Light-At-Night and Mood: Examining The Role Of Sex In C57Bl/6 Mice

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Thesis submitted to the Department of Psychology, Faculty of Science & Engineering, in fulfilment of the requirements for the degree of Master of Science (MSc), Maynooth University

October 2015
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Acknowledgements

I would like to thank my supervisor Dr. Andrew Coogan for all his help over the last three years. His passion for sleep and circadian rhythms was instilled into me when I was an undergraduate and made me want to peruse research in this area. Although, perusing a research career has been a steep learning curve for me, Dr Coogan's patience and knowledge has been extremely beneficial to me.

I would like to thank my close friends Mira Dobutowitsch, Páraic Kerrigan, Laura Rai, Penny Quinn, Simon Orrock, Katie Rogers, Emily Rogers and Kevin Ryan. Each of you have been a massive support over the last number of years. I would like to thanks my lab colleagues Niall McGowan, Saileog O'Keeffe and Sean Anderson.

Finally, I would like to thank my parents Connie and Michael for your endless love and support. In particular, my mother's constant encouragement and belief in me has been a significant driving force in my academic achievement thus far.

Abstract

Rates of major depression have increased substantially in recent years, although it is not currently clear what are the factors behind such increases. Environmental factors may be important, and it has recently been postulated that dim nocturnal light may contribute to depression symptoms in humans and in rodents. Sex is also a very important factor in affective disorders, with prevalence rates of major depression twice as high in females than in males. We set out to test the hypothesis that dim-light would interfere with the circadian rhythm of C57Bl/6 mice and induce both depressive-like and anxiety-like behaviours and that there would be sex-specific differences. Animals were either singly or group housed for a three week period where locomotor activity was measured. After this period they were tested on a range of tests of emotional behaviours. Animals were subsequently placed into either 12 h light: 12h dim nocturnal light (~5 lux) cycle or a 12:12 light/dark condition and retested on the behavioural battery after three weeks. Brains of the same animals were used to measure stem cell proliferation in the dentate gyrus using the biomarker Ki-67. Exposure to dim light-at-night did not lead to significant circadian disruption nor to significant changes in any of the parameters examined. Apart from the Forced Swim Test no sex-dependent effects were detected. Levels of neural progenitor cell as measured by Ki-67 were significantly decreased in the dentate gyrus of light-at-night animals. In the context of previous research in this area our results indicate that species and strain differences may be important in assessing the potential impact of dim nocturnal light on circadian and affective systems in rodents.

Introduction

Circadian Rhythms

Circadian rhythms are intrinsically driven biological oscillations of ~24 hours, which allow organisms to adapt and synchronise their physiology to the daily cycle of sunlight and darkness (Gallego & Virshup, 2007; Redlin, 2001). These rhythms are genetically encoded and are fundametal in governing cellular and physiological functions of the brain and body, (Buttgereit, Smolen, Coogan & Cajochen, 2015) the sleep-wake cycle, glucose, lipid, bone and drug metabolism, heart rate affective functioning, regulation of stress and growth hormones and immunity (Stevens & Zhu, 2015; Wright, Lowry, LeBourgeois, 2012). The circadian pacemaker oscillates to a period of near but not precisely 24-hours (Bray & Young, 2006), and is primarily generated as a result of daily light/dark (L/D) rhythms. This internalization of 24 hour L/D rhythms is advantageous for mammals to help predict daily recurring events, to organize and to coordinate interactions with the external environment (Albrecht, 2012). For instance, in diurnal species the daily reoccurrence of natural light stimulates energy intake and metabolism, physical activity and allows for superior cognitive performance levels. In contrast, the dark period promotes sleep and related functions during the darkness of night (Wright et al., 2013). Circadian rhythms also allow for the anticipation and preparation of environmental changes, which are fundamental for fitness and survival (DeCoursey & Krulas, 1998; Merrow, Spoelstra & Roenneberg, 2005; Ouyang, Andersson, Kondo, Golden & Johnson, 1998). For example, chipmunks who have a lesion to the master endogenous oscillator are significantly more prone to predators in the wild (DeCoursey & Krulas, 1998).

The primary function of the internal timekeeping system is that in the absence of of external cues, a temporal architecture on behaviour, physiology, and metabolism is

imposed (Buttgereit et al., 2015). This architecture results in temporal segregation of behavioural and physiological processes, which ensue an optimally timed interaction with the environment so that activity is limited to the correct temporal niche. For a circadian rhythm to occur it must have three properties. Firstly, in the absence of environmental cues the period of the rhythm should be ~24 hours. Secondly, although rhythms are synchronized/reset by external cues, the rhythms must be robust and buffered against changes in environment such as alterations in temperature and behavioural feedback (Ruby, Burns & Heller, 1999). Finally, although rhythms have the ability to shift when under the control of a range of different factors entrainment is restricted to a specific range (Ralph & Meneker, 1988).

The Master Oscillator

The suprachiasmatic nucleus (SCN) is the master circadian oscillator of the circadian system. It sends signals to other brain and body regions, which results in stable, robust and coherent oscillations throughout the whole body (Yamazaki et al., 2000). This consistency is fundamental for optimal output of physiology and behaviour (Reppert & Weaver, 2001; Shuboni & Yan, 2010). This is demonstrated in both *in vitro* and *in vivo* recordings of SCN firing frequency where SCN's electrical activity output expresses a smooth and repetitive oscillation (Inouye & Kawamura, 1979; Shibata, Oomura, Kita & Hattori, 1982). Extensive evidence suggests that the SCN is the master circadian oscillator of biological timekeeping. SCN lesions abolish circadian rhythms and result in impairment to both behavioural and neuroendocrine rhythmicity (Moore & Eichler, 1972; Richter, 1967; Stephen & Zucker, 1972; Stephen & Nunez, 1977). This desynchrony of non-SCN oscillations results in general loss of endogenous rhythms at the tissue and whole-animal levels. Nouye and Kawamura (1979) observed that when connections were severed between the SCN and the rest of the brain retention of rhythmic electrical activity was observed in the SCN. However, an elimination of

activity occurred outside of the SCN. This provides evidence that SCN projections transfer rhythmicity to neurons located outside of the SCN. Both electrical and chemical stimulation of the SCN result in the generation of phase-shifts (Albers, Ferris, Leeman, Goldman, 1984; Rusak & Gross, 1982). Transplanting an SCN into an animal whose own SCN has been ablated results in the restoration and generation of circadian activity (Lehman, Silver, Gladstone, Kahn, Gibson & Bittman, 1987; Silver, LeSauter, Tresco & Lehman, 1996). Rhymicity is even expressed in genetically non-rhythmic mice (Sujino, Masumoto, Yamaguchi, van der Horst, Okamura & Inouye, 2003). In animals, which express abnormally short or long circadian periods, it is found that the period of the restored rhythm is driven by the genotype of the SCN donor and not that of the SCN-lesioned host (Sujino et al., 2003; Ralph, Foster, Davis & Menaker, 1990). Finally, individual neurons, which are dissociated from the SCN, express self-sustaining oscillations long-term (Welsh, Logothetis, Meister & Reppert, 1995; Yamazaki et al., 2000).

The SCN is distinctive for a number of reasons. Firstly, it receives direct photic input from the retina allowing neurons within the SCN to synchronise to the environmental photoperiod of the LD cycle (Reppert & Weaver, 2001). Thus, the SCN is essential for photoperiodic time measurement and conveying information of day length information to the pineal gland and other areas of the CNS (VanderLeest, Rohling, Michel & Meijer, 2009). Secondly, the SCN neurons are comprised of distinctive topographically organised coupling mechanisms, which allow each neuron to become a coherent heterogeneous and highly robust oscillatory network becoming synchronised (Aton & Herzog, 2005; Herzog, 2007; Ko et al., 2010; Liu et al., 2007). At a single-cell level each of the SCN neurons exhibits a vast array of cell-autonomous circadian periods, which ranges from 22 hours to 30 hours (Ko et al., 2010; Welsh et al., 1995). This results in neurons inside the SCN having matching circadian periods and

phases (Herzog et al., 2004; Mohawk, Green & Takahashi, 2012; Welsch et al., 2010). Finally, the neurons produce a strong circadian rhythm of neuronal firing frequency through a range of different direct and indirect pathways and this results in the synchronisation of other cells throughout the body (Gachon, Nagoshi, Brown, Ripperger & Schibler, 2004).

Structure and Subdivisions of the SCN

The SCN is described as a paired neuronal structure located in the hypothalamus (See Figure 1.1). Specifically, it can be found at either side of the third ventricle just above the optic chiasm (Welsh, Takahashi & Kay, 2010). In mammals, each unilateral SCN contains circa 10,000 cells, which are believed to be SCN clock cells. Neuronal cell bodies located within the SCN are distinctively small and are comprised of dendritic arbors, which are in close proximity to one another (Welsh et al., 2010). The nuclei are placed strategically to receive visual input for L/D entrainment via direct and indirect retina-to-SCN pathways (Reppart & Weaver, 2001). Research provides strong evidence that the two SCN nuclei are coupled, as a unilateral lesion results in the disruption of behavioural circadian rhythms (Pickard & Turek, 1982). The two nuclei also project onto one another with both the core and the shell of each nucleus projecting contralaterally to the core and shell of the other nucleus (Leak et al., 1999). Additionally, it has been observed that the electrical activity is relatively similar with minor phase differences being observed in each nucleus (Inouye & Kawamura, 1982).

The SCN is anatomically divided into the ventral "core" region and the dorsal "shell" region. The core is adjacent to the optic chiasm and receives input from the retina with the shell region receiving input from the core (Abrahamson & Moore, 2001). SCN neurons are comprised of a number of neuropeptides (Dibner, Schibler, & Albrecht, 2009). Neurons located within either the core or the shell are characterised as belonging to either anatomical division of the SCN (Moore, Spech & Leak, 2002). The

core contains neurons which express vasoactive intestinal polypeptide (VIP) calretinin, neurotensin and gastrin releasing peptide (Vasalou & Henson, 2011). The shell contains neurons, which express argininge vasopressin (AVP), angiotensin II and met-enkephalin (Abrahamson & Moore, 2001). Apart from containing different neuropeptides the functions of the core and shell are different. The core, which contains light sensitive neurons, is essential for gathering and collecting light information from the retinohypothalamic tract (RHT). The core also collects information from the thalamus and raphe nucleus and ensures the maintenance of coupling within the SCN (Rosenwasser & Turek, 2011). Once all this information is organised, the core carries this activity to the shell. The shell's function is to generate the circadian timing system and regulates the outgoing circadian signal (Rosenwasser & Turek, 2011). The importance of the core in organising pacemaker function is observed when lesions to areas of the core result in the abolishment of circadian rhythms of locomotor activity, body temperature, heart rate, melatonin and cortisol (LeSauter & Silver, 1999). The relationship between the shell and the core is not bidirectional as the shell receives strong and significant input from the core SCN (Moore, Spech & Leak, 2002) whereas, the core SCN receives little input from the shell SCN (Romijn, Sluiter, Pool, Wortel, & Buijis, 1997). Experimental evidence demonstrates that each division of the SCN carry out different functions. Firstly, within the core of the SCN neurons project onto neurons in the shell whereas, the core does not project onto the shell (Rosenwasser, 2009). Secondly, there are a significant number of major SCN afferent pathways, which project precisely onto the core region while numerous SCN afferents project outwards from the shell to the thalamus and other extra-SCN targets (Rosenwasser, 2009; Moore, 1996; Moore & Silver, 1998). The gene expression of cFos and *Per*, neuropeptide release and circadian rhythmicity in neuronal activity is more commonly observed in the shell than the core (Inouye, 1996; Sumova, Tranvickova, Mikkelsen & Illnerova, 1998; Yan,

Takekida, Shigeyoshi & Okamura, 1999; Hamada, LeSauter, Venuti & Silver, 2001).

When the core neuropeptides VIP and gastrin-releasing peptide are administered they

induce light-like phase shifting and *Per* gene expression in the SCN *in vivo* and *in vitro*

(Albers, Liou, Stopa & Zoeller, 1991; Piggans, Antle & Rusak, 1995; McArthur et al.,

2000; Nielson, Hannibal & Fahrenkrug, 2002).
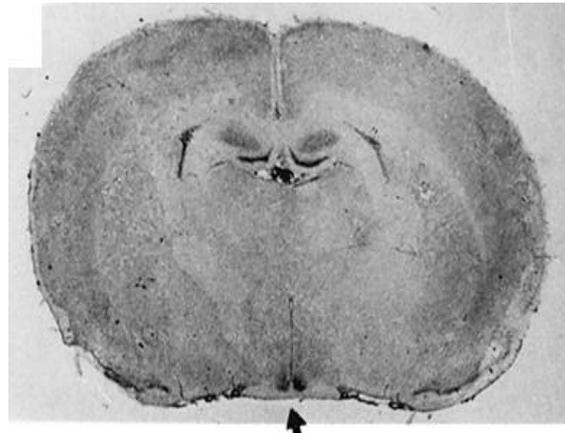
Figure 1.1. The location of the mammalian SCN.
Coronal Section through a rodent brain showing the
location of the SCN indicated by the black arrow
(Kriegsfeld & Silver, 2006).

Afferent Projections to the SCN

The three main afferent projections to the SCN as can be seen from the schematic

Figure 1.2 stem from the RHT, the thalamic intergeniculate leaflet (IGL), the raphe

nuclei. Each of these afferents concentrates in the SCN core.
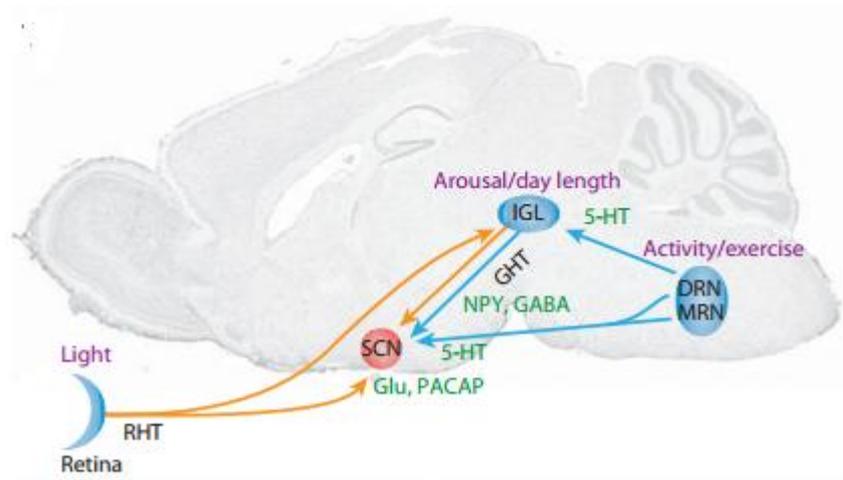
Figure 1.2. Main Afferent Pathways to the SCN.
Arrows which are in yellow illustrate the photic pathways to the SCN. Arrows in blue illustrate the non-photic pathways to the SCN (Dibner, Schibler & Albrecht, 2010).

Projections from the retina allow for the passing of signals to the SCN via a primary visual pathway known as the RHT. The RHT is essential to circadian rhythm regulation and plays a prominent role in non-image forming functions (Jonson et al., 1988). The RHT allows for photic information to be passed to the brain via the retina and along the optic nerve. The retina is comprised of a number of axons and retinal ganglion cells (RGCs) (Foster & Hankins, 2002). The RGCs are distinctively different to those involved in sensory vision and project directly to the SCN where they allow for photic entrainment to occur (Hattar et al., 2002; Moore et al., 1995; Rosenwasser, 2009).

The IGL is situated between the dorsal lateral and ventral lateral geniculate complex (LGN) in the retinorecipient region (Harrington, 1997; Moore & Card, 1994; Morin, 1994). The IGL receives input from the retinal cells whose axons make up the RHT (Pickard, 1982). The projection from the IGL to the SCN is known as the

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genicolohypothamic tract (GHT). Due to its position it is involved in the indirect integration of photic information to the SCN. The IGL contains the geniculate neurons of the GHT. These neurons contain the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and neuropeptide y (NPY), which transmits input to the SCN (Morin & Blanchard, 1995; Card & Moore, 1989; Mantyh & Kemp, 1983; Takatsuji, 1989; Morin & Allen 2006). The IGL plays a minor role of photic phase-shifting events, which signals events in the circadian clock (Moore & Card, 1994; Morin & Pace, 2002). However, it also plays a significant role in the non-photic regulation of the circadian system due to entrainment signals being carried from the SCN via the GHT. Lesions to the IGL results in attenuating the period lengthening response (Morin & Pace, 2002; Rosenwasser, 2009) and the abolishment of phase-shifting responses (Harrington & Rusak, 1986; Janik & Mrolovsky; 1994; Schuhler, et al., 1999), to novel induced wheel running (Wickland & Turek, 1994). Furthermore, the administration of anti-NPY antibodies to the SCN attenuates novel wheel running phase-shifts (Biello, Janik & Mrosovsky, 1994). This provides further evidence that the IGL and its neurotransmitters play a major role in non-photic resetting of the SCN.

The midbrain raphe nuclei provide another indirect pathway to the retinorecipient SCN. The neurotransmitters in the raphe nuclei pathways are involved in mediating non-photic phase shifts particularly arousal (Reppert & Weaver, 2001). Serotonergic (5-HT) is involved in regulating circadian rhymicity with administration of 5-HT receptor agonists attenuating/blocking photic phase shifts by suppressing the release of glutamate (Gannon & Millan, 2007; Rea & Pickard, 2010). Ablation to the serotonerigc afferent SCN pathways results in a reduction of phase-shifting effects for several non-photic cues (Challet, Pevet & Malan, 1997; Edgar, Reid & Dement, 1997).

Peripheral Clocks

In multicellular organisms, clocks and clock-controlled genes are located outside the SCN, in tissue and organs throughout out the body such as the liver, lungs, heart, liver, stomach, spleen and kidneys (Eismann et al., 2010; Yamamoto et al. 2004). As previously outlined the SCN is the master circadian clock at the top of a hierarchically organized system (Mohawk et al., 2012). When the circadian system is organized, the peripheral clocks, receive synchronisation assistance from the SCN. This is, through the SCN transmits signals to light insensitive peripheral clocks. This results in the generation of robust and coherent circadian oscillations among other oscillator populations within the peripheral area (Albrecht, 2012; Gallego & Virship, 2007; Mohawk, Green & Takahashi, 2012; Yoo et al., 2004). Communication signalling from the SCN to peripheral tissues results in the peripheral tissues becoming entrained to the environmental cycle through circadian regulation of gene transcription which allows peripheral tissues to perform appropriate functions at appropriate phases (Yamamoto et al., 2004; Storch et al., 2002). The tissue-specific clocks located within the organs and tissues have the molecular prerequisites required to express self-sustaining oscillations and express the molecular proteins, which are found in circadian oscillations in the SCN (Fonken & Nelson, 2014). The clock genes within the SCN induce circadian rhythms of physiology and behaviour through the synchronisation of peripheral oscillators and their expression of clock genes via neural and endocrine signalling (Guo, Brewer, Champhekar, Harris, & Bittman, 2005; McNamara, Seo, Rudic, Sehgal, Chakravarti & Fitzgerald, 2001; Reddy, Maywood & Karp, 2007). Additionally, as can be seen from Figure 1.3, non-photic zeitgebers signals, such as nutritional signals (Vollmers, Gill, Di Tacchio, Pulivarthy, Le & Panda, 2009) and temperature (Buhr et al., 2010; Son et al., 2011) have the ability to entrain the peripheral clock to the SCN. Although the peripheral clocks do not communicate with each other, they are coupled together via the

SCN. For instance, *in vivo* ablation of the SCN has a significant negative effect on the peripheral oscillators (Akhtar et al., 2002). Furthermore, Lamia et al. (2008) and Dibner et al. (2010) found that BMAL1 knockout mice were no longer able to produce a sufficient amount of glucose within the liver which is required for blood circulation. This resulted in the onset of hypoglycaemia. This illustrates the importance of the liver peripheral clock to the metabolic system. Although, *in vitro,* the SCN can express highly robust circadian rhythms over a long period of time (i.e. one month or more) (Yamazaki et al., 1995) the peripheral clock expresses quickly dampened circadian rhythms which diminish in between 2-7 cycles (Balsalobre, Damiola & Schibler, 1998).
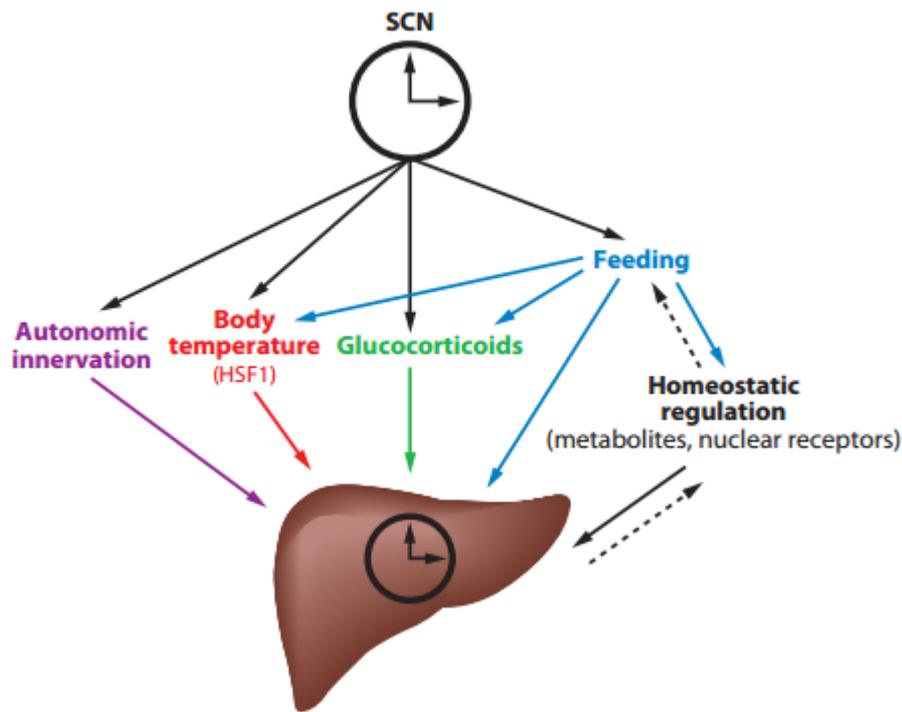
**Figure 1.3. Entrainment pathways from the SCN to peripheral clocks.** The SCN passes information to peripheral clocks via different cues. The peripheral clocks also entrain themselves endocrine signalling (Mohawk, Green & Takahashi, 2012).

In order for the circadian system to function optimally such that the clock information is useful for the entire organism, each of the circadian clocks within each of

the tissues are synchronized to one another and to the 24-hour day (Welsh, Takahashi & Kay, 2010). For this to occur, each tissue is maintained in a stable phase-relationship with each other (Albrecht, 2012). In order for the circadian system to function consistently, it requires (a) that the cellular clock be responsive to stimuli (e.g. input from other cells), (b) the integration of phase information regarding when the stimulus happened into the molecular intracellular clock information and (c) the transferring of clock information to other cells (output). For example, photic information is passed through the retina (input) into the SCN. Here the photic information is integrated to modify information relating to time. This in turn results in an alteration in the onset of certain behaviours and tissue activities such as initiating the sleep-wake cycle (Aubrecht, 2012; Eskin, 1979). It is fundamental that the circadian timing system continuously adapts and synchronises to both the environment and the internal signals of the body in order to organize individual cellular clocks and combine tissue sub-networks into a coherent functional network that regulates behaviour and physiology (Albrecht, 2012).

Molecular Clock

The main components of the molecular clock are the activating transcription factors Brain and Muscle ARNT-like protein 1 (BMAL1), Circadian Locomotor Output Cycles Kaput Protein (CLOCK), the negative regulatory feedback elements encoded by the Period genes (*Per 1,2,3*) and Cryptochrome genes (*Cry 1, 2*) (Gekakis et al., 1998; Zheng et al., 2001). Circadian clocks consist of a cell autonomous oscillator, which is generated by a transcription-translational negative-feedback loop with a delay between stimulus and response (Gallego & Virshup, 2007). This delay is critical in order to maintain periodicity to approximately 24 hours (Reppert & Weaver, 2002). Proteins CLOCK and BMAL1 are the transcription factors, which positively regulate the clock. These proteins dimerize and create a CLOCK-BMAL1 heterodimer. This combination

results in the transcription of the Period genes (*Per1* and *Per2*) and Cryptochrome genes (*CRY1* and *CRY2*) and *Rev-Erba* genes through E-box enhancers which are their own negative. During the first portion of the day transcription is increasing, which simultaneously results in respective repressors accumulating in the cytoplasm thereby restricting transcription. This process is mediated through *Per* and *CRY* being phosphorylated by casein kinase Ie (CKIe) and glycogen synthase kinase-3 (GSK3). They then translocate to the nucleus in a phosphorylation-regulated process and here they combine with CLOCK-BMAL1 heterodimer to inhibit it. For a new biological day to ensue the repressing proteins (*CRY & Per*) must be eliminated. This is achieved through post-translational modifications such as phosphorylation and degradation. The core clock is maintained by the conflicting functions of the orphan nuclear receptors *Rora* and *Rev-Erba* with the former activating BMAL1 expression and the latter inhibiting it. The inhibition of CLOCK-BMAL1 through Per and CRY in the nucleus indirectly results in the activation of BMAL1 which is mediated through inhibition of Rev-erba (Gallego & Virshup, 2007; Reppert et al., 2001; Welsh et al., 2010).

Entrainment

As previously outlined, in the absence of environmental cues the endogenous circadian clock operates with a clock cycle time that is not exactly 24-hours (Zheng et al., 2001). For instance, when conditions are constant the circadian clock expresses rhythms of more than 24 hours. This is referred to as "free-running." In order for 24-hour periodicity to occur the rhythm needs to be synchronised to the external environment (i.e. L/D cycle) to ensure that internal time coincides with external time. This synchronisation is referred to as entrainment and is the process in which circadian oscillators are synchronised to the environment (Colwell, 2011). Entrainment is a fundamental property of the circadian system by which the period of the internal clock (t) is synchronized to the period of the entraining stimuli (T cycle) (Duffy & Wright,

2005). The main feature of entrainment is the maintenance of an appropriate phase relationship between the circadian system, the timing of sleep and wakefulness and environmental time (Duffy & Wright, 2005). The principal *zeitgeber* ("time-giver" "synchronizer") which entrains an organism's activity to a 24-hour solar day is light. Therefore, circadian rhythms are mainly synchronised by the environmental LD cycle (Redlin, 2001). Light falls on the retina stimulating photoreceptors in the eye, which distinguish alterations in the quantity and quality of light over the 24-hour LD cycle. This results in synaptic transmission and innervations of the SCN's core clock cells, activating the proteins involved in the resetting of the transcription-translation feedback loop, which are the underlying foundation of circadian oscillations (Boyce & Barriball, 2010; Meijer & Schwartz, 2003; Reppert & Weaver, 2001). Other non-photic environmental time-cues act as zeitgebers for entraining the clock such as physical activity, social time and meals (Buttgereit et al., 2015).

Phase Response Curves (PRCs)

PRCs indicate the time across the circadian cycle in which a stimulus will have an impact on the circadian rhythmicity. This results in phase-shifts of circadian locomotor activity resetting the phase of the oscillator. PRCs can be characterised into either photic PRCs and non-photic PRCs. Photic PRCs functions elicit phase shifts during the subjective night for example, light stimulation. Non-photic PRCs functions display phase advances during the subjective day in response to forced locomotion and social stimuli (Golombek & Rosenstein, 2010).

Photic PRCs

The photic PRC explains when photic stimulation at different circadian phases result in either a phase-delay, a phase-advance or no alteration to the circadian phase. In nocturnal animals, photic PRCs elicit phase shifts of locomotor behaviour during the period of the subjective night. By contrast a "dead zone" occurs during the subjective

day. This is due to light falling during this phase of the circadian cycle and extra light leads to no alteration in behaviour (Daan & Pittendrigh, 1976). Pittendrigh and Daan (1976) argue for the existence of two mutually coupled oscillators. The first is a morning oscillator, which is accelerated by light and synchronised to dawn and an evening oscillator. It is also decelerated by light and synchronized to dusk. As can be seen, from Figure 1.3 the presention, of photic information during the late period of the subjective night results in the advancement of the morning oscillator with a later shift of the evening oscillator. Under these conditions the offset of locomotor behaviour is advanced (Meijer & Schwartz, 2003). Behaviourally, the presentation of light during the late part of the subjective night results in locomotor activity occurring earlier during the period of the next cycle (Daan & Pittendrigh, 1976; Rusak & Boulas, 1981). Presenting light information in the early part the subjective night results in delaying the evening oscillator which in-turn results in a later shift of the morning oscillator (Meijer & Schwartz, 2003). This results in delaying phase of locomotion which results in the onset of activity in the subsequent cycle occuring later.
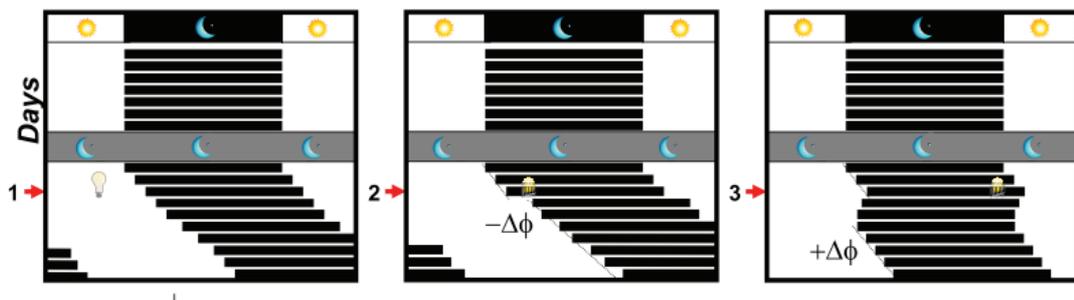
Figure 1.4. Photic Phase Response Curves. (1) Represents behavioural activity response to a light pulse during the subjective day. (2) Administration of a light pulse early during the subjective night which induces a phase delay. (3) Light pulse added late during the night resulting in a phase advance subjective night (Golombek, Rosenstein, 2010).

Non-photic PRCs

Although, the circadian clock does not produce responses to photic stimulation during the subjective day, a wide array of non-photic stimuli has been shown to induce phase

advances during this period (Burke et al., 2013; Hastings et al., 1998). Non-photic stimuli allow for behavioural activity. It is postulated that this behavioural activity results in phase-shifting responses in the SCN (Van & Turek, 1989). Conceptually, it is debated whether behavioural activity induces phase-shifting responses in the SCN or that behavioural activity activates the same pathways. In rodents, locomotor activity rhythms can be entrained by scheduled feeding, restricted exercise, acute exposure to sexual odours and social interaction. Ramkisoensing and Meijer (2015) report that substances and medications (serotonin antagonists, benzodiazapines, opioids) have been associated with increased behavioural activity and interactions with neurotransmitter pathways involved in non-photic resetting i.e., inducing a phase-shift. A phase-advance can be observed as a result of behavioural arousal or the administration of a saline injection during the subjective day period and the presentation of novel running wheels (Hastings et al., 1992). Additionally, as outlined earlier the afferent projections to the SCN such as the IGL and the serotonergic raphe nuclei and its respective constituent neurotransmitters are involved in inducing non-photic phase shifts (Ramkisoensing & Meijer, 2015; Rosenwasser, 2009).

The Visual System

The mammalian eye is a unique complex organ, which is integral to the detection of light. Its most notable function is to serve as an image forming visual system which involved in the conscious perception of images in one's visual field. Its second function is the detection of light for non-imaging forming behaviours, which occur outside of conscious awareness such as circadian photoentrainment, negative masking and pupillary light reflex (Schmidt et al., 2014). Thus, the eye is involved in the regulation of multiple behavioural and physiological functions, which are independent of image formation (LeGates, Fernandez & Hattar, 2014).
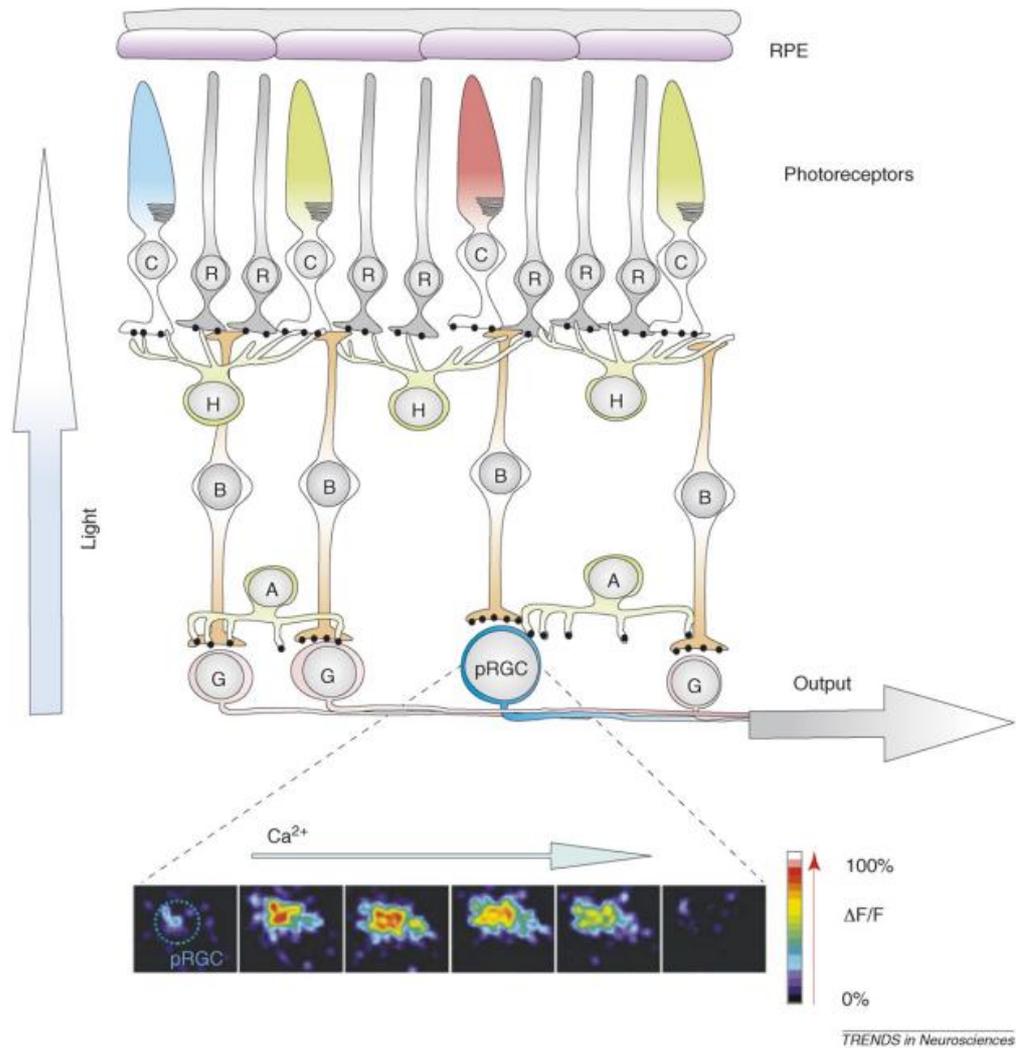
Figure 1.5. Structure of how non-image light is detected. Light passes through the traditional photoreceptors (rods and cones) to the retinal ganglion cells. The melanopsin expressing ipRGCs also detect light directly (Hankins, Peirson & Foster, 2008).

As can be seen from Figure 1.5 light is detected and the signals are passed through the rods and cones. The signals are processed in the retina and relayed via conventional RGCs to visual nuclei such as the dorsal lateral geniculate nucleus (dLGN) (Schmidt, Alam, Chen, Kofuji, Li, Prusky & Hattar, 2014). For many years it was believed that rods and cones were the only photoreceptors in the retina that carried out non-image forming behaviours. However, blind humans, who do not possess the ability to perceive image forming stimuli (not having rods and cones), display a circadian response to light such as the regulation of melatonin secretion (Czeisler, et al.,

1995; Lockley et al., 1997). Further evidence of an additional specialized class of photoreceptors came from animals that had retinal mutations (rd/rd). In these animals all rods were extinct within the retina. However, approximately 5% of cones existed beyond 18 months (Hattar et al., 2006). Interestingly, with this loss of photoreceptors, the rd/rd mice expressed a circadian response to light indistinguishable from those of congenic mice who have phenotypically intact retinas (Foster, Provencio, Hudosn, Fiske, De Grip & Menaker, 1991). These responses included locomotor activity being reduced after a light pulse was administered during the active period (Mrosovsky, 1994; Mrosovsky, Salmon, Foster, McCall, 2000), phase-shifting responses to light remaining intact (Foster et al., 1991) and the detection of light for circadian photoentrainment and pupillary light reflex (Freedman et al., 1999; Lucas et al., 2001). In order to ascertain whether the remaining cones in rd/rd animals were serving as photoreceptors for photoentrainment, animals that had a complete loss of rods and cones were used (rd/rd cl). These animals expressed an ability to photoentrain normally at both a behavioural and neuroendocrine level (Freedman et al., 1999; Lucas et al., 2001). This ability for mice and humans to maintain light-responsive behaviours when rods and cones were not present indicated that additional non-rod/non-cone photoreceptors were present in the mammalian retina (Provencio et al., 2002). A subset (~2%) of photosensitive RGCs, which contains the unique photopigmant melanopsin in response to light were located in the RGCs (Hattar et al., 2002). These cells displayed a light-evoked depoloarization in the absence of rod and/or cone signalling together with molecular alterations to SCN neurons (Hankins, Peirson & Foster, 2007). They also expressed intrinsic light responses when detached from adjacent retinal tissue (Berson et al., 2002). For this reason, these group of cells (known as intrinsically photosensitive retinal ganglion cells) (ipRGCs) were postulated to act as a third class of photoreceptors (Berson et al., 2002; Hattar et al., 2002). The ipRGCs project to many different brain regions involved in

regulating non-image forming functions such as to the SCN to photoentrain circadian rhythms or to the olivary pretectal nucleus (OPN) to control the pupillary light reflex (Hattar et al., 2006).

Evidence from melanopsin ablation studies provides a causal link between melanopsin and the ability of iRGCs to respond to light. For instance, melanopsin knockout mice (Opn4$^{-/-}$) demonstrate weakened phase-shifting, failure for pupil constriction and complete loss of photosensitivity (Lucas, et al., 2003; Ruby et al., 2002; Panda et al., 2002). This also provides evidence that ipRGCs receive light information secondarily via the RGCs from the rods and cones. Rods and cones play a role in photoentrainment. For instance, Opn$^{-/-}$ mice have an ability to photoentrain although at a weakened form (Hattar et al., 2003; Panda et al., 2003). This provides evidence that rods and cones express an ability to compensate for the loss of ipRGC photosensitivity. Results from the rd/rd cl animals suggests that iRGCs can fully compensate for ablation to rods and cones. When Opn4$^{-/-}$ mice are crossed with mice which have complete loss of rods and cones a complete loss of circadian response to light, pupillary reflex, light/dark cycle entrainment occurs (Hattar et al., 2003; Panda et al., 2003). This provides sufficient evidence that these receptors are essential for all non-visual photoentrainment.

After the discovery of the ipRGCs, it was believed that they were a uniform population. Their main function was argued to be for photoentrianment due to their projections to the SCN. However, as can be observed in Figure 1.6 there are at least 5 subtypes of ipRGCs. In rodents, each of these sub-types comprise of different morphological and electrophysiological components (Baver, Pickard & Soollars, 2008; Schmidt & Kofuji, 2009; Hu, Hill & Wong, 2013; Warren, Allen & Brown, 2003; Schmidt, & Kofuji, 2011). The three main characterized subtypes are the M1, M2, M3 ipRGCs (for full review of the subtypes sees Schmidt et al., 2011). These various

ipRGCs contribute differently to non-image and image forming behaviours (Schmidt, Chen & Hattar, 2011). It is, however, the M1 ipRGCs which are of main interest to chronobiologists because of their projections to the SCN and other brain areas involved in the regulation of circadian rhythms such as the subparaventricular zone, the IGL and the venteral lateral geniculate nucleus (vLGN) (Hattar et al. 2006; Schmidt, Chen & Hattar, 2011). Projections are also found at limbic areas of the brain involved with emotion such as the medial amygdala lateral habenula, which is associated with mood regulation (Hattar, Kumar, Park, Tong, Tung & Yau, 2006; Schmidt, Chen & Hattar, 2011). These projections give rise to speculation of an association between light and mood.
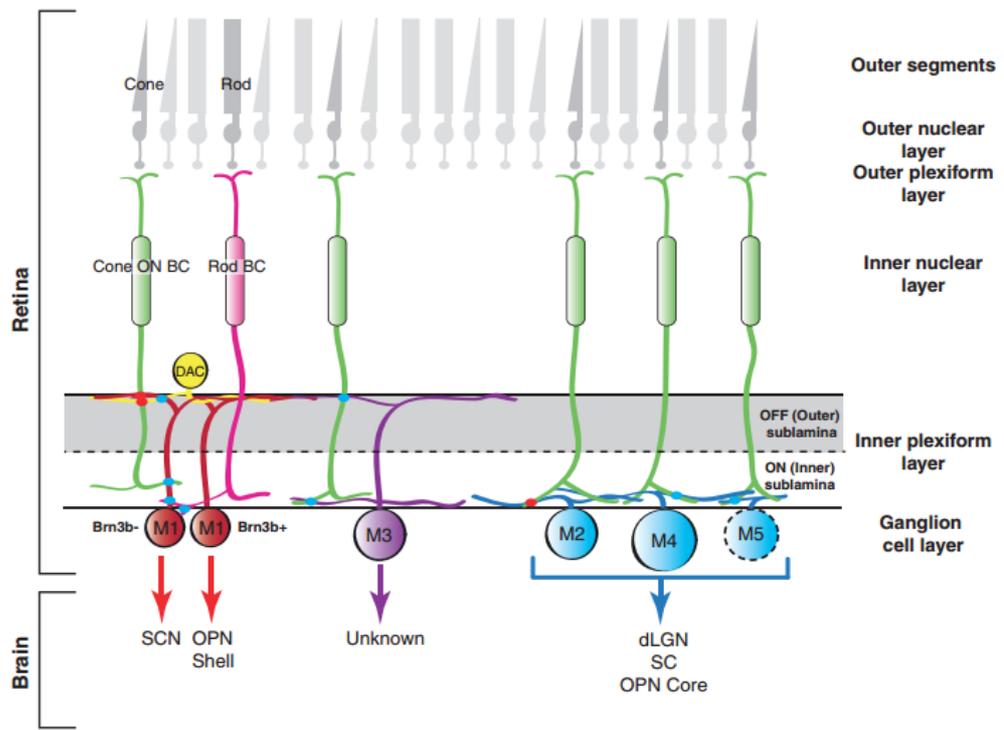
Figure 1.6. Diversity of ipRGCs. The ipRGCs are divided in 5 subtypes. The M1 ipRGCs are of significant importance given that they project to the SCN (Schmidt, Chen & Hattar 2011).

Molecular Visual System

Photic light information is passed through the SCN via the RHT, which arises from a widely distributed population of RGCs (Moore, Spech & Card, 1995). The RHT pathway allows for L/D cycles to be perceived, the entraining of SCN neural activity

which in in turn, allows for the rhythmic secretion of melatonin in the pineal gland (Brainard et al., 2001). The main neurotransmitter of the RHT is glutamate (Glu). The RHT peptides substance P (SP) and pituitary cyclise-activating peptide (PACAP) which are essential for the entrainment process (Chen, Buchanam, Ding, Hannibal & Gillette, 1999). Stimulation of light during the biological night period results in the release of neurotransmitters Glu and PACAP at the terminal synapses of the RHT. This signal is then transmitted to the SCN (Ecker at al., 2010).

The melanopsin-expressing RGCs are sensitive to non-photic light stimulation. When light reaches these cells during the night period, these cells generate action potentials that travel down the RHT innervate the SCN (Colwell, 2011; Ecker, 2010). In the RHT, axons of special photosensitive RGCs project to the SCN. As can be seen from Figure 4 stimulation of light during the night results in the RHT axons releasing Glu and PACAP at synaptic contacts with SCN neurons increasing firing too (Morin & Allen, 2006). The release of Glu induces kinase activation and the expression of early immediate genes cause a phase-shift (Albrecht, 2012). The neuropeptide PACAP augments the effects of Glu. Glu has an effect on n-methyl-d-aspartate receptor (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors resulting in depolarizing the membrane resulting in a calcium influx. The activation of calcium in the SCN neurons results in the arousal of various kinases leading to the phosphorylation of calcium response element binding protein (CREB). This activation of CREB results in transcription by binding calcium/cAMP response elements of genes and in collaboration with other proteins such as the CREB binding protein (Welsh, Takahashi & Kay, 2010). Within the SCN the phosphorylation of CREB at SER133 AND SER142 occurs within minutes of a light pulse where it is translocated into the nucleus. From there it attaches to the CREBs in the promotor regions of *c-Fos*, *Per1* and *Per2*, which induces transcription of these genes over a

number of hours (Colwell, 2011). However, this only occurs during the subjective night when light pulses have the power to shift the phase of the clock (Gau et al., 2002). This indicates that CREB-mediated transcription is required for photic resetting of the SCN clock.

Light and Circadian the Circadian Clock

As outlined, light is the most potent *zeitgeber* for entraining the circadian clock. Light exerts an ability to induce phase-shifting effects on the clock along with alteration of core clock proteins (Ramkisoensing & Meijer, 2015). These effects of light are most potent during the biological night when the clock is most sensitive to light (Pittendrigh & Daan, 1976). However, these effects are differential and are dependent on the timing of exposure to light (Porterfield, Piontkivska & Mintz, 2007). Due to the robustness of the clock's overt rhythms, the timing of the light stimulus imposes a critical influence on the direction and magnitude of response to the light stimulus (Duffy & Czeisler, 2009). For instance, when light is presented early or at the beginning of the subjective night, it has a phase-delaying effect, resulting in delaying the offset of *Per2*. In contrast, light presented during a period late into the subjective night induces a phase-advance of the molecular oscillator resulting in advancement in the quick onset of the *Per1* rhythm and acutely heightening mRNA transcription (Pittendrigh & Daan, 1976; Schwartz, Tavakoli-Nezhad, Lambert, Weaver & de la Iglesia, 2011; Shigeyoshi et al., 1997). Light presented during the late period of the night does not result in any readily detectable induction of *Per 2* (Zylka, Shearman, Weaver & Reppert, 1998). However, when light was presented during the day it had no effect (Meijer et al., 1998). These effects are observed in both diurnal and nocturnal rodents along with humans (Jewett et al., 1997).

After the phase-shift, the rhythm resumes the previous period (i.e., before a light pulse was applied). The phases-shifting capacity of a limit cycle oscillator is dependent

upon the amplitude. Low amplitude oscillators have more phase-shifting capacity compared to high-amplitude oscillations (Ramkiosoensing, Gu, van Engeldorp Gastelaars, Michel, Deboer, Rohling, & Meijer, 2014). The magnitude of the light-induced phase-delays and advances is dependent on light intensity and on the duration of light exposure (Meijer et al., 1992). These phase-dependent responses which are found in both uni- and multicellular organisms are fundamental to the organism's ability to entrain to a new light cycle.

Single-unit recordings have found that ~30% of SCN neurons, located in the retinorecipient, are light responsive. A majority are light-activated and a minority are light suppressed (Meijer et al., 1986). When retinal illumination occurs the light-responsive SCN neurons exhibit an increase in electrical impulse frequency (Meijer, Watanabe, Schaap, Albus, & Detari, 1998; Drouyer, Rieux, Hut, & Cooper 2007; Nakamura, Fujimura, Ebihara, & Shinohara, 2004; Mure, Rieux, Hattar & Cooper, 2007). When light exposure is provided the firing rate of SCN neurons increases significantly. However, more increases are found during the night compared to the day (Meijer, Watanabe, Schaap, Albus & Detari, 1998). The conceptual understanding for the difference in firing rates is that during the day period these circadian pacemaker neurons are already electrically active and the extra excitatory input from the RHT would not result in extra action potentials. However, in contrast during the night-time they are inactive and administration of light results the generation of action potentials (Meijer, et al., 1986).

Electronic Light

Before the advent of electronic light, the rotation of the earth about its axis gave rise to the predictive natural L/D cycle. However, with technological advances this gave rise to lighting devices which unnaturally extend the light period. Currently, 99% of individuals living in both the United States and Europe are exposed to light pollution

with a further 62% of the world's remaining population also exposed (Navara & Nelson, 2007; Fonken & Nelson, 2011). The increase of electronic light has brought about a significant number of advantages to modern society. Extending the period of light into darkness through the means of artificial light has taken away the negative stigma of night being a period associated with crime, sickness, sleep and death (Ekirch, 2005). Electronic lighting played a major role in the industrial revolution by developing industry and technology, allowing the extension of the workday into the night, further boosting economic development. Additionally, electronic lighting has given individuals a choice to self-select their sleep-wake cycles instead of being forced to comply with the illumination offered through the solar cycle. Despite these advantages, the widespread use of electronic light occurred before there was a strong understanding of chronobiology and the effects of light on the circadian system. Its widespread use occurred so rapidly that it was impossible for evolutional adaptations to occur to counter any of the known deleterious physiological and behavioural changes (Fonken & Nelson, 2013). As indicated earlier, LAN exerts the strongest influence in resetting the endogenous clock. This is achieved through phase-shifting the clock in order to adapt to the illumination signals provided to the SCN (Karatsoreos, 2014). This also results in the inhibition of melatonin and abnormal phase relationships in molecular, cellular and physiological responses (Bedrosian, Weil & Nelson, 2012). Light intensities less than 180 LUX cause phase-shifts (Boivin, Duffy, Kronauer & Czeisler 1996). and the circadian system is sensitive to light intensities below this threshold (Gorman, Evan & Elliott, 2006).

Wavelength-Sensitivity to Light

The types of lighting available have also changed in conjunction with the rise of artificial light. These changes occurred before our better understanding of how specific wavelength light emitted can disrupt the circadian system. Incandescent bulbs were the

first unnatural light emitting sources, which produced longer-yellow wavelength light. Due to their high-power consumption and short life span, these lights were replaced with fluorescent light bulbs that emit short-wavelength light. As can be seen from Figure 5 each of the photoreptors located within the retina having a unique spectrum of light to which they are most sensitive. (Lucas et al., 2014; Roecklein et al., 2009). Melanopsin, the photopigmant of the ipRGCs, which is involved in regulating circadian rhythmicity is most sensitive to short wavelength blue light (460-480nm) emitted from fluorescent bulbs and light emitting diodes (LED) light. It is less sensitive to red light (>600 nm) (Brainard et al., 2001; Dacey et al., 2005; Lockely et al., 2003; Newman et al., 2003). Lockley et al. (2006) demonstrated that the circadian system is sensitive to a specific range of light. When comparing light at 460nm and 555nm they observed that melatonin supersession was greatest under administration of 460nm light. At equal photon densities, compared to long wavelength light, 460nm short wavelength light evokes circadian phase-shifts (Lockley et al., 2003), suppresses and phase advances the offset of the human melatonin rhythm (Brainard et al., 2001; Chellappa et al., 2011; Revell, Arendy, Terman & Skene, 2005; Wright, Lack & Kennaway, 2004), reduces subjective sleepiness (Cajochen, Munch, Kobialka, Krauchi, Steiner & Oelhafen, 2005), increases alertness (Lockley, Evens, Scheer, Brainard, Czeisler & Aeschbach, 2006), increases body temperature (Cajochen, Munch, Kobialka, Krauchi, Sreiner, Oelhafen, Orgul & Wirz-Justice, 2005) and activates the expression of the circadian clock gene *Per2* in humans (Cajochen, Jud, Munch, Kobialka, Wirz-Justice & Albrecht, 2006). Light exposure even when they eyes are closed results in the suppression of melatonin (West et al., 2010). Even at dim levels the wavelength of light has the potent influence to reduce the amplitude, power and total activity of a circadian behavioural rhythm (Bedrosian et al., 2013). This indicated the potent influence that these spectral wavelengths have on the endogenous clock and its output.
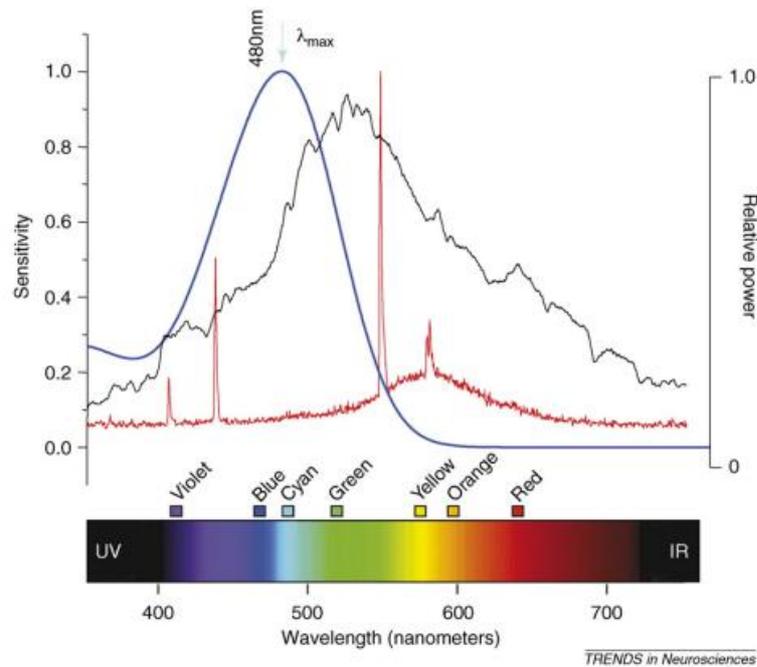
Figure 1.8. Melanopsin Spectral Wavelength Sensitivity. The melanopsin RGCs are most sensitive to short-wavelength blue light with peak sensitivity of around 480nm (see blue line). Peak sensitivity to natural daylight (indicated in black line) and red lighting (indicated in red line) is lower (Hankins, Peirson & Foster, 2007).

Electronic Light & Circadian Rhythms

Classic studies from Aschoff (1960) found that within both uni- and multicellular organisms the L/D cycle exerts the most potent influence in synchronising the endogenous clock to the external environment. Traditionally, human biological clocks were synchronised to a consistent pattern of light and dark that was related to the solar cycle. Although still present, individuals before electronic light individuals were reliant on natural daylight. During midday periods of natural daylight, colour temperatures range from 6000 K when conditions are overcast to 25000 K when skies are completely clear. When evening draws in, the colour temperature of the sun is reduced to 2000 K (Schubert & Kim, 2005). This variation in sunlight colour temperature of natural light entrained the internal clock to the external environment resulting in optimal rhythmicity. However, with a greater dependence on round the clock societies due to longer work and social schedules this has led to the environmental L/D cues becoming increasingly

absent due to human manipulation extending the period of the light period during natural darkness. This results in a misalignment between internal biological processes and the external environment.

In contrast to natural light, artificial light at night (LAN) is ill-timed and dim. It expresses a spectral wavelength exerting significant control over our circadian system resulting in altered schedules and a reduction in the numbers of sleep hours (Boivin, Duffy, Kronauer & Czeisler, 1996; Redlin, 2001). Wright et al. (2013) provided evidence that reduced exposure to natural sunlight and increased use of electrical lighting results in changes to circadian physiology, which induces changes to the timing of sleep and wakefulness, indicating that natural light is a more potent *zeitgeber* for the internal circadian clock than is electrical lighting. LAN obscures entrainment of biological processes to external conditions which is disruptive to all aspects of our endogenous circadian rhythmicity. This is due to artificial light having the ability to reset the circadian clock (Dean & Pittendrigh, 1976; Pauley, 2004) resulting in a misalignment of physiology, behaviour and environment. In nocturnal rodents, administration of a light pulse during the night time quickly reduces activity levels (Redlin, 2001), while in contrast to diurnal animals light has the ability to trigger activity (Rajaratnam & Redman, 1999). This indicates that for nocturnal animals the perception of light during active periods ensues a contradiction to the endogenous timing information. These behavioural observations in nocturnal rodents support the idea that light induces an immediate adjustment to behaviour towards a behavioural state which would normally be performed during daylight (Redlin, 2001). This indicates how critical it is that light information is correctly timed for optimal biological timekeeping to occur.

Light has a deleterious effect on the secretion of hormones which control the circadian system within the body. For instance, melatonin is rhythmically secreted from

the pineal gland during the dark period. However, its secretion is inhibited by light in an intensity-dependent manner demonstrating that its expression is influenced by light (McIntyre et al., 1989). In humans, exposure to ~45LUX of light for a one-hour period significantly reduces plasma melatonin concentrations by a significant 60% (Brainard et al., 1988). In Siberian hamsters, exposure to light at low levels such as 1.08LUX can inhibit pineal melatonin content (Brainard et al., 1982). Suppressed melatonin levels during the night period can have negative implications on physiological timekeeping. Multiple evidence couples melatonin in the regulation of peripheral clock gene oscillations, setting and maintaining circadian times in tissues throughout the body (van Gall, Weaver, Moek, Jilg, Stehle & Korf, 2005) and re-entraining disrupted rhythms (Pandi-Perumal et al., 2006). Thus, dysregulation of the melatonin rhythm mediated by light can lead to the disorganisation of the body's time of day information, disrupt circadian clock gene expression and hormone secretion in clocks outside of the SCN. As can be seen from the evidence presented above, artificial LAN has profound effects on the clock properties of the circadian system which manifests into aversive health conditions such as heart disease, cancer and metabolic dysfunction (Fonken, Kitsmiller, Smale, & Nelson, 2012).

Incidence of Depression

Depression is the world's most common mental illness (Ustan et al., 2004) with lifetime prevalence of around 12-20% in both the European Union and the United States of America (Alonso et al., 2004; Blendy, 2006; Kessler et al., 2003; Marneros, 2009). In 2012 the World Health Organisation (WHO) estimated that globally 350 million people suffer from the disorder. By 2030 the WHO predicts that depression will be the world's second most debilitating disorder with the first being HIV/AIDS and being followed by ischaemic heart disease. Economically, the cost of depression in the EU has been estimated at 1% of the total economy. A Report on Public Expenditure in Ireland (2012)

stated that an annual public expenditure of €32 million had been spent on SSRI antidepressants and €25 million on other antidepressant agents. Due to both the economic cost and the debilitating impact depression has on sufferer's lives it is a significant public health priority. By relying on novel drug treatments, psychological therapies and environmental lifestyle changes, individuals can prevent and reduce the burden of this disease.

Recently there has been a significant increase in the rate of clinical depression. However, the reasons for the rise in occurrence remain unknown. Some argue that better defined diagnostic criteria and improved diagnosis has resulted in the heightened rate of occurrence (Bedrosian, Fonken, Walton, Haim & Nelson, 2011). However, other variables are involved that account for the significant increase. Although, it is well accepted that genetic predisposition plays a significant role in the manifestation of the disorder, the heightening incidence has occurred too rapidly for genetic shifts in human populations to account for the increased incidence.

The environment also plays a critical role in the development and maintenance of the disorder. It is plausible that recent environmental changes may account and act as a catalyst in heightening presentation of the disorder. One such factor may be the increase of artificial LAN. LAN may induce a misalignment between the internal timing system and the external environment resulting in circadian dysfunction. This leads to the development of depressive behaviours. (Bedrosian & Nelson, 2013; Dumont & Beaulieu, 2007; LeGates et al., 2014). This plausible explanation comes from ecological evidence whereby mood disorders are significantly more prominent when rhythms are disrupted by alterations in day-night schedules (Rosenberg & Doghramji, 2011) and transmeridian travellers suffering from "jet lag" (Srinivasan et al., 2010).

One group of the population who experience significant circadian dysregulation and report a higher incidence of depressive behaviours relative to the rest of the

population are shift-workers (Driesen, Jansen, Kant, Mohren, & van Amelsvoort, 2010; Rajaratnam et al., 2013; Roesenberg & Doghramji, 2011; Scott et al., 1997). Shift-workers account for 20% of the workforce. They are exposed to chronic levels of bright artificial light during the biological night (Rajaratnam & Arendt, 2001). Healy, Minors and Waterhouse (1993) found that nurses who worked night shifts for three-month periods have a significantly higher incidence of feelings of hopelessness, loss of control and apathy. With long term exposure to shift work over a 20-year period there is an increased lifetime risk of depression (Scott, Monk & Brink, 1997; Healy, Minors & Waterhouse, 1993).

Relationship between the Circadian Clock & Depression

Abnormalities in sleep and circadian rhythms are prominent symptoms in depression (McClung, 2011). Emens, Arntz and Rough (2009) report that phase-delays are associated with depressive symptoms with the severity of depression corresponding with the extent of the phase-delay. Poor quality sleep is reported weeks before the recurrence of depressive symptoms (Perlis, Giles, Buysse, Tu & Kupfer, 1997). A significant proportion of patients frequently report having biological rhythm-related symptoms such as disturbances to the sleep-wake cycle, disturbed sleep-patterns (Boyce & Barriball 2010; Tsuno et al., 2005), diminished slow-wave sleep, lower rhythm amplitude and diurnal mood changes (Li et al., 2013). At a molecular level neurotransmitters and hormones are implicated in the development and maintenance of depression and exhibit circadian oscillations. For example, the prominent neurotransmitters associated with depression (such as serotonin, norepinephrine, and dopamine and their accommodating receptors) express circadian rhythm in their levels, release and synthesis-related enzymes (Weber et al., 2004; Malek et al., 2005; McClung, 2007). Disruption to circadian rhythms have a knock on effect on the circuit of these neurotransmitter rhythms which may potentially have exacerbating effects in

inducing depressive-symptoms (for full review see McClung, 2007). Additionally, depressed individuals exhibit increased levels of plasmatic cortisol which is associated with stressful conditions. This prolonged hypercortisolemia is coupled with anhedonic behviour a symptom reminiscent of depression. In depressed individuals, the circadian rhythm of cortisol is disrupted indicating that changes in rhythm may induce depressive like behaviour (Salgado-Delgado et al., 2011 Soria et al., 2010; McClung, 2007).

Clock genes, which are essential in creating and controlling circadian rhythms, are involved in the regulation of monoaminergic pathways (Hampp et al., 2008), whose implication in the pathophysiology and treatment of depression is well reported (Belmaker & Agam, 2008). Animals, which harbour mutations in one or more of the clock genes, exhibit symptoms reminiscent of depression (For review see Landgraf, McCarthy, & Welsh, 2014). Single nucleotide polymorphisms in circadian clock genes are linked to the onset depression. (Lavebratt et al., 2010; McCarthy, Nievergelt, Kelsoe & Welsh, 2012; Soria et al., 2010; Takahashi, Hong, Ko, McDearmon, 2008). Human genetic studies association studies couple clock genes with mood disorders (Benedetti, Serretti, Colombo, Barbini, Lorenzi, Campori & Smeraldi, 2003).

Most of the successful antidepressant medication (such as fluoxetine) and mood-stabilizing medication (such as lithium) have an effect on circadian rhythms. This is achieved through shifting, resetting and stabilising the circadian rhythm (Abe et al., 2000; Sprouse et al., 2006). A reduction in melatonin which is under direct control of light has been associated with mood (Crupi et al., 2010). Pharmacological effects of the antidepressant agomelatine, a melatonin-receptor agonist, and serotonin, a (5-HT$_{2C}$) receptor antagonist (Kennedy & Rizvi, 2010) have been shown to increase the amplitude of circadian rhythms in the rest-activity cycle along with effects on sleep while simultaneously reducing depressive symptoms (Kasper et al., 2010; Ramierez-Rodriguuez et al., 2009). Administration of melatonin has direct actions on the

hippocampus, which is an area of the brain vulnerable in individuals with depression (Musshoff et al., 2002). Melatonin administration restores the reduction in spine density (Ramierez-Rodriguuez et al., 2009) and increases the production of neurotrophin (Kong et al., 2008). Given this link between melatonin and depression, as well as LAN supressing melatonin levels, it is plausible that depressive behaviour may occur as a direct result of melatonin suppression. Additionally, chronotherapeutic interventions targeting biological rhythms, such as sleep deprivation, sleep hygiene, and Cognitive Behaviour Therapy for Insomnia (CBTI) have been effective for the treatment of major depression (Kripke, Nievergelt, Joo, Shekhtam & Kelsoe 2009). This provides evidence of the role of the circadian system in mood disorders.

LAN and Depression

A significant number of studies have investigated whether alterations to the light environment results in changes to circadian rhythms which in turn induce depressive behaviour (LeGates et al., 2014). During the summer months, countries located at northern latitudes experience sunlight for nearly 24h each day. During this period, however, a significantly higher proportion of violent suicides are observed, indicating that even ecological natural light occurring throughout the night can have deleterious consequences on mood and behaviour (Egeland & Hostetter, 1983). As stated, epidemiological studies demonstrate that shift-workers who are exposed to chronic levels of bright light during the biological night present more frequently with depression (Scott, 1997). Furthermore, fluctuations to the L/D cycle due to transmeridian travel can also catalyse the onset of depression (Salgado-Delgado, Tapia Osioria, Saderi & Escobar, 2011).

Alterations in the circadian rhythm in rodents due to constant light results in depressive-like behaviours such as anhedonia and behavioural despair and lower weight gain (Fonken et al., 2009; Ohta et al., 2005; Tapia-Osorio et al., 2013). Hormonal

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differences associated with depression, such as a reduction in melatonin and increased corticosterone, have been observed (Tapia-Osoria et al., 2013). These findings of depressive-like phenotypes have been mirrored by LeGates and colleagues (2012) who presented light to mice at abnormal times (L/D 7:7) and resulted in the increase of depressive-like behaviour. Interestingly, Fonken and collegues (2009) found that when animals were provided with a light escape tube during constant light paradigms these animals exhibited significantly more behavioural despair as measured by the Forced Swim Test (FST) and reduced anxiety as measured by the Elevated Plus Maze test (EPM). This indicates the effects that constant light has on depressive-like phenotype expression.

The effects that LAN has on inducing alterations to circadian rhythms which in turn results in the development of depression is argued to be mediated by the non-image forming ipRGCs. This association comes from two-lines of evidence. First, exposure to chronic levels of dLAN emitting short-wavelength light - as indicated earlier, had significant effects on the circadian system - resulted in alterations to locomotor behavioural activity and induced depressive-like behaviours. These results were not observed when animals were exposed to red dLAN lighting, which ipRGCs are minimally sensitive to (Bedrosian et al., 2013). Further evidence that ipRGCs are mediated in the development of depression come from LeGates and colleagues (2012) who observed that mice who were ipRGCs defective did not express depressive-like behaviour after an aberrant light schedule compared to wild type animals (LeGates, Altimus, Wang, Lee, Yang, Zhao, Kirkwood, Weber & Hattar, 2012).

Constant Light and Circadian Rhythmicity

In nocturnal animals, constant light has deleterious effects on circadian rhythmicity. This is due to exposure to light during the sleep-phase instilling locomotor activity. This results in disruption to the sleep-wake temporal patterns and induces circadian (Aschoff,

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1981; Fonken et al., 2009; Ohta et al., 2005; Pittendrigh & Daan, 1976; Tapia-Osorio, Salgado-Delgado & Angeles-Castellanos, 2013). Ohto, Yamazaki and McMahon (2005) investigated possible reasons why circadian arrhythmicity occurs during constant light. They observed that the clock neurons become desynchronized. However, that did not interfere with their ability to generate circadian rhythms. Molecularly, constant light results in the loss of c-Fos activity within the SCN. Tapia-Osorio et al. (2013) argued that the decreased levels of neuronal activity typically produced by light in the SCN are attenuated and possibly its capacity to drive rhythmicity as a biological clock.

An abundance of research has successfully demonstrated that alterations to the natural L/D cycle as a result of LAN exposure induce circadian dysregulation and in turn depressive behaviours. This evidence comes from epidemiological evidence of shift workers exposed to LAN (Scott, 1997) and experimental evidence from those travelling within different time zones who experience fluctuations to their L/D cycle (Selgado-Delgado et al., 2011). Further support has been provided by experimental evidence which demonstrated the deleterious effects of constant light both on circadian rhythmicity and the onset of depressive-like behaviour (Fonken et al., 2009; Ohta et al., 2005; Tapia-Osorio et al., 2013). However, few studies have investigated if similar effects are induced by dim light-at-night (dLAN) (Bedrosian et al., 2011; Bedrosian et al., 2012; Bedrosian et al., 2013; Fonken et al., 2012). This is of significant ecological interest given that throughout the biological night humans are routinely exposed to low level of light from various technology sources alongside exposure florescent lighting penetrating through the bedroom windows (Cho, Ryu, Lee, Jim, Lee & Choi, 2015). These light sources emit short wavelength light which the ipRGCs are most sensitive to (Brainard et al., 2001). Given the profound deleterious effects observed under bright light (For review see Cho et al., 2015), it could be possible that similar effects are observed under dim levels.

dLAN and Circadian Rhythmicity

A small number of studies have investigated the effects that dLAN exposure at levels 5LUX has on circadian rhythmicity. Findings observe that both Siberian hamsters and diurnal Nile Grass rats housed dLAN remain entrained to the light cycle and remain rhythmic (Bedrosian, Galan, Vaughn & Nelson 2013 Fonken, et al., 2010; Fonken, Kitsmiller, Smale, & Nelson, 2012). However, results have been inconsistent regarding whether the amplitude of the rhythm is altered during dLAN exposure. Siberian hamsters housed under dLAN expressed a reduction in the power of the rhythm (Bedrosian, Galan, Vaughn & Nelson, 2013; Bedrosian, Weil & Nelson, 2013; Fonken, Weil & Nelson, 2013) but no reduction has been observed in diurnal Grass rats (Fonken, Haim & Neslon, 2012). After one-week re-exposure to a L/D cycle the reduction in power of the locomotor activity rhythm was returned back to levels similar to that observed in animals housed in L/D. Inconsistent results have been observed in studies using Siberian hamsters regarding the effects dLAN has on total activity. Some studies observe reductions in total activity (Bedrosian, Weil & Nelson, 2013; Bedrosian, Vaughan, Galan, Daye, Vaughen, Weil & Nelson, 2013) while no reductions in total activity are observed in others (Bedrosian, Galan, Vaughn, Weil & Nelson, 2013; Fonken et al., 2012). When animals were housed under red dLAN no differences in the power of the rhythm or total activity were observed (Bedrosian, Vaughn, Galan, Weil & Nelson, 2013). This suggests that individuals can be exposed to a specific dLAN lighting without having deleterious consequences to circadian rhythmicity.

Although dLAN animals exhibited an ability to remain entrained to the light cycle exposure to dLAN elicits alterations to core clock genes and proteins in the SCN and peripheral clocks. Exposure to dLAN results in the weakening of the *Per1* and *Per2* rhythm and protein expression in the SCN of Swiss-Webster mice and Siberian hamsters (Bedrosian, Galan, Vaughn, Weil & Nelson, 2013; Ikeno, Weil & Nelson,

2014; Fonken, Aubrecht, Melendez-Ferandez, Weil & Nelson, 2013; Shuboni & Yan, 2010). Ikeno and colleagues (2014) found that activity counts were significantly different between the light and the dark phase however, no differences in activity counts were observed under L/dLAN. It is plausible that the presence of dLAN interferes and disrupts the typical regulation of the expression of *Per1* which results in blunting locomotor activity rhythms. This was due to average activity counts not being significantly different between the light and dLAN phase compared to animals housed in a L/D cycle. Additionally, dLAN results in a suppressed expression of BMAL1, *Per1*, *Per2, Cry1*, and *Rev-Erb* in the liver of male Swiss-Webster mice (Fonken et al., 2013). The alterations to clock gene expression as a result of dim light exposure do not lead to disruptions in locomotor activity rhythms (Fonken & Nelson, 2013). However, these alterations in clock genes in the peripheral areas may have deleterious implications for metabolism which will be discussed later.

dLAN and depressive-like Behaviours

A handful of animal studies have examined the deleterious consequences of dLAN in inducing depressive-like behaviours. A small number of studies conducted on ovariectomized female Siberian hamsters have demonstrated exposure to dLAN is sufficient to induce depressive-like phenotypes in the domains of anhedonia and behavioural despair (Bedrosian et al., 2011; Bedrosian et al., 2012; Bedrosian et al., 2013). Fonken and colleagues (2013) found that in melatonin secreting C3H/HeNHsd mice, dLAN exposure resulted in both increased expression of anhedonic-like behaviour and behavioural despair as measured by the FST. Furthermore, these animals displayed increased defecation which is interpreted as a stress-related autonomic activity (Julio-Pieper, O'Mahony, Clarke, Bravo, Dinan & Cryan, 2012). Fonken and colleagues (2012) observed that diurnal Nile Grass rats expressed anhedonic behavior but not elicit behavioural despair. Borniger, McHenry, Salloum and Nelson (2014) exposed both

male and female Swiss-Webster mice to dLAN prenatally and three weeks after they were born and found that these animals expressed higher anxiety-like behaviour and fearful responses however, they did not express any differences in behavioural despair or anhedonic-like behaviour. Interestingly, it is argued that depressive-like behaviour is mediated through the ipRGCs as chronic exposure to short-wavelength light, to which melonopsin is most sensitive to elicited depressive-like behaviours. These depressive phenotypes were not observed in animals housed under red dLAN (Bedrosian et al., 2013).

Bedrosian, Weil and Nelson (2012) found that administering the antidepressant citalopram to animals housed under dLAN conditions resulted in reduced behavioural despair as measured by the FST. This provided predictive validity to this environmental model of depression. Additionally, Bedrosian, Weil and Nelson (2013) reported that depressive-like behaviour was ameliorated within two weeks when animals were re-accommodated under a L/D cycle. Although, the above studies have suggested that dLAN can induce depressive-like phenotypes, findings between species have elicited different behavioural responses on the depressive measures. For example, while dLAN elicited behavioural despair in Siberian hamsters no differences were observed in Swiss-Webster mice. This calls into question the generalizability of results given the inconsistent findings.

dLAN and Molecular Mechanisms

The hippocampus is one of the primary sites involved in the pathophysiology of major depressive disorder (MDD)(Duman, Heninger & Nestler, 1997; Malberg, Eish, Nestler & Duman, 2000). Humans with a clinical diagnosis of depression demonstrate hippocampal atrophy, volume loss and grey matter alterations (Brenner, Narayan, Anderson, Staib, Miller & Charney, 2002; Frodl et al., 2002). In humans, a reduction in hippocampal spine density (Law, Weickert, Hyde, Kleinman & Harrison, 2004) has also

been linked to depression via the neurotrophic hypothesis, which suggests that alterations in the plasticity of neuronal pathways is associated with a depressive pathology (reviewed in Altar, 1999). In validated animal models of chronic stress and depression, a reduction in hippocampal dendritic spines and a decrease in dendritic complexity is observed (Hajszan et al., 2009; Hajszan et al., 2005; Hajszan et al., 2010). Administration of antidepressant treatment increases CA1 spine density in rats thus providing predictive validity to these models (Hajszan et al., 2005; Norrholm and Ouimet, 2001). In similar models, the expression of brain-derived neurotrophic factor (BDNF) is significantly inhibited within the hippocampus (Murakami, Imbe, Morikawa, Kuba & Senba, 2005). BDNF is fundamental for the survival of neurons and the proliferation of NPCs (Kim, Li, Hempstead & Madri, 2004; Sakata et al., 2012). Reductions in BDNF expression therefore have significant negative consequences for the structure of the brain. Decreased hippocampal BDNF expression is observed in the brains of suicide victims (Pandey et al., 2008). The administration of antidepressant medication results in an increase of BDNF expression (Altar, 1999; Duman & Monteggia, 2006).

The area of the brain most prone to inflammation is the hippocampus (Han & Yu, 2014). This is due to the high expression of receptors for pro-inflammatory cytokines, such as interleukin (IL) 1B and tumor necrosis factor (TNF). Inflammatory cytokine response is associated in the pathogenesis of depression (Bedrosian, Weil & Nelson, 2012). One third of human patients treated with recombinant human cytokines develop MDD. In addition to this, MDD presents more often in patients with inflammatory disorders in comparison with the general population. dLAN results in the increase of hippocampal cytokine expression and a reduction in BDNF expression (Bedrosian et al., 2013). These molecular changes occur simultaneously with an increase in depressive-like behaviours. Building upon these results, it has been found

that inhibitors to these cytokines may result in the reduction of depressive symptoms (Bedrosian et al., 2013).

Changes in the dendritic morphology of CA1 pyramidal neurons in the hippocampus are associated with the pathogenesis of affective disorders. Bedrosian, Fonken, Walton, Haim and Nelson (2011) found that in ovariectomized hamsters dLAN exposure was sufficient to reduce the density of dendritic spines. No alterations to the length and cell body size of the dendrites were observed. Studies have reported correlations between the density of CA1 dendritic spines and depressive–like phenotypes (Bedrosian et al., 2013; Fonken, Kitsmiller, Smale & Nelson 2012). This suggests a functional role for decreased CA1 spine density and depressive-like behaviours which is induced by dLAN exposure. As indicated earlier, the spectral wavelength of light also impacts on the endogenous clock which in-turn has effects on molecular on areas of the brain. Bedrosian, Vaughn, Galan, Dayle, Zachary, Weil and Nelson (2013) found that blue and red dim-light led to a reduction in dendritic spine density along with alterations indicating that the type of spectral wavelength is important. However, CA3 and DG neurons failed to show any morphological changes in response to dLAN. Bedrosian, Zachary, Weil and Nelson (2012) found that the administration of antidepressants medication resulted in CA1 dendritic spine density being moderately restored in animals housed under dLAN.

Neural Stem Proliferation & Neurogenesis

For most of the mammalian brain, the development of neurons is restricted to the discrete developmental period. However, two areas of the brain show exception (subgranular layer and subventricular zone of the dentate gyrus) and continue to generate new neurons throughout the post-natal period and into adult life (Gage et al., 1995; Lieberwith, Liu, Jia & Wong, 2012; Van Praag et al., 2002). This ability to generate new neurons from the replication and maturation of neural progenitor cells

(NPCs) is referred to as neurogenesis (Boldrini et al., 2012). Specifically, it occurs in the subgranular layer and subventricular zone of the dentate gyrus located within the hippocampus (Eriksson et al., 1998). In the rodent brain, circa 250,000 new neurons, or approximately 6% of the granule cell layer, are formed each month (Cameron and McKay 2001). The number of new neurons is smaller in primates than rodents (Gould et al 1999; Kornack & Rakic 2001).

Neural stem cell proliferation is the step before neurogenesis, as not all proliferating cells become neurons. Progenitor cells are accommodated and travel within the subgranular layer of the dentate gyrus where they divide and generate daughter cells. These form clusters within this subgranular layer. Progenitor cells then pass through a complex number of transitioning cell changes (Hsieh, 2012; Sierra et al., 2010). Half of the newly-formed cells undergo apoptosis (Wong & Herbert, 2004) and the surviving progenitors then differentiating into neurons, glia oligodendrocytes, astrocytes or granule neurons (Duan, Kang, Lui, Ming & Song, 2008; Monje et al., 2002). Those that become neurons within a month travel to the granule cell layer, differentiate, develop synaptic input, axon projections and dendritic efferent and express neuronal markers (Esposito et al., 2005; Gage et al., 1995; Gould & Gross, 2002). This process of neuronal differentiation, survival and incorporation is significantly complex and as a result is highly susceptible to pathological and environmental stimuli (Lugbert et al., 2010).

Relationship between NPCs, Neurogenesis and Depression

Although, the hippocampus is primarily implicated in being essential for learning and memory, depressive psychopathology involves hippocampal symptoms (McNaughton, 1996, Eichenbaum, 1999; Kempermann, 2002). This is supported by evidence that sufferers of depression have reduced hippocampal volume (Gueze, Vermetten & Bremner, 2005; Videbech & Ravnkilde, 2004). Decreased neurogenesis

has been associated in the pathophysiology of anxiety and depression (Benninghoff, Schmitt, Mossner & Lesch, 2002; Kempermann & Kronenberg, 2003; Snyder, Soumier, Brewer, Pickel & Cameron, 2011). In comparison to medicated depressed patients the hippocampi of un-medicated depressed patients express significantly less granule neurons in the dentate gyrus (Boldrini et al., 2013). A reduction in neurogenesis has also been observed in animal models of depression, such as olfactory bulbectomy in both mice and rats (Islam, Moriguchi, Tagashira & Fukunaga, 2014; Jaako-Movits, Zharkovsky, Pedersen & Zharkovsky, 2006). Neurogenesis-deficient mice express depressive-like phenotypes in the domains of behavioural despair and anhedonia. In addition they display increased food avoidance in a novel environment following acute stress (Snyder et al., 2011).

Stress, which is a strong antecedent for the development of depression in some individuals, has also been found to decrease the rate of neurogenesis (Tanti, Rainerm Minier, Surget, Belzung, 2012; Nollet et al., 2012), with the stress hormone glucocorticiouds having a significant negative effect (Huang & Herbert, 2006; Wong & Herbert, 2006). It is argued that the creation of new neurons in the adult brain is essential for mood control (Schnell, Albrecht & Sandreilli, 2014). The administration of antidepressant medication increases levels of neurogenesis in the hippocampus and simultaneously blocks the effects of stress (Malberg et al., 2000; Santarelli, et al., 2003; Sapolsky, 2004). Moreover, a blockade of hippocampal neurogenesis blocks the actions of antidepressants in behavioural models of depression. This demonstrates a direct link between behaviour and new cell birth (Duman, 2004).

It is unclear whether decreased neurogenesis is due to a reduction in proliferating cells or whether later stage NPC maturation is compromised (Alahmad & Herbert, 2008; Boldrini et al., 2012). Reif and colleagues (2006) found that NPCs were not decreased in patients with depression. This was surprising given that decreased cell

proliferation has also been reported in response to both acute and chronic stress paradigms (Fuchs et al., 1997; Yang et al., 2013). Administration of pharmacological treatment (Boldrini et al., 2009; Boldrini et al., 2012; Czeh et al., 2001; Malberg, Eich, Nestler and Duman 2000) and electroconvulsive therapy (Madsen et al., 2000; Scott et al., 2000) increases NPCs and neurogenesis. This suggests that through increasing cell proliferation and heightening neurogenesis through antidepressant medication, it overcomes the stress-induced atrophy and reduction of hippocampal neurons. However, some studies have reported that administration of antidepressant medication does not lead to increases in levels of NPCs (Lucassen, Stumpel, Wang & Aronica, 2010; Reif et al., 2006). However, age may be a factor in the inconsistent results with no pharmacological effects effects being observed in older mice (Couillard-Despres et al., 2009; Navailles, Hof & Schmauss, 2008).

Light and Reduction in NPCs

As previously outlined, LAN is a risk factor in the development of depression as evidenced by shift-workers (Scott et al., 1997) and constant light paradigms (Salgado-Delgado et al., 2011) in animal studies. Given that levels of NPCs has been increased after antidepressant medication in depressed individuals (Boldrini et al., 2009) it is reasonable to hypothesize that LAN may attenuate the production of NPCs in combination with inducing depressive behaviours. Presently, only one study has investigated the effects of constant lighting on NPC production (Fujioka et al., 2011). This is surprising, given that NPC production is sensitive to environmental stressors and constant light serves as a stressor in animal models (Abilio, Freitas, Dolnikoff, Castrucci & Frussa-Filho, 1999; Van der Meer & Van Loo, 2004). Although, not investigating constant light Gibson and colleagues (2010) observed that fluctuations to the L/D cycle due to jet-lag paradigms, resulting in exposure to light during the biological night, leads to a reduction in NPCs. The current study wishes to investigate

whether dLAN can simultaneously induce depressive-like behaviour and a reduction in NPCs.

Weight

The rate of obesity and metabolic disorders has risen significantly over the course of the 20th centaury. In 2000, for the first time in our history the proportion of adults who have excess body fat exceeded those who are underweight (Caballero, 2007). Obesity is a pathogenic condition characterized as the build-up of excess adipose tissue. It is linked to many chronic health issues which include diabetes, cardiovascular disease, hypertension, diabetes, asthma and cancer (Fonken & Nelson, 2014). Obesity significantly reduces the quality of life of the individual, induces a wide array of health related difficulties along with doubling healthcare costs. Given the depilating effects that obesity has on the individual and the financial burden it places on society understanding fully what are the factors involved in the development and maintenance of the condition are of significant interest.

Although, high-calorie diets and a sedentary lifestyle serve as contributing risk factors for the development and maintenance of obesity, other factors may also play a part in its development. As stated earlier, one such environmental factor is the increase of LAN which may have effects on our circadian biology, and, in turn leads to metabolic disorders. Fonken and Nelson (2014) indicate that the rise in exposure to LAN coincides with the increase of obesity and metabolic disorders in the USA.

Circadian Clock and Metabolism

A number of studies provide a link between metabolism and the molecular circadian clock (For full reviews see, Bruce & Young, 2006; Fonken & Nelson, 2014). For instance, mice expressing mutations to major circadian clock genes such as CLOCK are vulnerable to developing obesity and metabolic syndrome (Turek et al., 2005). Mutations to clock genes BMAL1, *Per1*, *Per2*, Vipr2, and *Rev-Erba* additionally result

in symptoms which are reminiscent of metabolic syndrome (Fonken et al., 2014; Marcheva et al., 2010; Carvas et al., 2012). Lesions to the SCN results in the onset of mild obesity compared to animals which are sham-operated upon. Although, only mild increases are observed, significant reductions in neuroendocrines (hepatic insulin and basal glucose) which are critical for metabolism are observed (Coomans, van den Berg, Lucassen, Houben, Pronk & van der Spek, 2013). This indicates that disruption to core clock genes and proteins in the central oscillator has deleterious effects on metabolism.

Exposure to LAN is correlated with alterations in metabolism. Shift-workers who are exposed to chronic levels of LAN are at a greater risk of developing metabolic syndrome (Wang, Armstrong, Cairns, Key & Travis, 2011), having an increase body mass index (Ha & Park, 2005; Parkes, 2002), expressing an attenuation of rhythms related to growth and melatonin and decreasing insulin sensitivity (Stevens et al., 2007). Obayashi and colleagues (2013) conducted a highly ecologically valid study which investigated LAN exposure home-settings. They found that those exposed to high levels of LAN had higher levels body weight, high levels BMI, and lower lipoprotein cholesterol levels.

Possible reasons for the increases in weight gain have been attributed to LAN suppressing the natural production of melatonin under circadian control (Tan, Manchester, Fuents-Broto, Paredes & Reiter, 244). Similar results are observed in photoperiodic animals who display a decrease in body mass during short-days when the circadian pattern of melatonin secretion is longer (Goldman, 2001). Administration of melatonin decreases body mass in rats (Terron, Delgado-Adamez, Pariente, Zen, Fernandez-Rodriguez, 2013) and improves glucose homeostasis in Zucker rats (Agil, Rosado, Ruiz, Figueroa, Zen, Fernandez, 2012).

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Constant Light and Metabolic Disorders

Exposure to constant light and dLAN is associated with circadian arrthmicty and metabolic alterations in rodents (Coomans et al., 2013). Swiss-Webster mice exposed to constant light display increased body mass size independent of changes in total caloric intake and behavioural output (Fonken et al., 2010). Similar results have been observed in non-photoperiodic C57Bl/6J mice (Fonken & Nelson, 2014).

Given that increases in weight are observed in both humans who perform shift-work and animals placed under constant light paradigms, it is plausible to suggest that constant dLAN may play a factor in weigh gain. Multiple studies conducted in albino Swiss-Webster mice, indicate that exposure to dLAN results in increases in body mass (Aubrechet et al., 2015; Borniger et al., 2014; Fonken et al., 2013). These effects are observed only after four days which indicates that minimum exposure has deleterious effects (Fonken et al., 2013). This increase in body mass occurs independently of total daily calorie intake and behavioural home cage activity. However, these increases in body mass are reversed when dLAN animals are returned to a L/D cycle (Fonken, Weil & Nelson, 2013). This suggests that alterations in metabolism which are induced by exposure to dLAN are not permanent. dLAN exposure has also be found to results in impaired glucose tolerance, which is symptomatic of a pre-diabetic like-state (Fonken et al., 2010).

The increase in body mass following dLAN exposure is attributed to the shifting in timing of food intake towards the light phase. It is only when food is restricted to the active phase (dim phase) that no significant increases in body mass observed (Fonken et al., 2010). This suggests that the timing of food intake is essential for prevention of weight gain. The timing of food intake is also important in humans with individuals who eat after 8pm express higher BMI (Baron, Reid, Kern & Zee, 2011). This is consistent with animal studies indicating that eating during what is typically the sleep

phase in diurnal and nocturnal animals results in higher weight gain. The importance in the timing of food intake may also be due to peripheral tissues (hepatic and adipose tissues), which are critical for metabolic regulation and are entrained by the timing of food intake (Meneker et al., 2013). Changes in timing of food intake as a result of dLAN exposure may result in abnormalities in peripheral clock gene expression resulting in mass gain (Marcheva et al., 2010).

Suppression of peripheral clock gene expression under dLAN exposure has also been attributable to weight gain. This is due to many organs and tissues involved in metabolism containing peripheral clocks which are under circadian control (Borniger et al., 2014; Mohawk, Green & Takahashi, 2012). Exposure to dLAN leads to a reduction in clock gene Rev-Erba expression in peripheral tissues which are critical for metabolic regulation such as the liver and adipose tissues. This attenuation of Rev-Erba under dLAN is of significant interest given its involvement not only in the circadian feed-back loop but also its association in the regulation of rhythmic metabolism (Fonken et al., 2013). As indicated earlier, peripheral clocks are kept entrained by receiving temporal information from the SCN which is, mediated through different signals (Mowhawk, Green & Takahashi, 2012). One such signal is body temperature which is reduced under dLAN exposure (Borniger et al., 2014). Borniger and colleagues (2014) argue that alteration to this entraining signal of peripheral tissues results in the onset of poor metabolism due to an internal desyncrony between the SCN and the temperature sensitive peripheral clocks.

Objectives and aims of the current study

The increasing prevalence of dLAN has simultaneously occurred with increasing rates of depression and obesity. The primary objective of this study is to investigate whether exposing C57Bl/6 mice to dLAN results in alterations in circadian rthymicity. We wish to examine whether the exposure to dLAN results in the development of depressive-like

behaviours, molecular alterations within the brain and increase in body mass. Animals in the dLAN condition will be housed under blue-enriched short wavelength LED lighting of 5LUX during the dark period. The particular wavelength light is emitted from smart phones, televisions, alarm-clocks and most common electrical devices, which remain illuminated during the subjective night period (Cho et al., 2015). This is the wavelength of light which the melanopsin-expressing ipRGCs are most sensitive to (Bedrosian, Vaughn, Galan, Daye, Weil & Nelson, 2013). The LUX level chosen provides validity to the study given that it is the level of light typically found in sleeping environments. This level of light exposure is five times brighter than that of maximal moonlight and is easily differentiated from daytime light levels. Furthermore, 5LUX of dLAN has been found to induce depressive behaviours in other animal species (Bedrosian et al., 2011; Bedrosian et al., 2012; Bedrosian et al., 2013; Fonken et al., 2012). This model of depression provides both face and predictive validity (Bedrosian et al., 2012).

Animals will be accomadated under dLAN conditions for a three-week period. This housing period has been employed in previous studies. It provides sufficient time to fully investigate the possible magnitude of pathogenic effects of dLAN in causing depressive-like symptoms.

Although, previous studies have used female Siberian hamsters or diurnal Swiss Webster mice, the current study will use C57Bl/6 mice. This is to provide more translatable findings to a human context given that hamsters are highly photoperiodic which may negatively impact on the efficacy of findings. The naturally melatonin-deficient C57BL/6 mouse strain was selected given there frequent use in animal models of depression and circadian research (Jung, Hong, Ma, Hwang, Kim, Lee, Seo, Lee & Jang, 2014; Kudryavtseva, Bakshtanovskaya & Koryakina, 1991). Furthermore, we believe this strain of animal will complement the previous research conducted, which

has yielded inconsistent results in regards to the power and period of the rhythm and on the different behavioural measures of depression. To investigate depressive-like behaviours, we will utilise a range of behavioural measures of depression which have face, concurrent and predicative validity.

Females are two times more susceptible to developing depression compared to men (Goodnick, Chaudry, Artadi, Arcey, 2000; Sloan & Kornstein, 2003; Piccinelli & Wilkinson, 2000). However, the previous studies investigating the effects of dLAN on circadian rhythmicity and inducing depressive-like behaviours have been predominately carried out on overectimsed female hamsters. No study has directly compared the sexes to examine whether similar effects are observed, or given that females are more susceptible to depression, whether they are more sensitive to dLAN exposure compared to males. The current study endeavours to investigate whether dLAN exposure has similiar deleterious effects in altering circadian rhythmicity and inducing depressive-like behaviours in male and female mice. The current study will investigate the magnitude in the effects that dLAN has on circadian rhythmicity and depressive-like behaviour. This will be achieved by first placing animals into a L/D cycle and testing depressive-like behaviour and then returning animals to an experimental lighting condition of either dLAN or L/D. This will allow for an examination into the severity of dLAN exposure has for each animal. Finally, although the exact mechanism that couples LAN and the prevalence of depressive-like behaviour is unknown, many argue that it is due to the suppression of melatonin. C67Bl/6 mice are melatonin-deficient meaning that they secrete little or no endogenous melatonin (Kasahara, Mekada, Yoshiki, Kato, 2010). By using melatonin-deficient animals we are able to examine whether the alterations in circadian rhythmicity and the manifestation of affective disorders under dLAN is due melatonin dysfunction.

As a result of the inconsistent results regarding the effects of dLAN exposure on circadian rhythmicity, the second overarching question is to define the effects of dLAN on circadian rhymicity. This will be investigated by examining the locomotor activity of animals. As indicated above, the timing of the sleep-wake cycle is influenced by the circadian clock. In rodents, the sleep-wake cycle is indicated by the presence or absence of motor activity. The timing of the locomotor activity provides an accurate indicator of the phase of the circadian clock and the sleep-wake cycle (Redlin, 2001). Furthermore, due to rodents being nocturnal animals the study will be able to separate the impact of sleep disruption from the effects of dLAN as these animals sleep during the light period of the subjective day. This will provide us with a better understanding of the effects of dLAN on circadian rhythmicity.

Given the association between depression and a reduction in the level of NPCs and a small number of studies demonstrating that constant light elicits depressive-like behaviours and reduces the level of NPCs (Fujioka et al., 2011; Gibson et al., 2010), the current study seeks to investigate the effects that dLAN has on the rate of stem cell proliferation. This will be investigated using the mitotic maker Ki-67, which is expressed during all phases of the cell cycle except G_0 (Kee, Sivalingam, Boonstra & Wojtowicz, 2002). We aim to examine whether dLAN reduces the rate of NPCs. Therefore, we hypothesize that:

1. dLAN will lead to circadian alterations that will in turn result in the manifestation of a depressive-like phenotypes in C57BL/6 animals.

2. The effects of dLAN exposure on circadian rhythmicity and onset of depressive-like behaviours will be more pronounced in female rather than male animals.

3. Animals housed in groups will exhibit less depressive-like behaviours compared to those housed singly. This is due to group housing acting as a non-photic

zeitgeber and entraining the clock to the L/D cycle despite the presence of dim light.

4. Animals housed under dLAN conditions will express a reduction in NPCs compared to animals accommodated under a L/D cycle.

5. dLAN will express increased body mass gain compared to animals housed under L/D cycles.

Method

Animals & Housing

87 C57BL/6 mice (*Mus musculus*) aged between 8 and 12 weeks at the onset of experiments and weighing between 19-25 grams were used in the current study. The C57BL/6 strain was selected as they are naturally melatonin-deficient, commonly employed in models of depression (Jung, Hong, Ma, Hwang, Kim, Lee, Seo, Lee & Jang, 2014; Kudryavtseva, Bakshtanovskaya & Koryakina, 1991) and have been extensively used for circadian research (Schwartz & Zimmerman, 1990). Furthermore, they have also been employed as a background strain for investigation of circadian-related targeted mutations (e.g., see Eckel-Mahon & Sassone-Corsi, 2013). Singly housed animals were accommodated in polypropylene cages (33cm long x 15 cm wide x 13cm high). These cages were equipped with steel running wheels (11.5cm diameter) which allowed for behavioural monitoring. Group housed animals were accommodated in groups of three according to their sex. For identification purposes group housed animals were individually ear punched. Group housed animals were accommodated in polypropylene cages. In order to provide environmental enrichment each of the cages were equipped with tunnels, nesting material and running wheels. For both single and group housed animals, food and water was made available *ad libitum*. The cages and animals were housed within an environmental isolation cabinet which allowed for complete control over the photic stimuli to which animals were exposed to. The lighting conditions were adjusted via a timer on the outside of the cabinet and therefore the cabinet was not unnecessarily opened. The light sources were standard fluorescent light bulbs with an average 150 LUX luminance in each individual cage. Animals were exposed to a 12:12 light/dark (L/D) photoperiod (lights on 06:00; lights off 18:00) for a minimum of three weeks prior to any experimentation. Temperature and humidity were kept constant with an average temperature of 21+/- °C and humidity 50 +/-%. The cages

were kept ventilated via axial fans which aided in preventing the build-up of pheromones, with the fans producing white noise at the level of 50dB. All protocols were approved by the Research Ethics Committee at the National University of Ireland, Maynooth (BSRESC-2013-0018) and licensed by the Health Products Regulatory Authority. All animals were treated in accordance with the European Union Directive 2010/63/EU (Protection of Animals Used for Scientific Purposes) Regulations 2012 (S.I. NO. 543 of 2012). Every effort was made to minimise the amount of animals used. Any suffering and discomfort was kept to a minimum. Experiments were conducted in sequence from the least to most stressful test.

Experimental Design

Animals were randomly assigned to singly housed (28 females, 27 males) or housed in groups of two/three (16 females, 16 males). For a more detailed demographic of animals in each condition see Table 2.1. As can be observed from Figure 2.1, which provides a illustrates the experimental design animals were first placed in a 12:12 L/D cycle for a three-week period. For animals that were singly housed measurements of general locomotor activity monitored were taken. At the end of the three-week period behavioural tests which assessed depressive-like behaviours were employed. This provided a baseline measure of behaviour. After baseline testing animals were then placed into a three-week period of either a 12:12 L/D (~150/0 LUX) cycle or into a 12:12 light/dim-light (L/dLAN) (~150/5 LUX) cycle (Lights on: 06:00, Lights off: 18:00). Light during the light phase was emitted from typical fluorescent bulb, whereas the dim-light was from strips of cool white LEDs. These strips were evenly glued to the ceiling of the environmental isolation unit in order to ensure even dim light distribution at cage level. After the three-week period behavioural measures were repeated in the same order. Animals were then returned to their cages in the environmental isolation cabinet for 3-5 days. This was to ensure that animals levels of stress which occurred as a

result of behavioural testing would be reduced after being tested. These steps were taken as stress would have deleterious molecular effects on the brain. Animals were then sacrificed and brains were harvested for immunohistochemistry.

Table 2.1.
Number of animals in each condition

|	Control Single	Single dLAN	Control Group	Control dLAN
Female	16	12	8	8
Male	11	16	8	8
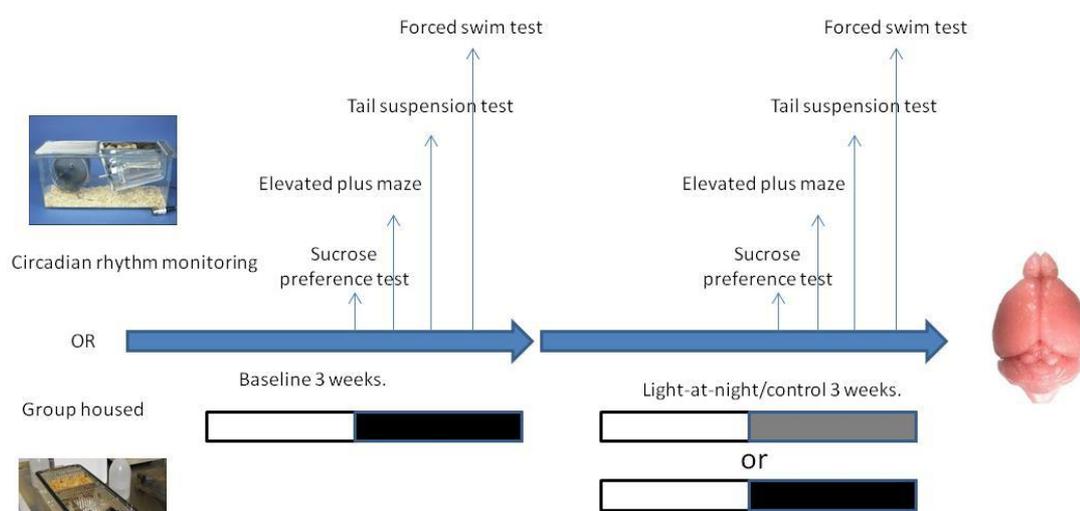
Figure 2.1. Schematic Image of experimental protocol. Animals were assigned single housing or group housing. They were then placed into a L/D cycle. After a three-week period animals completed a battery of behavioural tests. Animals were then placed into either a L/D cycle or L/dLAN cycle for a three-week period. After this period they completed the battery of tests. Animals were then sacrificed and their brains were harvested for immunohistochemistry.

Circadian Behavioural Analysis

Singly housed animals were accommodated in cages which were equipped with steel running wheels to monitor locomotor activity. Mircoswitches were attached to the cages which were connected to a data acquisition system computer system using the

Chronobiology Kit by the Stanford System (Santa Cruz, California). This provided a recording of daily rhythms of locomotor activity. Each wheel revolution created a switch closure measured by the data acquisition system collected every 5 minutes, which then generated double plotted actograms or actigraphs of activity rhythms for each animal, whom were singly housed. Using the actogram data generated from each animal, the phase angle of entrainment was calculated for the three-week period. The phase angle of entrainment is the time difference between the entraining external cue and the displayed internal rhythm which is accessed via timing of locomotor activity. This involved assessing the time in which the onset of main activity was conducted each day (see Figure 2.2). This time was then subtracted from the time in which lights turned off, or in the case of dLAN animals, when dLAN turned on. An average time of onset of activity was calculated using the mean of the individual scores over the 21-day period. This provided a separate mean phase angle of entrainment for each animal for baseline and after control conditions. To determine the period and amplitude of the rhythm, periodgrams were conducted over the 21-day period at baseline and after experimental conditions using Chronobiology Kit software (see Figure 2.3).
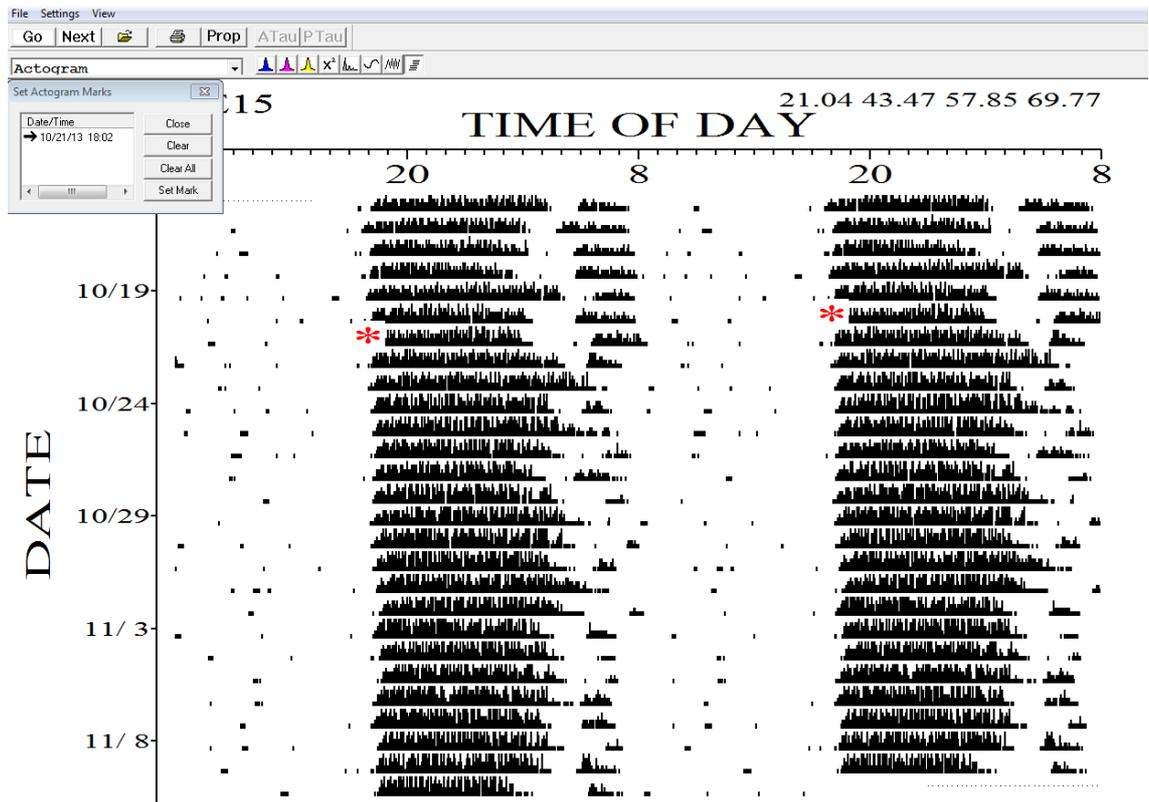
Figure 2.2. Sample actogram illustrating how the phase angle of entrainment is calculated. Red asterisks denotes the time in which the main onset of activity occurred.
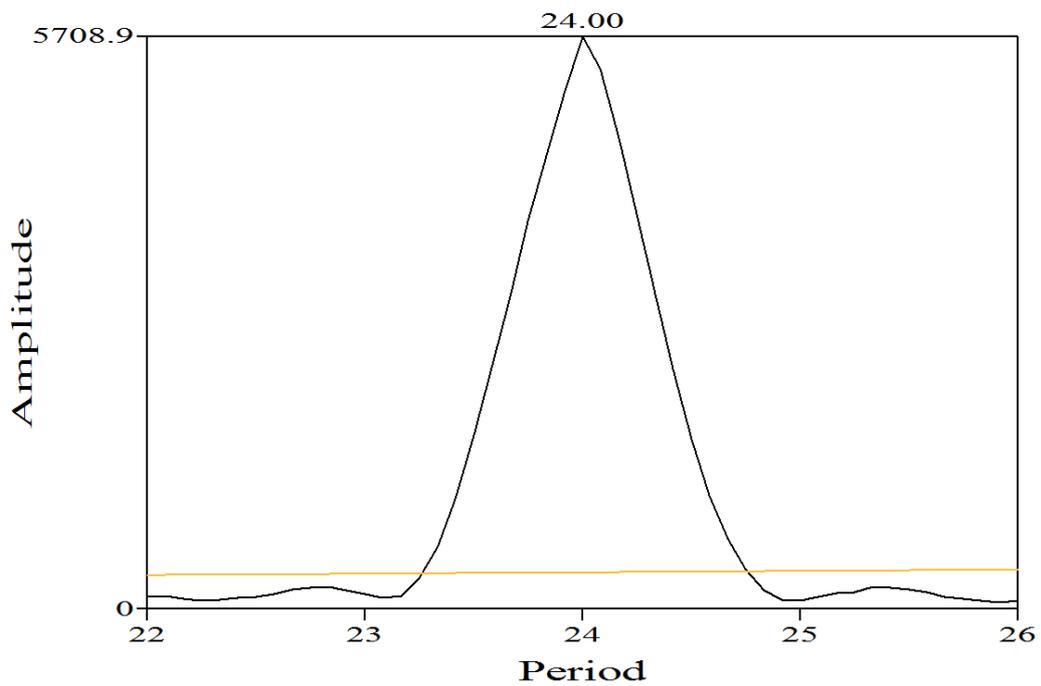

Figure 2.3. Sample Chi-Square Periodograms. Sample chi-square periodogram calculated by Chronobiology illustrating both the free running period and amplitude of the activity rhythm.

Body Weight

Animals were weighed at the start of the experiment and each week until they were sacrificed.

Behavioural measures to assess depressive-like behaviours

Behavioural testing took place in an experimental room where mice were given 24-hours to acclimate to the testing room before testing. In this room, the lighting conditions were similar to the environmental chamber unit. Mice performed a battery of behavioural tests in the following order: the sucrose preference test, EPM, TST and FST. This sequence was chosen to ensure that tests ranged from least stressful to most stressful. This minimised the effects of the previous test on later ones. All measures, apart from the TST have been used in previous studies investigating the effects of dLAN on on affective performance (Bedrosian et al., 2011; Bedrosian et al., 2012; Bedrosian et al., 2013; Fonken et al., 2012). All tests were conducted during the light phase between 11.00-15.00 hours for the following reasons: (i) it was expected that locomotor activity would be different between groups during the dark-phase but not the light phase and both the FST and TST are activity dependent measures of depression (ii) the lighting conditions were manipulated during the dark phase, but lighting conditions were similar during the light phase. All tests were scored by the researcher manually.

Sucrose Preference Test

Day 1: The sucrose preference test was employed to measure anhedonia. It is argued to be a reliable and valid measure to assess depressive-like behaviour without placing animals into stressful conditions and is minimally invasive. Typically, when rodents are repeatedly exposed to sucrose water they increase sucrose ingestion over fresh tap water. In contrast, animals which develop depressive-like behaviour decrease sucrose water intake (Willner, Muscat & Papp, 1992). This reduction in sucrose preference is interpreted as an anhedonic response, which is indicative of a depressive-like state

(Willner et al.,1992). In the present study, the amount of sucrose consumption was measured by looking at the amount of 1% sucrose solution consumed over a 24-hour period. Animals were presented simultaneously with two water bottles. One bottle contained ordinary water and another bottle contained 1% solution of sucrose. Both bottle caps were duct taped to ensure that the water/sucrose solution would not spill. To control for possible side preferences, placement of the bottles in the cage were counterbalanced after 12 hours. Bottles were weighed immediately before and after 24-h following placement to ascertain how much sucrose and water were consumed respectively. Sucrose preference was calculated as [sucrose consumed/(water consumed + sucrose consumed)]. This provided a % of sucrose consumption.

EPM

Day 2: This test was employed to assess anxiety-like behaviour. The protocol as set out by Walfe and Frye (2007) was employed in this study. The apparatus was plus-shaped in design and was elevated up above the floor. The apparatus comprised of a centre area of diameter 12.5 cm from which four arms extended of length 34.5cm, width 5cm. Two of the arms were open without walls while the other two arms were enclosed by high walls. Each mouse was placed in the centre of the test apparatus facing an open arm. Time spent in open arms, time spent in closed arms and latency to first entry to the open arms were the measures taken during the five-minute period in which the test was being conducted. In order for the test to be novel to animals when they were tested subsequently after experimental condition, the layout of the test was altered. Where previously the arms were open, the arms were closed and vice versa . Additionally, at the end of each test the whole area of the apparatus was sprayed with 70% ethanol solution to remove any scent of animals and wiped with dry paper towel to remove any mouse dropping and urine.

TST

Day 3: The TST was employed to assess learned helplessness which is behavioural response reported in those suffering with clinical depression (Strau et al., 1985). In this test, rodents were suspended from a burette clamp by adhesive tape attached -1cm 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 this period. The dependent measure to assess a depressive like response was the total time spent immobile. Mice were considered immobile only when they hung passively and completely motionless. After the test was completed the adhesive tape was taken carefully from the animal's tail ensuring not to harm the animal.

FST

Day 4: In order to assess learned helplessness the FST was employed. Animals were placed into an opaque cylinder jar which was filled with room temperature water (22+-1°C) for a period of six minutes. Dependent measures to assess depressive-like responses were the latency to first immobility and total period of time spent immobile (Porsolt, Bertin & Jalfre, 1977). Following the test, each animal was removed from the water, patted dry and placed under a heater to fully dry off. Containers were emptied and cleaned between each test. Increased immobility is regarded as learned helplessness. This is a symptom of depression which is classically seen in those with a clinical diagnosis of depression.

Transcardial perfusion and tissue preparation

All perfusions were carried out in the middle of the light phase. Animals were terminally anaesthetised with an intraperitoneal injection injection of sodium pentobarbital (Euthathal, Merial Animal Health, UK), the chest cavity opened and animals perfused transcardially with .9% saline, and then 4% parapformaldehyde (PFA, Sigma) in 0.1M Phosphate Buffer (PB) at 4°C. Brains were then removed, post-fixed

overnight in 4% PFA for 24hrs at 4°C. The brains were then placed in 30% sucrose. The brain was then cut caudally removing the cerebellum and rostrally to remove the pre-optic region with a single edged razor blade. Brains were mounted on a freezing stage microtome (Leica) with 0.1M PB acting as the mounting medium. The caudal cut surface was attached to the stage and the ventral aspect faced towards the blade. Brains were quick frozen with dry ice and 30um thick serial coronal sections were cut throughout the rostrocaudal extent of the SCN to the dentate gyrus. All sections were collected and divided into 4 series, allowing for the acquisitions of multiple areas of the brain. The sections were then stored in .01M PB at a pH of 7.4 (Sigma) with 0.1% sodium azide at 4°C, which prevented microbial growth prior to being processed for immuohistochemical staining.

Immunohistochemistry protocol

Free floating brain sections were placed onto culture plates and were washed twice at room temperature for a period of 10 minutes. Sections were subsequently washed in PBX, which consisted of .1M PB and .03% Triton X-100 (Sigma) for 10 minutes. PBX allows for the cell membrane to be perforated. Sections were then incubated in 0.1M PB and 1.5% Hydrogen peroxide (H202) for 20 minutes. H_2O_2 allows for the inactivation of endogenous peroxidases and reduces non-specific background staining. Sections were then repeatedly placed into another set of 10 minute washes of PB and PBX as outlined earlier. A non-specific antibody blocking step was then carried out in 0.1M PBX with 5% Normal Goat Serum (NGS, for rabbit polyclonals) for 60 minutes at room temperature. After this, sections were then incubated with the primary antibody Ki67 which is raised in rabbit diluted in 0.1M PBX and 2% NGS. Sections were placed in the fridge overnight at 4°C. Following this period of incubation, the sections were then put through a series of washes (2 x PB and x 1 PBX wash). Sections were then incubated in the appropriate biotinylated secondary antibody (1:400 biotinylated goat anti rabbit

Jackson Immune research Labs) diluted to .1M PBX and 2% NGS at room temperature for 70 minutes. As the biotinylated secondary antibody is photosensitive, the sections were covered from light for the remainder of the immunohistochemistry. Using the avidin-biotin method (0.4%) in 0.1M PBX sections were then treated with the Vectastain Elite Universal Kit (Vector Laboratories, Peterborough, Cambridgeshire) for 90 minutes at room temperature. Sections were then washed twice for 10 minutes in .01M PB and then in 0.1M Sodium Acetate (pH6, Sigma) for 10 minutes. Immunoreactivity was visualized with light sensitive nickel-enhanced diaminobenzidine (3, 3'-diaminobenzidene with ammonium nickel chloride, NiDAB, Ph6) and 60ul glucose oxidase (5mg/ml). This worked as a catalyst allowing for the visualization of protein products. In order to control for inter-run reliability, each of the sections, when being ran for a specific antibody were reacted at the same time, with each section developing for the same period of time.

When sufficient staining occurred, the sections were washed in 0.1M sodium acetate to stop the reaction and then underwent two final washes in .1M PB. Tissue sections were then stored .1M PB at 4°C and then mounted onto gelatine coated slides (1% gelatine and 0.05% chromium potassium sulphate) and were allowed to dry before being put through dehydrating and delipifying steps. Sections were dehydrated as they each passed through a series of graded ethanol washes (70%, 90% 100%) for a period of three minutes. After this, sections were then cleared for a period of three minutes in 2% Histoclear washes (National Diagnostics, UK). Slides were then left to air-dry overnight before they were coverslipped using mounting media (Eukitt, Fluka Analytical).

After the coverslip section had dried, photomicrographs of the sections were taken. These were taken under constant light intensity using a digital camera which was attached to an Olympus BX-51 light microscope at either 40x or 100x magnification. Stem cell proliferating cells, with Ki67, were counted by eye under 400x magnification.

Mircophotographs are presented in text with scale bars representing 100um unless otherwise noted. An analysis of between four and six sections of the mid dorso-ventral level of the hippocampus was conducted. Hippocampal sections were assessed across the dentate gyrus as described by Paxinos and Franklin (2004).

Data Analysis

All results were analysed in IBM SPSS Statistics version 20. All data is presented as means ± standard error of the mean (SEM). In terms of statistical analysis, 3-way mixed between-within ANOVAs was used to access the angle on entrainment, the period and amplitude of the rhythm. SEX and LIGHTING CONDTION acted as the between-subjects factor and TWO-TIMEPOINT SCORES as the within subjects factor. To investigate significant two way-interactions a Wilcoxon Signed Rank t-test was conducted.

When investigating the effects of housing and sex on depressive behaviours, as measured by the behavioural measures, 3-way mixed between-within ANOVAs were conducted. TIMEPOINT-SCORES acted as the between-subjects factor and LIGHTING CONDITION and HOUSING TYPE were the within-subjects factor. Two-way interactions were further investigated using the Wilcoxon Signed Rank t-test. When three-way interactions were observed, a change in performance scores across the two testing points were calculated (time2/time1). Change in score in the respective behavioural measure served as the dependent measure, lighting condition as the fixed factor and was split for housing type.

When investigating the effects of housing and sex on depressive behaviours as measured by the behavioural measures 3-way mixed between-within ANOVAs were conducted with TIMEPOINT-SCORES as the between-subjects factor and LIGHTING CONDITION and SEX as the within-subjects factor. Two-way interactions were further investigated using the Wilcoxon Signed Rank t-test. Where three-way interactions were

observed a change in performance scores across the two testing point were calculated (time2/time1). Change in score in the respective behavioural measures served as the dependent measure, lighting condition as the fixed factor and split for sex.

To investigate effects of light condition and sex on rates of stem-cell proliferation 2 way-between groups ANOVA was conducted.

<center>**Results**</center>

Effects of dLAN on Circadian Rhythmicity

For a three-week period, all animals were accommodated under a L/D cycle. Observation of the standard double-plotted actograms provided behavioural rhythms data (as can be seen in Figure 3.1), which demonstrated that animals when were accommodated under a L/D cycle they behaved in a similar way. Both groups of animals entrained to the L/D cycle with locomotor activity commencing during the dark phase and being kept to a minimum during the light phase.

After the three-week period animals were placed into their experimental condition of either 12:12 L/D cycle or 12:12 L/dLAN. Control animals continued to exhibit entrainment to the 12:12 L/D cycle. It would appear that when animals were first housed under 5LUX dLAN elicited a phase delay in the onset of their behavioural activity. During a six-day period this phase-delay diminishes and timing of activity onset returns to what was previously observed under the 12:12 L/D cycle.

(A) Control Animal

Time (h)

(B) dLAN Animals

Time (h)
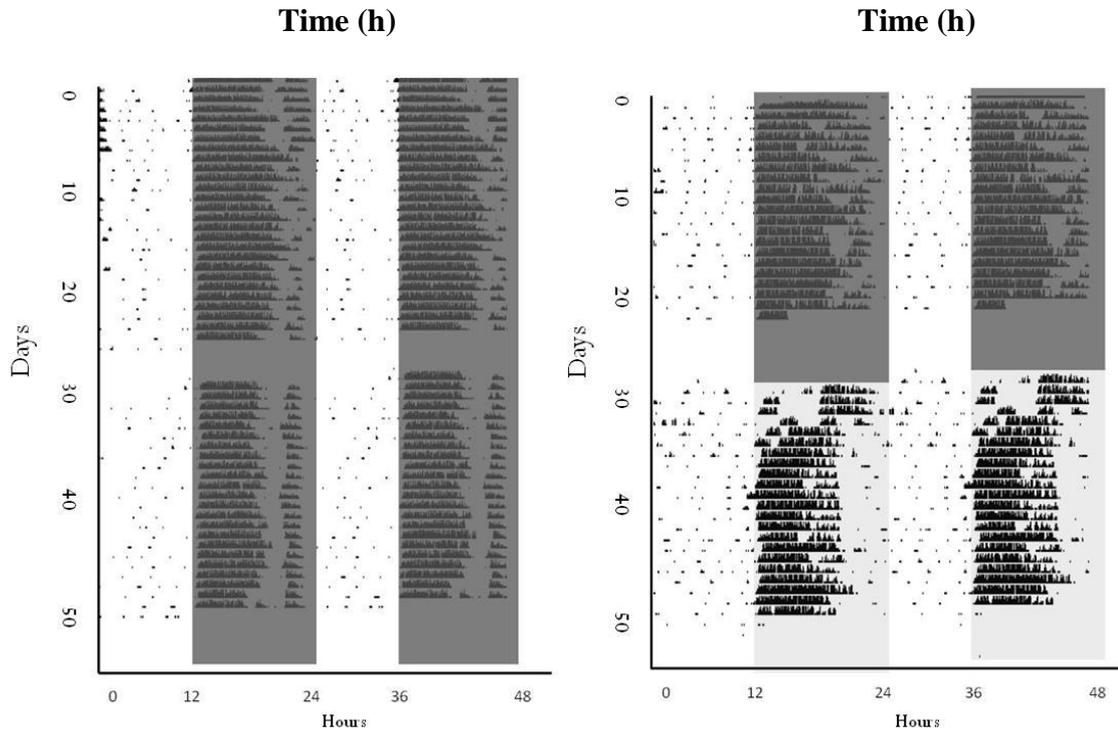
Figure 3.1. Locomotor Activity Rhythms under Different Lighting Conditions. (A) L/D and (B) L/dLAN are sample double-plotted actograms from control and dLAN animal respectively showing locomotor activity. The first half of the actogram represents animals housed in a 12:12 L/D cycle. The second half represents animals housed in the experimental lighting conditions of (A) 12:12 L/D and (B) 12:12 L/dLAN.

Phase Angle of Entrainment

A three-way mixed between-within subjects ANOVA was conducted to assess the impact of sex and lighting condition on the phase angle of entrainment at baseline and after the intervention (see Figure 3.2). The analysis found no significant three-interaction between time x lighting condition x sex, Wilks's Lambda = .981, F (1, 35) = .694, p = .410, partial eta squared = .019. No significant interaction for angle of entrainment and sex was observed, Wilks's Lambda = .975, F (1, 35) = .899, p = .350, partial eta squared = .019. No significant two-way interaction between time and lighting

condition was observed, Wilks's Lambda = .971, $F(1, 35) = 1.040$, $p = .315$, partial eta squared = .029.

(A)
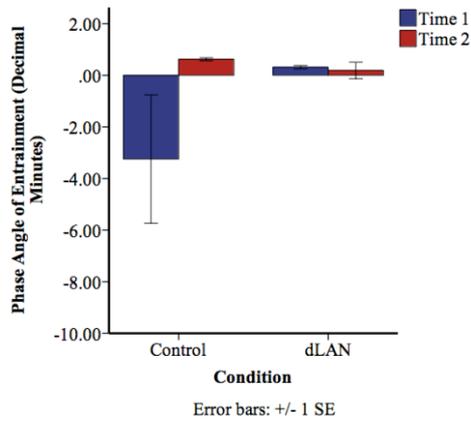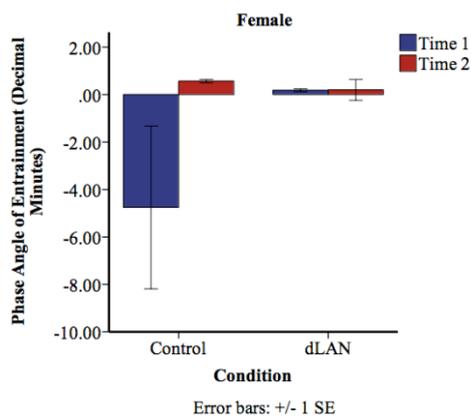
**(B)**   **(C)**
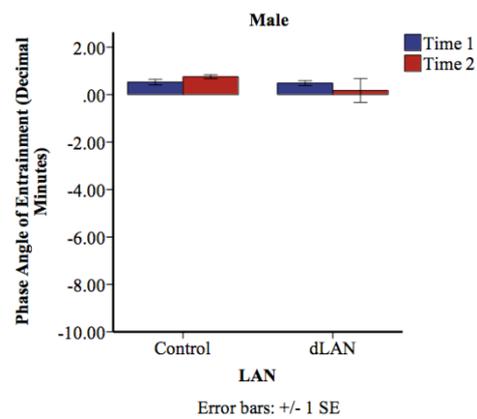
Figure 3.2. Phase Angle of Entrainment under Different Lighting Conditions. Each bar graph illustrates the average phase angle of entrainment under the different lighting conditions at baseline and after experimentation. Specifically, (A) illustrates the phase angle of entrainment under the different lighting conditions. The lower panel of graphs illustrates the phase angle of entrainment for each sex at each time-point under different lighting conditions with (B) representing females and (C) representing males. No differences were found between groups. Error bars represent ±SEM.

Period of the Rhythm

A three-way mixed between ANOVA was conducted to access the impact of sex and lighting condition on the period of the rhythm, across two time-points (baseline and

after experimentation). As can be seen in Figure 3.3, there was no significant three-way interaction between time x lighting condition and sex, Wilks's Lambda = .99, F (1, 37) = .105, p = .75, partial eta squared = .003. No interaction was observed between period x sex, Wilks's Lambda = .97, F(1, 37) = 1.10, p = .30, partial eta squared = .029. There was, however, a significant two-way interaction between period and lighting condition, Wilks's Lambda = .89, F(1, 37) = 4.37, p = .043, partial eta squared = .106, with the experimental group expressing a mean reduction in the period of their rhythm after being accommodated in dLAN. To further investigate this interaction a Wilcoxon Signed Rank test was conducted which found no statistical differences between control (z = -1.00, p = .317, r = .15) and dLAN (z = 1.86, p = .063, r = 3.41) and the power of their rhythm across the two time-points. There was no main effect observed for time, Wilks's Lambda = .95, F (37, 1) =1.87, p = .180, partial eta squared = .048. The main effect comparing lighting condition and sex was not significant, F (1, 37) = 1.28, p = .26, partial eta squared = 0.34.

(A)
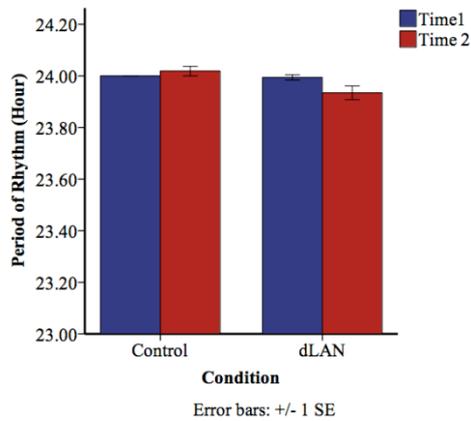
(B)
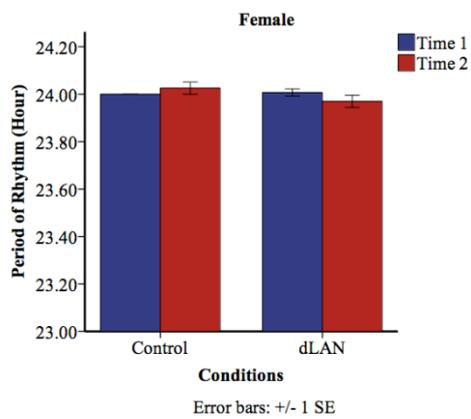
(C)
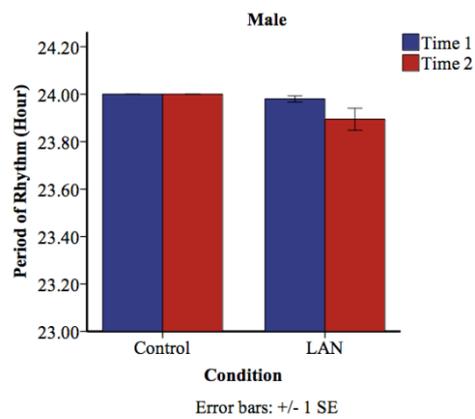
Figure 3.3. Period of the Rhythm Under Different Lighting Conditions. Each bar graph illustrates the period of the rhythm under the different lighting conditions at baseline and after experimentation. Specifically, (A) illustrates the period of rhythm for all animals under the different lighting conditions. The lower panel of graphs illustrates the period of the rhythm for each sex at each time point under different lighting conditions with (B) representing females and (C) representing males. No differences were found between groups. Error bars represent ±SEM.

Power of the Rhythm

In order to assess whether the power of the rhythm changed due to dLAN and sex a three-way mixed between-within ANOVA was conducted. No significant interaction between power x lighting condition x sex was observed, Wilks's Lambda = .988, $F(1, 32) = .076$, $p = .785$, partial eta squared = .002. As can be seen in Figure 3.4, no significant interaction was observed for period of the rhythm across the time points and

sex, Wilks's Lambda = .985, $F(1, 32)$ = .50, p = .48, partial eta squared = .015. Again no significant interaction was observed for period of rhythm across the two time points and lighting condition, Wilks's Lambda = .975, $F(1, 32)$ = .84, p = .37, partial eta squared = .025. Finally, no main effect for time was found, Wilks's Lambda = .971, $F(1, 32)$= .96, p = .335, partial eta squared = .029. The main effect comparing both sex and lighting condition was also non-significant, $F(1, 32)$ = .95, p = .336, partial eta squared = .029.

(A)
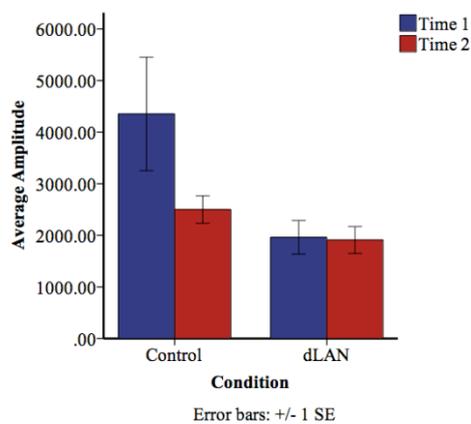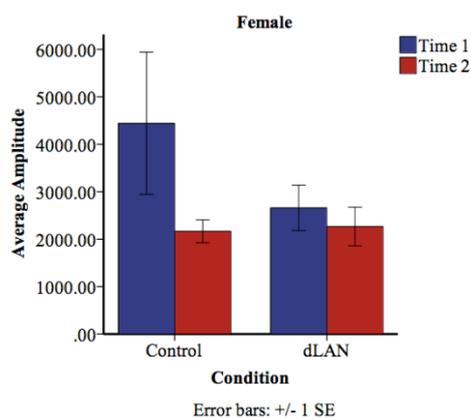
**(B)**   **(C)**
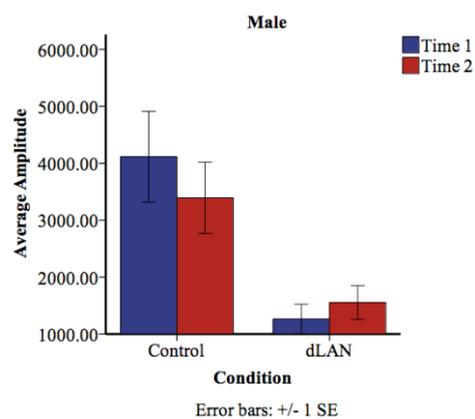
Figure 3.4. Power of the Rhythm Under Different Lighting Conditions. Each bar graph illustrates the average power of the rhythm under the different lighting conditions at baseline and after experimentation. Specifically, (A) illustrates the power of the rhythm for all animals under the different lighting conditions. The lower panel of graphs illustrates the power of the rhythm for each sex at each time-point under different lighting conditions with (B) representing females and (C) representing males. No differences were found between groups. Error bars represent ±SEM.

Investigation of Type of Housing and Lighting Condition on depressive behaviours

A series of 3-way mixed-between ANOVAs were conducted to investigate the effects of both housing condition and lighting condition on the development of depressive-like behaviours across two time-points (see table 3.1). Only measures which reached statistical significance will be discussed.

TST – Total Immobility

A three-way between-within subject's ANOVA was conducted to assess the effects of housing and lighting conditions on total time immobile on the tail suspension test. Animals were divided into two groups based on housing conditions (Group 1: singly housed; Group 2: group housed) and sex. There was a significant main effect for time, Wilks's Lambda = .907, $F(1, 76) = 7.81$, $p = .007$, partial eta squared = .093. For both groups the total time immobile increases across the two time-points (represented in Figure 3.5 (A) below). A moderate significant interaction effect was observed with time x lighting condition, Wilks's Lambda = .941, $F(1, 76) = 4.74$, $p = .033$, partial eta squared = .059. To investigate this interaction further a Wilcoxon Signed Rank Test revealed that animals housed singly demonstrated an increase in time spent immobile on the TST across the two time-points, $z = -3.235$, $p = .001$, $r = .31$. There was an increase in immobility from time one (Md = 22.71) to time two (Md 27.24). Figure 3.5(B) illustrates the mean increase in total immobility on the TST from time one ($M = 187.57$, $SD = 51.44$) to time two ($M = 211.67$, $SD = 47.56$).

The main effect comparing both lighting condition and housing was not significant with $F(1, 76) = 1.79$, $p = .185$, partial eta squared = .023. There was no significant interaction between time x housing x lighting condition, Wilks's Lambda = .98, $F(1, 76) = 4.74$, $p = .273$, partial eta squared = .16. No significant interaction was

observed between time and grouping condition, Wilks's Lambda = .99, $F(1, 76)$ = 1.073, p = .303, partial eta squared = .014.

(A)

(B)   **(C)**
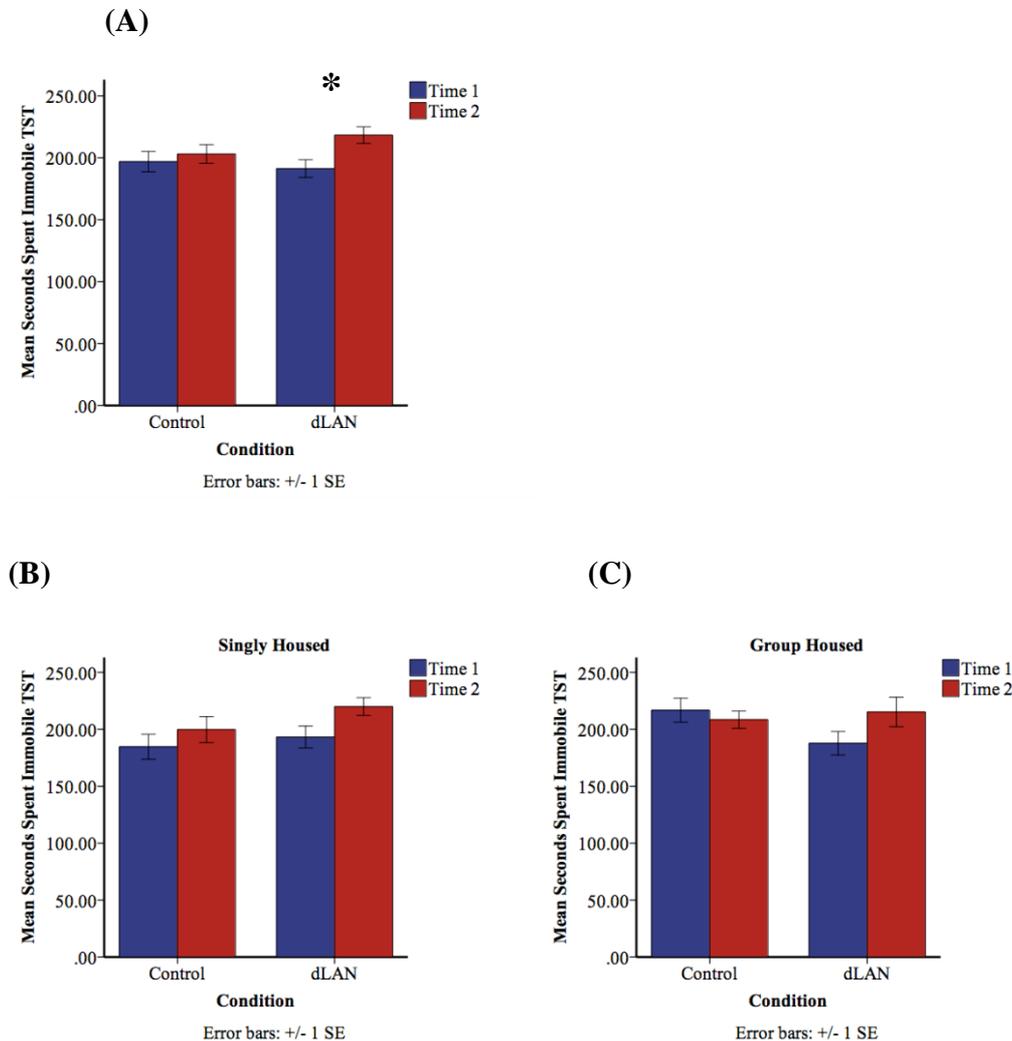
Figure 3.5. TST Total Immobility Latency Under Different Lighting Conditions. Each bar graph illustrates the mean latency to immobility under the different lighting conditions at baseline and after experimentation. Specifically, (A) illustrates the mean time spent immobile under the different lighting conditions. The lower panel of graphs illustrates the mean total amount of immobility for each sex at each time-point under different lighting conditions with (B) representing females and (C) representing males. Bar graph 3.5(A) illustrates that dLAN animals had a significant increase in immobility from time one to time two. Error bars represent ±SEM, * represent p<.05 in Wilcoxon Signed Rank Test.

FST Latency to Immobility

A large main effect for time was found, Wilks's Lambda = .752, $F(1, 80)$ = 28.54, p < .001, partial eta squared = .248, indicating that the time in which animals first became immobile changed from the baseline at time one as represented in Figure 3.6(B). A

interaction effect between time x housing was observed, Wilks's Lambda = .901, $F(1,80)$ = 8.82, p = .004, partial eta squared = .099. To further investigate this interaction a Wilcoxon Signed Rank Test was conducted which revealed a statistical decrease in latency to immobility in animals who were singly housed z = -5.96, p < .000, with a large effect size (r = .57). The median time in latency to immobility reduced from 69 at time one to 20 at time two. Figure 3.6(A) illustrates the mean decrease in latency to immobility on the FST from time one (M = 70.91, SD = 44.12) to time two (M = 25.16, SD = 22.76). No significant differences were found in animals group housed animals across time-points, z = -1.67, p = .094, r = .21. A significant two-way interaction between time x lighting conditions was observed, Wilks's Lambda = .94, $F(1, 80)$ = 5.04, p = .027, partial eta squared = .059. There was a significant interaction between time x housing x lighting condition, Wilks's Lambda = .95, $F(1, 80)$ = 9.51, p = .003, partial eta squared = .106. To investigate this interaction further, a two-way between-groups ANOVA was conducted with the dependent variable calculated as the change in time spent immobile on the TST across the two-time points (time2/time1). It was observed that group housed dLAN animals expressed a significantly faster latency to immobility compared to control animals $F(1, 27)$ = 6.49, p = .017, partial eta squared = .194. The mean change score across time-points for group housed controls was .74 (SD = .48) and .38 (SD = .28) for group housed dLAN animals.

(A)
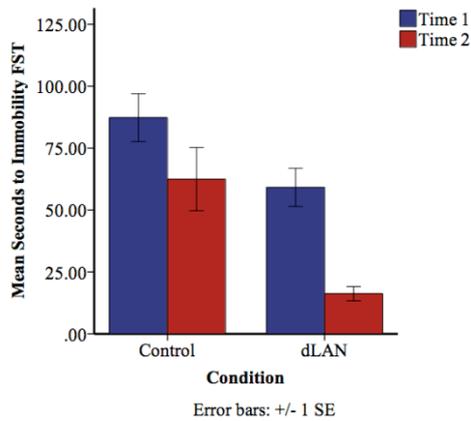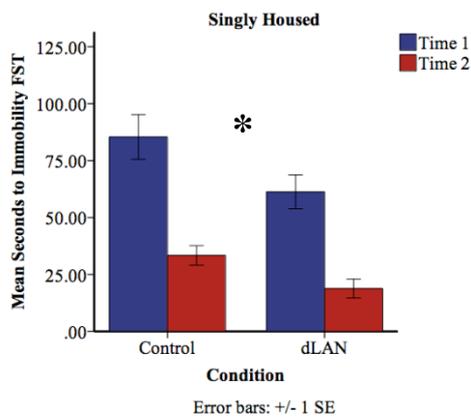
(B)   **(C)**
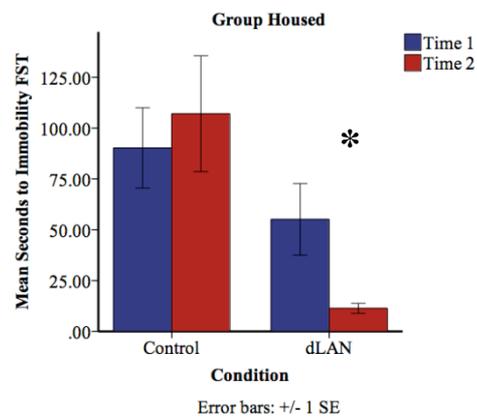

Figure 3.6. FST Latency to Immobility Under Different Lighting Conditions. Each bar graph illustrates the FST mean latency to immobility under the different lighting conditions at baseline and after experimentation. Specifically, (A) the mean FST latency to immobility under the different lighting conditions. The lower panel of graphs illustrates mean latency to immobility for each sex at each time-point under different lighting conditions with (B) representing females and (C) representing males. Bar graph 3.6(C) illustrates that dLAN animals had a decreased time to immobility from time one to time two. Error bars represent ±SEM, * represent p<.05 in two-way between groups ANOVA.

	Sucrose Preference		EPM Closed Arms		EPM Lat. Open Arm		EPM Total Open Arms		TST Total Immobility		FST Lat. to Immobility		FST Total Immobility	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Time	**$F_{(1,80)}$ = 13.94**	**.000**	**$F_{(1,79)}$ = 4.87**	**.030**	$F_{(1,79)}$ = .740	.392	**$F_{(1,79)}$ = 14.46**	**.000**	**$F_{(1,76)}$ = 7.81**	**.007**	**$F_{(1,80)}$ = 28.54**	**.000**	**$F_{(1,80)}$ = 52.11**	**.000**
Time x Housing	$F_{(1,80)}$ = 3.29	.073	$F_{(1,79)}$ = 2.18	.144	$F_{(1,79)}$ = .034	.854	$F_{(1,79)}$ = 1.26	.266	$F_{(1,76)}$ =1.07	.303	**$F_{(1,80)}$ = 8.22**	**.004**	$F_{(1,80)}$ = 2.06	.155
Time x Lighting Condition	$F_{(1,80)}$ = .937	.336	$F_{(1.79)}$ = .906	.344	**$F_{(1,79)}$ = 5.13**	**.026**	$F_{(1,79)}$ = .345	.558	**$F_{(1,76)}$ = 4.74**	**.033**	**$F_{(1,80)}$ = 5.04**	**.027**	$F_{(1,80)}$ = 2.71	.104
Time x Housing x Lighting Condition	$F_{(1,80)}$ = .076	.738	$F_{(1,79)}$ = 2.37	.130	$F_{(1,79)}$ = 2.02	.159	$F_{(1,79)}$ = .007	.934	$F_{(1,76)}$ = 4.74	.273	**$F_{(1,80)}$ = 9.50**	**.003**	$F_{(1,80)}$ = 1.13	.291
Housing xLighting Condition	$F_{(1,80)}$ = .700	.405	$F_{(1,79)}$ = 2.65	.108	$F_{(1,79)}$ = .004	.950	$F_{(1,79)}$ = 3.75	.057	$F_{(1,76)}$ = 1.79	.185	**$F_{(1,80)}$ = 4.73**	**.033**	$F_{(1,80)}$ = 1.51	.222

Effects of Lighting Condition and Sex on Onset of Depressive Behaviours

To investigate the effects of light and sex on the onset of depressive behaviours multiple mixed between-within ANOVAs were conducted (results represented in Table 3.2). Only tests yielding significant results are discussed in detail.

Table 3.2
Results from ANOVA. *F*-Ratios, degrees of freedom

Main effects and Interactions.	Sucrose Preference		EPM Closed Arms		EPM Lat. Open Arm		EPM Total Open Arms		TST Total Immobility		FST Lat. to Immobility		FST Total Immobility	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Time	**$F_{(1,80)}$ = 11.81**	**.001**	$F_{(1,79)}$ = 2.20	.141	$F_{(1,79)}$ = .019	.891	**$F_{(1,79)}$ = 11.70**	**.001**	**$F_{(1,76)}$ = 8.93**	**.004**	**$F_{(1,80)}$ = 26.97**	**.000**	**$F_{(1,80)}$ =53.19**	**.000**
Time x Sex	$F_{(1,80)}$ = .120	.730	$F_{(1,79)}$ =3.67	.059	**$F_{(1,70)}$ = 16.85**	**.000**	$F_{(1,79)}$ =.101	.751	$F_{(1,76)}$ = .231	.632	**$F_{(1,80)}$ = 5.02**	**.028**	$F_{(1,80)}$ = 2.49	.119
Timex Lighting Condition	$F_{(1,80)}$ = .776	.381	$F_{(1,79)}$ =.026	.872	**$F_{(1,79)}$ = 4.29**	**.042**	$F_{(1,79)}$ = .369	.546	**$F_{(1,76)}$ = 4.18**	**.044**	**$F_{(1,80)}$ = 4.62**	**.035**	$F_{(1,80)}$ = 3.79	.055
Time x Sex x Lighting Condition	$F_{(1,80)}$ = 1.40	.241	$F_{(1,79)}$ = 2.87	.094	**$F_{(1,79)}$ = 12.01**	**.001**	$F_{(1,79)}$ = .279	.599	$F_{(1,76)}$ = .421	.518	**$F_{(1,80)}$ = 5.51**	**.021**	**$F_{(1,80)}$ = 4.00**	**.049**
Sex x Lighting Condition	**$F_{(1,80)}$ = 4.80**	**.031**	$F_{(1,79)}$ = .803	.373	**$F_{(1,79)}$ =**	**.009**	$F_{(1,79)}$ = . 011	.916	$F_{(1,76)}$ = 1.98	.164	**$F_{(1,80)}$ = 10.48**	**.002**	$F_{(1,80)}$ = 3.68	.059

Sucrose Preference Test

When investigating sucrose preference, a main effect for time was found with Wilks's Lambda = .871, $F(1, 80) = 11.805$, $p = .001$, partial eta squared = .129. This indicates that regardless of experimental condition and sex both groups exhibited increased sucrose preference when tested at time two as represented in Figure 3.7(A). As can be seen from Table 3.2 no statistically significant two- or three-way interactions were yielded.

(A)
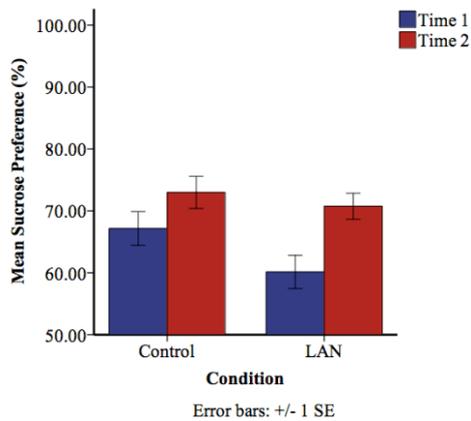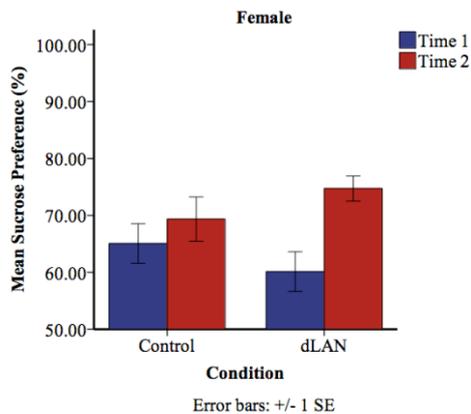
(B)   **(C)**
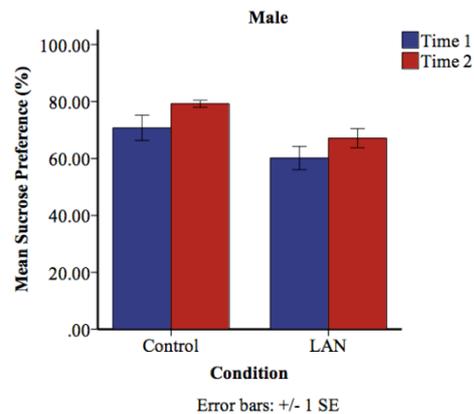
Figure 3.7. Sucrose Preference Under Different Lighting Conditions. Each bar graph illustrates the mean sucrose preference (in percentages) under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the mean % sucrose preference under the different lighting conditions. The lower panel of graphs illustrates the mean % sucrose preference for each sex at each time-point under different lighting conditions with (B) representing females and (C) representing males. No differences were found between groups. Error bars represent ±SEM.

EPM Open Arms

When investigating the total amount of time spent in open arms a main effect for time was observed, Wilks's Lambda = .871, $F(1, 79) = 11.70$, $p = .001$, partial eta squared = .29. As can be Figure 3.8(A) regardless of experimental condition and sex both groups, spent less time in open arms at time two compared to time one. As can be seen from

Table 3.2 no statistically significant interactions or main effects comparing sex and light condition were found.

(A)
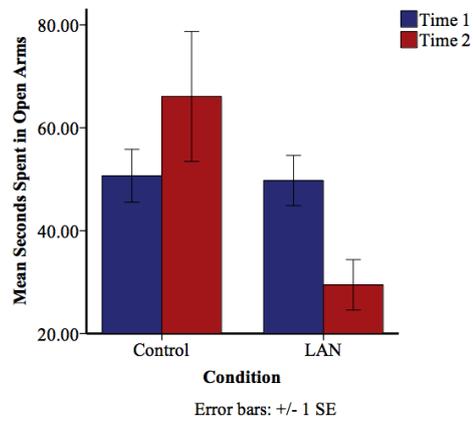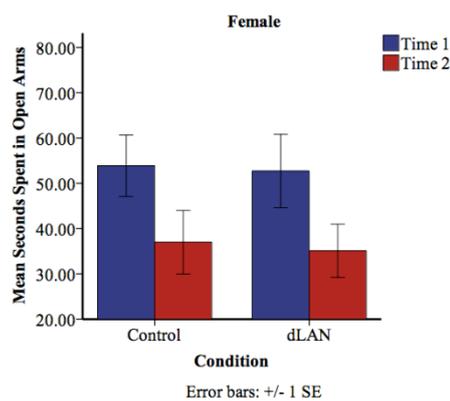
(B)   **(C)**
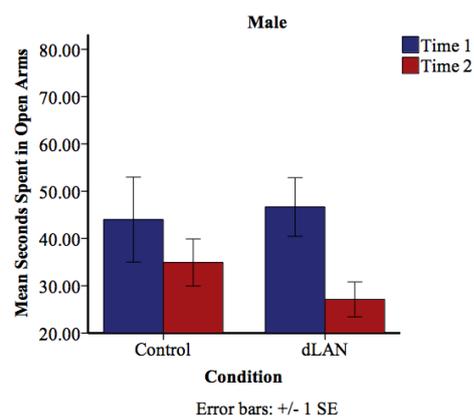
Figure 3.8. Total Time Spent in Open Arms Each bar graph illustrates the mean time spent in open arms under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the mean total time in open arms under the different lighting conditions. The lower panel of graphs illustrates the mean total time in open arms at each time-point under different lighting conditions with (B) representing females and (C) representing males. No differences were found between groups.   Error bars represent ±SEM

EPM Latency to Open Arms

When investigating the latency to first entry to open arms there was no effect for time, A two-way interaction effect was observed between time and light condition with Wilks's Lambda = .95, $F(1, 79) = 4.29$, $p = .042$, partial eta squared = .051. To further

investigate this interaction a Wilcoxon signed rank test was conducted which revealed a statistical decrease in time to first entry visit to open arms for the experimental group at time two, z = -2.038, p = .042, with a large effect size (r = .21). The median time to first entry to open arms reduced from 25.85 to 18.72 at time two. Figure 3.9(A) illustrates the mean decrease in latency to open arm on the EPM from time one (M = 45.44, SD = 49.24) to time two (M = 29.46, SD = 33.21). increase in total immobility on the TST from time one (M = 187.57, SD = 51.44) No significant differences were observed in the control condition across time-points, z = -81, p = .069, r = .20.

A significant interaction between time and sex was also found, Wilks's Lambda = .82, F(1, 79) = 16.85, p < .001, partial eta squared = .176. Females' first entry to the open arms increased when tested for the second time whereas male animals' time to first entry decreased. A large three-way interaction between time x sex x light condition was found, Wilks's Lambda = .87, F(1, 79) = 12.01, p = .001, partial eta squared = .132. dLAN males exhibited decreased latency to open arm entry from time 1 to time 2. To further understand the three-way interaction a two-way between groups ANOVA was conducted with change in latency time to open arm entry from baseline to after testing acting (Time 2/Time 1) as the dependent variable. No statistically significant differences were seen between females accommodated under an L/D cycle or dLAN, F(1, 41) = 2.74, p = .106, partial eta squared = .063 and males F(1, 36) = .49, p = .487, partial eta squared = .013.

(A)
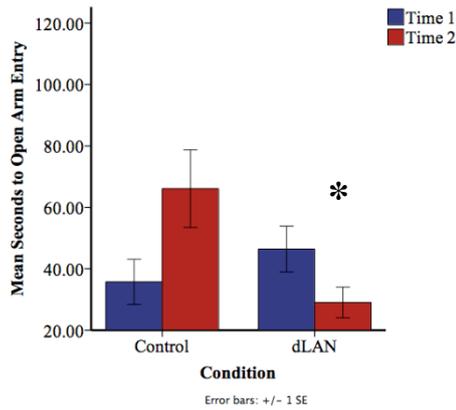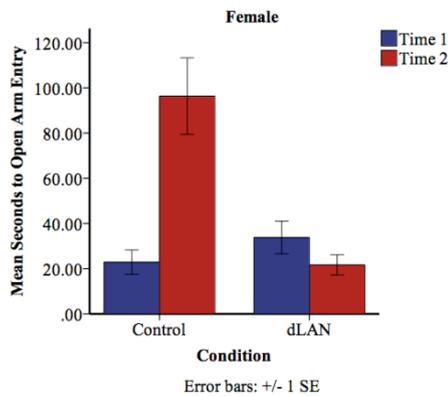
(B) **(C)**
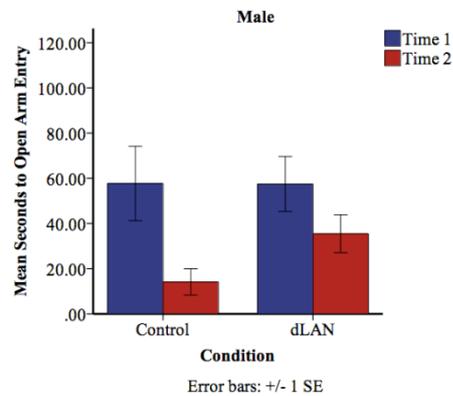
Figure 3.9. Latency to Open Arm Entry. Each bar graph illustrates the mean time taken for first entry to open arms under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the mean latency time to open arm under the different lighting conditions. The lower panel of graphs illustrates the mean latency to open arm entry at each time-point under different lighting conditions with (B) representing females and (C) representing males. Figure 3.9(A) represents a statistically significant reduction in time for first arm entry, * represents p<0.05 in Wilcoxon signed rank test.

Tail Suspension Test

When investigating the total time spent immobile on the Tail Suspension Test a significant main effect for time was found, Wilks's Lambda = .895, $F(1, 76) = 8.93$, $p = .004$, partial eta squared = .105. As can be seen from Figure 3.10(A) regardless of experimental condition and sex both groups expressed increase in immobility across the time-points. A moderately significant two-way interaction between time and light

condition was found, Wilks's Lambda = .948, $F(1, 76)$ = 4.19, p = .044, partial eta squared = .052. To further investigate these interactions a Wilcoxon Signed Rank Test was conducted and revealed a significant difference in immobility for dLAN animals across the two time-points, z = 3.49, p < .001, with a large effect size (r = .36). The median time for immobility increased from time one (Md =199) to time two (Md = 232.5). Figure 3.10 illustrates the mean increase in total time spent immobile on the TST from time one (M = 190.11, SD = 48.93) to time two (M = 218.34, SD = 45.67). No significant differences were observed within control conditions across the time-points, z = -.770, p = .441, r = .090. No significant three-way interaction between time x light and sex was observed, Wilks's Lambda .994, $F(1,76)$ = .421, p = .519, partial eta squared = .006. The main effect comparing sex and light condition did not yield a significant result, $F(1, 76)$ = 1.979, p = .164, partial eta squared = .025, suggesting no difference between light condition and sex on the total amount of time spent immobile. As can be seen from Table 3.2 no significant three-way interaction was observed between time x lighting condition x sex.

(A)

(B)   **(C)**
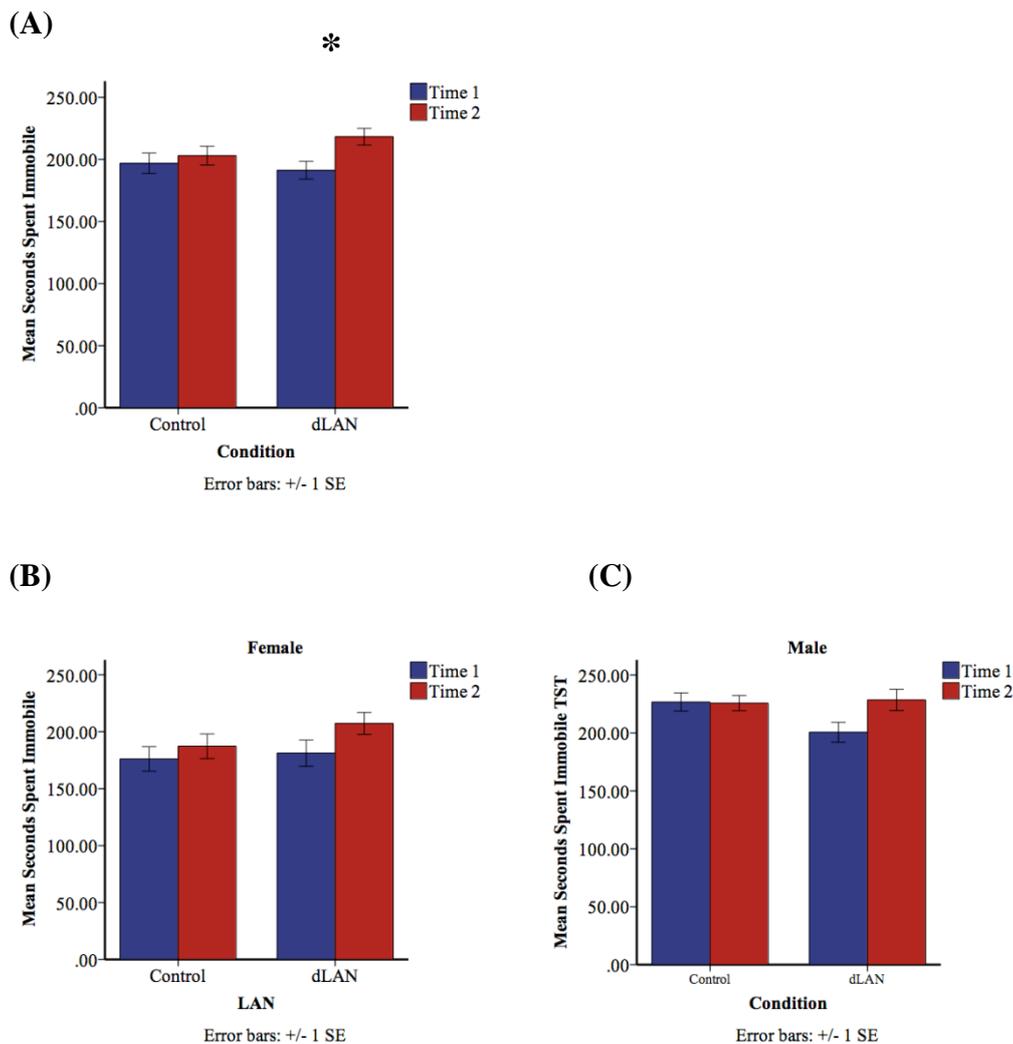
Figure 3.10. Total Time Spent Immobile TST. Each bar graph illustrates the total time spent immobile on the TST under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the total time spent immobile on the TST under the different lighting conditions. The lower panel of graphs illustrates the total time spent immobile on the TST at each time-point under different lighting conditions with (B) representing females and (C) representing males. Figure 3.10(A) represents a statistically significant increase in immobility in dLAN animals from across the timepoint, * represents p<0.05 in Wilcoxon signed rank test. No significant effects for sex and lighting on total time immobile were observed.

FST – Latency to Immobility

When investigating the latency to immobility on the FST a 3x2 ANOVA was conducted and a large main effect for time, Wilks's Lambda = .25, $F(1, 80) = 26.97$, $p < .0005$, partial eta squared = .252. This indicates that regardless of experimental condition both groups expressed a decreased latency to immobility from time one to time two (see

Figure 3.11) A two-way interaction between time and light condition was observed, Wilks's Lambda = .945, $F(1, 80) = 4.62$, $p = .035$, partial eta squared = .055. To investigate this interaction further a Wilcoxon Signed Rank Test was conducted which found that across the two time points both conditions expressed differences in their times to first latency. For control animals this reduction was $z = -2.73$, $p = .006$, partial eta squared = .30. The medium timing for control animals reduced from time one (Md = 70) to time two (Md = 33). As can be observed in Figure 3.11(A) control animals expressed a mean decrease in latency to immobility from time ($M = 87.60$, $SD = 58.67$) to time two ($M = 62.48$, $SD = 78.70$). Again, a significant decrease in time in latency to immobility between time one and time two was observed in animals exposed to dLAN, $z = -5.40$, $p = .000$, $r = .56$. The medium time for first onset latency reduced from time one (Md = 46) to time two (Md = 10). As can be observed in Figure 3.11(A) control animals expressed a mean decrease in latency to immobility from time ($M = 58.87$, $SD = 51.66$) to time two ($M = 16.24$, $SD = 19.40$). A mixed Factorial ANOVA indicated a moderate two-way interaction between time and sex, Wilks's Lambda = .941, $F(1, 80) = 5.02$, $p = .028$, partial eta squared = .059. Finally, from the 3x2 Factorial ANOVA a moderate significant three-way interaction between time x lighting condition x sex was observed, Wilks's Lambda = .93, $F(1, 80) = 5.5$, $p = .021$, partial eta squared = .048. To further investigate this, a two-way between groups ANOVA was conducted to investigate the impact of sex and lighting condition on latency to immobility (Time 2/Time 1). It was observed that for females, lighting conditions did not significantly change the latency to immobility with $F(1,42) = .484$ $p = .490$, partial eta squared = .011, as can be seen from Figure 3.11(B). However, lighting condition did have a statistically significant influence on males' latency to immobility, $F(1, 32) = 5.33$, $p = .028$ partial eta squared = .143 with dLAN animals having a faster latency (see Figure 3.11(C)), thus indicating that male animals accommodated under dLAN expressed

shorter latency to immobility from time one to time two.

(A)

(B)   **(C)**
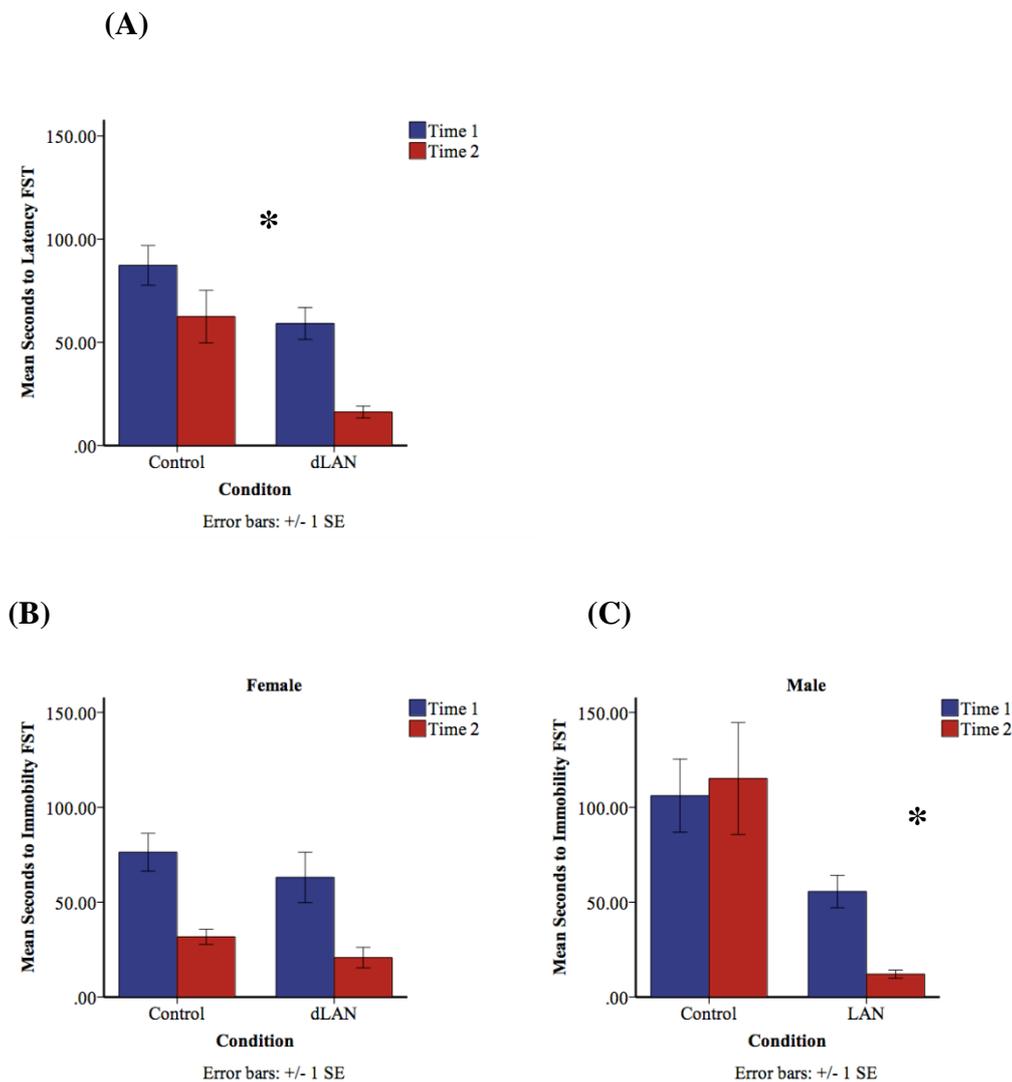
Figure 3.11. Latency to Immobility FST. Each bar graph illustrates the FST mean latency to immobility under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the mean latency to immobility under the different lighting conditions. The lower panel of graphs illustrates the mean latency to immobility at each time-point under different lighting conditions with (B) representing females and (C) representing males. Figure 3.10(A) represents a statistically significant increase in immobility in dLAN animals from across the time-points, * represents p<0.05 in Wilcoxon signed rank test. Figure 3.10(C) represents a statistically significant decrease in latency to immobility for males, * represents p<0.05 in two-way between groups ANOVA.

FST – Total Immobility

When investigating the total time spent immobile on the FST a main effect for time was found, Wilks's Lambda = .601, $F(1, 80) = 53.190$, $p < .001$, partial eta squared = .399 as

represented in Figure 3.12(A). This indicates that regardless of experimental condition and sex both groups exhibited increased total immobility at time two compared to time one. As can be seen from Table 3.2 no significant interactions between time and light condition and time and sex were observed. A borderline significant three-way interaction between time x light condition x sex interaction was observed, Wilks's Lambda = .93, $F(1, 80) = 4.00$, $p = .049$, however, the effect size was small (partial eta squared = .002). No main effects comparing light and sex were significant, $F(1, 80) = 3.681$, $p = .059$, partial eta squared = .044, suggesting no difference in sex and light condition on total time spent immobile. To further investigate the three-way interaction between time x sex x lighting condition a two-way between groups ANOVA was conducted. The dependent variable was change in total time spent immobile on TST (time two/time one), lighting condition and sex acted as fixed factors. For females, no statistically significant main effect for lighting condition was observed $F(1, 42) = .003$, $p = .959$, partial eta squared .000. However, an effect for lighting condition was found in males, $F(1, 34) = 7.29$, $p = .001$, partial eta squared = .177. The mean score for animals accomadated under a L/D cycle was .97 ($SD = .57$) and 1.5 ($SD = .61$) for dLAN animals.

(A)

(B)

(C)
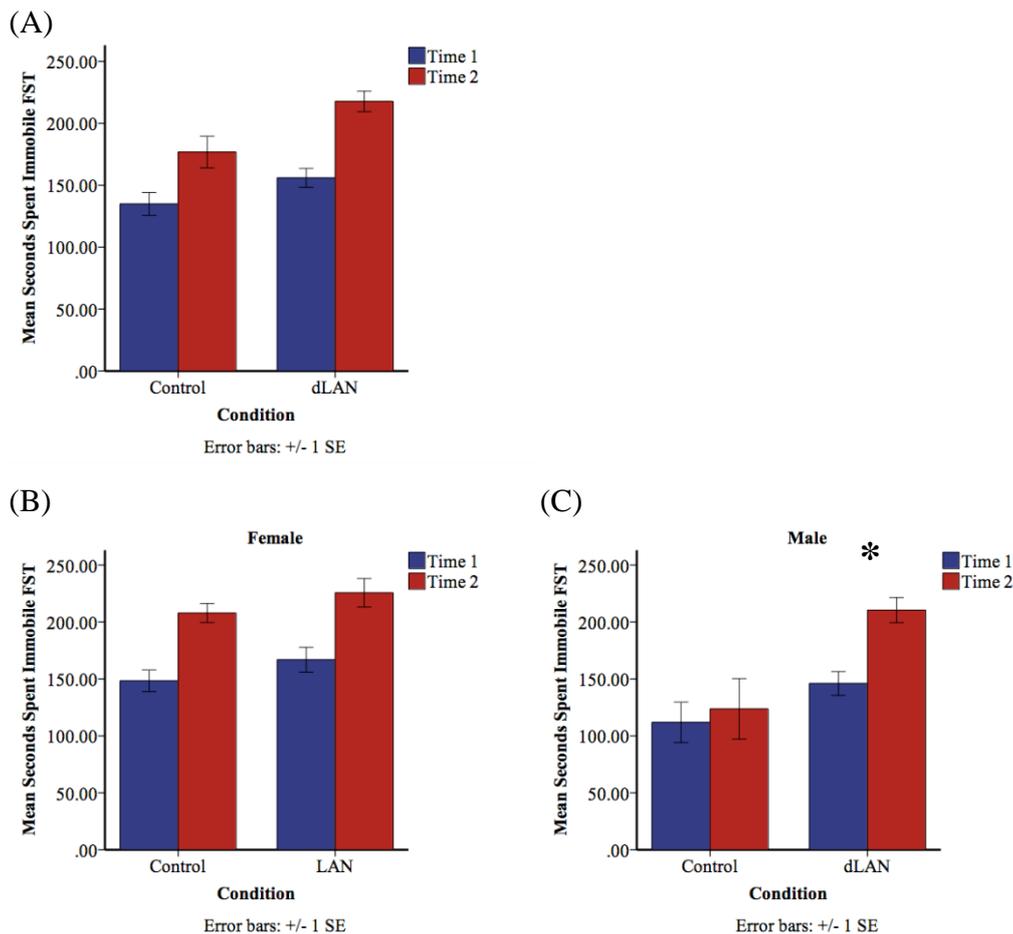
Figure 3.12. Total FST Immobility. Each bar graph illustrates the mean FST total time spent immobile under the different lighting conditions at baseline and after experimentation. Specifically, (A) represents the total time spent immobile under the different lighting conditions. The lower panel of graphs illustrates the total time spent immobile at each time-point under different lighting conditions with (B) representing females and (C) representing males. Figure 3.11(C) represents a statistically significant increase in immobility in dLAN animals from across the time-points, * represents p<0.05 two-way between ANOVA.

Body Weight

Due to sexual dimorphism in body size existing between C57Bl/6, body mass was

analysed within each sex separately. A mixed between-within ANOVA was conducted

to assess the impact of lighting conditions on body weight across a six-week period and

revealed a significant result for both females (Wilks's Lambda = .252, $F(5, 24) = 14.25$,

$p < .001$, partial eta squared = .748) and males (Wilks's Lambda = .215, $F(5, 16) =$

11.70, $p < .001$, partial eta squared = .784), indicating that the weight increased in both

groups over time (represented in Figure 3.12 below). For males no two-way interaction

between time and light was observed, Wilks's Lambda = .587, $F(5, 16) = 2.25$, $p = .099$, partial eta squared =. 413. For females a significant interaction between time and lighting condition was observed, Wilks's Lambda = .496, $F(1, 24) = 4.87$, $p = .003$, partial eta squared = .504. To further investigate this interaction a 2x2 between groups ANOVA was conducted with change in weight (week6/week1) serving as the dependent continuous variable. No significant effect for lighting condition on change in weight was found with $F(28,1) = 3.71$, $p = .064$, partial eta squared= .117. For males no significant interaction between lighting condition and times was observed, $F(16, 5) = .099$, $p = .099$, partial eta squared = .413.
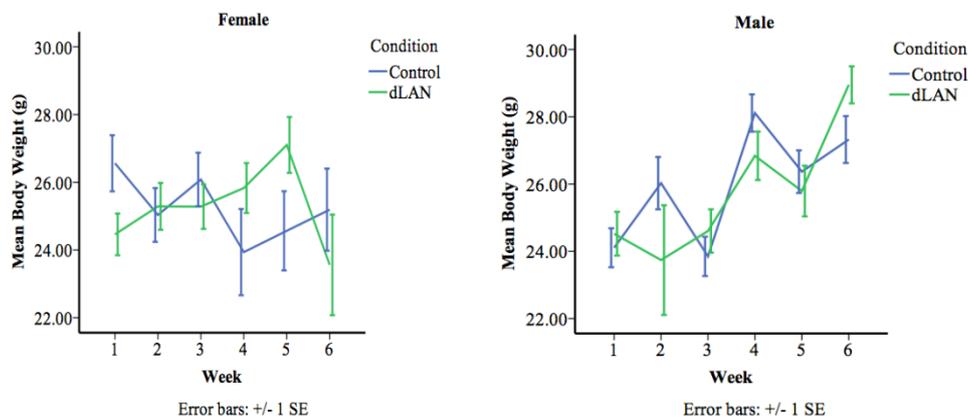
Figure 3.12. Growth Curve of Control and dLAN Mice. No significant differences were observed. Error bars represent ±SEM.

Neural Stem Cell Proliferation

A two-way between-groups ANOVA was conducted to explore the impact of sex and lighting condition on levels of proliferating cells in the dentate gyrus. No significant main effect for sex was returned, $F(1, 38) = .308$, $p = .582$ partial eta squared = .008. There was a significant main effect for lighting condition, $F(1, 38) = .5.71$, $p = .022$, partial eta squared = .131, with less proliferating cells identified in the dLAN condition ($M = 15.24$, $SD = 8.03$) compared to controls ($M = 23.78$, $SD = 11.29$) (as represented in the Figure 3.13(C) below).

| (A) Control | (B) dLAN |
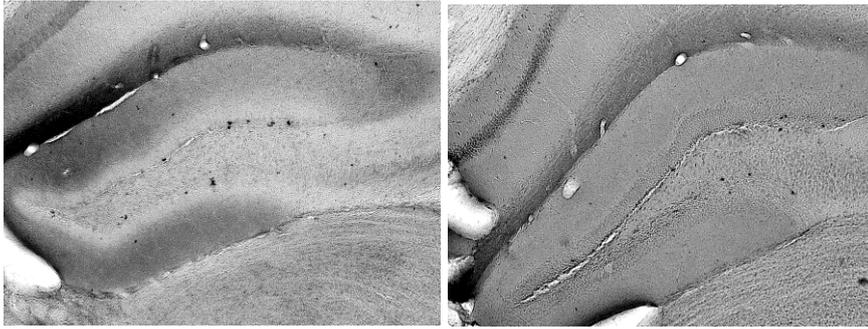
(C)
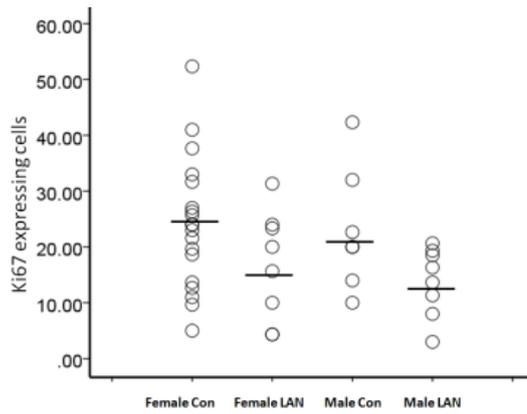
Figure 3.13. Neural Stem Cell Proliferation. Image (A) and (B) are micrographs representing the number of proliferating cells stained for Ki67 in the hippocampus. (A) is taken from an animal housed under L/D conditions. Representative photomicrographs illustrating Ki67 expression in the hippocampus at 100x magnification. Image (B) is taken from an animal housed under L/dLAN conditions. Figure 3.13(C) is a scatterplot illustrating the mean distribution of Ki67 expressing cells for female control animals, female dLAN animals, male control animals and male dLAN animals. The marking represents the mean Ki67 cell count.

Discussion

Rates of depression have increased significantly over recent decades, with the factors leading to its onset and maintenance remaining largely unspecified (Bedrosian et al., 2013). Although genetic vulnerability plays a significant role in the onset of depressive behaviour, the rise in incidence has occurred too quickly for genetics to be the sole explanation. Environmental factors also play a pivotal role in the manifestation and maintenance of depression. It is plausible, that recent environmental changes have resulted in the rise of the depression. One possible environmental change is the increased occurrence of dLAN which is emitted from most technological devices and outdoor lighting during the biological night (Cho et al., 2015). To investigate this, the current study employed a chronic dLAN paradigm to examine ,its effects on circadian rhythmicity, its contribution to the possible onset of depressive-like behaviours, and its effects on neural stem cell proliferation behaviours. Furthermore, given the higher incidence of depression in females compared to males (Goodnick, Chaudry, Artadi, Arcey, 2000; Sloan & Kornstein, 2003; Piccinelli & Wilkinson, 20000), the current study sought to examine whether dLAN exposure was more deleterious amongst females compared to males.

In summary, by using non-melatonin secreting C57Bl/6 mice the results from the current study found that dLAN exposure resulted in a temporary phase delay in activity but overall did not interfere with entrainment. dLAN only induced a depressive-like phenotype in one of the behavioural tests and placed a potent influence in reducing stem cell proliferation in the dentate gyrus. An effect for sex were only observed in males in the FST. Exposure to dLAN did not result in significant weight gain or loss. Results will now be discussed in more detail:

Circadian Rhythmicity

The current study wished to investigate whether dLAN exposure during the biological night affected circadian rhythmicity, as measured by locomotor activity. This is of significant importance given that light pulses during the biological night exert a significant effect on suppressing overt locomotor activity in nocturnal animals (Duffy & Czeisler, 2009). Specifically, the current study investigated whether dLAN exposure would result in circadian behaviour being no longer entrained to the L/D cycle, as a temporal cue of complete darkness would not be available under dLAN conditions. At a basic level both control and dLAN animals expressed maximum activity during the period of darkness/dLAN and had minimal activity during the light phase. This is typical behaviour of nocturnal animals, which are inactive/sleeping during the light phase and are active during the dark phase. It further indicates that chronic dLAN exposure does not alter light phase activity. This provides further evidence that dLAN does not disrupt sleep (Borniger, Weil, Zhang & Nelson, 2013).

At baseline, all animals were entrained to the L/D cycle with activity onset commencing at the start of the scotophase. In animals exposed to a 12/12 L/dLAN cycle a phase-delay in behaviour was expressed, with locomotor activity commencing later. Over ~7 days this phase-delaying response gradually diminished with the onset of main activity rhythms returning to what was previously observed at baseline. This is consistent with other studies which found that dLAN animals remained entrained to a L/D cycle despite being exposed to dLAN (Bedrosian et al., 2013; Borniger et al., 2013). No significant phase angle of entrainment differences were observed between control and dLAN animals, indicating that dLAN exposure did not interfere with the timing of the internal rhythm as indicated by locomotor activity. The continuation of

entrainment and no phase angle differences whilst being exposed to dLAN suggests no effects of dLAN on circadian rhythmicity.

The current study used LED emitting short-wavelength light. This light is of the spectral wavelength to which melanopsin ipRGCs which control non-image function behaviour is most sensitive (Brainard et al., 2002). The results suggest that at low levels this wavelength light does not interfere with photoentrainment. This maybe due to the intensity of light not being strong enough. Entrainment theory proposes that the range on entrainment is associated with the strength of *zeitgeber* signal (i.e. the light stimulus) (Roenneberg, Daan & Merrow, 2003; Jud, Schmutz, Oster, Hampp & Albrecht, 2005). It could be argued that the light intensity of 5LUX may not have the synchronising strength to induce major differences to the phase-dependent angle of entrainment or lead to significant alterations in entrainment. This is in contrast to studies which have found that exposure to constant light exposure at levels of 150LUX elicit circadian arrhythmicity as measured by overt behaviour and desynchronisation of clock neurons. These effects are due to no temporal cue being available to distinguish between light and dark (Martynhak et al., 2011; Ohto, Yamazaki & McMahon, 2005; Martynhak, Kanazawa, Nasimento & Andreatini, 2015; Tapia-Osorio et al., 2013). dLAN exposure may not interfere with circadian rhymicity as the light intensity is too low to have a negative impact on overt behaviour. Despite animals in the dLAN condition being continuously exposed to dim light during the dark-phase, their actigraph data indicated that they were able were able to differentiate between the continuation dim light after the light phase and commence locomotor activity similar to animals housed under L/D conditions.

The circadian clock without entraining cues has a period which is not precisely 24-hours (Reppert et al., 2001). Exposure to temporal cues of light and darkness synchronises the period to 24-hours (Daan & Pittendrigh, 1976). The current study

observed no significant differences in the period of the rhythm which is consistent with other reported work. This indicates that dLAN exposure still provided a temporal synchronising cue which the animals may have interpreted as the dark period of the L/D cycle. Only one study found that a proportion of Swiss-Webster mice housed under dLAN expressed disrupted patterns of wheel running with animals expressing a non 24-hour wheel running rhythm (Fonken et al., 2014).

No differences in the amplitude of the rhythm were observed between the different groups, which is consistent with work carried out on the diurnal male Grass rats (Fonken, Kitsmiller, Smale & Nelson, 2012). However, this is inconsistent with studies using Siberian hamsters where exposure to dLAN at the same level induces reductions in the power of the rhythm (Bedrosian, Weil & Nelson, 2013; Bedrosian, Galan, Vaughn, Weil & Nelson, 2013). Bedrosian and colleagues (2013) attribute the reduced amplitude to dLAN exposure, as re-accommodation to a L/D cycle results in the power of the rhythm returning to what would be typically exhibited under L/D conditions. The current study observed no significant differences in the period of the rhythm which is consistent with other reported work (Bedrosian et al., 2013).

Although, the findings from the current study are consistent with other studies deomonstraying dLAN exposure does not interfere with the entrainment of activity patterns (Bedrosian et al., 2013), evidence from Swiss-Webster mice and Siberian hamsters have demonstrated that dLAN induces alterations in the phase and amplitude of core clock gene expression and protein production such as changing the expression of *PER1*, *PER2* and BMAL1 in the the SCN (Bedrosian et al., 2013; Bedrosian, Galan, Vaughn, Weil & Nelson, 2013; Fonken, Aubrecht, Melandez-Fernandez, Weil & Nelson, 2013). This indicates that although dLAN is not placing a potent influence in altering circadian behaviour as measured through the locomotor activity, it is changing expression of clock genes which are integral to the clock.

dLAN and depressive behaviours

The current study investigated the effects of dLAN on the manifestation of affective behaviours. This is of significant interest given that rates of depression are increasing simultaneously with the increased presence of dLAN in sleeping environments. The current study investigated for the first time the the magnitude of change that dLAN exposure can have on animals performance on a battery of behavioural measures of depressive-like behaviour compared to when they were first tested after being accommodated under L/D conditions. Although, the current study found that dLAN did not affect the entrainment of activity patterns or induce significant changes to the angle of entrainment. However, depressive-like phenotypes can still be expressed even when there is no inference to entrainment. For instance, it has been observed that light manipulations which do not induce circadian arrhymicity, or sleep deprivation have a potent influence on the onset of depressive behaviours (LeGates et al., 2012). Nevertheless, it is observed that the ipRGCs play a significant function in mediating the association between dLAN and depressive-like behaviours. ipRGCs deficient animals fail to express depressive-like behaviour after an aberrant light schedule which did not cause circadian arrthmicity or sleep deprivation (LeGates et al., 2012).

EPM

The EPM provides a behavioural measure of high level anxiety. Within this test, rodents, which are exploratory animals, are placed with an in-built conflict between a fearful response to a novel or unpleasant environment and a natural tendency to explore and investigate new environments and surroundings. This inhibition of fearful response (as measured by quick time latency to first open arm entry and increase in total time spent in open arms) suggests reduced anxiety whereas the opposite response pattern is considered as reminiscent of high level anxiety (Crawley, 1985; Prendergast & Nelson, 2005). The results from the current study indicated that animals housed under dLAN

conditions had a statistical decrease in their time to first open arm entry which would not be interpreted as anxiety-like behaviour. In comparison control animals had an increase in their time to first open arm entry which is interpreted as anxiety-like behaviour. These findings are inconsistent with Borniger and colleagues (2014) who found that dLAN exposure increases in anxiety-like behaviour. However, our results are consistent with other studies, which have observed that dLAN exposure leads to reductions in time to first arm entry in Siberian hamsters (Bedrosian, Fonken, Walton, Haim & Nelson, 2011; Fonken et al., 2009). Furthermore, our results are consistent with studies employing constant light paradigms (Castro, Frussa-Filho, Fukushiro, Chinen, Abilio & Silva 2005; Fonken et al., 2009; Ma et al., 2009). This would suggest that under dLAN conditions, the EPM may not provide a measure of anxiety-like behaviour but instead investigate impulsivity and novelty-seeking behaviour. This putative link would be consistent with some subtypes of depression where individuals report more impulsive risk-taking behaviour such as suicidal behaviour (Bedrosian et al., 2012). In chronic stress animal models of depression, symptoms of anhedonia have been observed alongside increased novelty-seeking behaviours (Li et al., 2010). No significant differences were observed between groups on total amount of time spent in the open arms, which is inconsistent with other studies observing differential effects in opposite directions (Bedrosian et al., 2011; Borniger et al., 2014).

Forced Swim Test

Assessment of beavhoural despair was assessed using Porsolt's FST and the TST. From baseline to post-experimentation dLAN animals expressed a decreased time in latency to immobility and increased total time immobility on the FST, these effects were more pronounced in males. This indicates that exposure to dLAN results in more pronounced behavioural despair in males compared to females. Interpretation of the FST suggests that animals who become immobile faster and elicit an increased time in total

immobility are displaying behavioural despair, which is a core symptom of affective disorders (American Psychiatric Association, 2013). Similar results of quicker latency times and increased total immobility have been observed in melatonin producing C3H/HeNHsd mice (Fonken & Nelson, 2013) and Siberian hamsters (Bedrosian, Zachary, Weil and Nelson 2012; Bedrosian, Vaughn Galan, Daye, Weil & Nelson, 2013; Bedrosian, Weil & Nelson 2013; Bedrosian, Fonken, Walton, Haim & Nelson, 2013). However, these results have not been observed in Swiss-Webster mice (Borniger et al., 2014). Unlike the FST, no effects for sex were were observed in the TST across the two lightinh conditions. However, it was observed that dLAN animals expressed significantly higher total immobility when placed under dLAN.

Sucrose Preference Test

Anhedonic behaviour was assessed using the sucrose preference test, which has shown good validity and reliability (Forbes, Stewart, Matthews & Reid, 1996; D'Aquila, Newton, Willner, 1996). Within C57Bl/6 mice, chronic exposure to dLAN did not elicit any changes in sucrose preference. These findings are consistent with one study using Swiss-Webster mice (Borniger et al., 2014). However, our findings are inconsistent with a significant proportion of work which provides evidence of reductions in sucrose preference in melatonin producing C3H/HeNHsd mice (Fonken & Nelson, 2013), diurnal male Grass rats (Fonken, Kitsmiller, Smale, Nelson, 2012) and Siberian hamsters (Bedrosian, Vaughn Galan, Daye, Weil & Nelson, 2013; Bedrosian, Weil and Nelson 2013; Bedrosian, Fonken, Walton Haim & Nelson, 2011).

Melatonin – A Tentative Link

Although, the exact mechanism which couples LAN and mood are unknown, many argue for the role that melatonin may play in this association (Bedrosian et al., 2013). Some propose that the reduction of melatonin secretion through dLAN produces multiple downstream effects (Bedrosian et al., 2013). This is due to temporal

organisation of physiological processes being dependent upon the decoding of light information into hormonal signalling, which in turn are distributed throughout the body. In periods of darkness the SCN communicates with the pineal gland to synthesise, produce and secrete melatonin (Reppert & Weaver, 2001). Light information results in the SCN inhibiting the production of melatonin in the pineal gland, which in turn prevents its secretion into the bloodstream where it provides a physiological cue for night-time (Reiter, 1993). The association between melatonin and mood is supported with the efficacious antidepressant agomelatine which expresses a melatonin receptor agonist and serotonin receptor antagonist ($5-HT_{2C}$) (Goodwin, Emsley, Rembry & Rouillon, 2009; Kennedy & Rizvi, 2010). The current study casts doubt over the argument that the suppression of melatonin due to dLAN exposure elicits depressive-like behaviour. In the current study, melatonin-deficient C57Bl/6 animals developed depressive-like behaviour in the domain of behavioural despair. Although the effects sizes were small these symptoms were elicited in animals who do not express melatonin. It is therefore plausible, that melatonin may not be the primary mechanism for the relationship between LAN and mood.

Weight

Increases in levels of obesity have occurred over the last 20 years alongside the increased prevalence of Dlan (Fonken et al., 2014). The current study indicated that both females and male growth curves were not significantly altered under chronic exposure to dLAN. These findings are in contrast to multiple studies which have reported that body mass increased following the chronic exposure of dLAN in both male and female Swiss-Webster mice (Fonken et al., 2010; Fonken et al., 2013; Fonken et al., 2014; Aubrecht et al., 2015; Borniger et al., 2014). This is despite in each of the studies animals expressed equivalent levels of total daily locomotor activity and the same caloric intake. This shifting of food intake within these studies is significant given

that within nocturnal animals food consumption occurs predominantly during the dark phase (Zucker, 1971). Interestingly, Fonken and collegues (2010) observed that the amount of daytime food intake correlated with final body mass. Furthermore, when food was restricted to the dLAN phase no increases in body mass were observed. Within these studies increases in body mass are attributable to multiple factors. These include the shifting of food intake from the dark phase to the light phase (Fonken et al., 2010), the reduction in body temperature which is an entraining cue for peripheral tissues (Borniger et al., 2014), and finally, alterations to core clock gene expression and protein production in the peripheral tissues critical for metabolism, such as occurs the liver (Fonken, Aubrecht, Melandez-Fernadez, Weil & Nelson, 2013).

One possible reason for the contrasting results is due to all animals having access to a running wheel. Multiple evidence suggests that running wheel access is not only positively coupled with strengthening both circadian rhythms (Power, Hughes, Samuels & Piggins, 2010; Schroeder, Truong, Loh, Jordan, Roos & Colwell, 2012) but also limiting weight-gain through exercise (Fonken et al., 2014; Pattarson & Levin, 2008). The argument for the reduction in body mass due to access to a running wheel is supported by findings from Fonken and collegues (2014). They found that access prevented increases in body mass among Swiss-Webster mice housed under dLAN, compared to mice with locked wheels accommodated under the same conditions. In the current study the consumption and timing of food intake was not measured. All previous work investigating the effects of chronic dLAN exposure on metabolism have been conducted on albino Swiss-Webster Mice. This is not an optimal animal to employ given that they incur significant retinal damage to photoreceptors when exposed to intense light during the night period and their spectral sensitivity to light is different (Castelhano-Carlos & Baumans, 2009). Animals employed in the current study are pigmented which make the results more comparative to humans.

Photoperiod

We believe that the current findings investigating the effects of dLAN on circadian rhythmicity and development of depressive-like behaviour are more valid given that the majority of studies investigating this topic have been conducted in highly photoperiodic Siberian hamsters. In contrast, this study employed C567Bl/6 mice which are typically unresponsive to photoperiod (Goldman, 2001; Nelson & Moffatt, 1994; Rosenwasser et al., 2015). Siberian hamsters are a highly photoperiodic species of animal. In order for these species to survive and reproduce, the timing and duration of light is of critical importance as it provides a critical cue to determine the time of year (Aubrecht, Weil & Nelson, 2014; Einat, Kenfeld, Schor & Eilman, 2006; Ikeno et al., 2014). For these species melatonin, which is under control of the circadian clock, serves as the biological signal for day and night length. The sensitivity to light means that the duration of the production and secretion of melatonin corresponds with night length. As a result, photoperiodic information becomes encoded within the melatonin signal, with this signal becoming unravelled within specific tissues which are fundamental to seasonal, physiological and morphological behaviour responses (Goldman, 2001). For example, photoperiodic animals housed under long photoperiods express shorter periods of melatonin secretion (Ikeno et al., 2014).

These photoperiodic species sensitivity to dim-light is demonstrated in jet-lag paradigms where the intensity level of dim moonlight and starlight (<.005LUX) allows for the acceleration of re-entrainment to 4-hour phase advances and reduces time of re-entrainment by up to 29% in these species (Frank, Evans & Gorman, 2010; Gorman, Evans & Elliott, 2006). Furthermore, light exposure to levels as low as 1.08 LUX is sufficient to reduce pineal melatonin content in these species (Brainard, Richardson, Petterborg & Reiter, 1982). This sensitivity is not as apparent in humans, who require

exposure to ~45LUX for plasma melatonin concentration to be attenuated by ~60% (Brainard et al., 1988).

As a result of this sensitivity to dim light, it is reasonable to argue that Siberian hamsters are not optimal animals to employ when investigating the effects of dLAN on circadian rhythms and its possible manifestation on affective disorders. These species may not have the capacity to differentiate between dim-light and light and as a result perceive the onset of dLAN as a continuation of constant light. This may account for the depressive-like phenotypes within these species as constant light results in depressive-like behaviour being expressed in non-photoperiodic animals (Becker, Bilkei-Gorza & Zimmer, 2010; Fonken et al., 2009; Fujioka et al., 2011). Evidence supporting that photoperiodic animals are not able to differencite between dim-light and light has been observed by Ikeno and collegues (2014), who found that Siberian hamsters housed under dLAN for 20 weeks failed to exhibit rhythmic nocturnal activity. This indicates that over long periods of time the entrained rhythmicity which is first observed under dLAN (Bedrosian et al., 2013) becomes arrhythmic. This could be mediated by the long-term absence of the entraining zeitgeber melatonin on the SCN due to constant light, albeit at dim levels during the night period. In animal models of depression using Siberian hamsters it has been observed that long-photoperiods ameliorates depressive-like symptoms (Benabid, Mesfioui & Ouichou, 2008; Molina-Hernandez & Tellez-Alcantara, 2000) and short-photoperiods have opposite effects (Benabid, Mesfioui & Ouichou, 2008; Prendergast & Nelson, 2005; Einat, Kronfeld-Schor, Eilman, 2006). However, in these studies investigating long-photoperiods of 16/8 L/D cycles, the dark period was completely dark. These photoperiodic species may thus require a distinctive darkness cue given their sensitivity to light which may prevent depressive behaviours due to their perception of light. Although evidence has reported that photoperiodic animals remain entrained to 5LUX (Fonken et al., 2009; Ohta et al., 2005),

photoperiodic animals demonstrate entrainment under 5LUX dLAN (Bedrsosain et al., 2013) albeit with alterations to core clock genes and proteins (Bedrosian, Galan, Vaughn, Weil & Nelson, 2013). Studies of constant light and exposure to 22-h L/D cycles are linked to the onset of depressive-like phenotypes (Becker, Bilkei-Gorza & Zimmer, 2010; Fonken et al., 2009; Fujioka et al., 2011). Therefore, it may be possible that these photoperiodic species, who demonstrate sensitivity to light, perceive the presence of dLAN as constant light. Although the intensity of light dLAN is not strong enough to alter overt locomotor behaviour, its continuation does not provide a distinctive dark cue within these light sensitive species.

Test-retest Reliability

The current study measured depressive-like behaviours using a battery of behavioural measures across two time-points. These tests were conducted at baseline and after experimental the experimental period. This design was chosen to tease apart whether if and to what extent dLAN exposure changed behaviour at time-point two compared to time-point one. The behavioural tests used in this study have been found to express good face, construct and predictive validity and have widely used in psychiatric testing were utilised (van der Staay, Arndt, Nordquist, 2009). In the current study, significant effects for time were observed in both control and experimental animals when tested across the two timepoints. This poses difficulty in the interpretation of results, as across the two time-points control condition animals along with dLAN exposure animals simultaneously expressed changes in time spent immobile as measured by the FST/TST, expressed heightened preference for sucrose solution, and different timing behaviour with entry to open arm on the EPM. This results in performance scores being overestimated due to significant change scores being observed. These tests which are commonly used in psychiatric research, should express test-re-test reliability with

behavioural trait characteristics remaining stable and consistent for control animals across time-points.

Differences in behavioural traits across time-points have been reported in diseased, naïve and medicated animals in studies employing the FST and EPM across time-points (Andreatini & Bacellar, 2000; Su, Hato-Yamada, Araki & Yoshimura, 2013). One contributing factor to the differences in behaviour observed across two-time points particularly in the TST and FST may be that of implicit memory. Su and collegues (2013) argue that memory processes play a role when animals are tested on a paradigm multiple times. They argue that rodents recall the previous aversive testing and subsequently when tested again adopt an immobile posture for longer, which in turn increases immobility. Administration of the amnesic drug scopolamine provides further support for the role of memory with immobility times decreasing in animals tested multiple times (Su et al., 2013). This evidence suggests that performing the FST once is more appropriate for optimally assessing mood while the second testing may be associated to the unpleasant memory of the first testing. The results from the current study in conjunction with the aforementioned research indicate that these tests are not appropriate to evaluate trait characteristics which, without treatment, are meant to remain stable consistent over time.

Stem-Cell Proliferation

To our knowledge, this is the first reported study to demonstrate that chronic exposure to dLAN is sufficient to reduce adult proliferating cells within the dentate gyrus as evidenced by Ki-67. This finding is of significant importance given that neural stem cell proliferation is the first step in neurogenesis and decreased neurogenesis is implicated as a contributing factor in the pathogenesis of depression (Kempermann & Kronnenberg, 2003). Further, confirming evidence of its association with depression is provided with administration of antidepressant and electroconvulsive therapy resulting

in an increase of NPCs in sufferers of MDD (Boldrini et al., 2009; Chen, Pandey, Dwivedi, 2006; Zhao et al., 2013). The current findings are inconsistent with Reif and collegues (2006) who report no reduction in NPCs in human sufferers of MDD (Reif et al., 2006). The current findings provide support that dLAN serves as a key regulator of progenitor cell proliferation. This is further supported by evidence from Fujioka and colleagues (2011) who found that C57Bl/6 mice exposed to constant light expressed a reduction in NPCs in the granule layer. The precise mechanism which results in chronic exposure to constant L/dLAN resulting in proliferating cells remains unknown. Multiple research studies indicate that reductions in proliferation of NPCs are mediated by a variety of stressors (Czeh, et al., 2001; Chen, Pandey, Dwivedi, 2006; Duman, Malberg & Thome, 1999; Fuchs & Flugge, 1998; Gould & Tanapat, 1999; Tanapat et al., 1998, 1999; McEwan, 1998). This coupling comes from multiple studies which report that constant light exerts stressful effects in animal models (Abilio, Freitas, Dolnikoff, Castrucci & Frussa-Filho, 1999; Fonken et al., 2010; Fonken et al., 2009; Van der Meer, Van Loo & Baumans, 2004). During stressful situations, stimulation of neuroendocrine systems, such as the hypothalamic-pituitary-adrenal axis occurs. Thus, it may be the neuroendocrine system that mediates the reduction in proliferating cells. Fujioka, Fujioka, Ishilda, Maekawa and Nakamura (2006) observed that excessive glucocorticoids resulted in the attenuation of neurogenesis. However, this link should be taken with caution as some studies have reported that glucocorticoids rhythms are not altered under dLAN exposure (Fonken et al., 2010) Other authors have indicated that reduction in physical activity may cause reductions. However, this argument does not seem plausible considering in the current study the power of the rhythm was not reduced and previous studies have reported that chronic dLAN exposure does not induce differences in total daily locomotor activity and locomotor activity rhythms (Bedrosian et al., 2013; Bedrosian et al., 2012).

The decrease in the numbers NPCs when animals are housed under dLAN conditions is possibly due to the reduction in growth/neurotropic factors in extracellular fluid such as BDNF. BDNF is required for the replication, survival, and differentiation of NPCs (Boscia, Esposito, Crisci, Francisis, Annunziato, 2009; Sakata et al., 2013). Its expression is reduced under stress-induced animal models of depression and increased with antidepressant medication (Altar, 1999). Furthermore, models of depression indicate that amelioration of depressive-like behaviour and restoration of spine density occurs only when BDNF expression is returned to optimal levels (Bedrosian, Weil & Nelson, 2013). A number of studies in both mice (Fonken & Nelson, 2013) and hamsters have indicated that dLAN exposure leads to a reduction in BDNF mRNA expression (Bedrosian, Weil & Nelson, 2013 Bedrosian et al., 2012; Fonken et al., 2012). It is argued that this reduction in neurotrophic support (i.e. BDNF) due to chronic exposure to dLAN results in disruption within the brain (Angelucci et al., 2005), leading to impaired NPCs levels which results in attenuated neurogenesis.

Ki-67 additionally labels resting cells due to their involvement in DNA repair (Ohta & Ichimura, 2000). The number of cells counted may be overestimated as Ki-67 labels dying neurons (Reif et al., 2006). The antibody also does not differentiate between neural and glial cells due to the marker being induced ahead of terminal differentiation. As a result, the current results cannot infer the possible quantity of new functional neurons which would develop through neurogenesis. Only an indication of neural stem cell proliferation can be inferred. Despite this, evidence suggests that Ki-67 is a leading estimate marker for adult neurogenesis as a result of its colocalization with BrDU which has been demonstrated that 75-90% of BrDU-positive cells express NeuN (Kadama, Fujioka & Duman, 2004). The current results provide a speculative approximation level of adult neurogenesis. Young C57Bl/6 mice were employed in the current study therefore, it is uncertain whether similar effects be observed in older

animals. This caution to generalise results comes from findings that older individuals report fewer NPCs (Lucassen, et al., 2001) compared with younger individuals (Boldrni et al., 2009). Additionally pharmacological effects of treatment have not been observed in older patients (Lucassen et al., 2001) compared with younger patients (Boldrini et al., 2009). This indicates that the therapeutic effects of antidepressant medication on the process of NPCs and neurogenesis are age-dependent (Couillard-Despres et al., 2009; Navailles, Hof & Schmauss, 2008).

While the number of NPCs identified between chronically exposed dLAN animals and controls were different, the amount of NPCs identified in each group may be overestimated yet still remain significantly different. This is due to the fact that animals were placed in environmentally enriched cages which had access to a running wheel and furthermore some animals had social interaction due to being accommodated in group housing. These enriched environments with social interaction have been implicated with increasing cell proliferation in C57Bl/6 animals (van Praag, Kempermann & Gage, 1999). Future work should provide animals with less enriching environments, which will allow for a more valid understanding of the effects of dLAN on proliferation.

Implications of current work to human scenario

The current findings suggest that dLAN exposure, which is an innocuous-seeming manipulation to our environment, places a potent influence on inducing aspects of depressive-like behaviour and molecular changes within the brain. Our findings are consistent with previous work using blue-wavelength light, demonstrating that dLAN exposure elicits behavioural despair as a symptom of depression. In addition to this, our work builds upon previous findings to demonstrate that dLAN elicits more behavioural despair in males compared to females. However, these sex effects were not observed in the other behavioural measures. In the other behavioural measures no significant effects

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in dLAN eliciting depressive-like phenotypes were observed. Our work is inconsistent with pervious studies indicateing that dLAN elicits anhedonic and anxiety-like behaviour. It is possible that non-photoperiodic animals are significantly less sensitive to the development of depressive-like behaviours compared to photoperiodic animals where significant depressive-like phenotypes were elicited across all the behavioural measures. This interpretation is important for the generalizability of results to humans who are non-photoperiodic also. It is of critical importance that future research investigating the effects of lighting on circadian rhythmicity and the development of depressive behaviours choose animals where findings are not strain specific and provide translatable findings to humans. In addition, our results found that dLAN exposure did not interfere with circadian rhythmicity. This may be due to the intensity of the light zeitgeber not being strong enough to elicit changes to the entrainment of behavioural rhythms. This observation is of significant interest given that humans are routinely exposed to dLAN. Our results, demonstrate for the first time that dLAN exposure can lead to reduction of NPCs within the hippocampus. Although, the antibody used in the current study cannot infer that neurogenesis is reduced it does provide, the best possible tool for estimating neurogenesis levels. Impaired neurogenesis has been associated with impairing the architecture of the hippocampus leading to atrophy. Attenuation of levels of neurogenesis are found in individuals with a clinical diagnosis of depression. Although, it is too far ahead for a conclusion to be made on humans our findings indicate that that humans should avoid dLAN exposure in order to prevent attenuation NPCs levels. This is especially more pertinent as most individuals have a light emitting device in their sleeping environment (Cho et al., 2015).

Given the nature of the study, a number of limitations exist. Primarily, depression is a heterogeneous, multifaceted disorder with the symptoms manifesting from interaction between genetics, environment and social factors. As a result of this

complex interaction it is difficult to fully model depression in animals. Animals have an ability to reproduce key aspects of depressive-like phenotypes which mirror the symptoms of affective disorders in humans. However, animals fail to fully encapsulate the complete spectrum of the disorder which is expressed in humans. One may argue however, that holding one condition constant - in the case of the current study, dLAN exposure - allows one to separate and investigate its effects on manifestation which could not be achieved in humans where multiple contributing factors are at play.

Future Work

- As a result of our findings conflicting with previous studies which employed Siberian hamsters and Swiss Weber mice it is imperative that future work use other strains of animals. This will ensure that findings are not strain specific and that results translate to humans.

- The fundamental aim of the study was to investigate whether depressive-like behaviour would develop after animals were housed in L/dLAN conditions compared to those housed under a L/D cycle. However, the current study found that for the majority of tests used to measure depressive-like behaviour effects for time were observed. That is, across both conditions, the animals sensitised to tests when tested at baseline and after experimental conditions. In order to eliminate effects for time future studies should employ a battery of behavioural measures which closely resemble the each other. For example, when measuring anxiety-like behaviour at baseline testing employ the EPM after experimentation employ the dark/light box.

- In the current study dLAN resulted in the reduction of proliferating cells within the dentate gyrus of the hippocampus. Future studies should investigate whether glial cells are reduced in other brain areas which are structurally vulnerable in those with depression such as the prefrontal cortex and amygdala.

- Exposure to dLAN for a three-week period did not significantly disrupt circadian rhythmicity or induce anhedonic or anxiety-like behaviour. Future work should investigate whether exposure to dLAN over a long period of time (e.g. three months) may elicit changes in circadian rhythmicity and in affective behaviours. Becker and colleagues (2010) observed that under a 22/2 LD cycle depressive behaviours were only elicited after four weeks. Similarly, Ikeno and collegues (2014) found that over four weeks exposure to dLAN is required before the rhythm becomes arrhythmic. Before, the four week period the rhythm is similar to what is observed in animals housed under L/D.

- Studies have yielded inconsistent results regarding whether dLAN acts as a stressor. It is unknown what mediates the induction of depressive-like behaviours and the molecular and structural changes within the brain under dLAN. One plausible explanation is that dLAN elicits a stress response however, inconsistent results have been yielded. Future work should simultaneously carry out a chronic mild stress paradigm and dLAN paradigm and measure faecal glucocorticoid and compare glucocorticoids to investigate if comparable amounts of stress responses occur.

- In the current study, the body mass of animals remained unchanged. These findings may be due to animals' ability to access a running wheel. Further exploration should consider whether no wheel results in weight gain in C57Bl/6 mice. Furthermore, analysis of feeding habits, food intake, and glucose tolerance testing may provide further insight into further investigation compared to simple body mass measures.

- Future work should investigate whether the administration of antidepressant medication results in increasing the number of NPCs in animals housed under under dLAN

- When animals were first placed in dLAN conditions a phase-delay in behaviour was observed. The onset of activity was returned to what was previously observed under the L/D condition after a week. Future work should measure depressive-like behaviour just before the onset of activity returns to previous levels. This would be of significant interest given that in humans with depression phase-delays are associated with depressive symptoms. The severity of depressive symptoms corresponds with the extent of the phase-delay (Emens, Lewy, Kinzie, Arntz & Rough, 2009).

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