent. Importantly, newly born hippocampal cells have previously been associated with training on a hippocampus-dependent learning task (Gould *et al.,* 1999; Ambrogini *et al.,* 2002; Leuner, Gould, & Shors, 2006). It is thought that stress is an important factor here as the collaborators demonstrated that controlling for HPA axis activation in adrenalectomised animals resulted in abolition of the effects on the hippocampus.

Findings from Craig and McDonald (2008) suggest that the duration of time subjected to circadian desynchrony has a meaningful effect on hippocampal learning and memory. In their study the researchers demonstrated that chronically phase shifted rats, exposed to

4 blocks of 6 consecutive 2 d and 10 d re-entrainment, showed significant impairments on both acquisition and retention in a watermaze task while acutely phase shifted rats, exposed to only one block of the shift-lag paradigm, performed at control levels. Indeed previous reports have demonstrated the amnesic effects of circadian perturbation on other learned behaviours such as fear or adverse stimuli conditioning (Tapp & Holloway, 1981; Fekete *et al.,* 1985).

More recently Loh and colleagues (2010) were able to selectively identify that recall but not acquisition was negatively impacted an acute 12 h phase shift either before or immediately after behavioural training. Furthermore the study demonstrated that the larger the phase shift incurred the more severe the impact on recall suggesting a proportional relationship between circadian misalignment and memory.

Importantly, the hippocampus, forebrain and amygdalae, components of the neural circuits responsible for governing the types of learned behaviours in the aforementioned, demonstrate autonomous circadian rhythms in gene expression (Lamont *et al.,* 2005; Reick *et al.,* 2001; Wakamatsu *et al.,* 2001). Consequently a valid mechanistic hypothesis suggests that synchrony between these oscillators and the central pacemaker may be critical in imposing a temporal structure to such cognitive faculties.

Such studies finding impairments in learning and cognition are not uncontested however. Recently Leenaars and colleagues (2012) demonstrated no difference between experimental and control rats in on an instrumental learning task. The shift-work paradigm used in the study differed from models previously used in the formerly mentioned studies in that the researchers used apparatus which forced locomotor movement rather than phase shifting the LD cycle. Interestingly on released from the protocol rats were not found to shift their locomotor rhythms to match that of the shift-work period suggesting that not entraining circadian rhythm may be beneficial in reducing cognitive failure.

Thus there is a collection of evidence which suggests that in animal studies circadian insult modelling shift-work can produce maladaptive effects on learning and memory, though the type of learning evaluated may be a relevant factor to consider in this prediction. Importantly there is evidence to suggest that when such deficits are determined they are chronic in nature as they can persist even after LD cycle is corrected.

1.4.3 Immune regulation and chronodisruption

An important consequence of chronic shift-lag on the circadian system is the effect on immune function. It is known that several immune factors are rhythmic in nature and that alterations to the normal sleep-wake cycle can affect circulating leukocytes, natural killer (NK) cells, and Ab titer function in viruses and autoimmune disorders (Renegar *et al.,* 1998; Palma *et al.,* 2006; Everson, 2005) as well as increasing expression of pro-inflammatory cytokines such as IL-6 and TNF-α (Mullington *et al.,* 2009; Vgontzas *et al.,* 2004).

Indeed several immune factors are found to be implicated in the circadian system including interleukins IL-1β, IL-2, IL-6, IL-10, GM-CSF, CCR2, TNF-α, and IFN-γ (Young *et al.,* 1995; Lundkvist *et al.,* 1998; Hayashi, Shimba, & Tezuka, 2007). In a functional manner treatment with gram negative bacterial wall component *lipopolysaccharide* (LPS) can alter circadian timing adjustment at a behavioural (Coogan & Wyse, 2008) and molecular level (Takahasi *et al.,* 2001; Okada *et al.,* 2008).

The profound influence of the circadian system on immune function is made even more complex considering findings from studies investigating molecular circadian parameters in transgenic rodents and immune reciprocity. Reports have found that IFN-γ rhythmicity is abolished in *Per2* mutant mice (Arjona & Sarkar, 2006) and cytokine activity is deficient after immune challenge (Liu *et al.,* 2006). Another study demonstrates exacerbated cytokine production after immune challenge in mice lacking

Cry1 and *Cry2* circadian genes (Hashiramoto *et al.,* 2010). Furthermore *Bmal1* KO mice develop an immune deficient syndrome characterised by coronial inflammation and attenuated lymphocyte profiles (Kondratov *et al.,* 2006).

Given the interrelated nature of the circadian system and immune function, combined with epidemiological inquires which report increased incidence of chronic illness in shift-worker populations, a unique opportunity exists to exploit animal models of circadian desynchrony to further investigate the mechanisms of shift-lag and deleterious health. In one such model Castanon-Cervantes *et al.* (2010) manipulated LD cycle in such a fashion which produced a 6 h phase advance for 4 consecutive weeks. After re-entraining to a stable LD cycle mice were challenged with LPS treatment. Mortality rate in the shifted mice was 89% compared to 21% in control mice maintained under normal 12:12 h LD conditions. In addition polysomnographic recording and corticosterone measurement removed potential confounders arising from sleep deprivation or stress demonstrating that continuous circadian insult alone was sufficient to drastically reduce survival post septic shock.

It is known from the cancer literature that in patients with certain malignancies perturbed sleep-wake patterns is a significant predictor for risk of death (Mormont *et al.,* 2000). Consequently animal models of circadian desynchrony have been used to investigate the impact of shift-lag on tumour development. Rodent research involving inoculation with MADB106, a promoter of lung cancer tumour growth (Logan *et al.,* 2012), and Glasgow osteosarcoma (Filipski *et al.,* 2004) have demonstrated that chronic shift-lag facilitates increased tumour growth compared to controls. Mechanistic dissection into promoted tumour growth by circadian disruption in the Logan *et al.* (2012) study reveals that perturbed expression of IFN-γ and NK cell factor granzyme B, and complete abolition of rhythmicity in perforin, a cytolytic protein secreted by NK cells. Also of mechanistic relevance in the Filipski *et al.* (2004) model collaborators report that in

shifted mice mPER2 and mRev-erb alpha levels were suppressed in peripheral organs and tumour sites demonstrating internal circadian desynchrony may be an important risk factor.

In a model of cardiovascular injury too repeated phase shifts have been implicated with decreases in longevity in cardiomyopathic Syrian hamsters (Penev *et al.,* 1998). The study found that repeated phase shifting reduced lifespan in the experimental cohort by 11%. This finding suggests that adequate circadian synchronisation has a measurable impact on cardiovascular disease outcomes and is particularly interesting given the high co-morbidity of cardiovascular health complaints in shift-workers who repeatedly experience rotating phase changes.

Davidson *et al.* (2006) previously demonstrated that chronic jet-lag negatively impacted C57BL/6 mice longevity. The study found that among the factors likely to impede survival were age and direction of phase shift. Younger mice tolerated shifting cycles better than aged mice and in the aged sample phase advances were more injurious compared to phase delays which is consistent with the findings suggesting shift-work tolerance declines in older cohorts (Blok & de Looze, 2011).

Though all of the studies mentioned above demonstrate that circadian misalignment produces unfavourable immune outcomes there is surprisingly little evidence demonstrating that circadian desynchrony imposes a challenge to health in absence of other opportunistic factor (age, infection etc.). A study which demonstrates this point well was conducted by Preuss and colleagues (2008) in which mice exposed to 12 h phase shifts every consecutive 5 d over a period of 3 months experienced no disruptive effects to metabolism or intestinal health. In mice treated with dextran sodium sulphate, a laboratory method of inducing colitis, however, when phase shifted in the same manner exhibited reduced body weight, abnormal intestinal histopathology, and exacerbated

inflammatory response as compared to animals in which colitis was induced but did not experience phase shift.

1.5 Objectives of current study

The primary objective of this study is to determine how mammalian circadian rhythms of locomotor behaviour entrain to light-dark cycles which resemble rotating patterns of shift-work encountered in occupations worldwide. Principally the present study uses a mouse model of shift-work to elucidate the effects of rotation direction of rapidly rotating shift-work patterns on circadian entrainment. This is achieved via manipulating advances and delays in the light-dark phase similar to many other studies animal models of jet-lag and shift-work. This is the first time however directionality of different rapidly rotating patterns are investigated.

As previous studies have demonstrated maladaptive weight changes as a result of repeated circadian phase pertubration similar in fashion to our paradigm, mouse weights were measured weekly to determine potential differences in animal growth curves.

Given the epidemiological evidence pointing to increased incidences of affective complaints among shift-workers, animals were tested on assays which measure depressive and anxiety-like symptoms after shift-work paradigms had concluded to determine changes in these domains which persist even after circadian entrainment has been rescued.

Thus far a number of animal shift-work studies have demonstrated cognitive deficits in learning and memory tasks during the acute phase of circadian perturbation. In this study the possibility of a long term deficit in learning as a result of shift direction will be investigated

Finally after behavioural testing had concluded mouse brains were processed for immunohistochemistry and suprachiasmatic nucleus expression of pro-inflammatory immune factors and clock gene proteins. Pro-inflammatory cytokines were chosen to

investigate the hypothesis that shift-work exposure may result in priming of SCN immune factors which might in turn represent maladaption in immune response and a potential contributory factor to shift-worker related illness. Circadian gene proteins were chosen to evaluate if and how the circadian system may be chronically affected by shift-schedules at a molecular level.

The objective of this thesis is to address the following hypotheses/research questions:

- **To assess if behavioural entrainment of locomotor rhythms to rotating shift-work display different characteristics dependant on (i) the direction of rotation and (ii) the speed of phase change. It is expected that the light-dark alterations assessed will produce different patterns of behaviour. The degree to which circadian parameters of circadian period length and amplitude are affected will be examined.**

- **To probe for changes in animal body-mass as a result of shift-work treatment. Our model will investigate whether the weights of animals exposed to a rapidly rotating photoperiod deviate that of control animals. If changes in body-weight are present comparison of shift-worker groups will be able to identify which patterns of rotation are most deleterious as a risk factor for obesity.**

- **Long-term depressive and anxiety-like symptoms will be assessed to explore for neurobehavioural changes persisting after normal photoperiod has been recovered.**

- **Further to the above point, after shift-work intervention has concluded and animals have been maintained once again on a fixed LD schedule animals will be assessed for cognitive deficits in novel object recognition to determine whether exposure to shift-work impairs cognition.**

- **We will examine *ex vivo* function of the suprachiasmatic nuclei of mice exposed to the shift-work paradigms. Neuroinflammatory markers will be assessed to determine if the light/dark schedules animals are exposed to weaken the circadian pacemaker. Expression of two core clock proteins PER1 and PER2 will be examined at time-points representing normal peak and nadir levels to assess if environmental manipulations can influence molecular timekeeping.**

2. Method

2.1 Animals

Forty male CD-1 mice weighing 26 – 42 g (8 – 12 wk old) at the beginning of the experiment were obtained from Harlan Laboratories (Leicestershire, UK). Animals were individually housed in polypropylene cages (29 × 13 × 12 cm) equipped with steel running wheels (11.5cm diameter). Food and water were available *ad libitum* and animals were maintained in a constant environment; ambient temperature of 21±2°C, circulating air, constant humidity of 50±10%. Mice were maintained in a 12-h light, 12-h dark cycle (lights on 0700; lights off 1900) with the exception of when experimental conditions demanded otherwise. The time at which the light switched on was defined as Zeitgeber time zero (ZT0). Manipulation of light-dark environment was achieved via an environmental isolation cabinet which allowed for complete control over exposure to photic stimuli. The interior of the cabinet was of a black and non-reflective material and the light luminance inside the cabinet was provided by standard fluorescent lighting and of low intensity (~50 lux at cage level) to avoid the development of cataracts in the albino mouse (see figure 2.1. for light meter level of each cage). All protocols were approved by the Research Ethics Committee at the National University of Ireland Maynooth (BSRESC-2011-0018) and licensed by the Department of Health and Children. All animals were treated in accordance with the Cruelty to Animals Act, 1876 and the SI No.17 – European Communities (Amendment of Cruelty to Animals Act, 1876) regulations, 1994 (European Directive 86/609/EC). All efforts were made to minimise the number of animals used in this study and any suffering or discomfort.

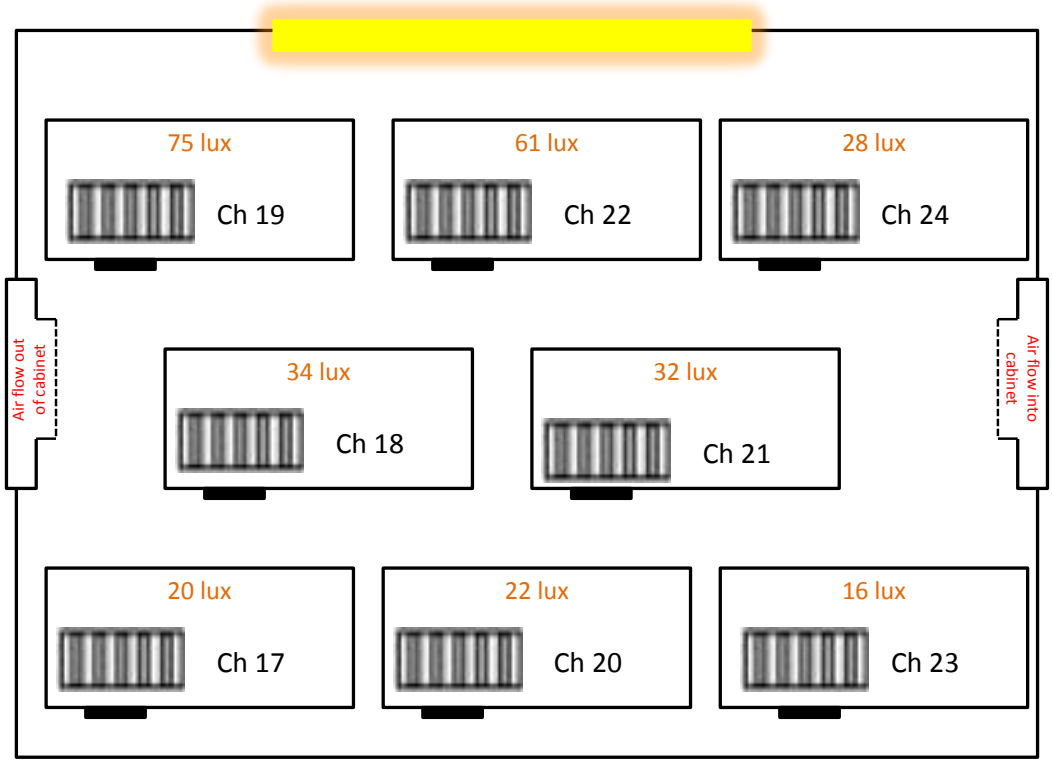


Figure 2.1. Schematic of environmental isolation cabinet. Each cage was equipped running wheel and data acquisition recording swithes. Photic input was controlled by fluorescent light source suspended from the ceiling of cabinet. Light meter recorded from animal level imposed over each cage. Flow of air was from right to left.

2.2 Experimental procedure and design

Twenty-four mice were assigned to three different experimental cohorts in this study (*n*=8 per group). The remaining sixteen animals were assigned to two control cohorts (*n*=8 per group).

The experimental conditions in this study were designed to mimic different patterns of rapidly rotating shift-work. Hence, three groups were selected to be put on shifting LD schedules which would resemble: (a) 'forward-rotating' or clockwise rotating shift-work patterns (SW-FWD); (b) 'reverse-rotating' or counterclockwise rotating shift-work patterns (SW-REV); and (c) within week alternating day and night shift-work patterns (SW-ADN).

In the SW-FWD LD protocol animals were exposed to 8 h phase delays every 2 d for six days followed by 2 d in DD to mimic *'days off'*. In the SW-REV LD protocol animals were exposed to 8 h phase advances every 2 d for six days followed by 2 d in DD. In the SW-ADN LD protocol animals were phase shifted by 12 h after 3 d on the equivalent of a day schedule, again after 6 'working' days animals were released into DD for 2 d. All experimental animals were maintained on a 12:12 h LD cycle initially before commencing the shift-work component of the study. See Figure 2.1 for timing of light changes during each shift-work protocol. After completing the respective shift-work protocols for 5 wk animals were released into DD for 2 wk to assess chronic changes in circadian factors in absence of the external light. To avoid any additional phase shifts, animals that were released into DD for 2 d after each block of work or at the end of the 5 wk protocol were done so in the animals' lights off phase.

Table 2.1. Hours of lights on during shift-work protocol

Light/dark shift-work schedule			
	'WORK HOURS'		
DAY	**SW-FWD**	**SW-REV**	**SW-ADN**
Monday	1700-0500	0700-2100	1900-0700
Tuesday	1700-0500	0700-2100	1900-0700
Wednesday	2300-1100	2300-1100	1900-0700
Thursday	2300-1100	2300-1100	0700-1900
Friday	0700-2100	1700-0500	0700-1900
Saturday	0700-2100	1700-0500	0700-1900
Sunday	DD	DD	DD
Monday	DD	DD	DD
Repeat ×5 wk			

Of the two control groups, one was maintained on standard 12:12 h LD cycle (lights on 0700) for 5 wk before being released into DD for 2 wk. These animals were later used for comparison of circadian parameters (period, amplitude etc.) in DD, weekly body-weights, and expression of immune factors in the SCN. The second control group was maintained on a standard 12:12 h LD cycle like the previous control animals; however after every 5 d on a normal LD cycle animals were exposed to 2 d in DD. Animals from this group were used for comparison on behavioural tests, weekly body-weights for SW-REV animals (i.e. animals were similar weight when beginning protocol ~ 26 g), and immunohistochemical expression of clock related proteins.

All animals were allowed to re-entrain to a standard 12:12 h LD schedule for 2 wk before behavioural testing took place. Upon concluding re-entrainment control and intervention animals were examined 5 d later on neurobehavioural assays of affective behaviour using the tail suspension test and, 5 d after that, the open field behaviour assay and an cognitive task which explored object recognition. After behavioural testing had taken place animals were euthanised and immediately after sacrifice processed for immunohistochemistry.

Controlling for any confounding effects of exercise, running wheels were made available outside of the experimental phase to both control and shifted animals. The different types of shift-work schedule are designed to be the only independent variables. The response variables investigated in this study are: observed entrainment of locomotor rhythms, change in circadian rhythm period and amplitude, changes in body weight, changes in affect and cognition, and data from immunohistochemistry.

2.3 Monitoring of behavioural rhythms

Locomotor activity (wheel running) was recorded via microswitches attached to the axis of the running wheels in the environmental cabinet. Activity was monitored

continuously using Chronobiology Kit (Stanford Software Systems, CA, USA) which digitally recorded behavioural events for later analysis. Actograms from each animal were double-plotted over 48 h to clearly represent behavioural patterns which were created by collecting the sum of activity over 5 min intervals. Daily onsets of activity were determined using a line that best fit and were recorded for the entire duration of time animals spent in environmental isolation cabinet (i.e. baseline – re-rentrainment). To determine chronic changes in animals' underlying endogenous rhythms, once shift-work protocols had concluded, photic signalling was removed from the environment and animals were released into DD. As the free-running circadian period of the mouse in DD is well characterised this allowed for comparison of plastic changes to the circadian system. To determine free-running period length and rhythm robustness in control and experimental groups, χ^2 periodograms were conducted over 7 d consecutive windows in DD using the Chronobiology Kit software.

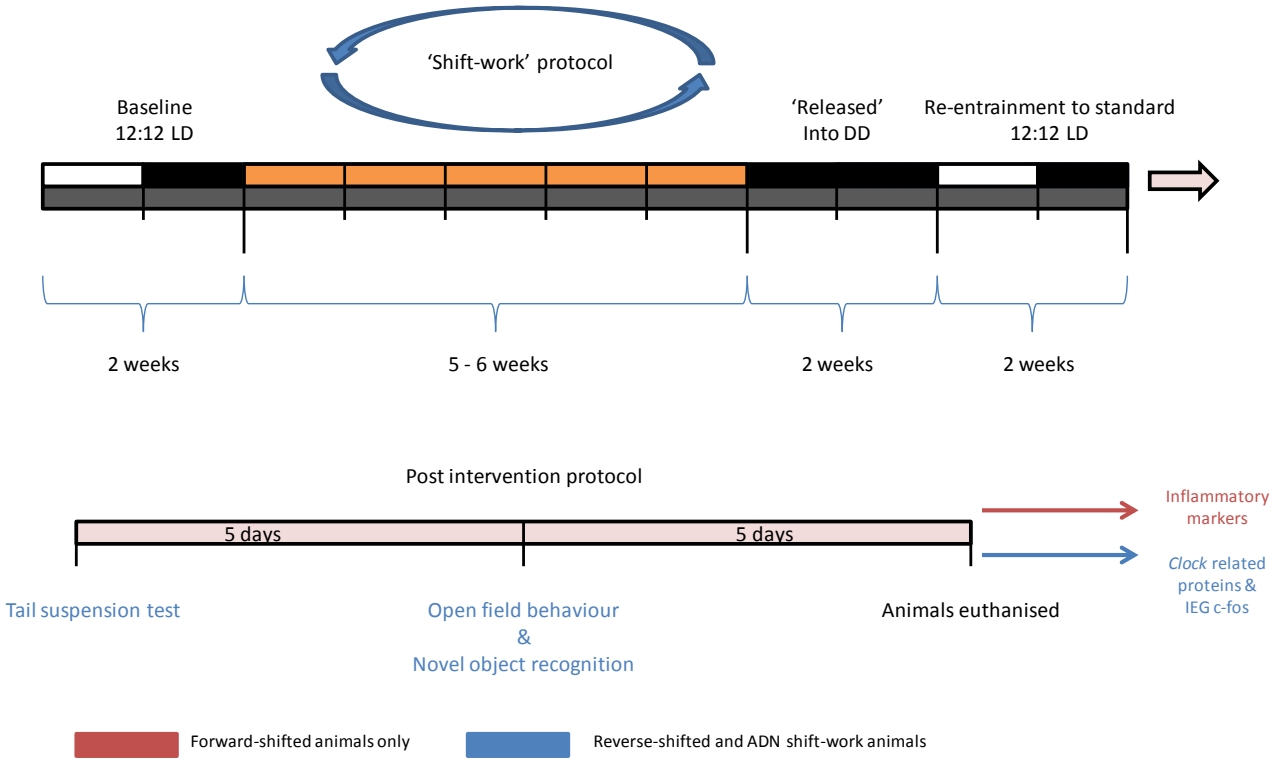


Figure 2.2. Schematic of experimental timeline of current study. Animals spend approximately 12 weeks in environmental isolation during which they first undergo stable LD entrainment before commencing one of three protocols designed to model the circadian element of rotating shift-work. Animals are released into DD to assess free running rhythms and are then re-entrained to standard LD cycle. Behavioural testing takes place after animals have concluded intervention and explore for potential chronic deficits.

2.4 Bodyweight

Mice were weighed before starting the baseline and subsequently once during each week of the working protocol until the entire time spent in the environmental isolation chamber concluded.

2.5 Tail suspension test

The tail suspension test (TST) was performed as described by Steru *et al.* (1985). Briefly, the method is based around learned helplessness and the observation that

alternating periods of mobility in mice suspended by the tail in a non-escapable circumstance is indicative of depressive-like behaviour in the rodent. Testing took place in the housing colony room to prevent changes in environment. Mice were suspended on the edge of a shelf 60 cm above floor level by adhesive tape attached ~1 cm from the tip of the animal's tail. Animals were suspended in the apparatus for a 6 min epoch and the duration of immobility was recorded during the last 240 s of the test by two independent observers on handheld start-stop timers. Mice were considered immobile only when they hung passively and completely motionless.

2.6 Open field behaviour

The open field arena was an aluminium circular area measuring 35 cm in diameter at the base of the arena (total arena area = 1017 cm²). The base of the arena was painted black to facilitate detection by recording equipment as the CD-1 mouse is an albino strain. The walls of the arena measured 15 cm and were sufficiently high enough to prevent escape from the open field (see figure 2.3 for arena dimensions). The test room was illuminated at the same intensity as the colony room. Each mouse was placed in the centre of the open field, and its behaviour was observed for 5 min. The recording equipment was connected to a camera suspended above the arena with a wide enough focus to record all locomotor activity within the open field area. The digital equipment was used to evaluate locomotor parameters such as distance travelled, velocity of each animal, and total amount of time spent mobile. To assess thigmotaxis the area was divided into two tracks defined by the *outside* area which was separated by a digitally imposed corridor which ran around the area 6 cm from the periphery, and the *inner* track which was the entire remaining area of the arena. The time spent by each animal in both corridors was recorded, in addition, locomotor factors (speed, distance etc.), the number of rearings (mouse standing on two hind paws not touching arena walls) and defecations

were recorded by two independent observers. At the end of each test the whole area of the arena was sprayed clean with an ethanol solution and wiped with a dry paper towel removing all traces of mouse droppings and urine.

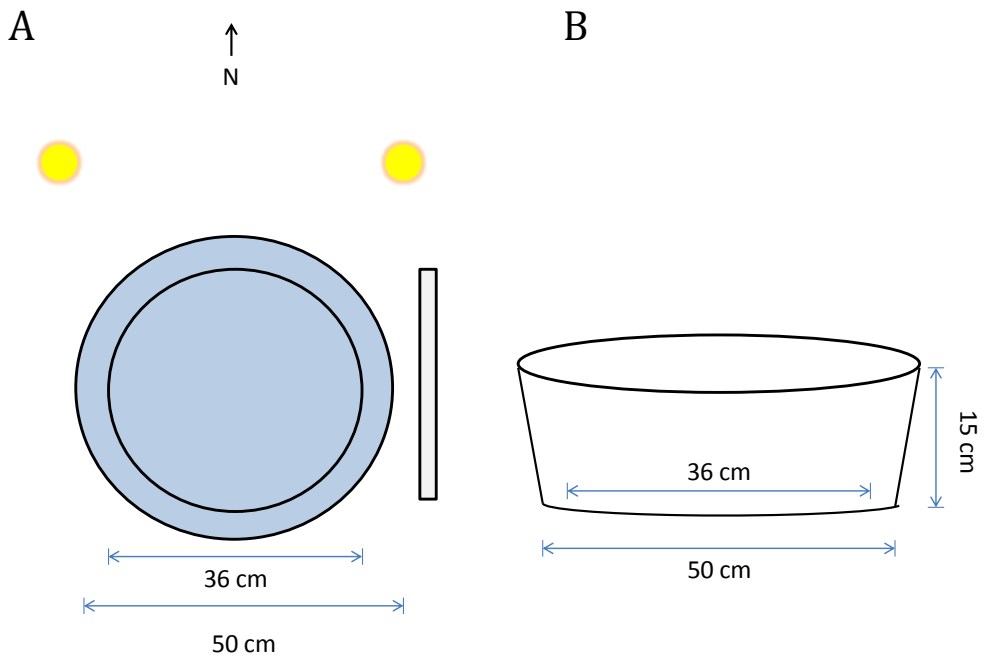


Figure 2.3. Schematic of open field behaviour apparatus. (a) Aerial view of open field arena. Two photic cues were present to the NE and NW of the arena and a visual cue card suspended to the E. (b) Dimensions of open field from a side view. Diameter of arena from top = 50 cm; diameter at base of arena = 36 cm; wall height = 15 cm.

2.7 Object exploration task

The object exploration task is a test used to assay differences in rodent exploratory behaviour and is based on the innate tendency of mice to explore moved objects for longer periods of time compared to non-moved ones. The protocol followed was similar to that published by Jürgenson, Aonurm-Helm, & Zharkovsky (2012). The protocol consisted of two experimental epochs each lasting 5 min. The first part of the test consisted of an *exploratory* trial in which animals were first exposed to the arena with two unfamiliar objects during which differences in exploratory behaviour functioned as an operational assessment of anxiety. The second trial took place 4 h after the initial trial and involved a second trial during which recognition of the object which underwent a novel spatial change measured animal cognition. The object exploration task took place in the same arena as the open field test (circular arena, diameter 36 cm, wall height 15 cm; figure 2.3). The task took place after animals had underwent the open field test. The objects chosen were a stone and a beaker lid with the latter being moved to a different point during the second trial (see figure 2.4. for a graphical representation of object placement and orientation changes between trials). All objects were sufficiently heavy that the mice did not move them during exploration. The amount of times each animal spent exploring both objects was recorded by two independent observers. Exploration was defined as the number of nose contacts or front paw touches each animal made with each object. Between trials both objects and the arena were wiped clean with an ethanol solution.

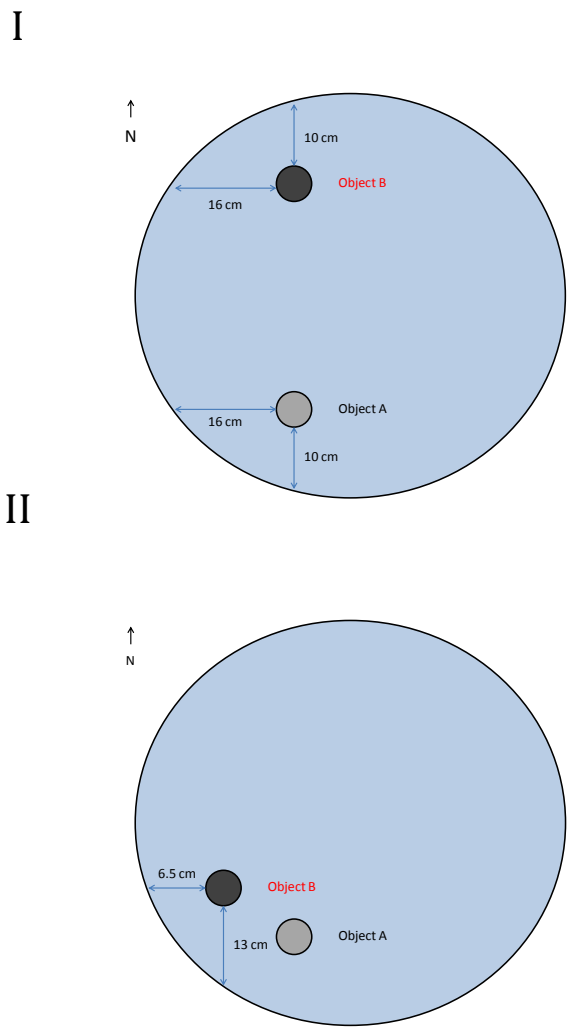


Figure 2.4. Schematic of novel object recognition task apparatus and procedure. (i) During the exploratory trial two objects, (a) a stone, and (b) a beaker lid, were placed adjacent to the arena walls at opposite locations in the arena. Exploration of each object was recorded over a trial period of 5 min. (ii) The second part of the experiment involved a trial which took place 4 h after the initial trial. Object (b) was relocated to a novel orientation in the open field which drastically differed from the original location.

2.8 Sectioning and immunohistochemistry

Animals were anaesthetised using haleothane and euthanised via cervical dislocation. Whole brains were harvested and immersion-fixed in 4% paraformaldehyde in 0.2 M PB for 48 – 72 h, before being cryoprotected in 30% sucrose. Brains were frozen on dry ice and cut serially into coronal sections (30 µm) through the rostrocaudal axis of the SCN (Bregma -0.22 to -0.82) using a freezing-stage microtome (Lecia). The location of the SCN was defined using the cortical features described in the Paxinos mouse brain stereotaxic coordinates (Paxinos & Franklin, 2004).

Free floating SCN sections were transferred to 0.1 M PB with 0.01% sodium azide to inhibit bacterial growth and prepared for immunohistochemistry. Sections were washed in 0.1 M PB and 0.1 M PBX (PB + 0.03% Triton X-100). Sections were then incubated in 0.1 M PB containing 1.5% hydrogen peroxide for 20 min, followed by another set of washes. Sections were incubated in 5% normal horse serum in 0.1 M PBX for 60 min. Sections were then incubated in primary antibody solution (primary antibody with 2% normal horse serum in 0.1 M PBX) for 36 – 48 h at a temperature of 4 ºC. Primary antibodies were against, markers of surface glial expression CD-11b (1:1000) and F4/80 (1:100), pro-inflammatory cytokines, IL-6 (1:200) and TNF-α (1:75), and clock proteins, PER1 (1:500) and PER2 (1:1000; see table 2.1 for additional antibody details). Another set of 0.1 M PB and 0.1 M PBX washes were applied and sections were incubated in secondary antibody solution (biotinylated anti-primary antibody with 2% normal horse serum in 0.1 M PBX). Another set of washes in 0.1 M PB and 0.1 M PBX was applied before sections were incubated in ABC solution for 90 min. This was followed with a set of washes in 0.1 M PB. To achieve immuno reactive (ir) staining, sections were first washed in 0.1 M sodium acetate. Substrate visualization was achieved by reacting free floating sections with a 3, 3 ’- diaminobenzidine/nickel sulphate solution (DAB; 0.02%, NiS; 0.08%) with the addition of glucose oxidase as a catalyst for reaction. All efforts were made to ensure that antigen staining took place for the different experimental groups at the same time however, where this was not practically possible, accurate comparability between sections was achieved by reacting sections in DAB for similar amount of time to ensure similar background staining density. Groups which were stained at the same time were as follows: SW-FWD and the first control group; SW-REV and the second control group; SW-ADN and additional control animals. Another set of washes in 0.1 M PB was applied before sections were mounted onto gelatine coated slides, dried overnight,

dehydrated in ethanol, and delipified in histoclear. Slides were cover slipped using Eukitt mounting medium (Sigma-Aldrich).

2.9 Quantification of immunoreactive staining

Microscopic images were captured using an Olympus BX51 microscope with an Olympus DP12 digital microscope camera interfaced with DP12-BSW imaging software. For each region of interest images were viewed at 10x magnification for identification and photography of regions. Immunostaining was quantified using ImageJ 1.43u (N.I.H., U.S.A.). In order to quantify the expression of ir particles in the SCN three representative sections of the nucleus were selected indicative of the rostral (Bregma 0.34); medial (Bregma 0.46); and caudal (Bregma 0.70) nucleus locations identified as described by Paxinos and Franklin (2004). Medial sections were furthermore compartmentalised into core and shell regions for discrimination of differences in staining (Figure 2.5).

To minimize the number of false positives for PER1 and PER2 ir particles a background optical density was established in a nearby area of the cortex with nonspecific staining and this value was subtracted from true protein particle staining observed in the SCN. In the cases where immune factors were examined in the SCN (i.e. CD-11b, F4/80, IL-6, TNF-α) the method described by (Viliplana et al., 2004) was used. Briefly this involved using the window/level feature in ImageJ to set an image threshold for ir particles after which the image was made binary and the features of interest were selected in the software. For each area of interest ir particles were measured bilaterally in the SCN and a mean value was recorded for each section. Data from rostral, caudal, and overall medial immunostain were collated under the label 'total' while medial core and shell regions were also categorised under their respective region for between location comparisons.

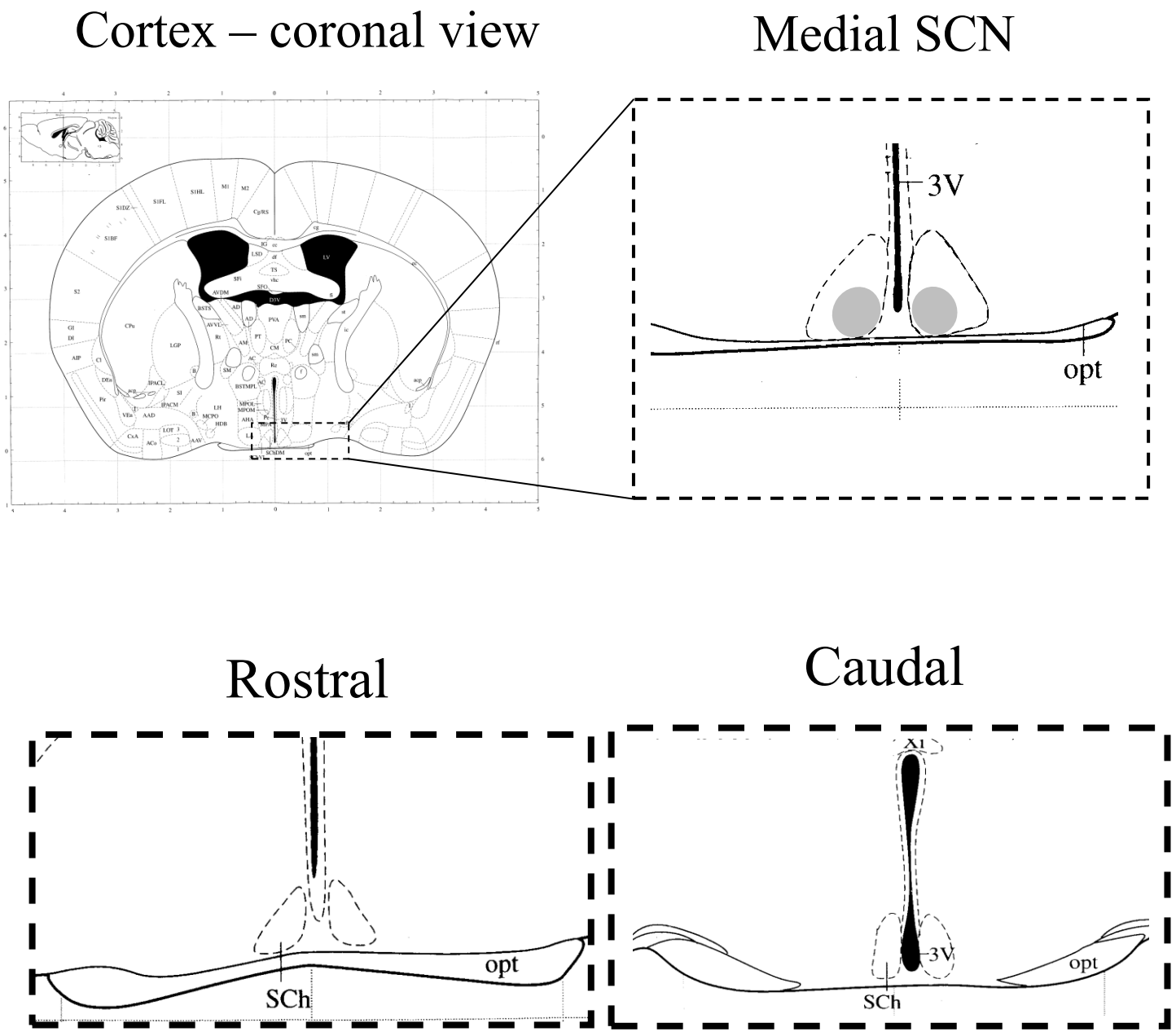


Figure 2.5 Stereotaxic coordinates used to define SCN regions. Clockwise from top left; coronal view of the cortex; schematic of medial SCN section, shaded area corresponds to core subnuclei; rostal and caudal section schematics for representation of how brain was cut through rostro-caudal axis. Pictures adapted from Paxinos and Franklin (2004).

Table 2.2. List of antibodies used in current study

	Dilution	Manufacturer	Polyclonal	Product no.
CD-11b [a]	1:1000	Serotec	Rat (anti-mouse)	MCA74GA
F4/80 [a]	1:100	Serotec	Rat (anti-mouse)	MCA497GA
IL-6 [a]	1:200	Santa Cruz	Goat	sc-1265
TNF-α [a]	1:75	Serotec	Rat (anti-mouse)	MCA2334
Per 1 [b, c]	1:500	Santa Cruz	Goat	sc-7724
Per 2 [b, c]	1:1000	Santa Cruz	Goat	sc-12429

Table 2.1. List of antibodies used in current study. Antibodies used for ir analysis of the following intervention groups: (a) SW-FWD; (b) SW-REV; and (c) SW-ADN.

2.10 Data analysis

One-way ANOVAs with Bonferroni *post-hoc* tests were used to determine differences in circadian period and rhythm between control and intervention groups. Circadian period and rhythm amplitude were derived using chi-squared periodograms in Chronobiology Kit. Body weight was analysed with an ANOVA for repeated measures for the main factor GROUP (two levels; control and intervention). ANOVAs were run separately for each control versus intervention comparison. Bonferroni *post-hoc* tests were used to make pairwise comparisons between groups. One-way ANOVA was used to determine differences in time spent immobile on TST and rearing, dropping, distance, velocity, and time spent mobile on OFB. Planned students t-tests were conducted to further explore differences limiting analysis to control and SW-ADN cohorts only for locomotor factors in OFB. Mixed between-within subjects ANOVA was used to assess habituation in the open field using GROUP as the between subjects factor and TIME-BIN as the within

subjects factor with Bonferroni *post-hoc* tests. A two-way between groups ANOVA was used to determine open field thigmotaxis with GROUP as the between subjects factor and AREA as the within subjects factor. Planned paired samples t-tests were conducted for each group to determine where animals spent most time. To assess object exploration task performance two-way between groups ANOVAs were conducted for each intervention group compared to controls with GROUP as the between subjects factor and TRIAL as the within groups variable. Two-way ANOVAs were used to determine differences in the SCN for immune factors using GROUP and REGION as factors. Two-way ANOVAs were used to assess remaining intervention groups and differences in PER1 and PER2 expression using GROUP and ZEITGEBER TIME as factors.

3. Results

3.1 Circadian rhythms under forward-shifting and reverse-shifting light/dark protocols

Observation of standard double-plotted actograms of animals belonging to SW-FWD and SW-REV shift-work protocols suggests that animals arrange locomotor rhythms in a similar fashion. Data of behavioural rhythms represented by actograms in figure 3.1 (SW-FWD) and figure 3.2 (SW-REV) and activity onset graphs (figure 3.3, SW-FWD; figure 3.4, SW-REV) can be discussed under the following headings: (I) it can be observed that initially in both groups that all animals entrain to the standard 12h:12h LD cycle; (II) it would appear that animals from each group may make an effort to entrain to environmental LD cycle changes, a trend which is better seen during the first two weeks of the time series plot; (III) Shortly after however in the latter weeks of each protocol behavioural tracking of light/dark cycle essentially capitulates and two distinct entrainment chronotypes can be observed. In both groups animals either (a) adopt a free-running rhythm period greater than 24 h in length or (b) develop a period shorter than 24 h, with the former being displayed in the majority of animals from each group (see figure 3.5 for within groups spread of this phenotype). Typically the free-running profile (a) is seen when mice are released into LL and the free-running profile (b) is observed in DD. It would appear therefore that these apparent free-running-like patterns of behaviour are indicative of mice ignoring photic changes in environment and relying on their endogenously potentiated circadian rhythms. After release into DD at the end of shift-work protocol (IV) animals from the forward-shifted group appear to retain a greater than 24 h circadian periodicy while reverse-shifted animals free-run as expected in DD or retain a period of ~24 h. (V) after manipulating LD cycle to match that of a 12h:12h cycle used during baseline animals are seen to re-entrain to a stable LD cycle with actograms suggesting that SW-FWD protocol mice may do so with a phase advance.

Control actograms – Animals maintained on a 12:12 h LD cycle

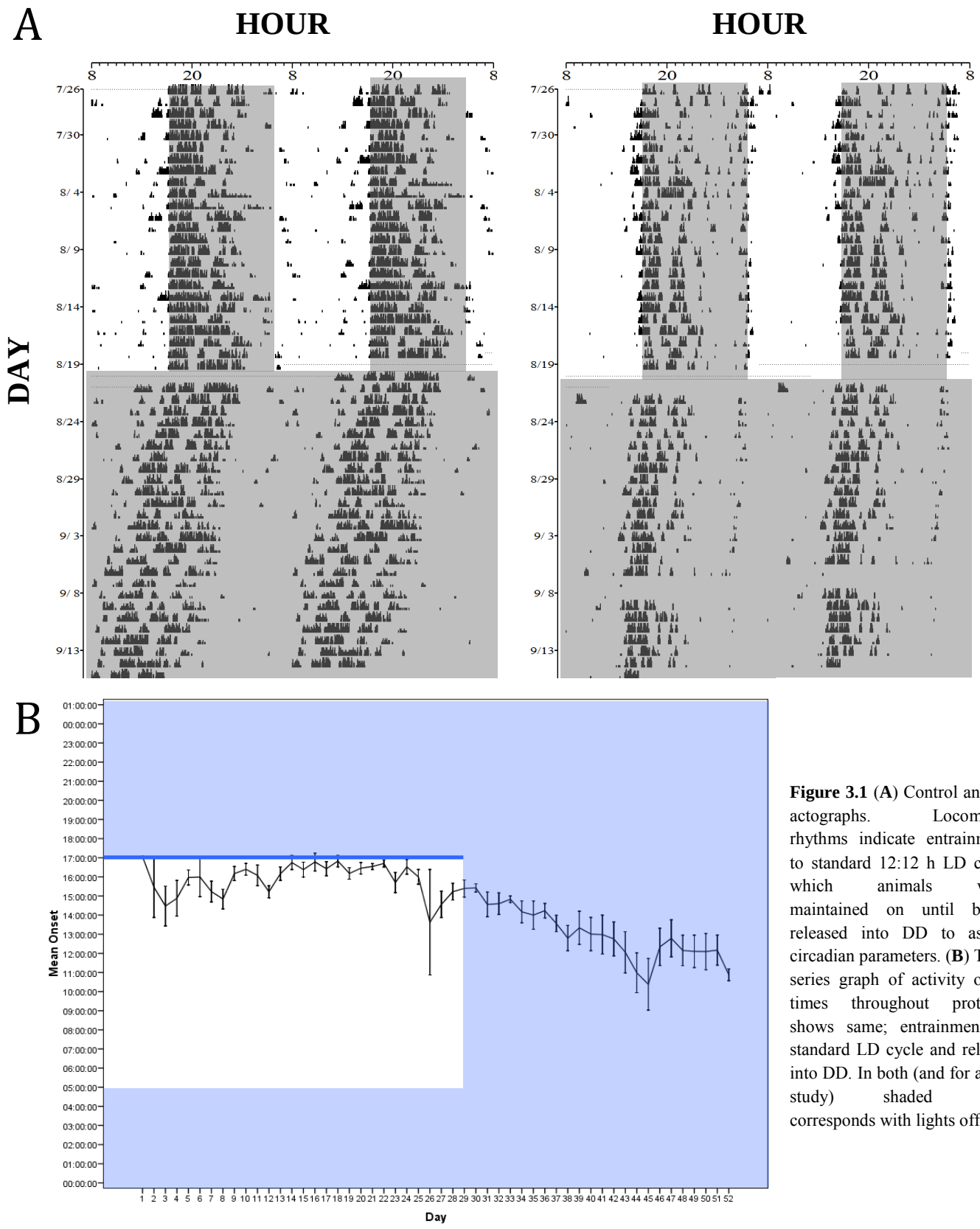


Figure 3.1 (**A**) Control animal actographs. Locomotor rhythms indicate entrainment to standard 12:12 h LD cycle which animals were maintained on until being released into DD to assess circadian parameters. (**B**) Time series graph of activity onset times throughout protocol shows same; entrainment to standard LD cycle and release into DD. In both (and for all in study) shaded area corresponds with lights off.

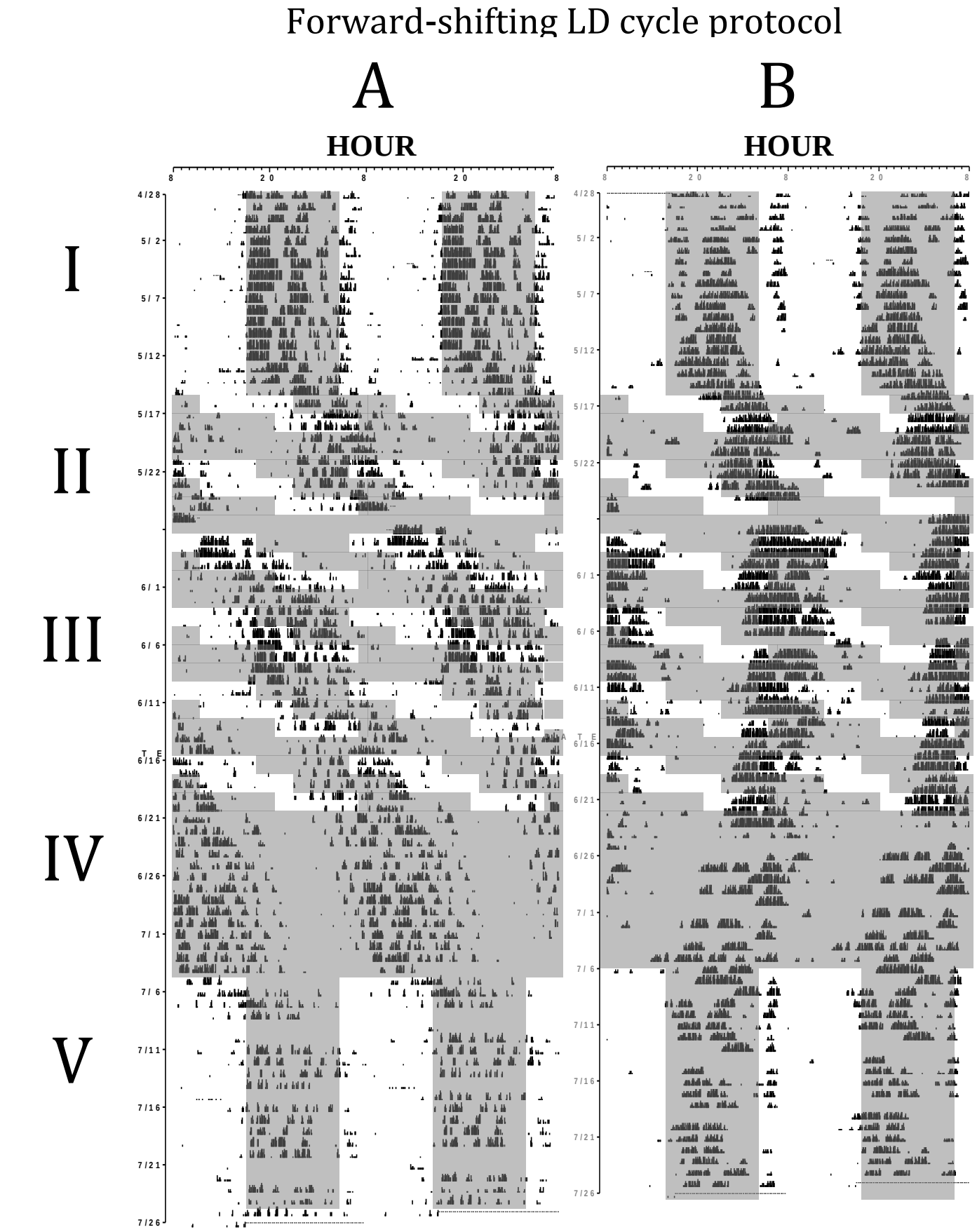


Figure 3.2 Forward-shifted animals actography. (A, B) Double-plotted actograms displaying two chronotypes in the SW-FWD intervention group. Observation suggests that animals follow a pattern of entrainment stages; (I) animals entrain to fixed 12h:12h LD cycle; (II) animals appear to briefly track the changes in environment; (III) ultimately animals capitulate and (A) begin to adopt a free running profile reminiscent of LL or (B) as in the case of one animal begin to free run as if placed in DD; (IV) animals placed in DD to assess differences in circadian period and rhythm; (v) animals entrain back to standard LD cycle after intervention has commenced

Reverse-shifting LD cycle protocol

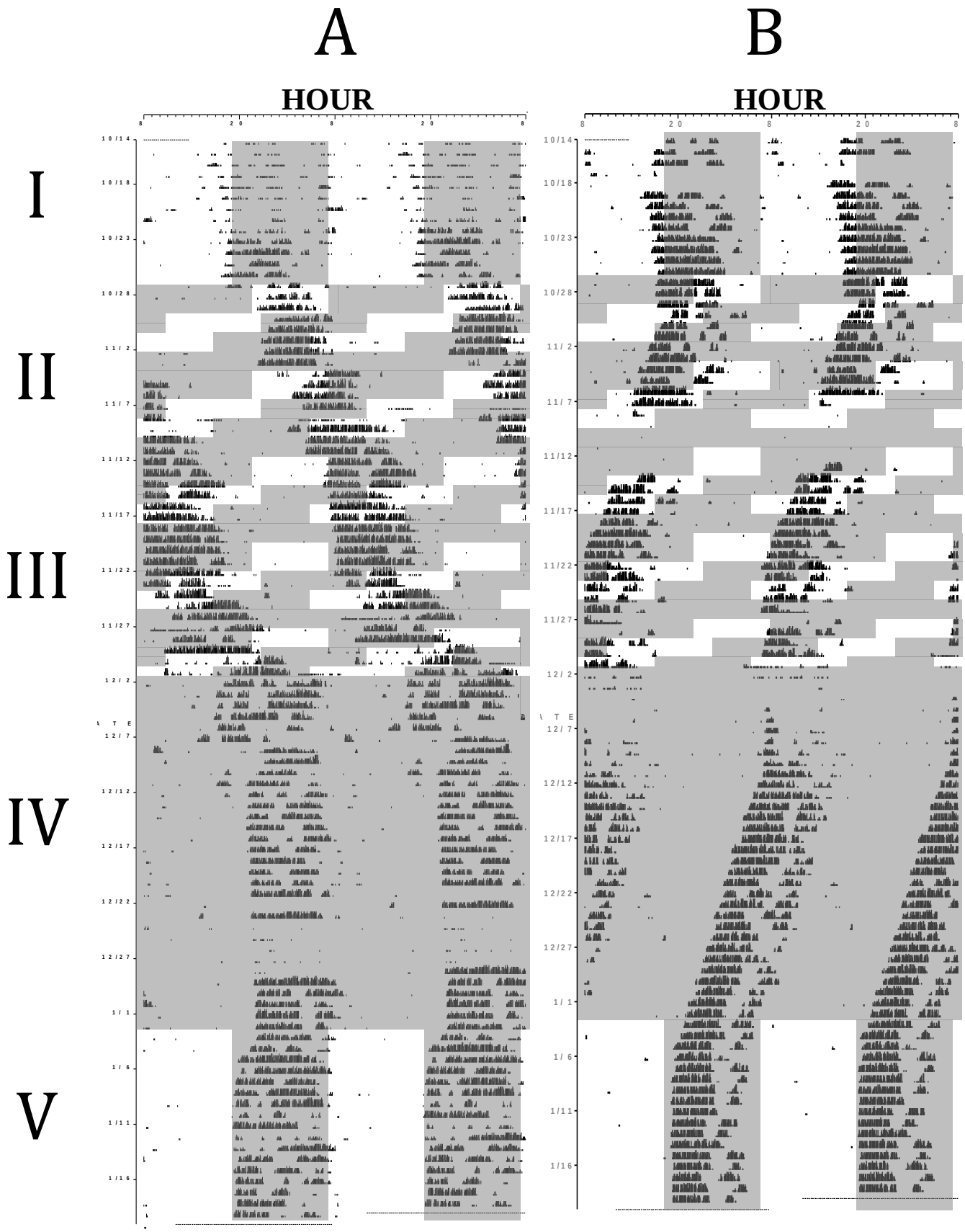


Figure 3.3 Reverse-shifted animals actography. (A, B) Double-plotted actograms displaying two chronotypes in the SW-REV intervention group. Entrainment stages may be discussed under the same headings previously described under figure 3.1. (IV) In contrast to SW-FWD animals however SW-REV animals adopt a period close to or less than 24 h when placed in DD

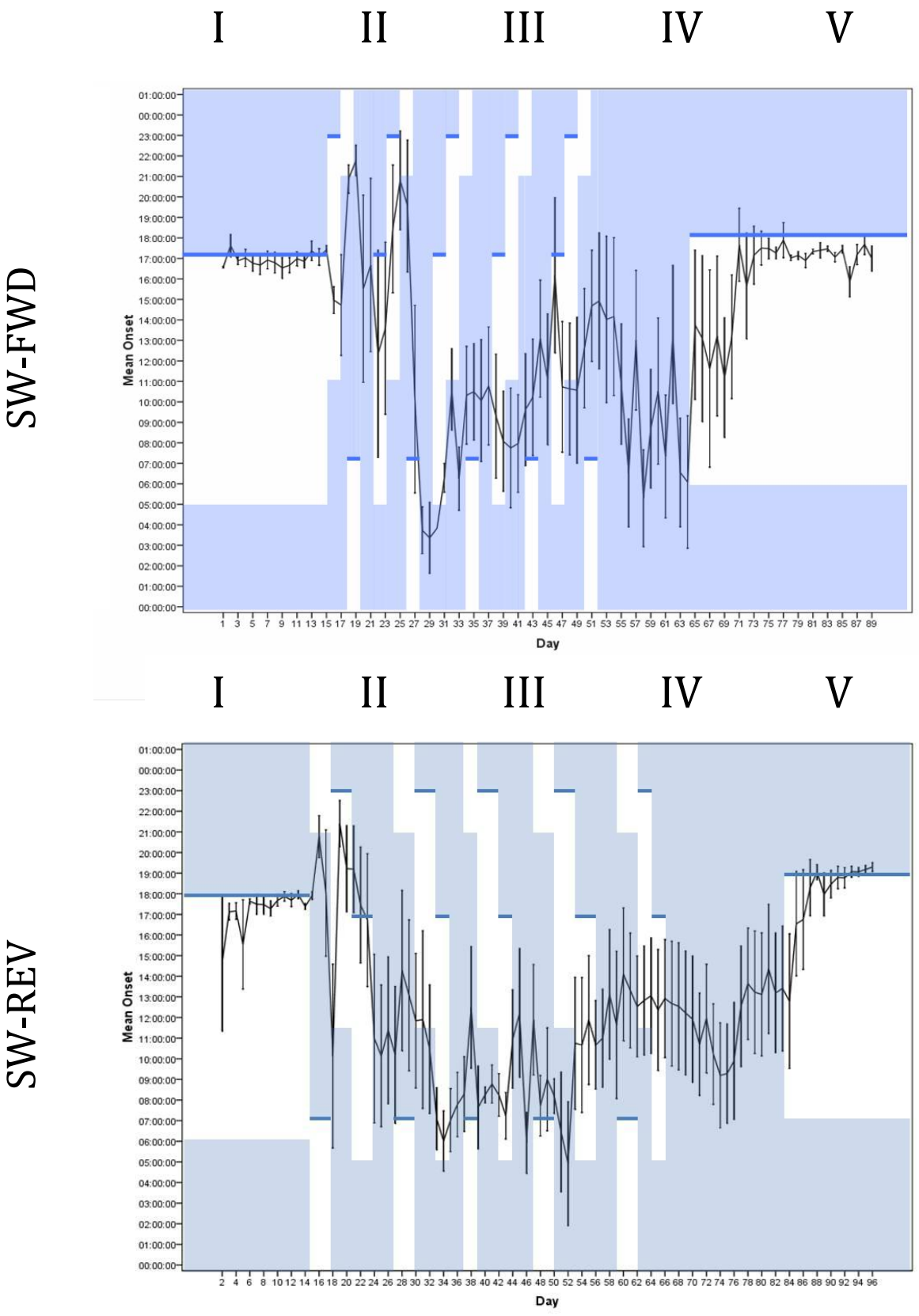


Figure 3.4 (Top) Time series graph of forward-shifted animals mean activity onset. / **Figure 3.5.** (Bottom) Time series graph of reverse-shifted animals mean activity onset. In both graphs (I) represents animals entrainment to standard 12:12 h LD; (II) brief change in locomotor rhythms matching LD changes; (III) cessation behavioural LD tracking occurs and animals begin to free-run completely ignoring photic time cues; (IV) animals released into DD; (V) animals re-introduced to stable 12:12 h LD schedules after 5 weeks on shift-work protocol and 2 weeks in DD.

Analysis of each animal's χ^2 periodogram over the 5 weeks on either the SW-FWD or SW-REV protocol reveals that in both intervention groups the majority of animals free run with a period of greater than 24 h with only two animals from each group adopting a less than or equal to 24 h period. Thus it is observed that in our animals the most common tracking cessation phenotype involves the previously described chronotype A which consists of a >24 h period that would typically resemble mouse free-running behaviour in LL conditions (see figure 3.6 below).

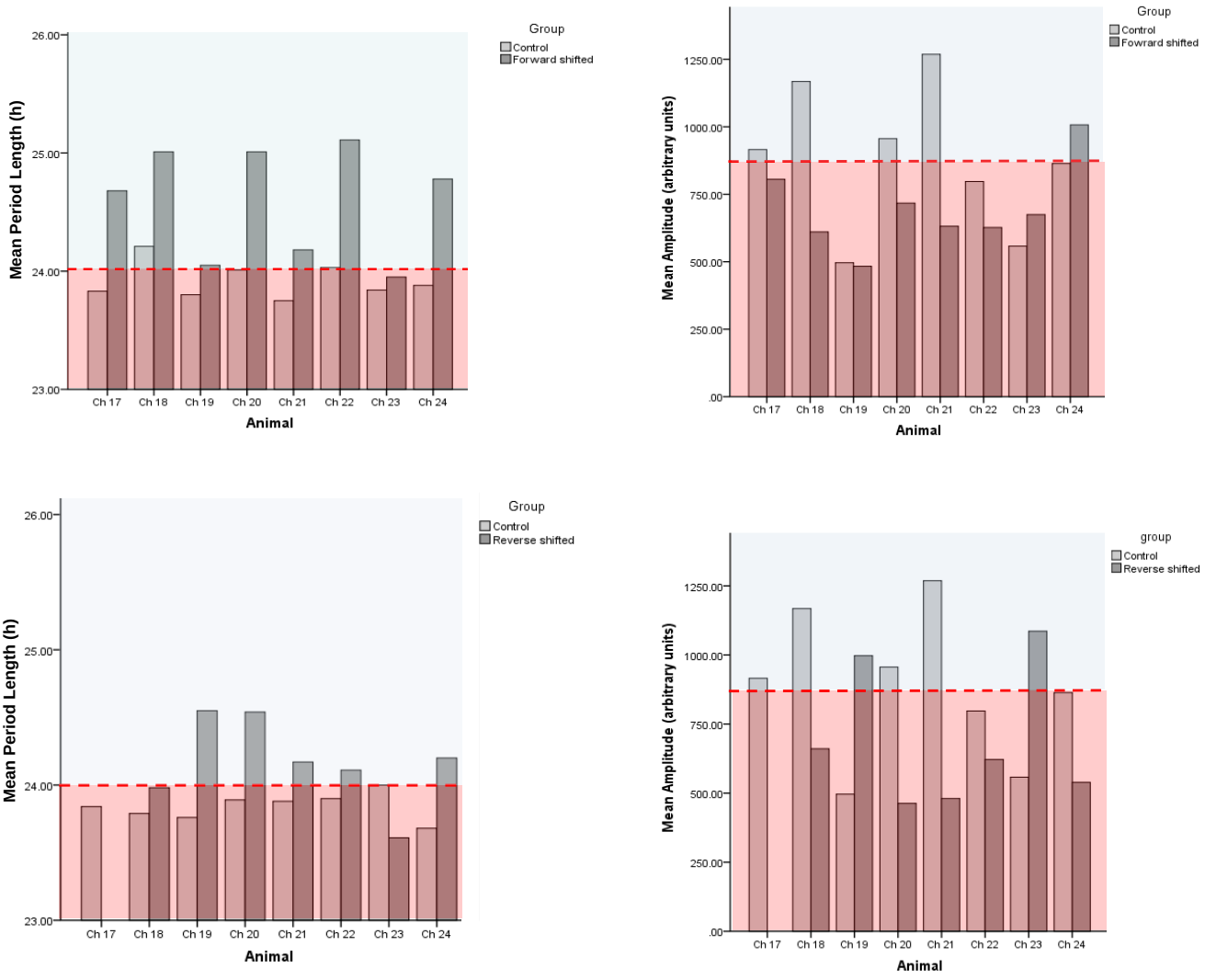


Figure 3.6 (Top) Mean period length and rhythm amplitude for SW-FWD animals. (Bottom) Mean period length and rhythm amplitude for SW-REV animals. Bars represent single animal cage during working protocol compared against control group maintained on 12:12 h LD cycle. Red line indicates period of 24 h in period length graphs and normalised control rhythm amplitude in amplitude graphs. SW-FWD and SW-FWD both appear to adopt periods of greater than 24 h during work protocol consistent with the free-running behaviour seen from actograms.

To further investigate whether the longer mean period length observed in SW-FWD animals was a function of animal chronotype the relationship between luminance levels at cage floor level in the environmental isolation cabinet and mean period length was examined using Pearson product-moment correlation coefficient. It was found that luminance level was not predictive of mean circadian rhythm period length, (r = -0.047, n = 8, p = 0.912), suggesting that the increase in period length is a mechanism of circadian phenotype in a turbulent LD schedule rather than light exposure. It was found that in the SW-FWD cohort also that luminance level was not predictive of mean circadian rhythm period length, (r = 0.383, n = 7, p = 0.396), suggesting that light exposure did not have a meaningful role in predicting free-running behaviour (figure 3.7).

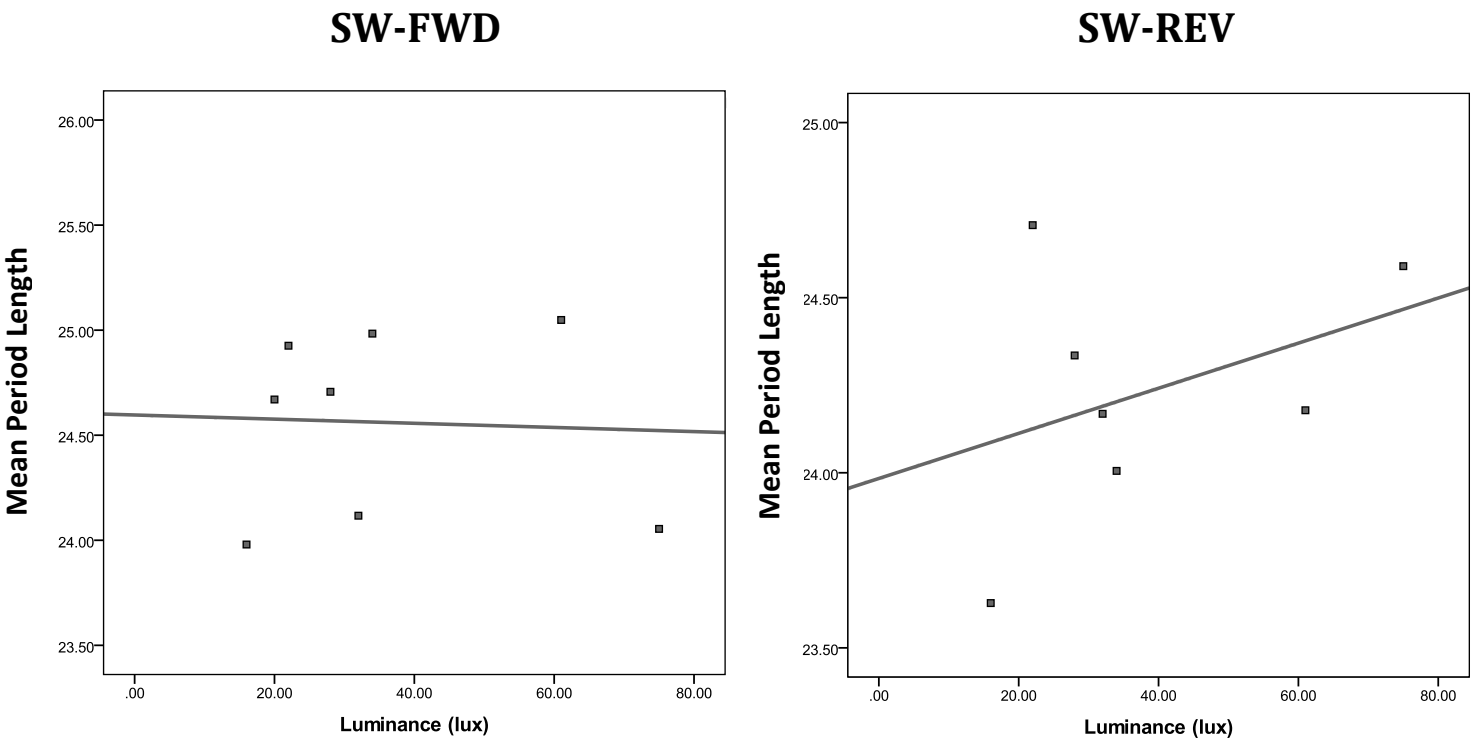


Figure 3.7 Pearson's correlation reveals no relationship between light luminance level and mean period length during shift work paradigm. SW-FWD, R^2 = 0.002; SW-REV, R^2 = 0.147.

3.2 Circadian entrainment observed under split day-night weekly schedules

Unlike the previous two shift-work protocols examined the SW-ADN animal group displays behavioural tracking of the rotating LD cycle. It can be seen from the double plotted actogram (Figure 3.8) that mice (I) entrain to a stable fixed 12-12 h LD cycle; (#) when the LD cycle is phase shifted by 12 h mice appear to delay their onset of activity to match the new LD pattern but before mice can fully entrain to the 'night-shift' component of the protocol the LD cycle is again phase shifted back to its original position for 3 d and animals revert their activity onsets similar to that seen during baseline. This pattern can be seen to persist throughout the shift-work protocol and is ubiquitous among all animals undergoing the SW-ADN schedule i.e. no individual phenotypes emerge as seen in the other shift-worker groups. (II) as animals are released into DD they adopt a free-running schedule with a period of less than 24 h as is typically expected. (III) Animals re-entrain to a fixed 12-12 h LD cycle. (Figure 3.9).

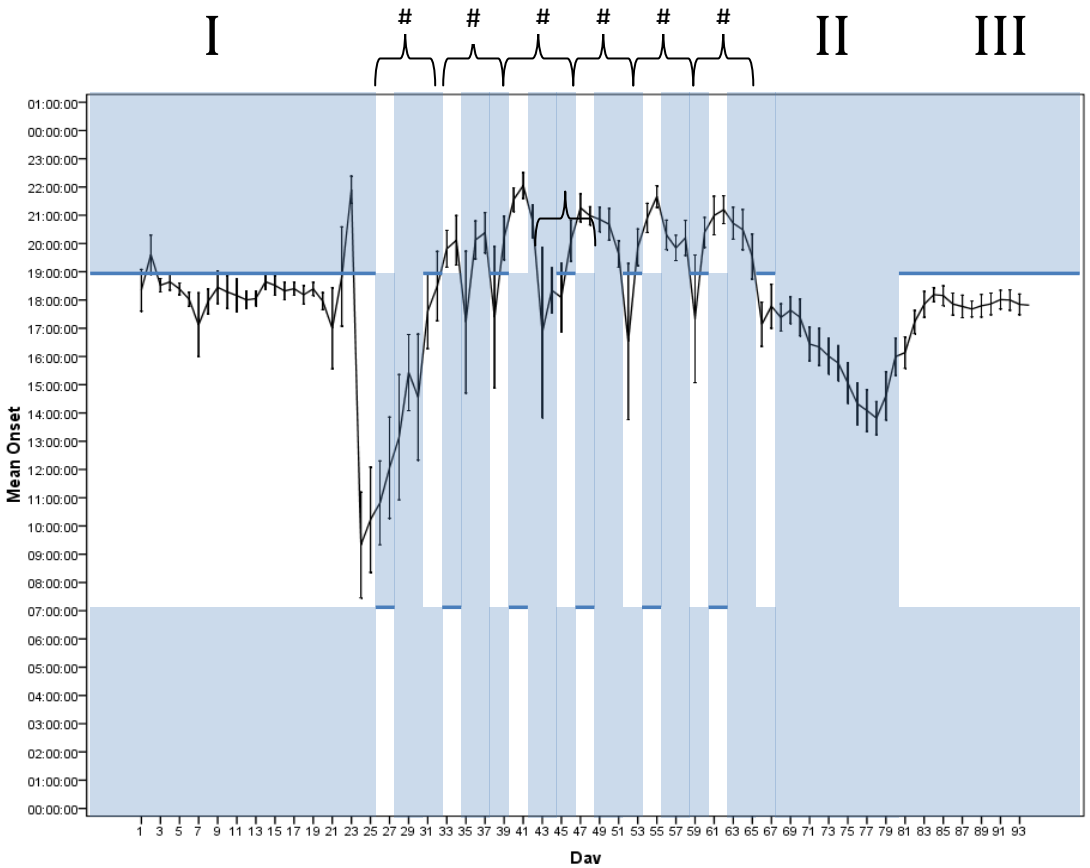


Figure 3.8 Time series plot of onset of mean activity of SW-ADN animals. Animals appear to (I) entrain to fixed LD cycle but as shift-work component of the protocol commences animals partially entrain to the phase shifted 'night-work' pattern before reverting back when the LD cycle is shifted back to baseline. (II) In DD animals free-run and (III) when presented with a stable 12:12 h LD cycle animals once again entrain.

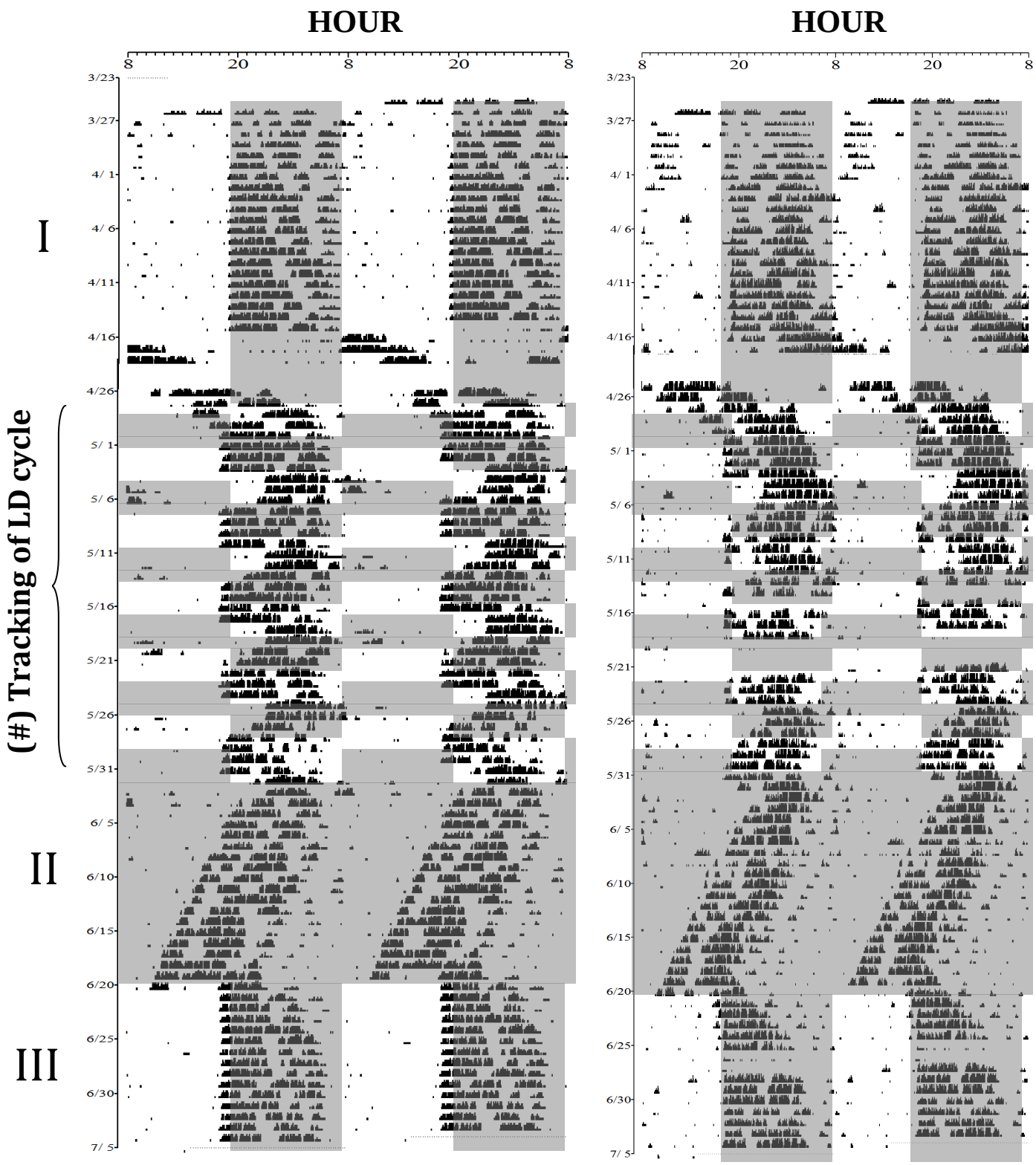


Figure 3.9 SW-ADN double-plotted actograms. Two actograms from the alternating day-night shift-work paradigm demonstrate entrainment patterns across the different stages of the protocol. (I) Animals entrain to a fixed 12:12 h LD cycle. Animals then can be seen to alter their locomotor rhythms appropriately to the phase shifted LD cycle. Activity onset in this manner corresponds to the delayed lights off phase of the day and appears to snap back accordingly when animals work their subjective day shifts. (II) when animals are released into DD they free-run as is normally anticipated. (III) when presented with a stable 12:12 h LD cycle once more animals re-entrain.

3.3 Between groups comparison of circadian factors in constant darkness

One-way between-groups ANOVAs were conducted to compare the impact of shift-work protocols on animals' free-running period and rhythm amplitude over 7 days in DD determined by χ^2 periodogram (figure 3.10). Period length in constant darkness of mice undergoing forward-shifting shift-work schedules, reverse-shifting shift-work schedules, and split day-night schedules failed to meet the assumption of homogeneity of variance (Levene = 5.285, p = 0.002) though groups were significantly different when analysed using a statistic robust to violations of homogeneity of variance; F (3, 193) = 15.886, (Welch = 26.106, p<0.001). Post-hoc comparisons using the Tukey HSD statistic revealed that SW-FWD protocol mice have a significantly longer free-running period (23.92±0.15, p<0.001) compared to controls and that SW-ADN mice have a significantly shorter free-running period (23.51±0.15, p = 0.004) compared to controls. Rhythm amplitude of animal groups released into DD similarly failed to meet the assumption of homogeneity of variance (Levene = 4.415, p = 0.005) therefore the same more robust statistic was utilised revealing significant differences between groups; F (3, 193) = 17.15, (Welch = 18.044, p<0.001). Bonferroni post-hoc comparisons revealed that SW-ADN protocol mice had a significantly greater rhythm amplitude (1015.5±191.6, p<0.001) compared to control animals.

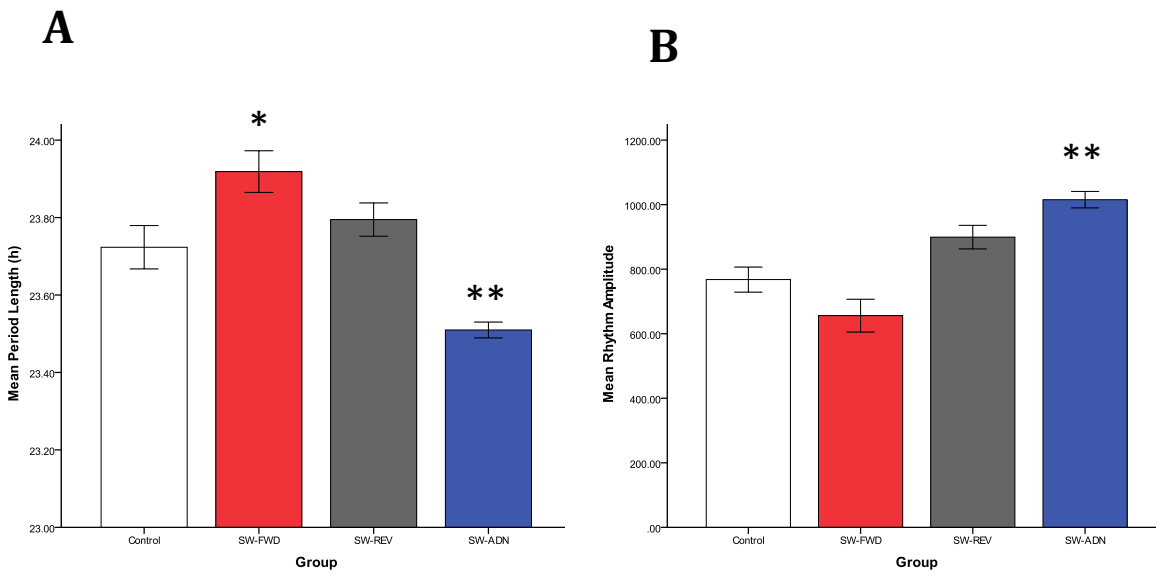


Figure 3.10 Circadian parameters in constant darkness **(A)** Mean period length between groups over 7 d in DD. **(B)** Mean rhythm amplitude between groups over 7 d in DD. Error bars represent ±SEM; * denotes p<0.05 vs control group, ** denotes p<0.001 vs control group.

3.4 Body-weight remains unaffected by rotating shift-lag schedules

A mixed factorial ANOVA was conducted to assess body-weight of SW-FWD protocol animals compared to age matched controls. Examination revealed that there was a significant main effect for time; F (6, 9) = 7.245, p = 0.005, indicating that the weight changes fluctuated in both groups over time (represented in figure 3.11 below). The group × time interaction effect was found to be statistically significant; F (6, 9) = 5.766, p = 0.01. Tukey HSD *post-hoc* pairwise comparisons revealed no differences between groups. The main effect for shift work paradigm comparing mean weights of age-matched control (control-a) and shifted animals was not found to be statistically significant, F (1, 14) = 0.755, p = 0.4. Thus no discernible differences were found between animal growth cuvres.

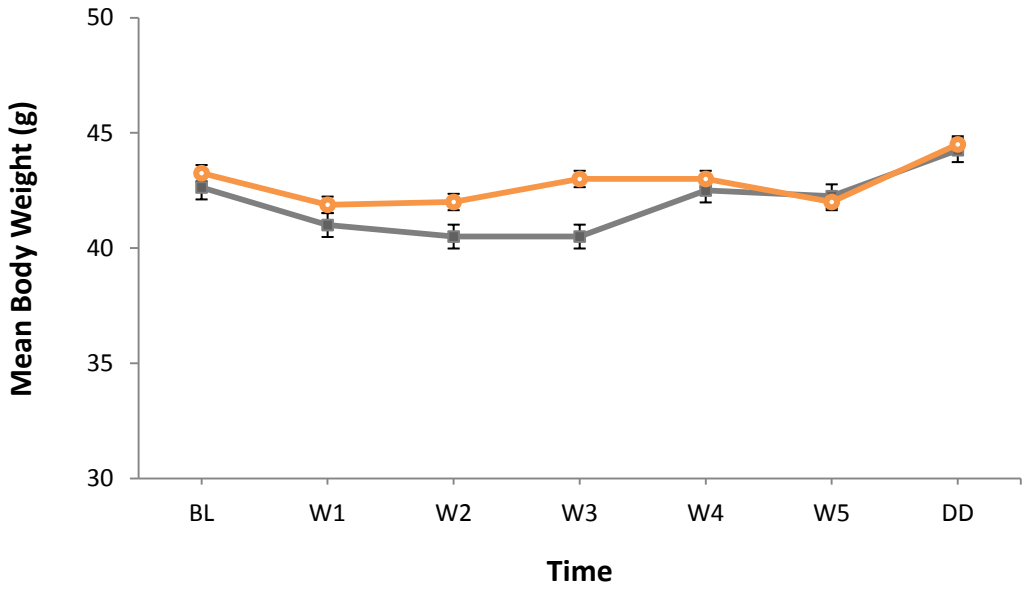


Figure 3.11 Growth curve of control and forward-shifted mice. Animal weight 40 – 45 g at beginning of experiment. Error bars represent ±SEM. Grey curve = control; orange curve = SW-FWD. * denotes $p < 0.05$.

In the SW-REV animals repeated measures ANOVA revealed a significant main effect for time; F (6, 9) = 8.670, p = 0.003. There was also a significant group × time interaction effect found between the two variables, F (6, 9) = 4.826, p = 0.018. Tukey HSD *post-hoc* pairwise

comparisons failed to find any significant differences between points however. The main effect comparing control and shifted group was not found to be significant, F (1, 14) = 1.041, p = 0.325. In this comparison the second control group was chosen as SW-REV and control-b protocol groups were the most similar in weight at the beginning of the protocol (age ~8 weeks). Growth curves represented in figure 3.12 below.

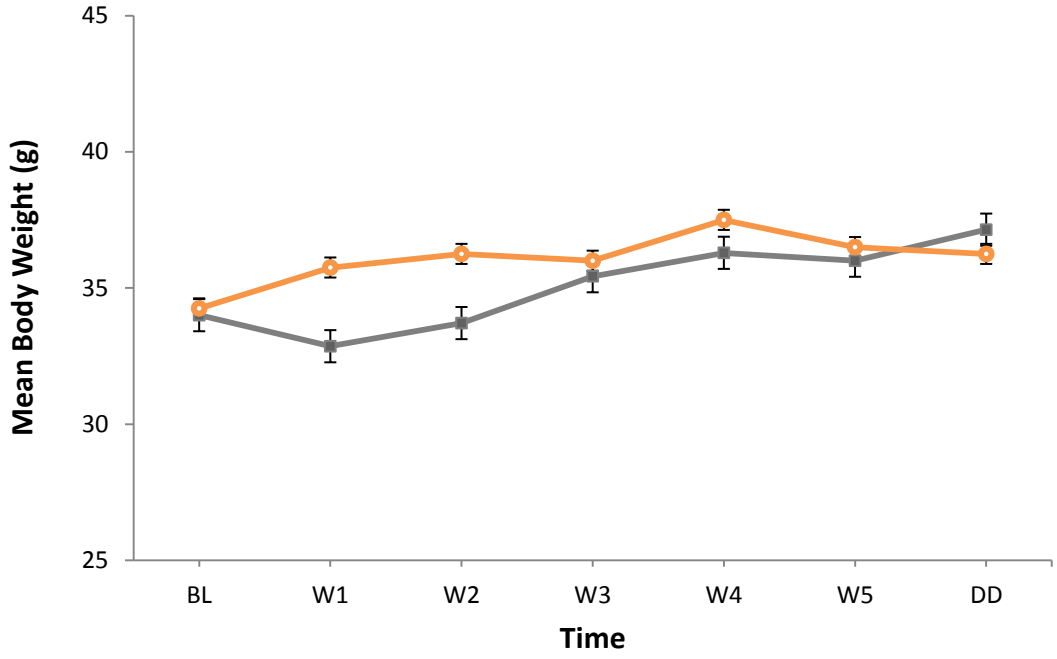


Figure 3.12 Growth curve of control and reverse-shifted mice. Animals weighed 32 – 35 g at beginning of experiment. Error bars represent ±SEM. Grey curve = control; orange curve = SW-REV. * denotes $p < 0.05$.

Mixed factorial ANONVA comparing control-a and SW-ADN animals body weight revealed a significant main effect for time; F (6, 9) = 5.653, p = 0.011 indicating that the control group grew slightly over time. There was a significant group × time interaction effect found between the two variables, F (6, 9) = 4.482, p = 0.022. Tukey HSD *post-hoc* pairwise comparisons again failed to delineate any differences between groups. The main effect comparing control and shifted

group was not found to be significant, F (1, 14) = 0.643, p = 0.436. Growth curves represented in figure 3.13 below.

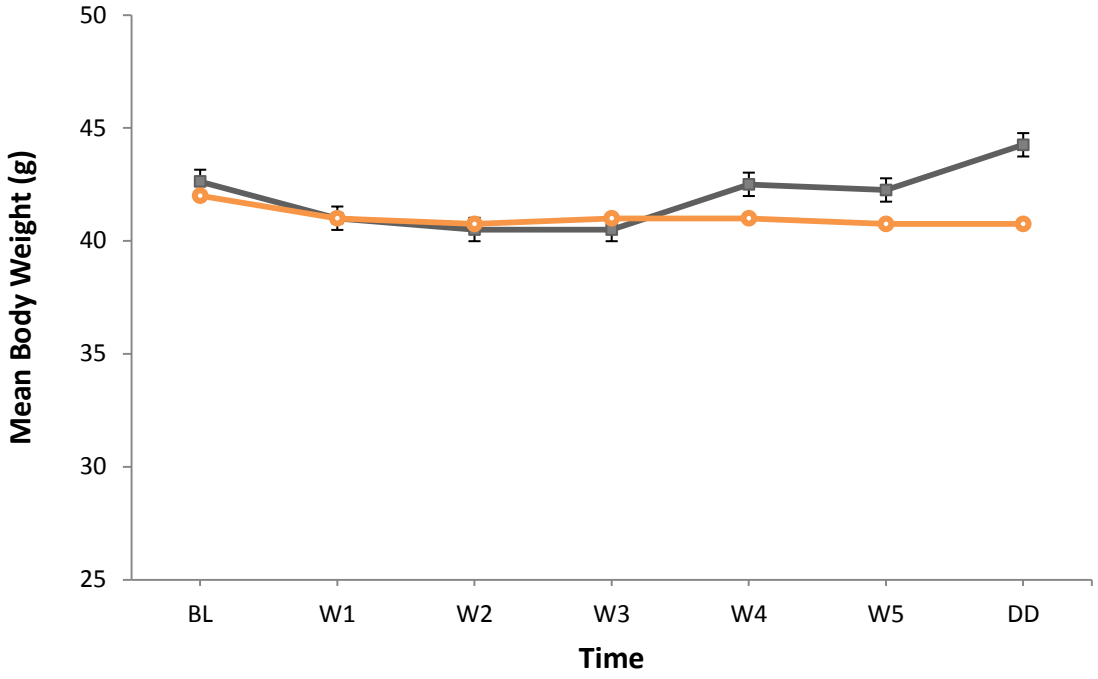


Figure 3.13 Growth curve of control and alternating day-night shifted mice. Animal weight 40 – 45 g at beginning of experiment. Error bars represent ±SEM. Grey curve = control; orange curve = SW-ADN. * denotes $p < 0.05$.

Table 3.1 Summary of animal weights throughout all protocols

Group	BL	W1	W2	W3	W4	W5	DD
Control a	42.63±2.7	41.0±2.8	40.5±3.3	40.5±1.8	42.5±2.1	42.25±2.6	44.25±2.9
Control b	34.25±2.3	32.75±1.8	34.0±1.5	35.25+1.0	35.63±2.3	36.0±2.1	37.5±1.4
SW-FWD *	43.25±1.5	41.88±1.4	42.0±2.1	43.0±1.9*	43.0±2.6	42.0±1.9	44.5±3.3
SW-REV **	34.25±4.2	35.75±2.7	36.25±3.1	36.0±3.2	37.5±2.8	36.5±2.8	36.25±3.3
SW-ADN *	42.0±2.4	41.0±2.4	40.75±2.1	41±2.4	41±3.2	40.75±2.8	40.75±2.8

Table 3.1 Summary of animal weight throughout all protocols. Animals weighed weekly, weights presented occur from second week of baseline, each successive week of 'shift-work' protocol, and the first week of being placed in constant darkness and are presented ±SD. * denotes groups compared to control-a in analysis; ** denotes group compared to control-b in analysis.

3.5 Tail suspension test results

To assay depressive-like behaviour in the mouse the tail suspension test was conducted on control and experimental groups. As the purpose of this inquiry was to assess the chronic changes in affect which might be caused by shift-work induced circadian desynchrony all experimental animals were tested 3 weeks after coming out of DD during which time they were maintained on a fixed 12:12 h LD cycle. One-way between-groups ANOVA indicated that there were no significant differences between groups; F (2, 22) = 1.53, p = 0.241. Control; (M=44, SEM=19.42), REV; (M=93.75, SEM=30.44), ADN; (94.75, SEM=15.08). Tukey *post-hoc* comparisons revealed no significant effects.

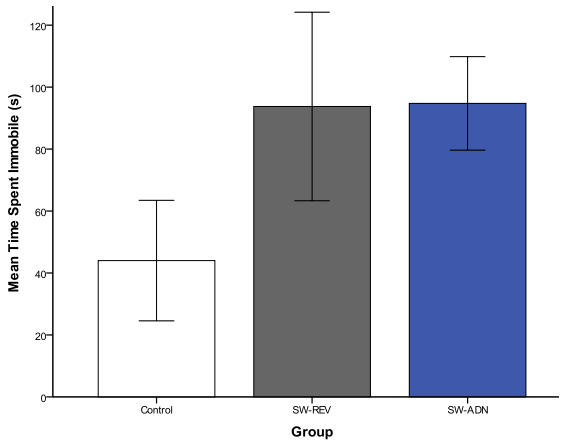


Figure 3.14 Tail suspension test in experimental and control animals. Intervention groups compared were reverse-shifting and alternating day-night patterns of rotating shift-work. No differences found between groups. Error bars represent ±SEM.

3.6 Open field behaviour results

To assess open field behaviour one-way between-groups ANOVAs were conducted to explore for differences in rearing behaviour and defecation in experimental groups (figure 3.15). Results revealed there were no significant differences found in rearing behaviour between groups; control: (M=16.71, SEM=4.68), REV: (M=6.38, SEM=3.21), ADN: (M=9.5, SEM=2.15); F (2, 22) = 2.36, p = 0.120 or the amount of droppings produced during testing; control: (M=4.43, SEM=0.87), REV: (M=2.75, SEM=0.86), ADN: (M=4.38, SEM=0.6); F (2, 22) = 1.53, p = 0.241. Tukey *post-hoc* comparisons revealed no significant effects.

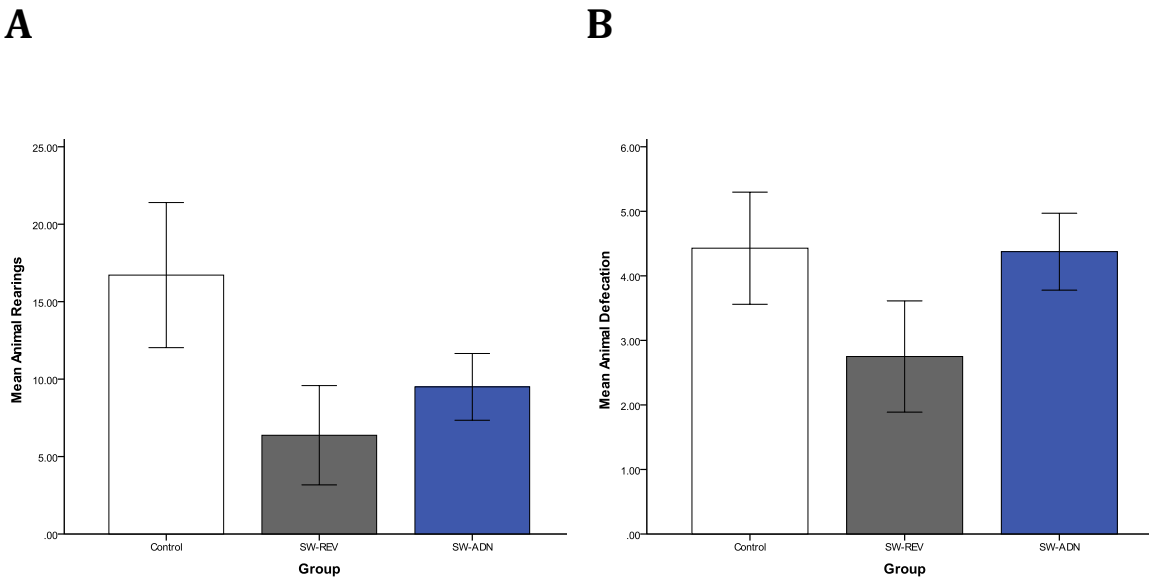


Figure 3.15 Open field behaviour in the mouse. Behavioural parameters scored were **(A)** number of times animal spent rearing and **(B)** number of droppings produced during the open field test. No significant differences were noted. Error bars represent ±SEM

Further ANOVAs were conducted for distance travelled, animal velocity, and per cent of time spent mobile (figure 3.16). In each test the SW-REV and SW-ADN protocol animals were compared to controls. Results indicate that there was no significant between groups differences found for mean distance travelled; F (2, 22) = 3.06, p = 0.071, or mean animal velocity; F (2, 22) = 2.76, p = 0.090. Examining total per cent of time spent mobile in the open field yielded a significant main effect for group; F (2, 22) = 4.24, p = 0.029. *Post-hoc* multiple comparisons using bonfferoni correction indicated no difference between groups. Planned independent samples t-tests were conducted to determine differences between control and SW-REV animals and control and SW-ADN animals. In SW-REV animals correcting for multiple comparisons there were no differences in distance travelled, t (13) = 0.77, p = 0.45, mean velocity, t (13) = 0.76, p = 0.46, or mean time spent mobile, t (13) = 0.003, p = 0.99. In the SW-ADN cohort there was a significant increase in mean distance travelled, t (13) = 2.95, p = 0.011, an increase in mean velocity, t (13) = 2.86, p = 0.013, and an increase in time spent mobile t (13) = 2.95, p = 0.011.

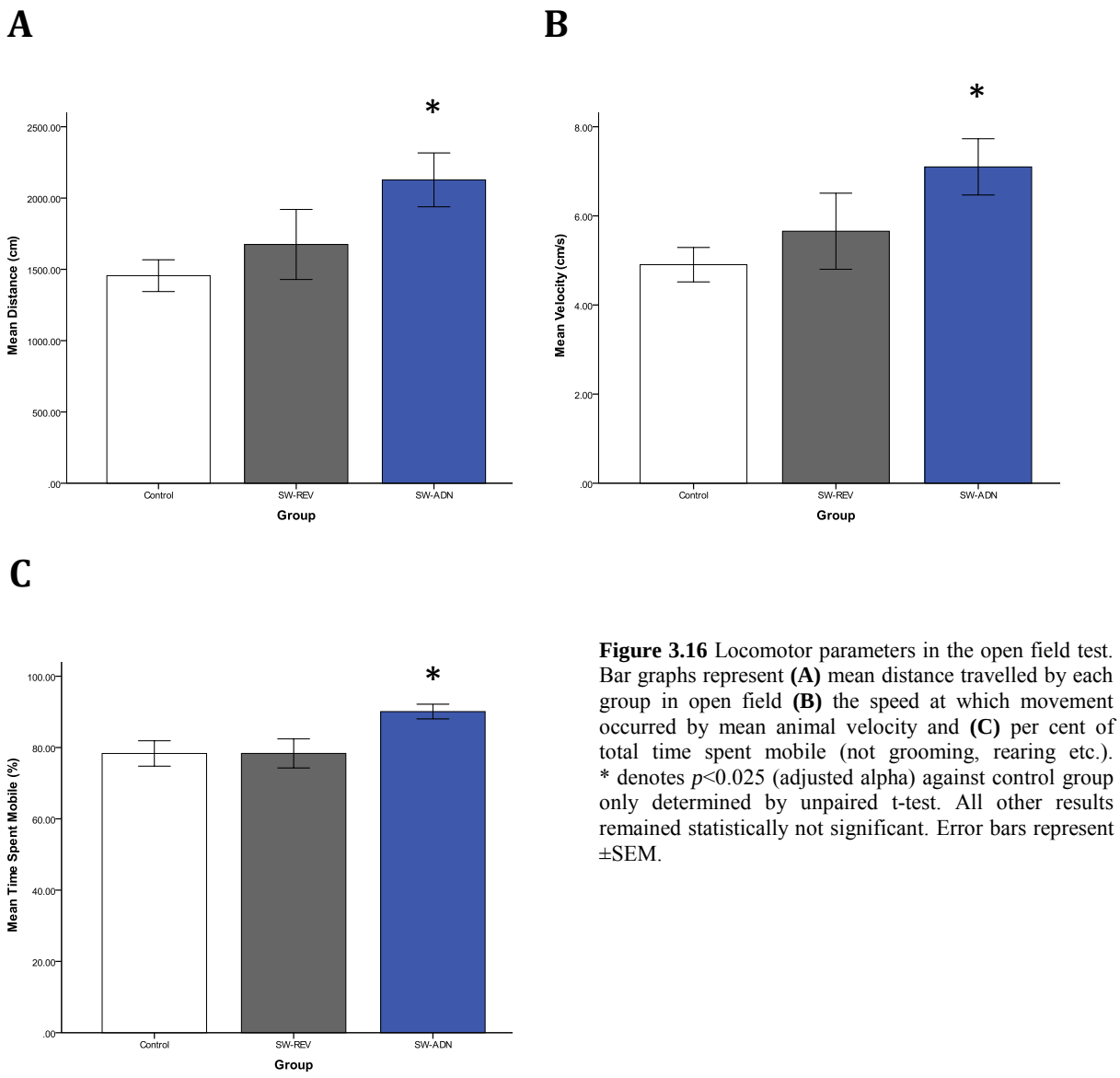


Figure 3.16 Locomotor parameters in the open field test. Bar graphs represent **(A)** mean distance travelled by each group in open field **(B)** the speed at which movement occurred by mean animal velocity and **(C)** per cent of total time spent mobile (not grooming, rearing etc.). * denotes $p<0.025$ (adjusted alpha) against control group only determined by unpaired t-test. All other results remained statistically not significant. Error bars represent ±SEM.

Under normal circumstances habituation is expected in the mouse over time after having been placed in a novel environment. To assess differences in habituation between control and experiment groups a mixed between-within subjects ANOVA was conducted with group as the between subjects variable and time as the within subjects variable (figure 3.17). The total time spent in the arena was divided into five time bins for analysis (T1: 0:00-0:59; T2: 01:00-01:59; T3: 02:00-02:59; T4: 03:00-03:59; and T5: 04:00-05:00). Results indicate that animals' total activity decreased over time as the within groups effect for time was statistically significant; F (4, 17) = 5.711, p = 0.004. There was no interaction effect found between the two variables; F (8,

34) = 0.418, p = 0.902. The main effect for group was not statistically significant; F (2, 20) = 3.025, p = 0.071. Bonferroni post-hoc comparisons revealed no differences between any groups.

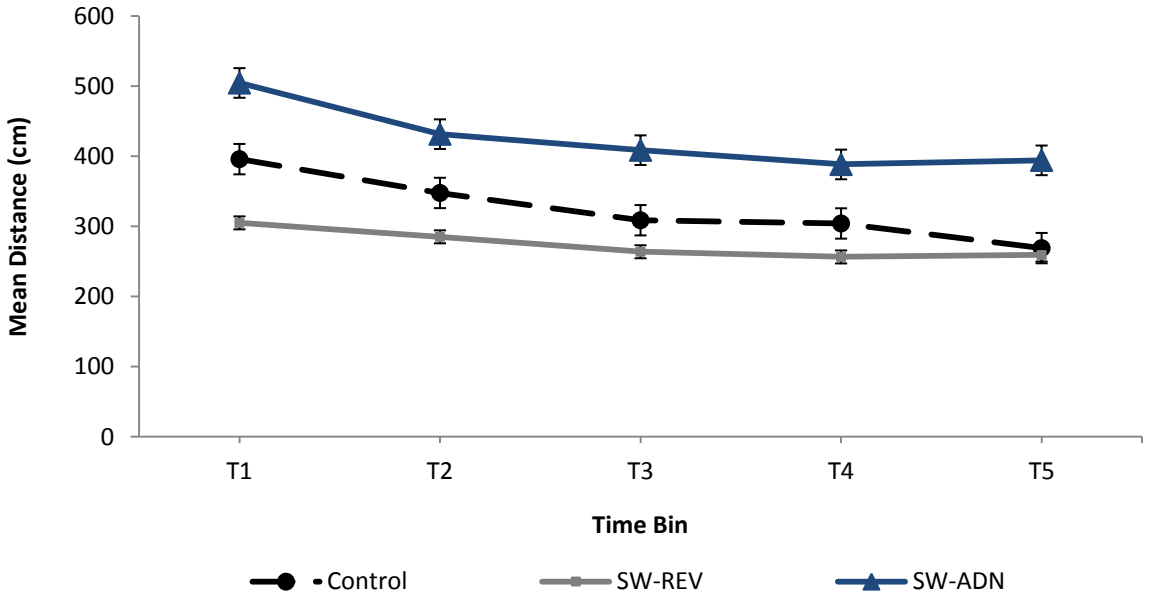


Figure 3.17 Habituation to open field. Comparison of control and experimental groups distance travelled over time reveal that all groups travelled significantly less distance as time elapsed. There were no differences between groups however indicating that all groups take a normal amount of time to habituate to the novel arena. Error bars represent ±SEM

3.7 Open field thigmotaxic preference

A two-way between groups ANOVA was conducted to assess the effect of shift-work protocol on open field thigmotaxis. The total area of the open field arena was divided into two tracks representing the inner and outer segments for analysis. The outer track was a corridor within the circumference of the open field which measured 6 cm from the periphery of the arena while the inner track was defined as the remaining area of the arena (see figure 3.18). There was an overall significant effect for area where grouped together animals demonstrated a thigmotaxic preference, F (2, 40) = 96.12, p<0.001). There was also a significant interaction effect reported between group and area, F (2, 40) = 10.42, p<0.001). The main effect for group could not be determined by ANOVA so to further investigate the interaction effect paired samples t-tests were conducted to determine the differences between control and shift-lag groups for thigmotaxis.

There was no significant difference for the control group on time spent in inside of the arena (133.69±40.27) compared to the outside (166.31±40.27), t (6) = 1.07, p = 0.325. In the intervention groups it was found that the SW-REV animals spent significantly more time in the outer corridor (213.03±23.78) compared to the inner area (86.97±23.78);), t (7) = 7.49, p< 0.001, similarly, the SW-ADN animals also spent significantly more time in the outer corridor (217.9± 36.51) compared to the inner area (82.10±36.40); t (7) = 5.28, p<0.001.

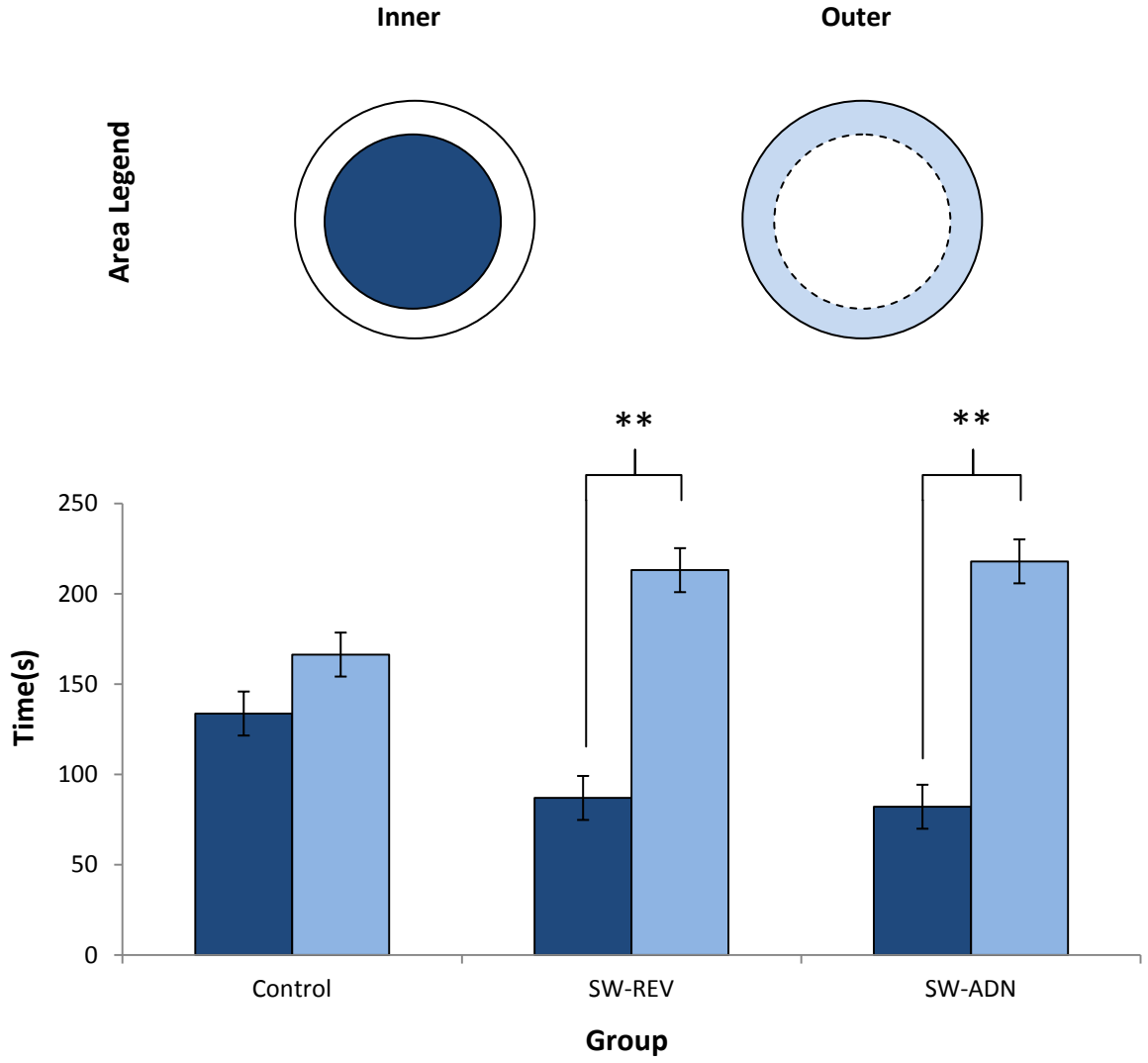


Figure 3.18 Open field thigmotaxis. Segregation of the open field arena into inner and outer corridors revealed that in both SW-REV and SW-ADN experimental groups, animals preferred the area closer to the container walls. This thigmotaxic preference was not noted in the control mice suggested shift-lagged are chronically more anxious. ** denotes p<0.001. Error bars represent ±SEM

3. 8 Object exploration task

To assess how all mice responded to the novel object recognition task a two-way repeated measures ANOVA was conducted for the factors trial and object. There was a main effect for trial found indicating that altogether animals explored objects less in the re-test trial (mean number of total object contacts in exploration trial; 9.7±0.5, compared to mean number of total object contacts in re-rest trial 6.9±0.5; mean ±SEM) F (1, 92) = 16.297, p<0.001. The main effect for object was not statistically significant; F (1, 92) = 2.812, p = 0.097, though there was a significant trial × object interaction effect reported; F (1, 92) = 4.201, p = 0.043. To further investigate this effect paired samples t-tests were conducted to compare object exploration between each trial. It was revealed that exploration of object A was significantly reduced during the re-test trial (9.9±3.9 in initial trial compared to 5.6±2.7 during re-test); t (21) = 5.579, p<0.001 (adjusted α = 0.025). There was no significant difference in object B exploration between trials; t (21) = 1.341, p = 0.194 indicating that animals explored the non-moved object less during the re-test trial but not the moved object (figure 3.19).

To compare groups individually for differences in the object recognition task separate two-way between groups ANOVAs were conducting using the same factors as previously mentioned. Paired-samples t-tests were used post-hoc to delineate where effects lay.

In the control group there was a significant main effect for trial; F (1, 28) = 9.630, p<0.01. There was no significant main effect for object; F (1, 28) = 3.797, p = 0.063, and no significant trial × object interaction effect; F (1, 28) = 0.880, p = 0.357. Paired-samples t-tests revealed that animals explored the stationary object significantly less during re-test; t (6) = 3.742, p = 0.01, but no significant difference was found for the moved object; t (6) = 1.81, p = 0.12 (adjusted α = 0.025) (figure 3.20).

In the SW-REV group there was a significant main effect for trial; F (1, 32) = 4.498, p = 0.043. There was a significant main effect for object; F (1, 32) = 7.692, p = 0.01, and a significant trial × object interaction effect; F (1, 32) = 11.738, p = 0.002. Paired-samples t-tests revealed that,

similar to the control group, animals explored the stationary object significantly less during re-test; t (7) = 5.060, p <0.01, but no significant difference was found for the moved object; t (7) = 1.038, p = 0.334 (adjusted α = 0.025) (figure 3.20). In the SW-ADN animals there was a significant main effect for trial; F (1, 32) = 4.837, p = 0.036. There was no significant main effect for object; F (1, 32) = 0.387, p = 0.539, and no significant trial × object interaction effect; F (1, 32) = 0.622, p = 0.437. Paired-samples t-tests revealed that, in contrast to previous groups, there were no between trial differences in exploratory behaviour towards the stationary object; t (7) = 0.985, p = 0.358, nor towards the moved object; t (7) = 1.889, p = 0.101 (adjusted α = 0.025), indicating that animals could not discriminate between objects which were moved and those that remained stationary (figure 3.20).

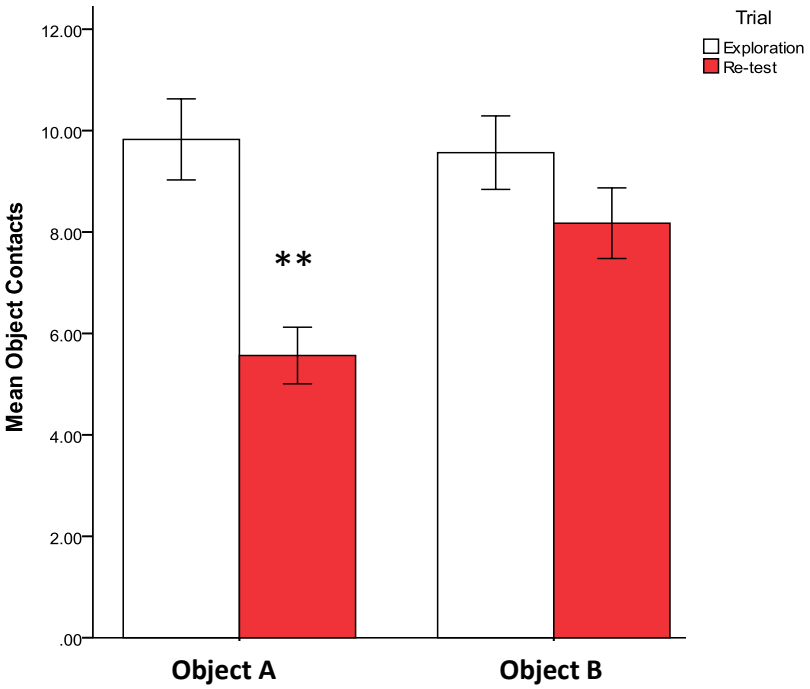


Figure 3.19 Overall animal object exploration. In all groups combined animals exhibit more exploratory behaviour in the initial trial where animals are naive to both objects. There exists between object discrimination however as animals explore the stationary object significantly less during the re-test trial whereas this difference is not observed in the moved object. ** denotes p<0.001. Error bars represent ±SEM

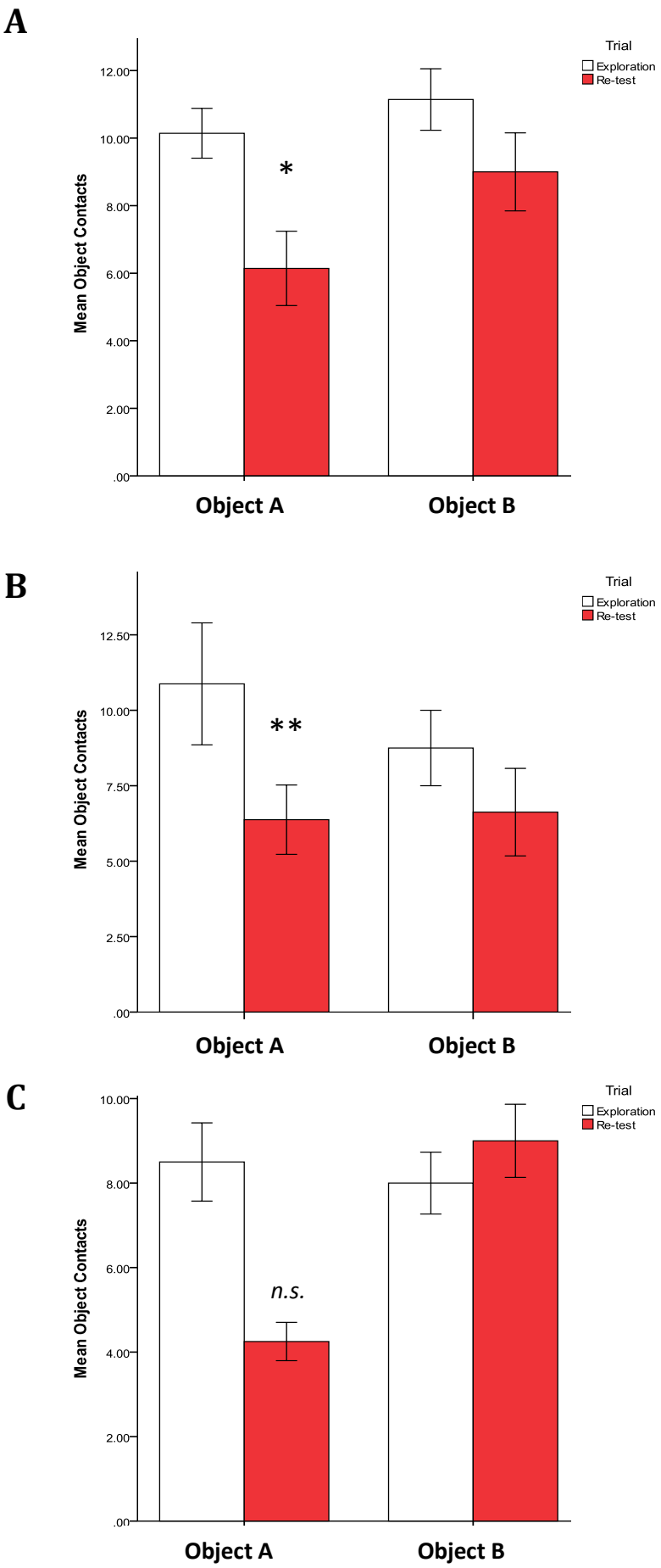
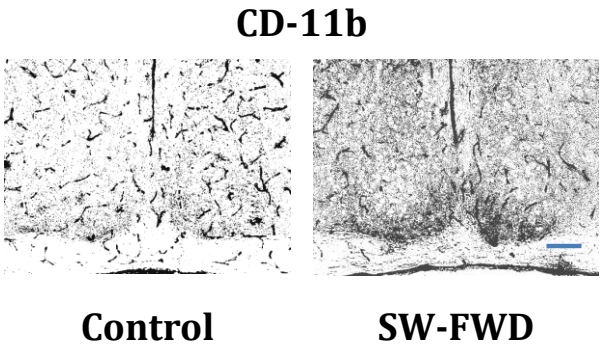


Figure 3.20 Novel object exploration performance between groups. Graphs are laid out as follows (a) Control animals' performance on task (b) SW-REV animals' performance on task and (c) SW-ADN animals' performance on task. Testing reveals that both control and SW-REV animals explore the non-moved object significantly less in the re-test tiral compared to initial exposure whereas this effect was not found for exploration of the moved object. In SW-ADN animals there is no between trial differences in exploration for either of the objects. The results suggest that control and reverse shift-lag animals noticeably discriminate between changes in their environment whereas the SW-ADN animals cannot. * denotes $p<0.05$. ** denotes $p<0.001$. Error bears represent ±SEM.

3.9 Forward rotating shift-work patterns do not produce neuroinflammation

To assess the potential outcome of neuroinflammation in the SCN as a result of chronic shift-lag schedules, surface markers of glial activation, CD-11b and F4/80, were compared between SW-FWD and control groups (figure 3.21). Two-way between-groups ANOVAs were used to analyse antigen particle data with group and region selected as the between groups variables. Results indicate that for CD-11b assayed sections there was no main effect for group found; F (1, 22) = 1.046, p = 0.322. The main effect for region was not significant; F (2, 22) = 0.351, p = 0.709, and there was no interaction effect found between the two variables; F (2, 22) = 1.984, p = 0.173.



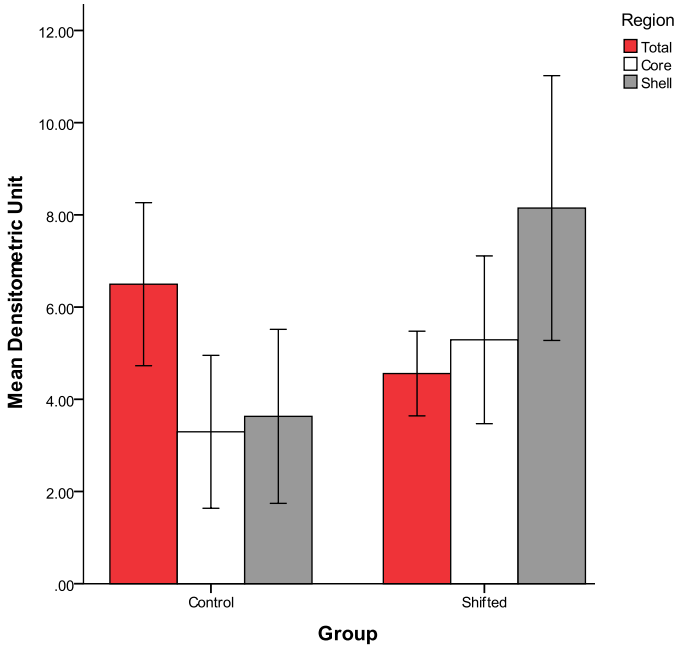
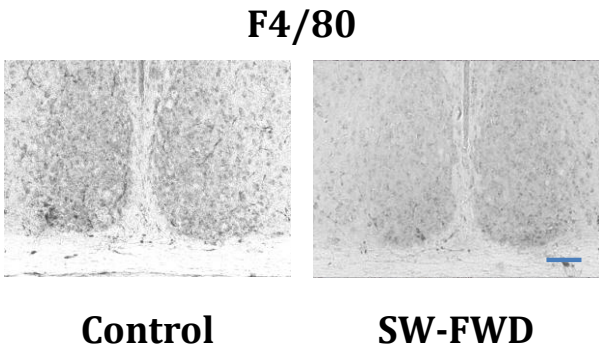


Figure 3.21 CD-11b expression in the SCN. Scale = OD, arbitrary units. No differences found between control and SW-FWD groups. Error bars represent ±SEM. Microphotgraphs taken at x10. Scale bar = 100µm

In sections stained for F4/80 marker expression there was no main effect for group found; F (1, 26) = 2.44, p = 0.134. The main effect for region was not significant; F (2, 26) = 0.63, p = 0.939, and there was no interaction effect found between the two variables; F (2, 26) = 1.649, p = 0.533 (figure 3.22).



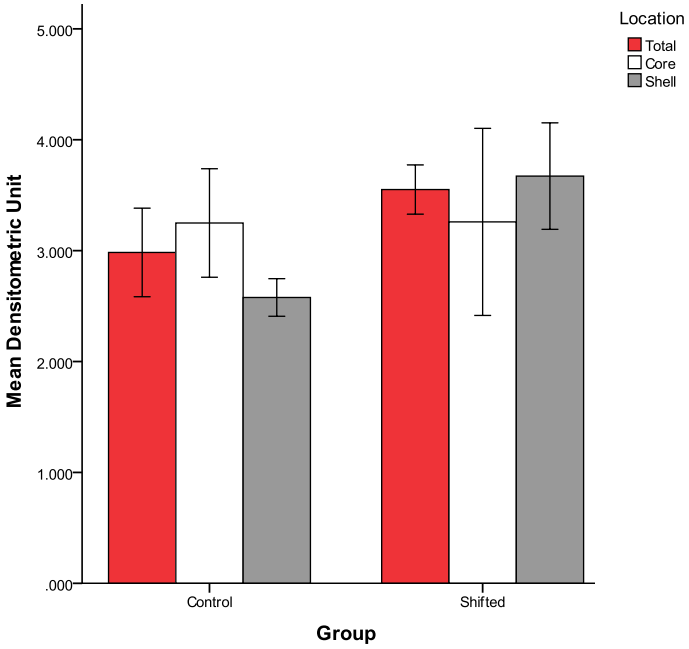


Figure 3.22 F4/80 expression in the SCN. Scale = OD, arbitrary units. No differences found between control and SW-FWD groups. Error bars represent ±SEM. Microphotgraphs taken at x10. Scale bar = 100µm

Sections which were stained for expression of proinflammatory cytokine TNF-α were analysed the same as previously described using two-way between-groups ANOVA with group and region factors. Results indicate that there was no main effect for group; F (1, 26) = 0.303, p = 0.588. The main effect for region was not significant; F (2, 26) = 1.579, p = 0.231, and there was no interaction effect found between the two variables; F (2, 26) = 0.726, p = 0.496. Sections were also stained to assess IL-6 expression in the SCN but due to poor results these IHC data could not be analysed and were not included in this study (figure 3.23).

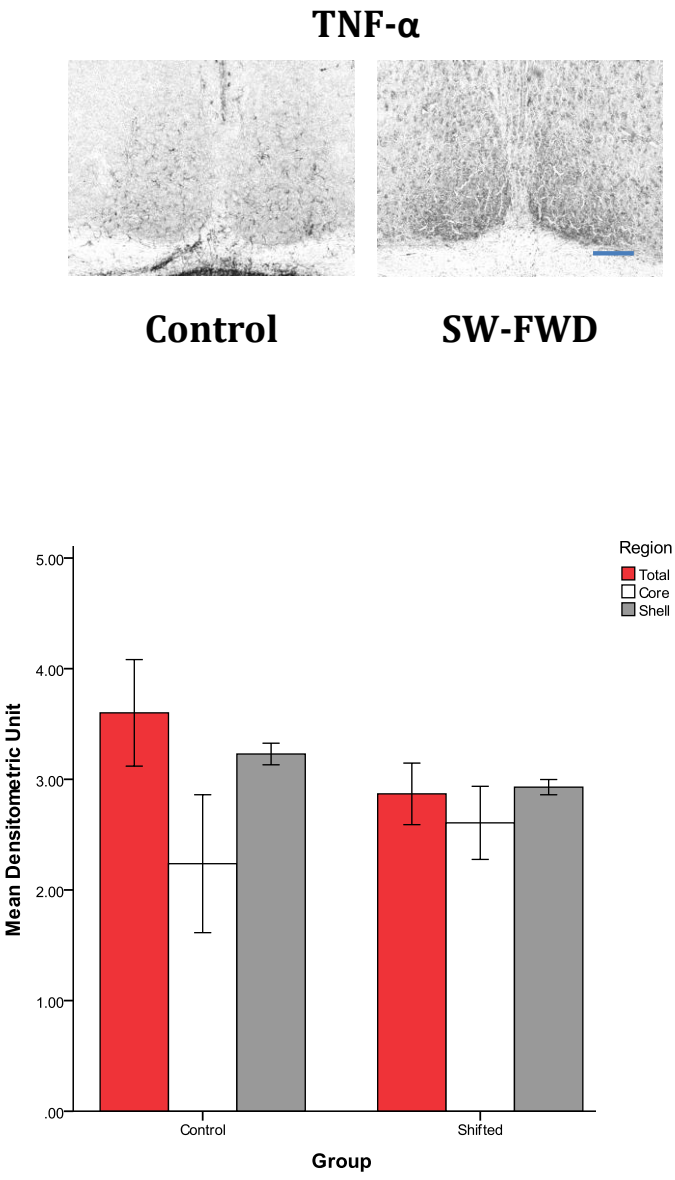


Figure 3.23 TNF-α expression in the SCN. Scale = OD, arbitrary units. No differences found between control and SW-FWD groups. Error bars represent ±SEM Microphotgraphs taken at x10. Scale bar = 100µm

3.10 PER1 suprachiasmatic nucleus expression

A two-way between-groups ANOVA was conducted to determine the impact of shift-work protocol and the zeitgeber time (ZT) animals was sacrificed at on entire SCN PER1 densitometry score (figure 3.24). The main effect for ZT was statistically significant; F (1, 65) = 88.513, p<0.001. There was no significant differences between groups found; F (2, 65) = 51.069, p = 0.350, and there was a significant group × ZT interaction effect found; F (2, 65) = 5.971, p = 0.004. Post-hoc investigation using tukey HSD *post-hoc* pairwise confirmed no significant differences between groups. To probe for potential differences at SCN core and shell regions additional ANOVAs were conducted limiting analysis to medial sections (see Figures 3.25 and 3.26 respectively) where these areas could be defined (see method section). In the SCN core it was found that there was a significant main effect for time; F (1, 30) = 40.271, p<0.001. There was no significant differences between groups found; F (2, 30) = 0.846, p = 0.439, and there was no significant group × ZT interaction effect found; F (2, 30) = 0.659, p = 0.525. In the SCN shell region there was a main effect for time found; F (1, 30) = 26.842, p<0.001. There was no significant differences between groups found; F (2, 30) = 0.189, p = 0.829, and there was no significant group × ZT interaction effect found; F (2, 30) = 0.8, p = 0.459.

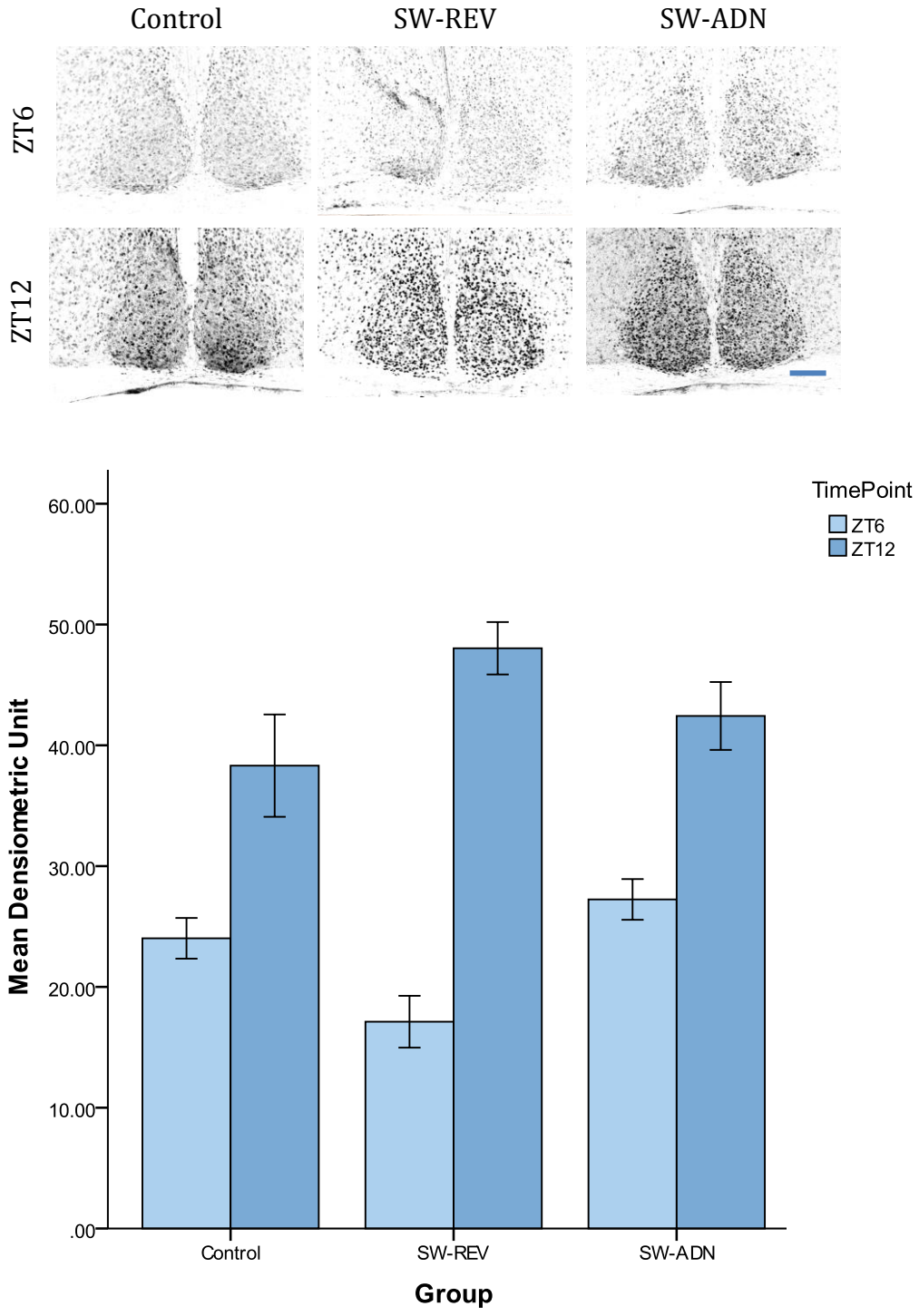


Figure 3.24 Overall SCN expression of PER1. There was a significant main effect for ZT (p<0.001). Between group comparisons yield no differences. Scale = OD, arbitrary units. Error bars represent ±SEM Microphotgraphs taken at x10. Scale bar = 100µm

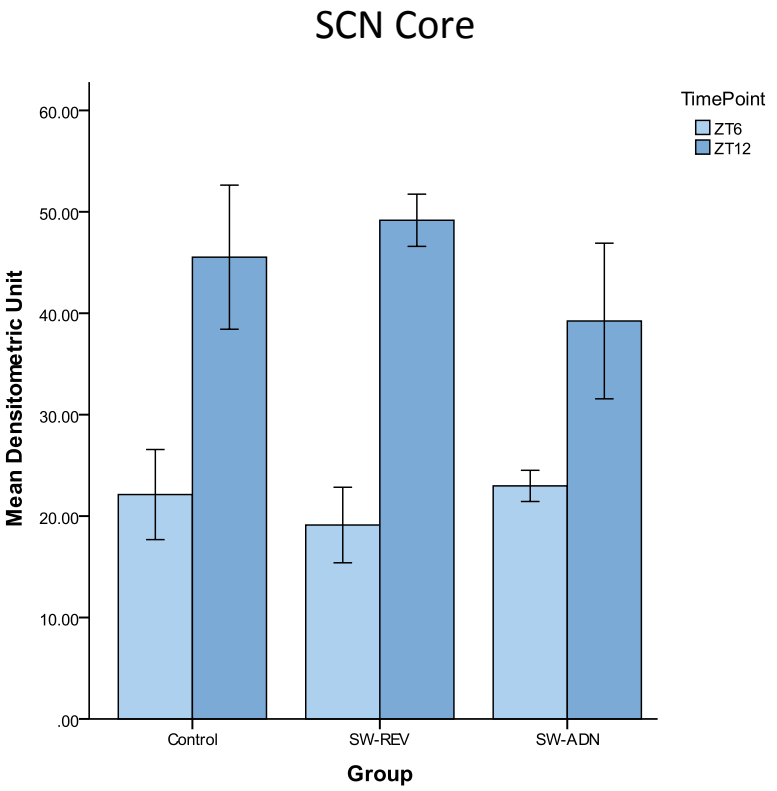


Figure 3.25 SCN core expression of PER1 at ZT6 and ZT12. Scale =OD, arbitrary unit. Significant effect found for ZT (p<0.001). Between groups comparisons reveal no significant differences. Error bars represent ±SEM.

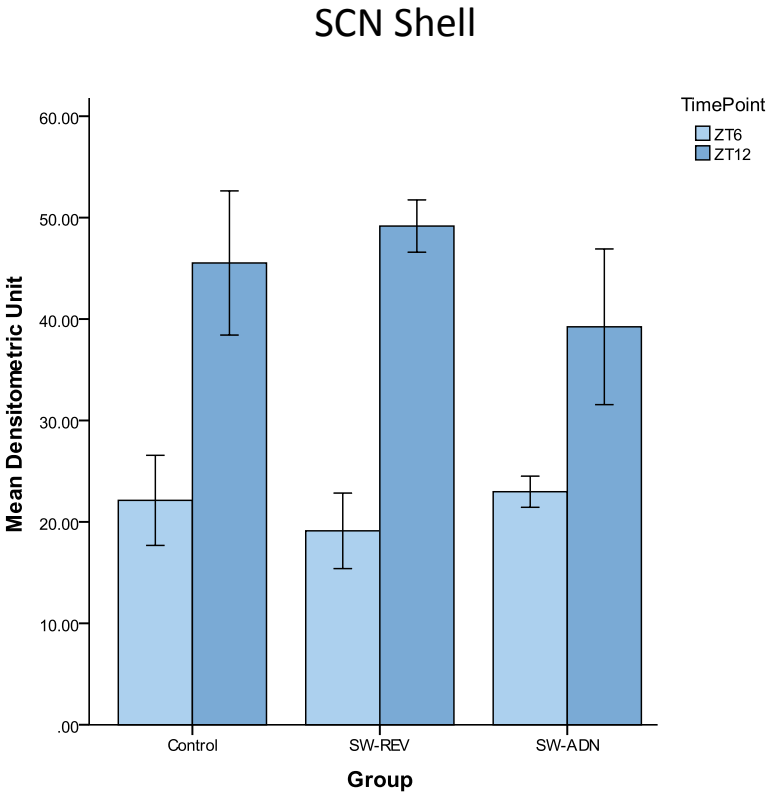


Figure 3.26 SCN shell expression of PER1 at ZT6 and ZT12. Scale =OD, arbitrary unit. Significant effect found for ZT (p<0.001). Between groups comparisons reveal no significant differences. Error bars represent ±SEM.

3.11 PER2 suprachiasmatic nucleus expression

A two-way between-groups ANOVA was conducted to determine the impact of shift-work protocol and the zeitgeber time (ZT) animals were sacrificed at on entire SCN PER2 densitometry score (figure 3.27). The main effect for ZT was statistically significant, F (1, 52) = 63.275, p<0.001. There was no significant differences between groups found, F (2, 52) = 2.783, p = 0.071, and there was no significant group × ZT interaction effect reported, F (2, 52) = 1.246, p = 0.296. As with the previous PER1 sections to probe for potential differences at SCN core and shell regions ANOVAs were conducted considering PER2 expression in these regions separately (figures 3.28 & 3.29). In the SCN core analysis revealed that there was a significant main effect for ZT; F (1, 31) = 25.208, p<0.001. There was no significant differences between groups found; F (2, 31) = 0.623, p = 0.542, and there was no significant group × ZT interaction effect found; F (2, 31) = 0.103, p = 0.903. In the SCN shell analysis revealed that there was a significant main effect for ZT; F (1, 31) = 29.690, p<0.001. There was no significant differences between groups found; F (2, 31) = 0.723, p = 0.495, and there was no significant group × ZT interaction effect found; F (2, 31) = 0.367, p = 0.696.

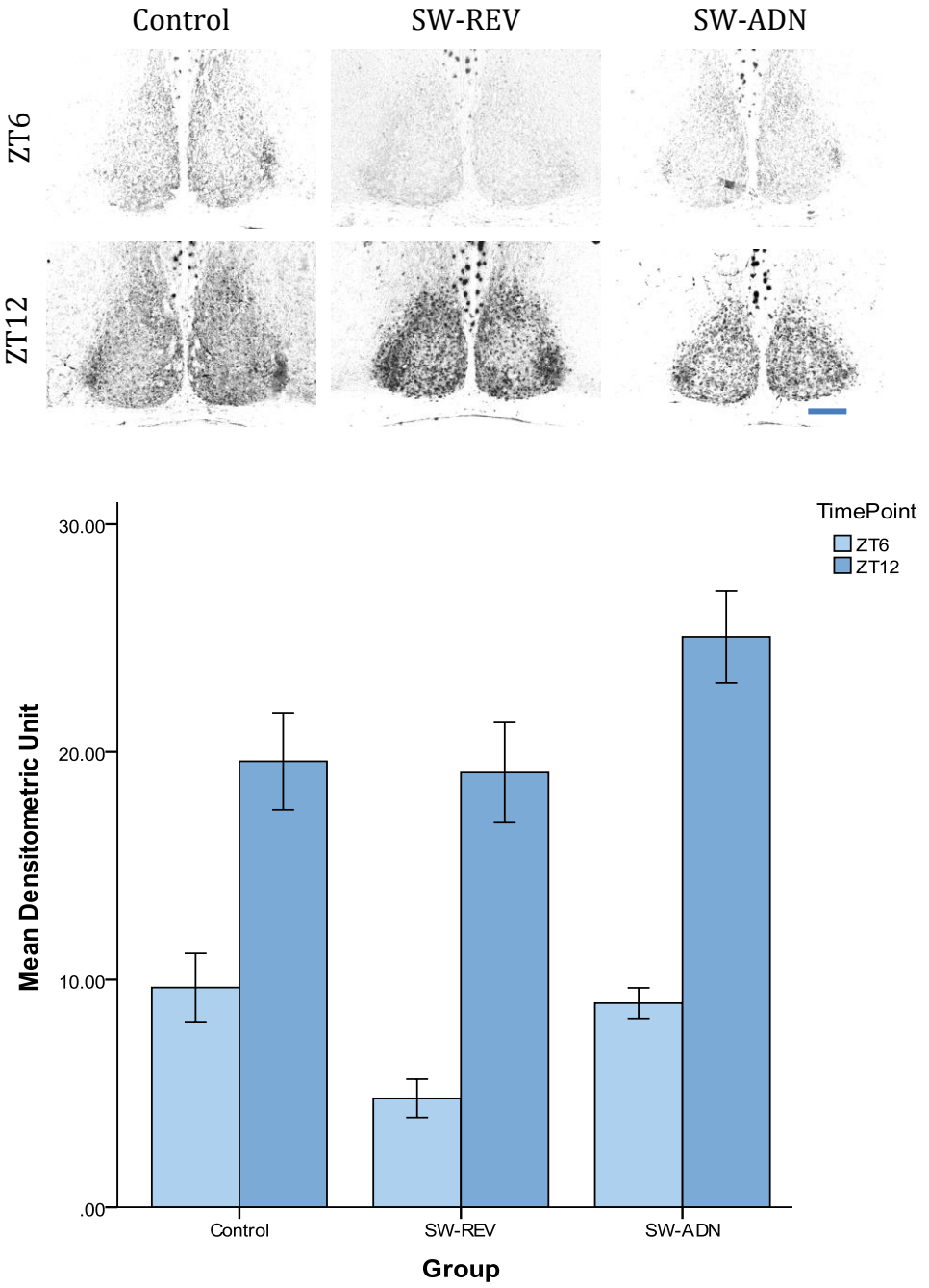


Figure 3.27 Overall SCN expression of PER2. There was a significant main effect for ZT (p<0.001). Between group comparisons yield no differences. Scale = OD, arbitrary units. Error bars represent ±SEM Microphotgraphs taken at x10. Scale bar = 100µm

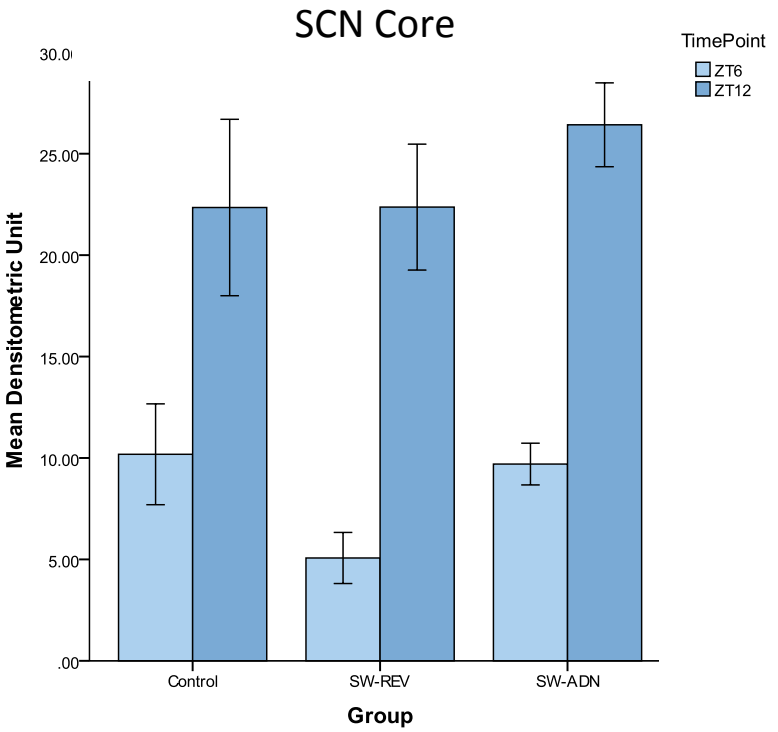


Figure 3.28 SCN core expression of PER2 at ZT6 and ZT12. Scale =OD, arbitrary unit. Significant effect found for ZT (p<0.001). Between groups comparisons reveal no significant differences. Error bars represent ±SEM.

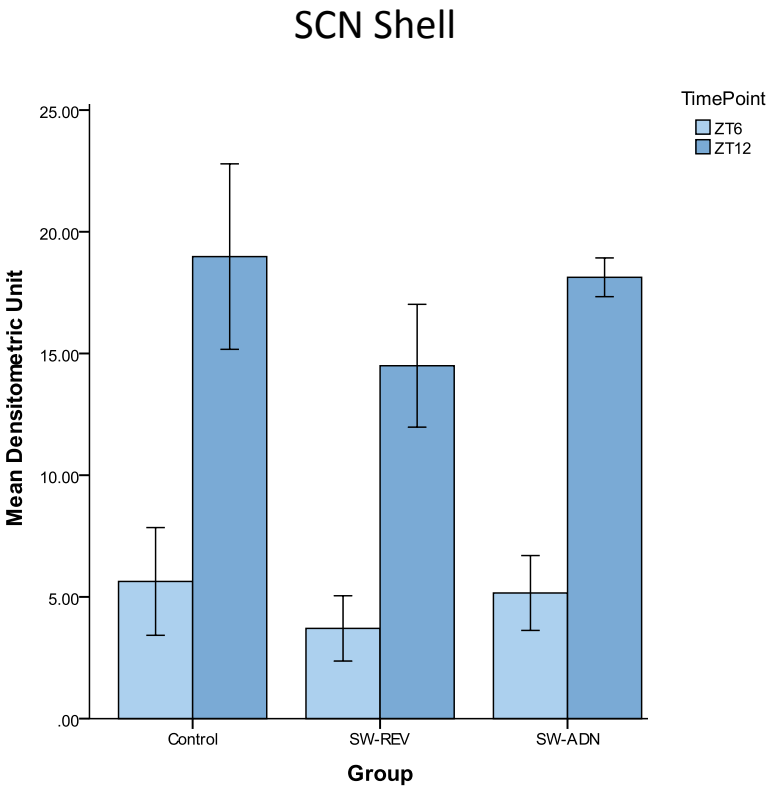


Figure 3.29 SCN shell expression of PER2 at ZT6 and ZT12. Scale =OD, arbitrary unit. Significant effect found for ZT (p<0.001). Between groups comparisons reveal no significant differences. Error bars represent ±SEM.

4. Discussion

4.1 Preamble

In the present study we exposed mice to different schedules of rapidly rotating shift-work to assess circadian entrainment and neurobehavioural affects. The results indicate different mice cohorts display chronotypically distinct patterns of locomotor entrainment, with entrainment typology being predictive of more profound plastic changes in locomotor parameters. Behavioural testing revealed chronic changes in anxiety but not in depressive-like behaviour with a possible hyperactivity phenotype being presented in one sample. Importantly these behavioural data were recorded at a time when turbulent photoperiod had been remedied and animals had re-entrained to baseline conditions. Immunohistochemical analysis yielded no meaningful differences between groups in the SCN suggesting the central pacemaker may not be an important substrate underlying the observed aberrant factors.

4.2 Successful circadian entrainment dependent on severity of shift-work

The results of this study suggest that with respect to rapidly rotating forward and reverse rotating patterns of shift-work the direction of shift rotation is not predictive of successful circadian entrainment. In both of these groups we demonstrate that genetically intact male mice appear to ignore rapidly rotating photic time signallers as suggested by apparent free-running of behavioural rhythms early on in each paradigm.

It has been hypothesised that in shift-workers a similar type of phenotype may serve as an adaptive mechanism in the long term preventing the maladaptive consequences of rhythms having to constantly re-entrain to rotating patterns of shift-work (Smith, Fogg, & Eastmann, 2009). Indeed re-entrainment is associated with internal circadian desynchrony at a molecular and physiological level (Yan, 2011; Yamazaki *et al.,* 2000) and thus capitulation of entrainment is hypothesised to be beneficial with respect to reducing circadian insult and thereby facilitating

health in the organism. There are preliminary findings indicating that in animals exposed to similar patterns of LD cycle rotation those that free-run rather than re-entrain have better outcomes after inoculation with a lung tumour inducing agent (Logan *et al.,* 2012). Thus it is hypothesised that at the level of immune regulation adaptive chronotype may have a meaningful effect on development of illness in shift-workers

The type of free-running of behaviour observed in the present study is normally only seen when photic exposure is static and therefore cannot act as a zeitgeber or when changes in the LD cycle are beyond the limits of entrainment. It would appear that the latter is what causes capitulation in our model of rapidly rotating 2-2-2 day shift-lag. Inspection of animal actograms reveals two distinct patterns of free-running activity within forward rotating and reverse rotating mice. It would appear that the majority of animals in each cohort adopt a period greater than 24 h whilst a few free-run with a period shorter than 24 h. It was determined that light exposure within the photic cabinet apparatus was not an influencing factor on free-running orientation as correlation coefficients between cage luminance level and circadian period length find no relationship. These variations within experimental animals are might therefore be internally motivated. In similar models of circadian desynchrony individual differences in entrainment type have also been found (Logan *et al.,* 2012; Gibson *et al.,* 2010). A possible contributory factor to the variation in phenotype observed in the present study is that the CD-1 mouse chosen is an outbred strain and therefore more susceptible to behavioural variability.

Of the three patterns of rotating shift-work examined in this study the SW-ADN animals were the only cohort to exhibit partial entrainment. This seems to suggest that with respect to entrainment of the mammalian circadian system tracking of the LD cycle is more responsive to 12 h phase shifts every 3-4 d than to 8 h phase shifts every 2 d which does not appear to facilitate entrainment at all. To date the majority of animal studies designed to mimic circadian desynchrony resulting from shift-work or jet-lag have been slower rotating than the present

paradigms tested or are acute in timespan rather than chronic in length (Tsai *et al.,* 2005; Bartol-Munier *et al.,* 2006).

Thus the finding that SW-ADN animals attempt to entrain locomotor rhythms are consistent with findings which suggest that the slower the rotation of LD the better entrainment and consequently re-entrainment is facilitated. It is reasonable then to purport that, together with previous findings which denote circadian entrainment is predictive of poorer health outcomes, SW-ADN animal health may be less robust than the other cohorts examined. Future research could perhaps focus on elucidating the effects of shift-work rotation direction and entrainment propensity on animal longevity after immune challenge to examine this prediction.

Analysis of behavioural circadian rhythm period length and amplitude when animals are released into constant darkness revealed that forward-rotating patterns of shift-work significantly lengthened period length while SW-ADN patterns significantly shortened period length and attenuated rhythm amplitude. No changes in these locomotor parameters were observed in the reverse rotating shift-work pattern indicating that animals undergoing this schedule were less susceptible to modulation of circadian rhythms. These observations are reminiscent of 'period aftereffect', a phenomenon seen in animals exposed to non-24 h light-dark cycles in which the long-term free-running period of rodent locomotor activity undergoes plastic changes (Pittendrigh & Daan, 1976). Aton and colleagues (2004) demonstrate also that aftereffects involve long-lasting period changes in the SCN in PER1 luciferase expressing mice tentatively indicating that neural plasticity is also involved in this mechanism. The adaptation of the circadian system in this manner highlights the ability of the organism to modulate activity to photoperiod exposure in a responsive manner. It is unclear whether the aftereffects observed in these parameters may have beneficial or deleterious consequences however.

Interestingly the most intense changes in period and amplitude were observed in the SW-ADN cohort which is consistent with the finding that this was the only group to demonstrate partial entrainment suggesting that repeated challenge to the circadian entraining mechanism is

causative of these modulations. Despite shift-work induced alterations in circadian period and amplitude all cohorts appear rhythmic throughout intervention suggesting that SCN oscillations persist in a coherent manner during exposure to rapidly rotating patterns of shift-work.

4.3 Animal body-weight unaffected by rapidly rotating shift-work

Epidemiological evidence suggests that shift-workers are at increased risk of becoming obese and developing illnesses such as diabetes and metabolic syndrome (Niedhammer, Lert, & Marne, 1996; Karlsson, Knutsson, & Lindahl, 2001). In line with prior human investigation animal models of non-rotating night-work undertaken by Salgado-Delgado and colleagues have reported increased weight gain in shift-worker animals compared to control groups (Salgado-Delgado *et al.,* 2008; Salgado-Delgado *et al.,* 2010). Such findings are not uncontested however as a similar simulation of non-rotating shift-work in the rat rather reports attenuation of normal weight gain compared to controls (Leenaars *et al.,* 2012).

In contrast to previous animal models of shift-work the present results indicate that mice growth curves remain unchanged by alternating LD schedules compared to those maintained under a stable LD cycle. Interestingly, our model of shift-work simulated three different work schedules which were rapidly rotated. Considered amongst previous studies, which have primarily focused on non-rotating work schedules, our observations suggest that subjects may be less susceptible to changes in body-weight while undergoing shift-work schedules that are frequently rotated.

It has been demonstrated in the Salgado-Delgado study (2008) that rats gradually shifted their feeding schedules to match the shift-work period. In the authors' later study it was found that rats' propensity to overweight was rescued when food access was restricted to the animals' normal active phase (Salgado-Delgado, 2010). Given these findings it is tempting to speculate that in the present study mice completing our shift-work protocol perhaps appropriate feeding habits in such a manner that may circumvent maladaptive weight gain which is facilitated by a

shift pattern which is rapidly rotating rather than previous findings which utilise a slower rotation. Food intake was not measured as part of this study however and thus it cannot be determined how feeding patterns entrain to the shift-lag schedules used in our model. Actigraphic measures of locomotor activity indicate that entrainment capitulates in SW-FWD and SW-REV animals suggesting that feeding time does not entrain to rapidly delaying or advancing LD schedules. As SW-ADN mice do display partial entrainment to LD cycle changes and also display normal growth curves however, the phase during which animals feed may not be an important factor for weight change in our model of animal shift-lag. Further, obesity in the Salgado-Delgado *et al.* (2010) model was also accompanied by changes in other metabolic parameters including perturbation of glucose and triglyceride rhythms, a finding agreeable with studies describing the causal role of circadian desynchrony in obesity (Tsai *et al.,* 2005; Barclay *et al.,* 2012) and impairments in insulin regulation (Bartol-Munier *et al.,* 2006).

Alternatively other reports have demonstrated changes in metabolic parameters which do not necessarily equate to changes in body-weight. In a recent mouse model of sleep fragmentation researchers found that 14 days of enforced locomotor activity during animals' sleep phase resulted in hyperphagia and impaired glucose tolerance but did not yield any increase in weight (Baud, Magistretti, & Petit, 2012). The researchers suggest that increased energy expenditure as a result of sleep disturbance may be the reason for no apparent changes in body-weight (Baud *et al.,* 2012). Interestingly in the present study SW-REV mice and mice undergoing SW-ADN schedules display significantly greater amplitude of locomotor rhythms when released into DD compared to controls. This possibly suggests that entrainment of circadian factors involved in shift-work schedule design may influence increased locomotor activity; perhaps as a mechanism which safeguards against maladaptive weight change.

While our model was not found to produce a weight-change phenotype in the CD-1 mouse it should be noted that other potentially compromised physiological and molecular metabolic parameters associated with metabolic syndrome in shift-workers were not assessed. In order to

present a comprehensive model of metabolic outcomes which may arise as a result of rotating shift-work future work should focus on other factors for metabolic syndrome and obesity such as hyperphagia, insulin resistance, glucose tolerance, hyperglycaemia, and hypertriglyceridemia.

4.4 Hyperactive and anxiogenic consequences of paradigm

It is known that experimental models of circadian disruption or sleep fragmentation can adversely affect mood and exacerbate anxiety in man (Bonnet, 1985; Scott *et al.,* 2006; Sagaspe *et al.,* 2005). This is also reflected in the epidemiology which finds that shift-work is a significant risk factor for mood disorders in occupational groups (Driesen *et al.,* 2010). Consequently we were interested in investigating if shift work-like rotating LD cycle manipulations produced chronic changes in measures of depressive and anxiety-like behaviour.

Rodents are exploratory animals when placed in a novel environment yet this innate characteristic is contrasted by the tendency to avoid open areas in which they are exposed to predation. In rodent models assessing anxiety-like traits open field thigmotaxis (i.e. where exploration predominantly restricted to the periphery) is considered an important indicator of anxious state. In studies involving pharmacological manipulation of behaviour anxiolytic and anxiogenic treatments respectively increase and decrease exploration of the centre area in the open field and thus reduce time spent at the periphery (Stefanski *et al.,* 1992; Plaznik *et al.,* 1994; Simon *et al.,* 1994). In the current study mice that had been exposed to rapidly rotating patterns of shift-work displayed significantly greater thigmotactic proclivity in the open field which was not observed in controls. This finding suggests that exposure to rotating patterns of shift-work causes an increase in anxiety-like behaviour. Importantly these differences were measured after circadian stress has been eliminated suggesting that these changes in affect possess some degree of chronicity.

To the authors' knowledge this is the first time such an investigation was undertaken using a rapidly rotating model shift-work as an experimental variable. Our data are reminiscent of

previous work which links sleep deprivation to anxiety-like behaviour in mice tested on the elevated plus maze (Silva *et al.,* 2004) suggesting a potential sleep dependant mechanism may be involved in the differences noted in the present study. In another model paradoxical sleep deprivation resulted in a reduction of anxiety-like behaviour, however contesting this assumption (Suchecki, Tiba, & Tufik, 2002). Importantly behavioural testing in our animals took place approximately 3 weeks after animals came out of DD and were maintained on a fixed 12:12 h LD cycle. If acute sleep disturbances were induced as a result of our paradigm it is expected that this is would be ample recovery time. Nevertheless the results indicate that a residual effect on anxiety-like behaviour is induced by our paradigm after manipulation is returned to baseline. Whether this is a result of chronic circadian desynchrony or sleep deprivation or a factor of the two remains to be investigated.

Comparison of locomotor parameters in control and experimental animals exposed to the open field indicate that SW-ADN mice travelled a significantly greater distance, moved significantly faster, and were hyperlocomotive compared to controls. We do not consider these findings representative of increased exploratory behaviour given (i) animals' thigmotaxic tendency, (ii) rearing behaviour matched control levels, and (iii) during testing mice appeared noticeably more agitated compared to other groups (observation, not quantified). The apparent enhancement of locomotor activity experienced in these animals is concluded instead to be as a result of hyperactivity within the SW-ADN cohort. Indeed increased spontaneous locomotor activity has previously been observed in rodents subjected to perturbations in sleep (Pokk & Vali, 2001; Pokk & Zharkoversusky, 1995).

It is interesting to speculate that activation of the stress axis may be a causal factor here. Previous experimental models of shift-work have demonstrated that increases in plasma corticosterone accompany perturbations in sleep-wake cycle (Weibel *et al.,* 1996). Findings from other studies contest this however reporting that corticosterone levels remain unaltered (Logan *et al.,* 2012; Barclay *et al.,* 2012). Interestingly in a model of sleep restriction animals tested using a

modified version of the open field a similar hyperactive phenotype was observed in experimental rats without any associated corticosterone elevations (Tartar *et al.,* 2009) suggesting a mechanism separate from corticosterone may be responsible for the hyperactive behaviour observed. Because we did not carry out any endocrine assays in the present study it is uncertain whether these behavioural differences are a result of HPA activity.

Another line of speculation implicates the possibility of REM sleep disturbance producing hyperactivity in SW-ADN animals. In experimental models paradoxical sleep deprivation is known to cause an increase in locomotor activity in rats (van Hulzen & Coenen, 1981) and in some cases may function as a valid model of manic behaviour (Gessa *et al.,* 1995). Thus rather than attributing hyperactivity to corticosterone elevations an alternative hypothesis may be that REM sleep disturbance as a result of our SW-ADN paradigm contributes to hyperactive behaviour observed in the open field. Of particular note was that SW-ADN animals were the only group affected in this way and subsequently this group experienced the most disrupted sleep-wake cycle by way of repeatedly attempting to re-entrain to the rotating LD schedule. This may perhaps be an indicator of disrupted periods of REM sleep in these animals. As previously discussed however it is unknown if at the time examined animals experience any residual disruptions in sleep. Future work exploiting polysomnographic measurement may illuminate a putative mechanism for hyperactivity in the SW-ADN cohort and additionally may investigate whether rotation directionality is a predictive factor in REM sleep deprivation severity.

Examination of tail suspension test performance indicated that shift-work patterns did not produce depressive-like symptoms. An important note in interpreting these findings is that there was a considerable amount of variability within groups. This possibly suggests that within our cohort sensitivity of affective change to shift-work is subject to independent differences. Our sample may therefore be underpowered to delineate any meaningful differences.

4.5 Partial circadian entrainment associated with cognitive deficits

In the current study we demonstrate that animals treated with the SW-ADN pattern of shift-work could not discriminate between objects which underwent spatial change between trials and those that did not. Performance on the cognitive task was distinguishable from other groups in which mice explored the stationary object significantly less in the re-test trial but explored the moved object equally between both trials. The model used here is similar to other hippocampal-mediated cognitive tasks such as the novel object recognition assay (NOR) which explores the tendency of rodents to seek novel changes in environment (Squire, Stark, & Clark, 2004). Numerous studies involving manipulation of circadian rhythms reveal profound learning and memory deficits on different batteries of cognition in rodents which are subjected to repeated phase shifts (Craig & McDonald *et al.,* 2008; Loh *et al.,* 2010; Gibson *et al.,* 2010). Specifically hippocampal dependent learning is known to require a coherent circadian system as studies examining the effects of circadian disruption in rodents report an injurious effect on hippocampal function (Gibson *et al.,* 2010; Kott, Leach, & Yan, 2012). In a functional manner circadian perturbation translates to pronounced deficits in tasks which examine memory.

In relation to the current study we consider several hypotheses to explain the effects observed in our model. It is well known that circadian modulation of learning effects performance on tasks which use time of day as a discriminatory cue (Pahl *et al.,* 2007; Pizzo & Crystal, 2002) implying that circadian mediated behaviour may act as a context feature for learning (Cain & Ralph, 2009). Ruby *et al.* (2008) further suggest that as well as imposing a temporal organisation on memory the pacemaker modulates learning via cyclical release of GABA as learning performance in arrhythmic animals is rescued after treatment with the GABA antagonist pentylenetetrazol. In light of the findings implicating the circadian system with successful learning and memory it is purported that deficits in memory persisting after the circadian system has re-entrained to a stable LD cycle might be as a result of plastic changes in the mechanism which is responsible for circadian mediated memory. This may be analogous to the manner in which locomotor

behavioural rhythm parameters undergo plastic changes in the same cohort of animals. While we can only speculate without investigation into molecular rhythms in the hippocampus inertia in the cognitive system as a result of prior exposure to a turbulent photoperiod might be a contributory factor. Additionally drawing from findings in the Kott *et al.* (2012) study we suggest that injury to hippocampal cells during the acute timescale of the shift-work paradigm might have long lasting harmful consequences which translate to persisting cognitive deficits in hippocampal-dependent learning.

Moreover we consider that animal stress might also be a relevant factor in our model. It is known that in experimental conditions constant light exposure can produce increased stress hormone secretion which can interfere with learning and memory (Wen-Pei *et al.,* 2007). Additionally in the study undertaken by Gibson and colleagues (2010) elevations in corticosterone accompanied circadian insult supporting the previous findings that stress is a dependent detrimental factor in memory performance (McEwen *et al.,* 1993). It had been demonstrated also in the Gibson *et al.* (2010) that an adrenalectomised cohort were preserved from hippocampal injury providing strong evidence for the role of stress hormone mediating chronodisruptive memory impairment. As our model uses the LD cycle to mimic shift work stressors resulting from this alternating LD cycle might explain the deficits observed, though to reiterate a point made earlier at the time examined animals had re-entrained and were maintained on a stable LD cycle.

Previous studies have implicated effective periods of REM sleep with successful performance on cognitive tasks (reviewed in Stickgold, 2005). Particularly noteworthy is the finding that object recognition task performance specifically appears to profit from uninterrupted sleep though only mice which were sleep deprived after acquisition, and not before, fail to discriminate between familiar and novel objects (Polchykova *et al.,* 2006). Models examining the biological substrates of learning and memory in the hippocampus have found that, controlling for the effects of corticosterone, REM sleep deprivation had the potential to independently suppress cell

proliferation (Mueller, Mear, & Mistlberger, 2011; Mueller *et al.,* 2008). While animals in the current study were not sleep deprived at the time of testing if, as suggested previously, SW-ADN schedules do produce REM sleep disturbances the harmful effects of such on cognition may be residual in nature. Supportive of this is the findings that shift-workers' sleep architecture is observed to undergo chronic changes producing decreased REM sleep duration but no effect is found on SWS (Åkerstedt, 1998). Changes in sleep architecture which possess some degree of permanence may result in reduced sleep quality and effectiveness leading to detriments in hippocampal memory persisting after environment has been ameliorated.

4.6 Molecular pacemaker undisturbed

It is a well documented finding that circadian disruption is linked to exacerbated immune response and can adversely impact longevity in animal models of chronic illness (Castanon-Cervantes *et al.,* 2010; Logan *et al.,* 2012; Filipski *et al.,* 2004; Silver *et al.,* 2012). Furthermore the SCN is known to express molecular components important in immune signalling (Beynon & Coogan, 2010; Lundkvist *et al.,* 1999). Additionally immune stimulation via treatment with endotoxin (Marpegán *et al.,* 2005) or pro-inflammatory cytokines (Boggio *et al.,* 2003) is known to have a pervasive effect on behavioural rhythms. Recently a model mimicking sepsis demonstrated that LPS treatment can result in persistent upregulation of microglial factors in the SCN well after the acute response had abated (O'Callaghan *et al.,* 2012).The aforementioned evidence points to an interrelated relationship between circadian desynchrony together with a weakened SCN and impaired systemic immune function, which, given the findings of increased inflammatory illnesses in shift-workers, it is reasonable to suggest that on-going disruption of circadian rhythms may be an important risk factor for disease.

Given these findings we addressed the hypothesis that repeatedly rotating LD patterns might themselves be sufficient to produce an adverse upregulation of glial markers and pro-inflammatory cytokines in the master-pacemaker which might in turn translate to impaired animal

health. Between controls and the forward shifted cohort that we selected to examine the presence of neuroinflammatory markers surface glial expression and pro-inflammatory particle data were scarce and no distinguishable differences were found. To date ours is the only study examining if an unstable ambient photic zeitgeber could produce an altered SCN microglial profile in absence of immune stimulation. These results indicate that circadian perturbation alone is not sufficient enough to produce adverse priming of SCN immune cells. Given the prior evidence in the literature the effect of circadian disruption on weakening central pacemaker immune regulation may be limited rather to an interaction with sickness.

Previously Bentivoglio *et al.* (2006) showed that aged mice exhibited a low-grade chronic inflammatory condition compared to younger mice. Such differences are hallmarks of natural senescence and are observed systemically without any immune intervention. Taking into consideration that age is an important factor in aberrant SCN priming, as well as overall resilience, but also the finding that the amount of time working shifts is predictive of a greater risk to health, ageing is an unavoidable concomitant factor which may enable shift-work to cause harm. Therefore it could be of potential interest to compare younger and older cohorts of mice subjected to the same photic manipulations in this study to elucidate if age in addition to shift-work could produce neuroinflammatory injury. Further to this, low-dose immune challenge combined with the present intervention may produce the undesirable changes in SCN morphology we wish to demonstrate and would serve as a model of the interplay of opportunistic infection which must be taken into account in assessing risk of occupational disease.

Due to the lack of noteworthy upregulation of immune factors in the pacemaker for the remaining experimental groups and one supplementary control group we focused our investigation on rhythmic circadian factors in the SCN. As rhythmic locomotor parameters are a reliable indicator of molecular circadian oscillators in the master pacemaker, and distinguishable differences were evident in the behavioural data, we asked if different patterns of shift-work could produce changes in PER1 and PER2 expression in the SCN predictive of a

potential substrate of the behavioural rhythm changes. Previously it has been shown that aftereffects of exposure to non-typical photoperiods produce changes in pacemaker function (Aton *et al.,* 2004; Bosler *et al.,* 2009). At the two timepoints tested there were no between group differences of either of these products. This is consistent with other studies which find that in animal models of shift-work peak expression of PER1 and PER2 remain coupled to the fixed LD cycle to which they were entrained (Salgado-Delgado *et al.,* 2008). Importantly other studies have examined pacemaker function after exposure to replications of shift-work rather than perturbations in the LD cycle to which entrainment of these molecular parameters is most sensitive.

Furthermore in our model of pacemaker functioning was assessed after animals had re-entrained to a fixed LD cycle for more than 3 weeks. While we can infer that peak and nadir SCN PER1 and PER2 expression remain within normal range at the time tested we cannot confirm if molecular rhythms may be disrupted during the acute timescale of the rotating LD cycle. A significant limitation in our study is the lack of animal numbers required to plot a daily curve of rhythmic circadian expression. It is possible that at timepoints which were not assessed changes in the intervention groups may be present. Indeed analysis revealed a group × zeitgeber time interaction effect in the reverse rotated group which perhaps merits further investigation in future studies.

Despite this no meaningful differences between groups at the timepoints selected implies that the patterns of rotating shift-work assessed in the current model do not disrupt phase or amplitude in a chronic fashion. Nevertheless, while it may be reasonable to concur that temporal coherency is maintained in the SCN, peripheral oscillators may be disrupted as a result of our paradigms. Clocks in peripheral tissues which are compromised as a result of LD disruption are known to re-entrain at a slower rate than the master pacemaker (Davidson *et al.,* 2009; Yamazaki *et al.,* 2000). It is possible that internal desynchrony may be induced in experimental animals but as our investigation only involved SCN tissues further research is required to determine this potential.

4.7 Conclusions

In conclusion, the present study demonstrates that different schedules of rotating shift-work produce distinct entrainment patterns and distinguishable aftereffects in circadian rhythm parameters. Accompanying these changes are adverse effects on mood and cognition with animals that attempt to partially entrain to a rapidly moving photoperiod at increased risk of negative outcomes. In contrast with other animal models using shifts of the LD cycle, animals in the present study did not gain weight, perhaps indicating that a slower rotating photoperiod is necessary to produce risk of obesity. Importantly no chronic changes in pacemaker function were observed suggesting that modulation of other sites mediate the behavioural effects observed. The present model adds to the growing literature exploiting animal work to determine how mammalian circadian rhythms entrain to an alternating LD cycle such as shift-work in humans.

4.8 Implications of current study in a human scenario

There are a number of items revealed in the current study which may be relevant to a human shift-work scenario. The finding that on fast rotating schedules there appears to be minimal entrainment in animals may be of interest as it has been hypothesised that in shift workers a similar lack of entrainment may serve as a mechanism preventing the maladaptive consequences of rhythms having to constantly re-entrain circadian rhythms to rotating patterns of shift work in the long term (Smith *et al.,* 2009).

Re-entrainment is associated with internal circadian desynchrony at a molecular and physiological level (Yan, 2011; Yamazaki *et al.,* 2000) and thus can contribute to many of the deleterious effects highlighted in the shift-work epidemiology. It has been hypothesised that a lack of entrainment might preserve health by limiting the maladaptive health consequences of repeated circadian challenge. According to the results of the current study, a condition resembling forward or reverse rotating shift work schedules would theoretically be beneficial as it might mitigate the health consequences of frequent circadian re-entrainment.

It is also of interest to note that cognitive performance was worse in the alternating shift-work group. If these results are transferable to a human condition it would be recommended that when designing work rosters this finding might be considered. Indeed the epidemiology of mental health and performance in shift-workers reveals that there currently exists a problem where workplace accidents more frequently occur during non-typical work arrangements (Folkard & Tucker, 2003). Thus designing shift-work rosters which limit the amount of cognitive impairment experienced by workers would be beneficial.

An important caveat exists when interpreting the results of this study. Notably, we chose to examine nocturnal mice in the design of this experiment whereas man is a diurnal species. In effect this means that period of darkness represents 'daytime' for the mouse and light the 'night-time', or resting phase. One may argue however that is it the speed and direction in which the zeitgeber moves which is relevant in the study of circadian rhythm entrainment and not the absolute relationship to the active/inactive phase which is important. Future work using a diurnal rodent model might be informative in this regard as the active and inactive phases of day more closely resemble that of humans. Additionally a mouse model capable of producing melatonin (e.g. C3H strain) might be beneficial for probing into the effects of this hormone on health. For example the *'light-at-night'* hypothesis places important emphasis on the role of melatonin suppression in the development of several cancers and immune disorders (Stevens, 2009).

Ideally a more naturalistic model should be employed when modelling human shift-work. It is difficult to extrapolate the findings of this study to the human condition as several social factors and individual responsibilities determine circadian entrainment. Where human experimental models of shift-work are unfeasible however, the controlled design of animal models affords an opportunity to study the effects of a turbulent photoperiod on mammalian circadian entrainment from both a behavioural and molecular perspective.

4.9 Future work

Based on observations in the current study, it is proposed that future investigations could compliment this work by undertaking the following:

- As previous reports indicate that age is an important factor in exploring the deleterious effects of shift-work on health (i.e. individuals exposed to shift-work from a young age are at increased risk of developing autoimmune disease, while tolerance of shift-work is known to decline with age) it may be of interest to explore similar LD manipulations comparing younger and older cohorts to determine differences in locomotor entrainment and pacemaker coherency.
- Although weights remain unchanged in the current study, further exploration is required to unequivocally rule out any harmful metabolic consequences. Analysis of feeding habits, food intake, and glucose tolerance testing might illuminate the full spectrum of effects.
- Corticosterone endocrine assay may determine if stress is an important factor with respect to the observed changes in mood. Alternatively polysomnographic measurement might validate the hypothesis that long-lasting changes in sleep architecture may be accountable as the literature points to NREM sleep deprivation and psychiatric symptoms.
- The above point applies to the effects on learning and memory we observed also. In addition, examination of other cognitive faculties such as conditioned place preference (CPP) or passive avoidance (PA) might distinguish between types of memory that are more severely impaired as a result of chronodisruption.
- Given that only two timepoints were used to assess SCN function our data cannot present a full picture of potential differences in molecular rhythms between groups. The use of transgenic animals carrying a *Per2* luciferase reporter gene may provide a more comprehensive analysis. Bioluminescent oscillation assay of PER2::luc mice in *ex vivo*

tissue cultures could be used to determine internal desynchrony within animals and overall coherence quality between experimental groups.

- Although PER2 function appeared to be no different between control and shift-worker cohorts, future studies might probe for differences in protein expression of other canonical genes involved in the molecular clock such as CLOCK, BMAL1, CRY, REV-ERB, and CK1E.
- Finally, larger sample sizes of experimental groups would enable comparison of the zeitgeber capitulation phenotypes we observe. It may be of interest to distinguish whether direction of free-running rhythms is a meaningful predicator of adverse health or behavioural outcomes. Moreover, analysis of pacemaker function could help elucidate what biological mechanisms underlie this phenotype and account for the variability within shift-worker cohorts.

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