PHYSIOLOGICAL CONSEQUENCES OF CIRCADIAN DISRUPTION BY NIGHTTIME LIGHT EXPOSURE

DISSERTATION

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By

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ABSTRACT

For more than 3 billion years, life outside the highest latitudes has evolved under brightly illuminated days and dark nights. Most organisms have developed endogenously driven circadian rhythms which are synchronized to this light/dark cycle. In recent years, daily light schedules have become artificial and irregular due to the use of electric lighting. In this dissertation, I propose that exposure to light at night (LAN) disrupts the circadian system altering metabolic, immunological, and behavioral functions.

The global increase in the prevalence of obesity and metabolic disorders coincides with increases in exposure to LAN and shift work. Therefore, my first experiments examined whether exposure to LAN affects metabolism. Mice exposed to dimly lit (5 lux) as compared to dark nights increased body mass and reduced glucose processing without changing caloric intake or activity. Exposure to dim light at night diminished the daily rhythm in food intake and restricting food access to the dark phase prevented weight gain in mice exposed to dimly lit nights (Chapter 2). Furthermore, metabolic changes associated with exposure to LAN are not permanent; placing mice back in dark nights partially reversed increases in body mass caused by exposure to dim light at night (Chapter 3). In Chapters 4 & 5, I investigated the interactions among LAN and more traditional risk factors for obesity such as high fat diet and lethargy.

Because light is the most potent synchronizing factor for the circadian system and
disruption in clock genes is associated with significant changes in metabolism, I next investigated the effects of exposure to LAN on the circadian system (Chapter 6). Exposure to dimly lit nights attenuated core circadian clock rhythms in both the master circadian pacemaker and peripheral tissues.

In addition to altering metabolism, exposure to LAN is implicated as a contributing factor to several diseases involving dysregulation of the immune system. This led to experiments examining the effects of acute exposure to dim LAN on recovery following cardiac arrest (Chapter 7). Exposure to dimly lit as compared to dark nights following global ischemia increased hippocampal inflammation, neuronal cell death, and mortality. Selectively inhibiting inflammation and altering the spectrum of nighttime light to which mice were exposed reduced damage among mice exposed to dim LAN.

In the experiments described above, I worked with nocturnal mice in order to assess the effects of nighttime light exposure independent of changes in sleep architecture. However, the secretion patterns of many hormones and immune parameters are different in nocturnal and diurnal species. In the final set of experiments, I demonstrated that diurnal Nile Grass rats (Arvicanthus Niloticus) exposed to dim LAN increased immunological measures (Chapter 8) and altered hippocampal connectivity in addition to changing cognitive and affective behaviors (Chapter 9). Taken together, these studies indicate that exposure to ecologically relevant levels of dim LAN attenuate core circadian clock mechanisms in rodents resulting in physiological and behavioral consequences.
DEDICATION

To my family – for their love, encouragement, and support.
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CHAPTER 1

THE “SKINNY” ON LIGHT AT NIGHT, CIRCADIAN CLOCKS, AND METABOLISM

Over the course of the 20th century the prevalence of obesity and metabolic disorders rapidly increased worldwide. By the year 2000 the number of adults with excess adiposity surpassed those who were underweight for the first time in evolutionary history (Caballero, 2007). The growth in obesity has been exponential in recent decades particularly for the highest weight categories. From 2000 to 2005 the number of individuals qualifying as morbidly obese (BMI over 50) increased by 75% in the United States (Sturm, 2007). Obesity is a pathogenic condition defined by the accumulation of excess adipose tissue and is associated with serious health complications including diabetes, cardiovascular disease, hypertension, asthma, and reproductive dysfunction (Guh et al., 2009). Obesity reduces quality of life, results in significant health related complications, and more than doubles healthcare costs. In addition to typical obesogenic factors such as high calorie diet and sedentary lifestyles contributing to obesity, other environmental factors are likely involved in the development and maintenance of this condition (Symonds, Sebert, & Budge, 2011).
Unprecedented transitions in human lifestyle occurred during the past century, such as advances in travel and communication, greater urbanization, and the eradication of multiple diseases (Engineering, 2000). One environmental change that had a mostly unappreciated, yet dramatic, effect on human lifestyle was the widespread adoption of electric lighting. Electric lights have provided many societal advances. Brightening the night has shed the negative stigma of nighttime as a time solely for crime, sickness, and death (Ekirch, 2005). The use of electric light at night played a large role in the industrial revolution allowing for the creation of shift work. Furthermore, electric lighting has given individuals the freedom to function on a self-selected sleep/wake schedule. Because the invention of electric lighting occurred prior to an understanding of circadian biology, little concern was given to the potential effects exposure to unnatural light schedules may have on human health. It is becoming apparent, however, that there are significant physiological repercussions associated with exposure to light at night (Fonken & Nelson, 2011; Navara & Nelson, 2007). In a sense, shift workers, who are exposed to high levels of light at night in the workplace, have served as society’s ‘canaries in the coal mine’ for maladaptive consequences of nighttime light exposure. Epidemiological evidence from shift workers demonstrates that prolonged exposure to light at night increases the risk of developing cancer (Stevens, 2009b), sleep disturbances (Kohyama, 2009), mood disorders (Driesen, Jansen, Kant, Mohren, & van Amelsvoort, 2010), metabolic dysfunction (B. H. Karlsson, Knutsson, Lindahl, & Alfredsson, 2003; Knutsson, 2003; Obayashi et al., 2013; Parkes, 2002; Puttonen, Viitasalo, & Harma, 2011; van
Amelsvoort, Schouten, & Kok, 1999), and cognitive impairments (K. Cho, 2001; Vetter, Juda, & Roenneberg, 2012).

The reason that exposure to electric lights likely affects physiology is because many organism have developed endogenously driven 24 h rhythms, termed circadian rhythms, that are most potently synchronized by light information. The rotation of the Earth about its axis produces a highly consistent cycle of light and dark that varies latitudinally and on a seasonal basis. For more than 3 billion years, life on Earth has evolved under bright days and dark nights. In order to optimally time physiological, behavioral, and metabolic functions many organisms developed circadian rhythms. Circadian rhythms allow organisms to anticipate predictable daily events such as food availability and rest. Here I propose that increases in exposure to light at night during the 20th century and concomitant changes in lifestyle are associated with alterations in metabolism. In this introductory chapter, I will first provide an introduction to the circadian system, with a specific emphasis on the effects of light on circadian rhythms. Next I address interactions between the circadian system and metabolism. Animal models have provided a vast amount of knowledge about the effects of circadian rhythm disruption on metabolism, as well as the effects of disrupted metabolism on the circadian system. Finally, I will tie in current experimental and epidemiological work associating exposure to light at night and metabolism.

2. Circadian clock work

Most organisms, from unicellular cyanobacterium, to fruit flies and humans, have developed an endogenous timekeeping system that synchronizes physiological and
behavioral processes to the external solar cycle (Bell-Pedersen et al., 2005). Biological clocks have the ability to both coordinate interactions among animals (e.g., knowing the time of day can help animals avoid predation or engage in mating) and synchronize internal physiological and biochemical processes within an individual (e.g., rhythmic hormone release in anticipation of food availability and sleep). Circadian rhythms are defined specifically as internally driven oscillation that meet several characteristics including: (1) the period of the rhythm is about 24 h in the absence of environmental cues, (2) rhythms are buffered against changes in environment such as temperature fluctuations and behavioral feedback, and (3) rhythms can shift under the influence of certain factors but entrainment is limited to a specific range (Dunlap, Loros, & DeCoursey, 2004).

One common example of a circadian rhythm in mammals is the presence of a sleep/wake cycle. In diurnal species, such as humans, sleep typically occurs during the dark portion of the day, whereas nocturnal animals, such as house mice (Mus musculus), generally sleep during the light. The propensity for sleep and activity is influenced by endogenously controlled rhythmic hormone release. For example, cortisol secretion spikes in the early morning directly prior to awakening in humans and then drops throughout the day, reaching its nadir around the time of sleep onset (S. L. Bailey & Heitkemper, 2001). Overall, circadian rhythms exist in many facets of physiology and the importance of the circadian system is clearly demonstrated by considering pathogenic conditions that result from altering circadian physiology (Takahashi, Hong, Ko, & McDearmon, 2008). Circadian rhythm disruptions contribute to a wide range of disorders
including cognitive impairments, mood disturbances, and increased risk of cardiometabolic disorders (Zee, Attarian, & Videnovic, 2013). In order to understand why these pathogenic conditions arise from disruption of the circadian clock it is first important to understand where circadian oscillations originate.

2.1. The suprachiasmatic nucleus is the master circadian oscillator

The suprachiasmatic nuclei (SCN) of the hypothalamus comprise the master circadian clock in mammals, at the top of a hierarchy of independent self-sustaining oscillators. The SCN is located in the anterior hypothalamus directly above the optic chiasm and is composed of approximately 50,000 densely packed small neurons in humans and 10-20,000 neurons in rodents (Dunlap, Loros, & DeCoursey, 2004). There are several lines of evidence confirming that the SCN is the master circadian oscillator in mammals: (1) SCN lesions abolish circadian rhythms (Stephan & Zucker, 1972), (2) electrical and chemical stimulation of the SCN induce phase shifts (Michel et al., 2013; Rusak & Groos, 1982), (3) transplanting an SCN into an animal whose own SCN has been ablated restores circadian activity (R. Silver, LeSauter, Tresco, & Lehman, 1996), and (4) individual neurons dissociated from the SCN display long term self-sustaining oscillations (Welsh, Logothetis, Meister, & Reppert, 1995). Cellular synchrony within the SCN is established through multiple mechanisms such as sodium dependent action potentials (Yamaguchi et al., 2003) and humoral signals (R. Silver, LeSauter, Tresco, & Lehman, 1996).

2.2. Molecular mechanisms of the circadian clock
The circadian clock in mammals is driven by an autoregulatory feedback loop of transcriptional activators and repressors (reviewed in (Mohawk, Green, & Takahashi, 2012; Reppert & Weaver, 2002). CLOCK and BMAL1 form heterodimers that induce expression of *Period* (*Per1, Per2, and Per3*) and *Cryptochrome* (*Cry1 and Cry2*) through E-box enhancers (Gekakis et al., 1998). PER and CRY proteins accumulate in the cytoplasm throughout the circadian day. Upon reaching a critical amount, PER and CRY form a complex that translocates back to the nucleus to associate with CLOCK and BMAL and repress their own transcription (Mohawk, Green, & Takahashi, 2012; Reppert & Weaver, 2002). This process takes approximately 24 h to complete a full cycle. In addition to the primary feedback loop, other regulatory loops influence the circadian clockwork. For example, the CLOCK:BMAL1 heterodimer also activates transcription of retinoic acid-related orphan nuclear receptors, *Rev-erba* and *Rora*, which have feedback effects primarily on *Bmal1* (Preitner et al., 2002).

Core clock components are defined as genes with protein products that are essential for the generation and regulation of circadian rhythms (Takahashi, 2004). Ablation of the core clock genes *Clock, Bmal1* (Bunger et al., 2000), *Per1, Per2* (Bae et al., 2001), *Cry1*, and *Cry2* (van der Horst et al., 1999) all disrupt circadian physiology (see Table 1 in (Ko & Takahashi, 2006). The line between what constitutes a core clock gene is constantly evolving. *Rev-Erb* and *Per3* were not initially considered critical for maintaining clock function; however, the importance of these genes for circadian regulation is now widely accepted (H. Cho et al., 2012; Pendergast, Niswender, & Yamazaki, 2012).
2.3. Additional clocks persist outside the SCN

In multicellular organisms, circadian oscillators are present in most if not all tissues. The SCN serves as the master circadian clock at the top of a hierarchically organized system (Mohawk, Green, & Takahashi, 2012). Tissue specific clocks contain the molecular machinery necessary for self sustaining oscillations (King et al., 1997) and have virtually the same molecular makeup as circadian oscillators in the SCN. Peripheral clocks are entrained by the SCN through both neural and hormonal signals (Guo, Brewer, Champhekar, Harris, & Bittman, 2005; McNamara et al., 2001; Reddy et al., 2007), as well as local factors such as nutritional signals (Vollmers et al., 2009). Peripheral clocks do not appear to communicate with each other but they are coupled to the SCN (A. C. Liu et al., 2007). Ablation of the SCN in vivo has profound effects on peripheral oscillators (Akhtar et al., 2002) and peripheral oscillators show more rapid dampening of circadian rhythms in vitro (Balsalobre, Damiola, & Schibler, 1998). Whereas SCN rhythms can persist for more than one month in vitro, peripheral rhythms are not as robust and diminish within two to seven cycles (Yamazaki et al., 2000).

2.4. Light entrains the circadian system

In most organisms, the circadian system functions at approximately but not exactly 24 h. Therefore, circadian clocks require external input to entrain them to the environment (Golombek & Rosenstein, 2010). Light is the most potent synchronizing factor for the circadian system. Light information travels directly from intrinsically photo-sensitive melanopsin containing retinal ganglion cells (ipRGCs), through the retinohypothalmic tract, to the SCN (Hattar, Liao, Takao, Berson, & Yau, 2002). Within
the SCN, light induces rapid changes in cellular activity that have been extensively characterized by examining the expression of immediate early genes (Rusak, Robertson, Wisden, & Hunt, 1990).

At the molecular level, exposure to light results in rapid induction of *Per1* (Albrecht, Sun, Eichele, & Lee, 1997; Shigeyoshi et al., 1997). A pulse of light during the night can phase advance or delay the circadian clock depending on the strength and time of the light signal (Miyake et al., 2000). For example, light at dawn advances the clock through advancing the onset of the *Per1* rhythm and acutely increasing mRNA transcription, whereas light at dusk delays the clock through delaying the offset of *Per2* (Schwartz, Tavakoli-Nezhad, Lambert, Weaver, & de la Iglesia, 2012). The SCN can rapidly adjust to light shifts, whereas peripheral tissues shift more slowly and in different ways (Yamazaki et al., 2000). Although light is the most potent signal for the mammalian circadian system, other factors such as food availability and locomotor activity can feedback and influence circadian clock function (Fuller, Lu, & Saper, 2008; Mistlberger & Antle, 2011). These types of stimuli can leave SCN rhythms intact, specifically altering clock gene expression in peripheral tissues (Damiola et al., 2000; Vollmers et al., 2009).

Multiple characteristics of the circadian system are conserved between rodents and humans. In humans, specifically-timed light pulses can also shift the circadian clock (Czeisler et al., 1989; Smith, Revell, & Eastman, 2009). Moreover, both humans and rodents are most responsive to 460 nm nighttime light exposure (Brainard, Richardson, Petterborg, & Reiter, 1982; Ruger et al., 2013). Longer wavelengths of lighting, such as
red light, do not activate the melanopsin containing retinal ganglion cells that project to the SCN and therefore minimally influence the circadian system (Brainard et al., 2008; Figueiro & Rea, 2010).

Exposure to constant dim light or total darkness results in a free-running circadian system. This knowledge has been used to test the effects of different factors, such as melatonin, on synchronizing circadian activity (Redman, Armstrong, & Ng, 1983). In contrast, the effects of dim and bright light at night on the circadian system are less well documented. In Chapter 2 of this dissertation I demonstrate that exposure to constant light alters activity rhythms and flattens circadian rhythms in glucocorticoids (Coomans et al., 2013; Fonken et al., 2010), two principle outputs of the circadian system.

There is evidence that very dim levels of light at night can influence circadian rhythms in both rodents and humans (Evans, Elliott, & Gorman, 2005; Jasser, Hanifin, Rollag, & Brainard, 2006). However, the effects of physiologically relevant levels of light exposure on the circadian system are not well characterized. Therefore, in this dissertation I evaluate the effects of chronic exposure to dim light at night (~5 lux) on the murine circadian system. The level of dim light used in my studies is ecologically relevant and comparable to levels of light pollution found in urban areas (Gaston, Davies, Bennie, & Hopkins, 2012; Kloog, Haim, & Portnov, 2009) and sleeping environments (Obayashi et al., 2013). In Chapter 5, I document that exposure to chronic low levels of light at night alters circadian clock genes in both the SCN and peripheral tissue (Fonken, L.K., Aubrecht, T.G., Meléndez-Fernández, O.H., Weil, Z.M., & Nelson, R.J., unpublished observations). Exposure to dim light at night specifically attenuates the rhythm in Per1
and Per2 gene and protein expression in the SCN around the light/dark transition. Furthermore, expression of Bmal1, Per1, Per2, Cry1, Cry2, and Rev-Erb are all suppressed in the liver. Nocturnal light may affect the liver through both autonomic and hormonal pathways (Cailotto et al., 2009). The changes in clock gene expression associated with exposure to dim light at night do not result in disruption of either the glucocorticoid rhythm or locomotor activity rhythm (Chapter 2). Similar changes in circadian clock function are also apparent in the SCN of hamsters exposed to low levels of light at night (Bedrosian, T.A., Galan, A., Vaughn, C.A., Weil, Z.M., & Nelson, R.J., unpublished observations). Hamsters exposed to dim light suppress PER1 and PER2 protein rhythms in the SCN independent of changes in activity rhythm. Overall, these results indicate that exposure to levels of nighttime lighting that are commonly found in urban settings can affect the circadian system.

3. The circadian system regulates metabolism and vice versa

Approximately 10% of the mammalian transcriptome displays circadian regulation (K. L. Eckel-Mahan et al., 2012; Panda et al., 2002; Storch et al., 2002). Among the rhythmic genes identified, many have a specific role in coordinating nutrient metabolism (K. Eckel-Mahan & Sassone-Corsi, 2009). For example, there is circadian expression of glucose transporters and the glucagon receptor (Panda et al., 2002), multiple enzymes involved in the metabolism of sugars and the biosynthesis of cholesterols display circadian oscillation (la Fleur, Kalsbeek, Wortel, Fekkes, & Buijs, 2001; Panda et al., 2002), and PCG-1α, an essential activator of gluconeogenesis, has a key role in regulating circadian rhythms (C. Liu, Li, Liu, Borjigin, & Lin, 2007).
Metabolically related hormones such as glucagon, insulin, ghrelin, leptin, and corticosterone also oscillate in a circadian fashion (Kalsbeek et al., 2001; Ruiter et al., 2003; Sinha et al., 1996). There is rhythmic expression of orexigenic signals including neuropeptide Y, galanin, and pre-opiomelanocortin within the hypothalamus, an area critical for coordinating metabolic signals (Jhanwar-Uniyal, Beck, Burlet, & Leibowitz, 1990; B. Xu, Kalra, Farmerie, & Kalra, 1999). Moreover, neuroanatomical organization provides evidence of interactions between metabolism and the circadian system; hypothalamic nuclei such as the paraventricular nucleus receive direct neuronal input from the SCN (reviewed in (Kalra et al., 1999).

3.1. Knocking out the circadian clock and obesity

In addition to fluctuations in metabolic processes suggesting an association between the circadian and metabolic systems, disrupting the clock network through genetic manipulations has provided insight into the role of the circadian system in maintaining metabolic homeostasis. Mice harboring mutations in various components of the circadian clock are susceptible to obesity and metabolic syndrome. Turek and colleagues were the first to report that Clock mutant mice on a BALB/c and C57BL/6J background are susceptible to diet induced obesity. Clock mutants show marked changes in circadian rhythmicity, as well as disruptions in diurnal food intake and increased body mass (Turek et al., 2005). This phenotype may partially result from changes in endocrine regulation as serum leptin, glucose, cholesterol, and triglyceride levels are all increased in Clock mutants compared to wild type mice. Deletion of Clock on an ICR background also results in metabolic alterations. In contrast to Clock deletion in a BALB/c and C57BL/6J
background, ICR Clock deficient mice are protected against weight gain due to impairments in dietary fat absorption (Oishi et al., 2006).

Disruption of other core circadian clock genes similarly affects metabolism. Mice deficient in Bmal1 alter insulin and glucose secretion (Marcheva et al., 2010) and rescuing Bmal1 in the central nervous system restores activity rhythms but not changes in metabolism (McDearmon et al., 2006). Mutation of Cry1 produces symptoms of diabetes mellitus in mice (Okano, Akashi, Hayasaka, & Nakajima, 2009). Furthermore, mice deficient in mPer1/2/3 increased weight gain on a high fat diet (Dallmann & Weaver, 2010) and single disruption of the Per2 gene alters glucose homeostasis (Carvas et al., 2012).

Loss of clock function in peripheral tissues also affects metabolism. Deletion of pancreatic Clock or Bmal1 reduces glucose and insulin processing abilities, independent of changes in activity or feeding rhythms in mice (Marcheva et al., 2010). Mice with liver-specific deletion of Bmal1 exhibit hypoglycemia during the fasting phase, exaggerated glucose clearance, and loss of rhythmic expression of hepatic glucose regulatory genes (Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007; Lamia, Storch, & Weitz, 2008). Changes in glucose processing with liver specific Bmal1 deletion occur independently of alterations in feeding behavior or locomotor activity, indicating a primary defect in metabolic responses (Lamia, Storch, & Weitz, 2008). In contrast, deletion of Bmal1 in adipocytes alters daily feeding rhythms and results in obesity (Paschos et al., 2012). Finally, Bmal1 deletion in the central nervous system produces
deficits in locomotor activity entrainment by periodic feeding, reductions in food intake, and subsequent loss of body weight (Mieda & Sakurai, 2011).

Alterations in secondary clock genes are also associated with metabolic changes in mice. Modulation of Per3 may be responsible for weight gain in Per1/2/3/- mice, as single deletion of Per3 results in significant weight gain (Dallmann & Weaver, 2010). Mice lacking the VIP-VPAC2 pathway, which plays an important role in SCN communication (Reppert & Weaver, 2001), dampen feeding rhythms and show reductions in metabolic rate (Bechtold, Brown, Luckman, & Piggins, 2008). Furthermore, mice lacking Nocturnin, a gene involved in the posttranscriptional regulation of rhythmic gene expression (Baggs & Green, 2003), remain lean on a high fat diet. This appears to be due to either changes in lipid uptake or utilization as genes important for lipid pathways lose rhythmicity in Nocturnin/- mice (Douris et al., 2011; Green et al., 2007). Recently, Rev-Erb has been implicated in the modulation of both metabolism and the circadian system (Yin et al., 2007). Dual deletion of Reb-Erba and Rev-Erbβ disrupts gene networks involved in lipid metabolism (H. Cho et al., 2012) and markedly affects circadian rhythms. Rev-Erb likely regulates hepatic lipid homeostasis through the recruitment of histone deacetylase 3 (Feng et al., 2011). Treatment with a Rev-Erb agonist increases energy expenditure and promotes weight loss in mice fed a high fat diet (Solt et al., 2012).

Associations between changes in clock genes and metabolism are also apparent in humans. Body mass index correlates with clock gene expression in peripheral adipose tissue depots (Zanquetta et al., 2012). Per2 expression levels in visceral adipose tissue
inversely correlate with waist circumference (Gomez-Abellan, Hernandez-Morante, Lujan, Madrid, & Garaulet, 2008) and \textit{Bmal}, \textit{Per2}, and \textit{Cry1} levels negatively correlate with total cholesterol and LDL concentrations. The methylation pattern of difference CpG sites of \textit{Clock}, \textit{Bmal}, and \textit{Per1}, are significantly associated with metabolic parameters including body mass index, adiposity, and metabolic syndrome score (Milagro et al., 2012). Moreover, a common clock polymorphism is coupled to the presence of metabolic syndrome in humans (E. M. Scott, Carter, & Grant, 2008).

3.2. \textit{Metabolism and the circadian clock are reciprocally related}

The relationship between the circadian system and metabolism appears to be bi-directional. In addition to circadian system disruptions causing obesity, metabolic abnormalities alter circadian rhythms. For example, in humans, metabolically related diseases such as obesity and anorexia nervosa are associated with altered hormone and body temperature rhythms (Ferrari, Fraschini, & Brambilla, 1990). Daily rhythms in glucose and insulin sensitivity are apparent in non-obese but not obese subjects (Lee, Ader, Bray, & Bergman, 1992). Moreover, obese women display significantly lower wrist temperature and a more flattened temperature rhythm compared to normal-weight control women (Corbalan-Tutau et al., 2011).

An intriguing interaction between high fat diets and clock genes has been discovered. Placing mice on a high fat diet lengthens the circadian period of activity and attenuates the diurnal pattern of feeding (Kohsaka et al., 2007; Stucchi et al., 2012). Mice fed a high fat diet dampen circadian rhythms in clock gene expression, specifically in peripheral metabolically related tissues. These changes appear to occur rapidly, as liver
rhythms are phase advanced 5 h, within one week of initiating a high fat diet (Pendergast et al., 2013). Furthermore, mice fed a high fat diet show a slower rate of re-entrainment of behavioral and physiological rhythms after a 6 h phase shift (Mendoza, Pevet, & Challet, 2008).

Modulation of leptin in mice and rats provides further support for the hypothesis that disrupting metabolism affects the circadian system. Zucker obese rats, a widely used obesity model produced by a single mutation in the gene encoding the leptin receptor (Phillips et al., 1996), exhibit phase advanced circadian rhythms and an attenuated amplitude of body temperature, activity, and sleep (Mistlberger, Lukman, & Nadeau, 1998; Murakami, Horwitz, & Fuller, 1995). Zucker rats increase daytime food consumption when compared to lean controls (Becker & Grinker, 1977; Wangsness, Dilettuso, & Martin, 1978) and preventing food intake during the light phase ameliorates weight gain in obese rats (Mistlberger, Lukman, & Nadeau, 1998). Furthermore, db/db mice, which lack the leptin receptor, show changes in the diurnal rhythms of multiple metabolically related hormones (Roesler, Helgason, Gulka, & Khandelwal, 1985) and clock genes in peripheral tissues (Su et al., 2012). ob/ob leptin deficient mice attenuate rhythms in activity, heat production, and clock gene expression (Dauncey & Brown, 1987). Disruptions in circadian rhythms, however, may occur prior to the onset of obesity in ob/ob mice (Ando et al., 2011).

Other obesity models display similar changes in circadian rhythms. Rats with ventromedial hypothalamic lesions become obese and alter the daily pattern of food intake and circadian gene expression (Balagura & Devenport, 1970). KK-Ay mice, a
mouse model of obesity and type II diabetes, attenuate clock gene rhythms in adipose tissue and the liver (Ando et al., 2005; Hashinaga et al., 2012). Moreover, a subset of Volcano mice are naturally susceptible to obesity and display phase advanced activity-onset and attenuated locomotor activity rhythms compared to non-obese Volcano mice (Carmona-Alcocer et al., 2012). Of note, one common feature in many of the obesity models discussed above is that rodents shift diurnal rhythms in food intake.

3.3. **You are what when you eat?**

Food is an entraining signal for the circadian system. Restricting feeding to certain times of day can lead to anticipatory increases in wakefulness, locomotor activity, body temperature, and glucocorticoid secretion (Krieger, 1974). Food entrainable oscillators appear dependent on extra-SCN signaling and likely involve the dorsomedial hypothalamus (Gooley, Schomer, & Saper, 2006). Indeed, while the SCN remains phase locked to light/dark cues, restricting feeding to certain times of day rapidly entrains circadian rhythms in the liver (Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001). In the absence of light cues, however, restricted feeding is capable of affecting the SCN. For example, in mice made arrhythmic by constant light exposure, restricted feeding rescues both activity rhythms and *Per2* rhythms in the SCN (Lamont, Diaz, Barry-Shaw, Stewart, & Amir, 2005).

Timing of food intake is now recognized as a critical factor in energy acquisition, storage, and expenditure. Mice fed a high fat diet only during the 12 h light (resting) phase gain significantly more weight than mice fed only during the dark (active) phase (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). This change in body mass may be
dependent on leptin signaling (Arble, Vitaterna, & Turek, 2011). Importantly, in this model total daily caloric intake and activity do not differ between groups, indicating changes in body mass can occur independently of changes in energy intake (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). A more thorough examination of metabolic characteristics in food restricted mice revealed light-phase fed mice exhibit a higher respiratory exchange ratio (indicating decreased reliance on fat oxidation), tissue-specific alterations in metabolically-related genes and circadian clock genes, changes in the diurnal variations in humoral factors (i.e. corticosterone), and increased weight gain within 9 days of restricting feeding (Bray et al., 2012). In the study by Bray and coauthors, mice did show changes in food intake; mice fed during the light phase had increases in food intake as well as a larger meal on presentation of food (Bray et al., 2012). The time of day at which dietary fat is consumed also influences multiple cardiometric parameters (Bray et al., 2010). Consumption of a high fat meal at the end, as compared to the beginning of the active phase, leads to features of metabolic syndrome including increased weight gain, elevated adiposity, reductions in glucose processing, hyperinsulemia, hyperleptinemia, and hypertrigluceridemia (Bray et al., 2010).

As discussed above, multiple obesity models show increases in rest-phase food intake. This has led to recent research assessing whether preventing rest-phase feeding can ameliorate weight gain. Mice fed a high fat diet that are food restricted to either 4 (Sherman et al., 2012) or 8 (Hatori et al., 2012) h per day during the active phase are protected against diet induced obesity. Food restricted mice consume equivalent calories as mice with ad libitum food access and yet are buffered against obesity, inflammation,
hepatic steatosis, hypercholesterolemia, and hyperinsulinemia. Changes in metabolism in food restricted mice are associated with improved intracellular signaling and nutrient utilization as well as greater circadian clock oscillations (Hatori et al., 2012; Sherman et al., 2012). Similarly, restricting food access to the 14 h dark phase reduces weight gain in Zucker obese rats (Mistlberger, Lukman, & Nadeau, 1998).

Accumulating epidemiological and experimental evidence in humans, supports the hypothesis that when energy intake occurs is important in determining energy utilization. Timing of food intake predicts body mass index in humans, even when controlling for variables such as sleep timing and duration. People who consume more food after 2000 h tend to have higher body mass index (Baron, Reid, Horn, & Zee, 2013; Baron, Reid, Kern, & Zee, 2011). Moreover, weight loss therapy is more effective for individuals who eat early as compared to late eaters (Garaulet et al., 2013). Short duration sleepers also have increased risk for obesity, weight gain over time, and higher body fat composition (Weiss et al., 2010). Controlling for factors such as preference for fatty food, skipping breakfast, snacking, and eating out only partially accounts for the effects of short duration sleep on obesity, suggesting that changes in metabolic homeostasis at different times of day may partially account for different body weight regulation (Nishiura, Noguchi, & Hashimoto, 2010). Indeed, people with Night Eating Syndrome, a disorder characterized by evening hyperphasia and nocturnal awakenings accompanied by food intake (Allison et al., 2010), show alterations in metabolically related hormones and are more likely to be obese (Birketvedt et al., 1999; Goel et al., 2009).

4. Exposure to light at night and obesity
Given the well established role of light in modulating the circadian system, and the relationship between circadian and metabolic functions, it is not surprising that exposure to light at unnatural times affects metabolism. Increases in nocturnal illumination parallel increases in obesity and metabolic syndrome worldwide. Here I propose that exposure to light at night may be contributing to increasing rates of obesity. In this section I will discuss evidence from animal models and epidemiological studies implicating exposure to light at night in the growing obesity epidemic.

4.1. Light at night and obesity: evidence from animal models

Exposure to continuous light, non-24 h light schedules, and dim light at night are all associated with metabolic changes in rodents. First, exposure to constant light desynchronizes circadian activity in rodents (Coomans et al., 2013). In Chapter 2 of this dissertation I describe the effects of exposure to constant light on metabolism in mice. Swiss Webster mice exposed to constant light as compared to a standard light/dark cycle show increases in body mass and reductions in glucose processing without altering total daily activity or food intake (Fonken et al., 2010). C57/Bl/6J mice show similar metabolic changes in constant light, with immediate body weight gain upon placement in constant light and reductions in insulin sensitivity. Following 4 weeks of exposure to constant light, mice lack a circadian rhythm in both food intake and energy expenditure. These changes are associated with reductions in circadian rhythm amplitude as measured by in vivo electrophysiological recordings of the SCN (Coomans et al., 2013). Rats also change metabolism with continuous light exposure. Rats exposed to constant light...
increase visceral adiposity and demonstrate higher feed efficiency than rats exposed to either a standard light/dark cycle or constant dim light (Wideman & Murphy, 2009).

One limitation to studying the effects of constant light exposure on metabolism is that the circadian system either free-runs or becomes arrhythmic under constant light conditions (Fonken et al., 2010; Lamont, Diaz, Barry-Shaw, Stewart, & Amir, 2005). Furthermore, with the exception of high latitudes, exposure to constant light in natural settings is rare. For this reason, in Chapters 2-6 of this dissertation I evaluate the effects of exposure to ecologically relevant levels of dim nighttime light (~5 lux) exposure on metabolic function. Mice exposed to dimly lit, as compared to dark nights, impair glucose processing, increase white adipose tissue, and elevate body mass gain (Chapter 2, Fonken et al., 2010). Changes in metabolism occur independently of changes in total daily food intake or locomotor activity. However, mice exposed to dim light at night shift timing of food intake, consuming more during the light phase. Much as other obesity models, restricting food intake to the dark phase prevents weight gain in mice exposed to dim light at night.

Metabolic changes associated with exposure to dim light at night are not permanent; placing mice back in dark nights partially reverses increases in body mass caused by dim light at night exposure (Chapter 3, Fonken, Weil, & Nelson, 2013). Moreover, exposure to dim light at night interacts with more traditional obesogenic risk factors to affect weight gain. Exposure to dim nights exaggerates weight gain on a high fat diet, increasing peripheral but not hypothalamic inflammation (Chapter 4). Increases in weight gain associated with exposure to light at night are also reduced by providing a
running wheel for voluntary exercise (Chapter 5). Providing mice a running wheel in dim light at night prevents increases in weight gain without restoring feeding rhythms. Models of circadian desynchrony provide further support for an association between exposure to altered light schedules and changes in metabolism. Placing mice in a 20 h light/dark cycles incongruous with their endogenous ~24 h circadian period, accelerates weight gain and alters metabolically related hormones (Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011). Circadian desynchrony in mouse models of shift work, demonstrate altered light or activity patterns, can disrupt liver transcriptome rhythms (Barclay et al., 2012), flatten glucose rhythms, increase abdominal fat (Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010), and cause reductions in body mass when shift work is combined with other environmental challenges (Preuss et al., 2008). Notably, preventing daytime food intake may rescue metabolic changes in mice undergoing a shift work paradigm (Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010).

4.2. Exposure to light at night and obesity: Evidence in humans

Multiple epidemiological studies in shift working populations have linked exposure to light at night to altered metabolism (X. S. Wang, Armstrong, Cairns, Key, & Travis, 2011). Health care personnel that work night, as compared to day shifts, show elevated risk for metabolic syndrome (Pietroiusti et al., 2010). Shift work is associated with increased blood pressure, cholesterol, obesity, and hypertriglyceridemia in males (Ha & Park, 2005; B. Karlsson, Knutsson, & Lindahl, 2001) and increased risk for obesity, hypertension, and hypertriglyceridemia in females (B. Karlsson, Knutsson, &
Lindahl, 2001). A study of offshore personnel either chronically working day shifts or
transitioning between day and nights shifts, demonstrated that the number of years of
shift work is positively associated with body mass index in the day/night workers. In
contrast, age is the greatest predictor for body mass index in the day shift population
(Parkes, 2002). This suggests that chronic exposure to light at night can affect body mass
index to a more appreciable extent than typical predictors of body mass, such as age.
Several other studies indicate that working rotating shift schedules as compared to day
schedules increases risk for developing metabolic syndrome (De Bacquer et al., 2009;
Esquirol et al., 2009; Lin, Hsiao, & Chen, 2009a, , 2009b; Sookoian et al., 2007).
Importantly, the effects of shift work on metabolism may be long lasting as former shift
workers show increases in obesity (Puttonen, Viitasalo, & Harma, 2011). Changes in
metabolic signals may contribute to increased body mass in shift workers. For example,
brief behavioral and endogenous circadian misalignment in a controlled laboratory setting
alters leptin, insulin, and cortisol secretion and elevates blood pressure (Scheer, Hilton,
Mantzoros, & Shea, 2009). Moreover, forced desynchrony can produce postprandial
glucose responses comparable to a prediabetic state (Scheer, Hilton, Mantzoros, & Shea,
2009).

In industrialized economies, approximately 20% of the population are shift
workers (Monk, 2000; Rajaratnam & Arendt, 2001). However, exposure to light at night
occurs beyond the scope of shift work. Over 99% of the population in US and Europe
experiences nighttime light exposure (Cinzano, Falchi, & Elvidge, 2001). Associations
between exposure to light at night and increases in body mass index are also apparent
outside the shift working population. A recent study by Obayashi et al reported that increased light exposure in an uncontrolled home setting is associated with obesity and other metabolic consequences. People with nocturnal light levels above 3 lux display significantly higher body weight, elevated body mass index, increased waist circumference, and elevated triglyceride and low-density lipoprotein cholesterol levels (Obayashi et al., 2013). Moreover, a large scale epidemiological study demonstrated that “social jetlag” is associated with increased BMI, even when controlling for factors such as sleep duration (Roenneberg, Allebrandt, Merrow, & Vetter, 2012).

One final piece of evidence linking exposure to light at night and obesity comes from a population where nighttime light exposure is minimal, the Amish. The Old Order Amish abstain from using public power, and therefore, are not exposed to common sources of light at night such as televisions, computers, and electric lights (S. Scott & Pellman, 1990). The prevalence of obesity among the Amish is far lower than the general population in the United States (Tremblay, Esliger, Copeland, Barnes, & Bassett, 2008). Lower obesity rates among the Amish are for the most part attributed to changes in activity and diet (Esliger et al., 2010; Tremblay, Esliger, Copeland, Barnes, & Bassett, 2008). However, other environmental factors are likely involved in limiting disease rates among the Amish. For example, the Amish also suffer disproportionately lower rates of breast and prostate cancer (Westman et al., 2010). Controlling for known carcinogenic variables such as tobacco use does not completely account for the disparate rates of cancer. Importantly, exposure to light at night is associated with increased risk for
developing both breast and prostate cancer (Kloog, Haim, Stevens, Barachana, & Portnov, 2008; Kloog, Haim, Stevens, & Portnov, 2009).

4. Conclusions

In this introduction, I specifically focused on how light at night may influence metabolism through disruption of the circadian clock genes. There are, however, several additional pathways through which light can affect metabolism, including disruptions in melatonin production, alterations in glucocorticoids, and sleep disturbances (Reiter, Tan, Korkmaz, & Ma, 2011). Exposure to sufficient levels and duration of nighttime lighting can suppress pineal melatonin secretion (Brainard, Rollag, & Hanifin, 1997). Melatonin is an endogenously synthesized molecule that is secreted by the pineal gland during the night in both nocturnal and diurnal mammals (Reiter, 1991). Melatonin has recently been reported to have anti-obesity effects (Mantele et al., 2012; Tan, Manchester, Fuentes-Broto, Paredes, & Reiter, 2011). Although there is compelling evidence that suppression of melatonin secretion can contribute to weight gain, I specifically did not focus on melatonin for several reasons: (1) pineal melatonin suppression requires high and sustained levels of nighttime light exposure (Brainard, Rollag, & Hanifin, 1997), (2) nighttime light exposure below the threshold for melatonin suppression is associated with changes in metabolism (Obayashi et al., 2013), and (3) multiple strains of laboratory mice that lack pineal melatonin demonstrate changes in metabolism with nighttime light exposure (Coomans et al., 2013; Fonken et al., 2010). Additionally, I did not focus on the effects of exposure to light at night on sleep or glucocorticoids because nighttime light
exposure affects metabolism in mice independently of changes in sleep architecture or corticosterone release.

**Chapters 2-7** of this dissertation are all conducted in nocturnal laboratory mice. A nocturnal rodent was selected for the studies in order to separate the effects of exposure to light at night from sleep disruption. Indeed, exposure to dimly lit nights does not interrupt sleep in Swiss Webster mice (Borniger, J., Weil, Z.M., & Nelson, R.J., unpublished observations). However, both circadian influences on behavior and the masking effects of light are very different in diurnal as compared to nocturnal species (R. Cohen, Kronfeld-Schor, Ramanathan, Baumgras, & Smale, 2010; Shuboni, Cramm, Yan, Nunez, & Smale, 2012). Rhythmic release of multiple hormones also occurs 180° degrees out of phase between diurnal and nocturnal rodents. Thus, in the final two chapters of this dissertation (**Chapters 8 & 9**), I consider the physiological implications of exposing diurnal Nile grass rats (*Arvicanthis niloticus*) to dim light at night. Grass rats exposed to dimly lit as compared to dark nights, enhance cell-mediated immune function as measured by delayed-type hypersensitivity, elevate antibody production following inoculation with keyhole lymphocyte hemocyanin, and increase bactericidal capacity (**Chapter 8** (Fonken, Haim, & Nelson, 2011)). Furthermore, Grass rats exposed to dim light at night display cognitive impairments in a Barnes maze test and increases in depressive-like behavior (**Chapter 9** (Fonken, Haim, & Nelson, 2011)). Changes in cognitive and affective responses are associated with altered hippocampal connectivity in grass rats exposed to dimly lit as compared to dark nights. In contrast to nocturnal rodents exposed to dim light at night (Fonken et al., 2010), grass rats chronically exposed to
dimly lit nights elevate corticosterone concentrations compared to rats exposed to dark nights.

One important population that is often neglected when considering light at night is patients in hospitals. Although multiple epidemiological studies have been conducted on nurses, there are no studies on the affects of light at night on the patients with whom they work. Many in-patients are already at high risk of increased inflammation and disrupted physiology, which may be exacerbated by light at night. For example, the circadian system regulates multiple immune related functions (Lange, Dimitrov, & Born, 2010). Antigen presentation, toll-like receptor function, cytokine gene expression, and lymphocyte proliferation all occur in a circadian pattern (Arjona & Sarkar, 2006; A. C. Silver, Arjona, Walker, & Fikrig, 2012). Furthermore, circadian clock proteins directly regulate the expression of pro-inflammatory cytokines (Narasimamurthy et al., 2012). Immune cells such as natural killer cells, macrophages, dendritic cells, and B cells possess molecular clock mechanisms necessary for self-sustaining oscillations (Arjona & Sarkar, 2005; Keller et al., 2009; A. C. Silver, Arjona, Hughes, Nitabach, & Fikrig, 2012). Because recent research has demonstrated that light at night may detrimentally affect the immune system (Bedrosian, Fonken, Walton, & Nelson, 2011a) in Chapter 7 of this dissertation I investigate the effects of exposure to light at night on recovery from global cerebral ischemia.

Most permanent damage to the central nervous system that occurs post-ischemia is mediated by endogenous secondary processes, typically involving inflammation. The delay in damage following cerebral ischemia suggests that the immediate post-recovery
environment may affect the trajectory of recovery. Because the circadian system is controlled by an endogenous biological clock, and physiological processes such as inflammation become dysregulated in disruptive lighting conditions, I hypothesized that cardiac arrest outcome may be negatively affected by light at night. In agreement with the hypothesis, mice exposed to dim light at night in the week following a cardiac arrest and cardiopulmonary resuscitation procedure increase mortality compared to mice exposed to dark nights. Moreover, mice exposed to dim light at night increase hippocampal cell death, microglia activation, and pro-inflammatory cytokine expression. Selectively inhibiting IL-1β or TNFα ameliorate damage in mice exposed to dim light at night, suggesting increases in inflammation are critical for light-associated damage. Restricting the wavelength of the nighttime light exposure to ~640 nm also reduces neuroinflammation and eliminates the detrimental effects of light at night on CA outcome. These findings implicate the involvement of the circadian system in dim light at night-associated damage.

Modern society now functions on a 24 h schedule. Although there are many economic and other societal benefits to such a schedule, there is converging evidence from epidemiological and experimental work that light at night has unintended, maladaptive consequences. In many ways, this field of study is just beginning; further characterization of the biological and psychological effects of light at night is needed along with effective interventions to ameliorate the unintended negative effects of light at night on health.
Overall, preventing the general population from excessive exposure to light at night can be achieved with relatively low-cost manipulations, such as using curtains to block out street lights, turning off hallway lights, and removing all light sources, including televisions and computers, from bedrooms. However, these methods do not prevent the social jet lag that many of us experience and exposure to light at night is often unavoidable in shift-working populations. To that end, there are studies currently comparing visual aids that may alleviate some of the maladaptive effects of exposure to light at night in shift workers. Specifically, manipulation of wavelength may prove effective in blocking out some of the light-induced physiological changes.
CHAPTER 2

LIGHT AT NIGHT INCREASES BODY MASS THROUGH ALTERED TIMING OF FOOD INTAKE

During the past two decades obesity has shifted from a US-centered epidemic to a global issue. Although well-documented factors such as caloric intake, dietary choices, and lack of exercise are known to contribute to the prevalence of obesity and metabolic disorders, additional environmental factors are now considered critical in the development and maintenance of obesity (Hill, Wyatt, Reed, & Peters, 2003). The increase of light at night (LAN) during the 20th century coincides with increasing rates of obesity and metabolic disorders throughout the world. Artificial lighting allows people to extend daytime activities into the night, but as a consequence produces significant environmental light pollution caused by light straying into the atmosphere and brightening the nighttime sky.

Circadian regulation of energy homeostasis is controlled by an endogenous biological clock, located in the suprachiasmatic nuclei (SCN) of the hypothalamus that are synchronized by photic information that travels directly from light-sensitive ganglion cells in the retina to the SCN, thereby entraining individuals’ physiology and behavior to
the external day-night cycle (Golombek & Rosenstein, 2010). Importantly, light is the most potent entraining signal for the circadian clock, although other factors such as food consumption influence clock signaling (Fuller, Lu, & Saper, 2008). To promote optimal adaptive functioning, the circadian clock prepares individuals for predictable events such as food availability and sleep. Shift work disrupts clock function and is linked to circadian and metabolic consequences including sleep disturbances (Kohyama, 2009), elevated body mass index (BMI) (Parkes, 2002; van Amelsvoort, Schouten, & Kok, 1999), altered plasma lipid metabolism and adiposity (B. H. Karlsson, Knutsson, Lindahl, & Alfredsson, 2003), and increased risk for cardiovascular disease (Ha & Park, 2005).

Multiple studies suggest a link between the molecular circadian clock and metabolism (for review see (Bray & Young, 2007). Mice harboring a mutation in their clock genes are susceptible to obesity and metabolic syndrome (Turek et al., 2005). Clock mutants show profound changes in circadian rhythmicity, as well as disrupted diurnal food intake and increased body mass. Serum leptin, glucose, cholesterol, and triglyceride levels are also increased in Clock mutants compared to wild type mice. Mice lacking the VIP-VPAC2 pathway which plays an important role in SCN communication (Reppert & Weaver, 2001) have metabolic abnormalities similar to those in Clock mutant mice (Bechtold, Brown, Luckman, & Piggins, 2008). Furthermore, consumption of a high-fat diet alters circadian rhythmicity and the cycling of circadian clock genes in mice (Kohsaka et al., 2007).

Because multiple studies have linked disruption of the molecular circadian clock and metabolic disorders (Bechtold, Brown, Luckman, & Piggins, 2008; Rudic et al.,
2004; Turek et al., 2005), I hypothesized that exposure to light at night alters circadian organization and affects metabolic parameters. I investigated the possibility of a direct link between altered light cycles and metabolic disorder by housing mice in either a standard light/dark cycle (LD; 16 h light at ~150 lux/8 h dark at ~0 lux), a light/dim light cycle (dLAN; 16h light at ~150 lux/ 8h dim light at ~5 lux), or 24 h of continuous lighting (LL; constant ~150 lux) and assessed metabolic parameters. I included a dLAN group in addition to LL because mice in constant lighting have no temporal cue to distinguish time of day, and their biological clocks free-run. The dLAN group may more directly model environmental light pollution experienced in industrialized nations; however, I predicted circadian alterations in daily activity would not be as profound in a dLAN environment. By including the LL group, this study not only focused on the effects of light pollution, but also on the effects of a desynchronized circadian system on metabolism. I hypothesized that mice housed in dLAN and LL conditions would alter metabolic parameters compared to mice housed in LD. More specifically, I hypothesized that housing mice in dLAN and LL would result in reduced glucose tolerance and increased body mass in comparison to LD-housed mice. I also hypothesized that mice housed in LL and dLAN would alter stress levels as evaluated by circulating corticosterone and that LL would induce locomotor arrhythmicity indicative of circadian disruption.

Methods

Animals
Eighty male Swiss Webster mice (~8 weeks of age) were obtained from Charles River Labs (Kingston, NY) for use in these studies. The mice were individually housed in propylene cages (30 x 15 x 14 cm) at an ambient temperature of 22 ±2°C and provided food (D12450B: 10% kcal% fat, 70% kcal% carbohydrate, 20% kcal protein; Research Diets Inc., New Brunswick, NJ, USA) and water ad libitum. Upon arrival, all mice were maintained under a 16:8 light/dark (lights on at 23:00 Eastern Standard Time [EST] ~130 lux) cycle for one week to allow them to entrain to local conditions and recover from the effects of shipping. A 16:8 light dark cycle was used rather than 12:12 to avoid providing a seasonally ambiguous signal. Because Swiss Websters are an outbred species and may retain some responsiveness to photoperiod, mice might interpret a 12:12 light cycle as either a long or short photoperiod, and thus impose higher variability in phenotype (Nelson, 1990). All experimental procedures in this dissertation were approved by The Ohio State University Institutional Animal Care and Use Committee, and animals were maintained in accordance with the recommendations of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals.

Experiment 1

Following the habituation period, mice were pseudo-randomly assigned to one of three groups (n=10/group); mice were housed in either a standard light/dark cycle, constant light, or a light/dim light cycle. Mice were weighed during group assignment to establish that all groups had a similar baseline body mass. All mice weighing >35 grams were excluded from the experiments. After group assignment, the LL mice were placed in a constant light room (LL) where they were exposed to a constant amount of
continuous light (~150 lux). The dLAN mice were placed in a room with a 16:8 light/dim cycle; during the light period they were exposed to ~150 lux of light and during the dim period they were exposed to ~5 lux of light at cage level.

Food intake was measured daily immediately before the onset of the dark period (15:00 EST) throughout the study and body mass was measured weekly. After 6 weeks in light conditions food intake was measure twice daily, at the onset of the dark period (15:00 EST) and onset of the light period (23:00 EST) in order to quantify timing of food consumption (expressed as percentage: 100 x consumption light/(consumption light + consumption dark)). Home cage activity was monitored during the 5th and 6th weeks in light conditions; each room was monitored for 4 consecutive days including a weekend. At week 4 mice underwent an intra-peritoneal glucose tolerance test (GTT). After 8 weeks in light conditions mice were killed by cervical dislocation at one of two time points, either directly after the onset of darkness (between 15:00 and 17:00 EST) or during the middle of the light phase (5:00 to 7:00 EST), in order to collect blood samples at the peak and nadir of locomotor activity. Epididymal fat pads were also collected as an index of white adipose tissue.

Experiment 2

Following the habituation period, mice were pseudo-randomly assigned a number and divided into one of six groups (n=8-9/group); mice were housed in either LD or dLAN and had either 24 h/day food access (FA; food weighed twice daily at 9:00 and 19:00 EST), food access during the dark phase (FD; food in: 9:00 EST, food out: 19:00 EST), or food access during the light phase (FL; food in 19:00 EST, food out: 9:00 EST).
After 7 weeks in light conditions mice underwent a series of three retro-orbital blood collections with at least 48 h between collection points to assess serum corticosterone concentrations. After 8 weeks in light conditions mice were killed by cervical dislocation at one of two time points, either directly after the onset of darkness (10:00-12:00 EST) or during the middle of the light phase (23:00-1:00 EST). Epididymal fat pads were collected. All entrances into the animal rooms and collections after lights off were made under dim red illumination.

Glucose tolerance test

The glucose tolerance test was given after four weeks in experimental light conditions. Mice were administered a 1.5 g/kg body mass intra-peritoneal glucose bolus at 10:00 EST after an 18 h fast. Blood samples of ~5 µL were collected via submandibular bleed before injection, and at 15, 30, 60, 90, and 120 min following injection. Blood glucose was immediately measured with the Contour blood glucose monitoring system and corresponding test strips (Bayer HealthCare, Mishawaka, IN).

Activity analyses

Locomotor activity was tracked in 8 mice per group using OPTO M3 animal activity monitors (Columbus Instruments, Columbus, OH) that continuously compile data using MDI software system. Results from locomotor activity were used to determine whether lack of physical activity or altered rhythmicity may have affected metabolism (Laposky, Bass, Kohsaka, & Turek, 2008).

Blood collection and hormone analyses
Blood samples were then allowed to clot, had the clot removed, and were centrifuged at 4°C for 30 min at 3300 g. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -80°C for subsequent analysis. Total serum corticosterone concentrations, for mice, was determined in duplicate in an assay using an ICN Diagnostics ¹²⁵I double antibody kit (Costa Mesa, CA USA). The high and low limits of detectability of the assay were 1200 and 3 ng/ml, respectively. The intra-assay coefficient of variation was 7.00% for experiment 1 and 16.83% for experiment 2.

**Statistical analyses**

Hormone concentrations, total daily food consumption and total daily activity were analyzed using a one-way analysis of variance (ANOVA). Following a significant F score, multiple comparisons were conducted with Tukey’s HSD tests. Glucose tolerance test results were analyzed with a repeated measures ANOVA with lighting condition as the within-subject factor and time as the between subject variable. A repeated measures ANOVA was also used to analyze change in body mass over time. Following a significant result on repeated measures ANOVA, single time point comparisons were made using Student’s t-tests. The above statistical analyses were conducted with StatView software (v. 5.0.1, Cary, NC). Fourier analysis was used to determine whether locomotor activity was rhythmic and followed 24 h periodicity using Clocklab software from Actimetrics (Wilmette, IL). Mice were considered rhythmic when the highest peak occurred at ~1 cycle per day with an absolute power of at least 0.005 mV/Hz as previously described (Kriegsfeld et al., 2008). Nonlinear regression analysis was used in GraphPad Prism software (v. 4 La Jolla, CA). In all cases,
differences between group means and correlation coefficients were considered statistically significant if \( p \leq 0.05 \).

**Results**

**Body mass**

Body mass was differentially affected by light condition over the 8 experimental weeks (\( F_{24,192} = 3.457; p < 0.0001 \); Fig. 1a). A significant increase in body mass among LL and dLAM mice, relative to the LD control group, was evident beginning one week after onset of light treatment and continuing throughout the 8 week study (\( F_{2,24} = 4.441, 10.187, 12.660, 12.232, 6.561, 4.568, 4.293 \) respectively, \( p \leq 0.01 \)). The elevated body mass among LL and dLAN groups was due to increased body mass gain (\( F_{2,24} = 4.291; p < 0.05 \); Fig 1b), rather than initial differences in body mass among groups. Furthermore, epididymal fat pad mass was significantly greater at the end of the study in mice with light at night suggesting increased body mass reflected increases in white adipose tissue (\( F_{2,24} = 4.767; p < 0.05 \); Fig. 1b).

**Glucose tolerance test**

After four weeks in experimental light conditions, LL and dLAN mice displayed impaired glucose tolerance during an intra-peritoneal glucose tolerance test (GTT). Injection of glucose increased blood glucose levels in all groups following an 18 hour fast (\( F_{5,23} = 151.015; p < 0.0001 \); Fig. 1c). LL and dLAN mice failed to recover glucose levels as rapidly as the LD group (\( F_{10,115} = 2.514; p < 0.01 \)). dLAN mice significantly elevated glucose levels after 60 min and glucose levels remained elevated after 90 and 120 min in dLAN and LL mice compared to the LD group (post hoc; \( p < 0.05 \)).

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Furthermore, final glucose levels in the GTT positively correlated with body mass ($R = 0.5236; p < 0.05$; Fig. 1d).

**Locomotor Activity**

In contrast to the LD and dLAN groups, which displayed the typical circadian rhythm in locomotor activity, as measured in the home cage via an infrared beam crossing system, the LL mice were arrhythmic (7 out of 8 arrhythmic as measured by Fourier analysis; Fig. 2a-c). However, total daily locomotor activity was similar for all groups ($F_{2,153} = 0.0002; p > 0.95$; Fig. 2d).

**Glucocorticoids**

Corticosterone was decreased in the LL ($p < 0.05$, Fig. 3a), but not dLAN mice; these results suggest that changes in glucocorticoid concentrations were not necessary for altered metabolism.

**Energy Intake**

Total 24 h food consumption did not differ between groups ($F_{2,24} = 0.107; p > 0.85$). Although no differences in total food consumption and home cage locomotor activity were detected between groups, feeding behavior was altered in the dLAN group ($F_{1,38} = 29.315; p \leq 0.05$; Fig. 3b). The dLAN mice consumed 55.5% of their food during the light phase as compared to 36.5% in LD mice, indicating that mice exposed to LAN ate more food during the day, rather than at night. LL mice were not considered in this comparison because they had no temporal signal to distinguish the light and dark phase. Furthermore, correlation analyses confirmed that percentage of daytime food
consumption was positively related to final body mass and final glucose levels in the GTT (\( R = 0.5058 \) and \( R = 0.6066 \) respectively; \( p < 0.01 \); Fig. 3c/d).

**Timed feeding**

Because altered timing of food consumption may mediate changes in body mass in the dLAN mice, I performed an additional experiment with mice housed in either LD or dLAN with either continuous access to food (FA) or with food availability limited to either the light (FL) or dark (FD) phase, respectively. Timed feeding affected body and epididymal fat pad mass gain (\( F_{2,44} = 5.392 \) and \( 4.372 \) respectively; \( p < 0.05 \); Fig. 4a/b).

Within dLAN mice limiting food access to the dark phase prevented weight and fat gain (weight: \( t_{14}=1.940 \), fat: \( t_{14}=2.526 \), \( p \leq 0.05 \)). dLAN-FD mice gained an equivalent amount of weight and fat as LD-FD mice and LD mice with 24h food access (weight: \( t_{14}=-0.250 \), \( t_{14}=-1.176 \) fat: \( t_{14}=-0.076 \), \( t_{14}=-1.004 \); \( p > 0.05 \)). Furthermore, FL mice increased body and fat pad masses (post hoc, \( p < 0.05 \)); this increase was not dependent on light/dark cycle and LD- and dLAN-FL had comparable weight gain. There was no effect of light on corticosterone concentrations at the 6 time points measured (\( p > 0.05 \)). However, timed feeding altered corticosterone concentrations at ZT 16 (\( F_{2,24} = 15.316 \); \( p < 0.05 \); Fig. 4c), such that timed feeding during the dark phase increased corticosterone concentrations (post hoc, \( p < 0.05 \)). Food consumption changed over time such that consumption decreased throughout the study (\( F_{6,258}=664.474 \); \( p < 0.05 \); Table 1). Furthermore, there was an interaction between timed feeding and food consumption over time (\( F_{12,258} = 98.051 \); \( p < 0.05 \), with timed feeding groups initially consuming more than mice with food access all the time.
Discussion

Swiss-Webster mice were housed in either standard light/dark cycles, 24 h of continuous lighting, or in light/dim cycles. A significant increase in body mass among LL and dLAN mice, relative to the LD control group, was evident beginning one week after onset of light treatment and continuing throughout the 8-week study. After four weeks, LL and dLAN mice displayed impaired glucose tolerance during an intraperitoneal glucose tolerance test; the mice with dLAN failed to recover glucose levels as effectively as the LD group. Increased body mass and reduced glucose tolerance are indicative of a pre-diabetic-like state (Kahn, Hull, & Utzschneider, 2006). Thus, as little as 5 lux of light exposure during the typical dark period is sufficient to increase body mass and compromise glucose regulation.

Total daily locomotor activity, as measured in the home cage via an infrared beam crossing system, was similar for all groups. However, in contrast to the LD and dLAN groups, that displayed the typical circadian rhythm in locomotor activity, the LL mice were arrhythmic. Although no differences in total daily food consumption were detected between groups, feeding behavior was altered in the dLAN group. The dLAN mice consumed 55.5% of their food during the light phase as compared to 36.5% in LD mice, indicating that mice exposed to dLAN ate more food during the day, rather than at night. LL mice were not considered in this comparison because they had no temporal signal to distinguish the light and dark phase. Again, total 24 h consumption did not differ among groups, but food intake is substantially higher at night among nocturnal rodents (Zucker, 1971) and altered timing of food consumption has been associated with metabolic...
syndrome in other animal models (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Turek et al., 2005). Correlation analyses confirmed that percentage of daytime food consumption was positively related to final body mass and final glucose levels in the GTT.

To establish whether the altered timing of food intake contributed to the increased weight gain, I performed an additional study with a timed feeding schedule. Because mice housed in LL would likely entrain to the time of food access, they were omitted from the food access iteration (Mistlberger, 2009). Mice were housed in either LD or dLAN with either 24 h ad libitum access to food, food access only during the light phase, or food access only during the dark/dim phase. Restricting food availability to the dark phase prevented weight and fat gain among dLAN mice. dLAN-FD mice gained an equivalent amount of weight and fat as LD-FD mice and LD mice with no food restriction. This further suggests that altered timing of food consumption in the dLAN mice leads to increased body mass gain. As previously reported for rats and mice, FL mice increased body mass; this increase was not dependent on light/dark cycle and LD- and dLAN-FL had comparable weight gain. Mice with limited access to food displayed an initial spike in consumption that decreased over time. When food access was limited to the light phase, mice displayed an increased elevation in food consumption during week one; however, mice with access to food limited to the dark phase consumed more food in subsequent weeks. Again, overall food intake for the study was comparable among all groups suggesting that the timing of food intake is a critical factor mediating increased weight gain.
Alterations in light cycles are typically considered to be stressful; the results of previous research on the effects of LAN on glucocorticoid concentrations, however, are equivocal (Abilio, Freitas, Dolnikoff, Castrucci, & Frussa-Filho, 1999; Fonken et al., 2009). Because high glucocorticoid concentrations can alter metabolism resulting in obesity I measured circulating glucocorticoids (Dallman et al., 2004). Contrary to my predictions, corticosterone was decreased in the LL, but not dLAN mice, which suggests that changes in glucocorticoid concentrations were unnecessary for altered metabolism. The decreased corticosterone concentrations in the LL mice likely reflect masking of the glucocorticoid rhythm. Glucocorticoid concentrations were affected by food restriction. Mice with food access during the dark phase had elevated peak glucocorticoid concentrations. There were no differences in glucocorticoid concentrations between dLAN and LD mice that were on the FA or FL feeding schedules.

These results establish that night-time illumination at a level as low as 5 lux is sufficient to uncouple timing of food consumption and locomotor activity resulting in metabolic abnormalities. dLAN mice display desynchrony between internal metabolic activity and food intake, as demonstrated by altered timing of food consumption; this may be the primary factor leading to increased weight gain. Similarly, in LL mice the arrhythmic home cage activity suggests that there may be desynchrony between food intake and metabolic parameters leading to increased weight gain by a similar but distinct mechanism. Mice exposed to LAN may have disrupted melatonin signaling leading to a misalignment of food intake and activity resulting in altered fuel metabolism. Melatonin concentrations have been relatively unexplored in Swiss-Webster mice, however, retinal
melatonin levels were undetectable in one previous study in common with melatonin values in common strains of laboratory mice such as C57Bl/6 (Tosini & Menaker, 1998). Although previous research failed to detect melatonin in many strains of mice (Goto, Oshima, Tomita, & Ebihara, 1989), more recent studies report attenuated, but rhythmic, melatonin expression in strains such as C57Bl/6 which were previously thought void of melatonin (Kennaway, Voultsios, Varcoe, & Moyer, 2002). Melatonin rhythmicity, rather than absolute quantities of nightly melatonin secretion, plays a crucial role in metabolic function (Korkmaz, Topal, Tan, & Reiter, 2009). For example blunted nighttime melatonin rhythms due to LL increased visceral adiposity in rats (Wideman & Murphy, 2009) and daily administration of melatonin suppressed abdominal fat and plasma leptin levels (Rasmussen, Boldt, Wilkinson, Yellon, & Matsumoto, 1999). Furthermore, melatonin influences clock gene expression in peripheral tissues such as the heart (Zeman, Szantoova, Stebelova, Mravec, & Herichova, 2009) and may similarly modulate clock gene expression in peripheral tissue involved in metabolism.

Mice exposed to LAN may also have disrupted clock expression leading to altered metabolism. The SCN are the primary pacemaker at the top of a hierarchy of temporal regulatory systems wherein multiple peripheral tissues contain molecular machinery necessary for self-sustaining circadian oscillation (Kohsaka & Bass, 2007). In addition to becoming obese, mice fed a high fat diet have disrupted mClock expression in the liver (Kohsaka et al., 2007) and mice with mutations in clock genes have altered energy homeostasis (Turek et al., 2005). Moreover, mice with mutations in either Clock or Bmal1 show impaired glucose tolerance, reduced insulin secretion, and defects in the
proliferation and size of pancreatic islets (Marcheva et al., 2010). Several models of obesity have reported attenuated amplitude of circadian clock gene expression and changes in the phase and daily rhythm of clock genes may cause obesity (Barnea, Madar, & Froy, 2009).

Metabolism and the circadian clock are intrinsically related (K. Eckel-Mahan & Sassone-Corsi, 2009) with desynchrony of feeding and activity causing metabolic alterations (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010). In humans even brief circadian misalignment results in adverse metabolic and cardiovascular consequences (Scheer, Hilton, Mantzoros, & Shea, 2009). The seemingly innocuous environmental light manipulation used in this study that changed feeding behavior resulting in obesity may have important implications for humans. Patients with night-eating syndrome are obese and appear to display circadian rhythm disruption (Benca et al., 2009; Stunkard, Grace, & Wolff, 1955). More generally, prolonged computer use and television viewing have been identified as risk factors for obesity, diabetes, and metabolic disorders (Fung et al., 2000). For the most part, researchers considering this correlation have focused on the lack of physical activity associated with television and computer use; however the results from the current study suggest that exposure to nighttime lighting and the resulting changes in the daily pattern of food intake and activity may also be contributing factors.
Mean ± SEM daily food intake (g)

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<tr>
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**Table 2.1.** Food intake in food restricted mice exposed to dimly lit or dark nights.
Figure 2.1. Light at night affects body mass and glucose tolerance.

Body mass, fat pad mass, and glucose tolerance in mice exposed dark or lit nights. (A) Mice with light at night elevated body mass beginning one week after placement in light conditions and continuing throughout the remainder of the study. (B) Body mass gain and epididymal fat pad mass differed among groups at the conclusion of the study suggesting increases in body mass may be due to changes in body fat composition (C) Mice exposed to either dim or bright light at night had reduced glucose tolerance; dLAN and LL mice failed to recover blood glucose as rapidly as LD mice (D) Furthermore, body mass at the time of the glucose tolerance test positively correlated with final blood glucose levels.

*p≤0.05 when LD differs from both groups, &p≤0.05 between all groups.
Figure 2.2. Activity is comparable between mice exposed to dimly lit and dark nights.

Representative activity records from mice held in (A) a standard light/dark cycle (B) a light/dim cycle and (C) constant lighting. Homecage locomotor activity was measured in an infrared beam crossing system and is shown in a double plotted actogram. LL mice became arrhythmic in constant light, however dLAN mice maintained rhythmicity. (D) All mice had equivalent total 24 h activity (p>0.05).
Figure 2.3. Time of food intake but not corticosterone is altered by dim light at night.

(A) Serum corticosterone concentrations were reduced in the LL but not dLAN mice at the two time points measured suggesting that glucocorticoids did not mediate the changes in body mass (*p≤0.05 from LL and dLAN). (B) Mice exposed to bright and dim light at night ate more food during the light phase than in the dark phase which is atypical in nocturnal animals (*p≤0.05). (C) Body mass and (D) blood glucose levels are associated with percentage of daytime food consumption.
Figure 2.4. Restricting feeding to the dark phase prevents dim light at night induced body mass gain.

Body mass gain and epididymal fat pad mass differed between groups at the conclusion of the study (A) Total body mass gain and (B) epididymal fat pad mass (*p≤0.05 when group differs from LD, †p≤0.05 when FD differs from both). Timed feeding during the dark in mice with light at night prevented increased weight gain and elevated epididymal fat pad mass. (C) Serum corticosterone concentrations are altered by timed feeding. Feeding mice during the dark resulted in increased corticosterone concentrations at Zeitgeber Time (ZT) 16 irrespective of light condition (*p≤0.05 FD differs from FA and FL).
CHAPTER 3

DARK NIGHTS REVERSE METABOLIC CHANGES CAUSED BY DIM LIGHT AT NIGHT

Metabolic disorders are increasing in prevalence worldwide and represent a major global health threat. The metabolic syndrome is categorized by the development of several metabolic abnormalities that increase the risk of coronary artery disease, stroke, and diabetes. Hypercaloric food intake and physical lethargy are known to underlie the development of metabolic syndrome and obesity. However, additional nontraditional factors are likely involved. The increasing prevalence of metabolic disorders coincides with increasing exposure to light at night (Fonken & Nelson, 2011; Reiter, Tan, Korkmaz, & Ma, 2011; Wyse, Selman, Page, Coogan, & Hazlerigg, 2011). Recent epidemiological and experimental studies implicate the introduction of artificial light in the development of metabolic syndrome (Maury, Ramsey, & Bass, 2010). Indeed, shift-workers who experience high levels of light at night are at increased risk for cardiovascular disease (Ha & Park, 2005; Knutsson, 2003) and elevated body mass index (Parkes, 2002). Even brief behavioral and circadian misalignment alters metabolic homeostasis in humans, resulting in hyperglycemia, hyperinsulinemia, and postprandial
glucose levels comparable to a pre-diabetic state (Scheer, Hilton, Mantzoros, & Shea, 2009). Moreover, in rodent models exposure to light at night produces changes in metabolism (Fonken et al., 2010; Vinogradova, Anisimov, Bukalev, Semenchenko, & Zabezhinski, 2009; Wideman & Murphy, 2009).

Metabolic processes fluctuate throughout the day. The suprachiasmatic nuclei (SCN) of the hypothalamus comprise the master circadian clock in mammals and control physiological and behavioral circadian rhythms. Photic input to the SCN is the dominant cue for entraining the circadian clock. Light travels directly from intrinsically photosensitive retinal ganglion cells (ipRGCs) to the SCN via the retino-hypothalamic tract (RHT). Prior to the wide-spread adoption of electric lighting the circadian system was principally synchronized to the solar cycle. In contrast, modern light exposure occurs in a variety of patterns. Because of the importance of light in synchronizing the circadian system, exposure to aberrant light schedules disrupt circadian activity. Disruption in the clock gene network is linked to changes in sleep, body mass, locomotor activity, and food intake. Homozygous Clock mutant mice have significant increases in energy intake and body weight, and total arrhythmicity when housed in constant darkness (Turek et al., 2005). These mutants also showed dyslipidemia, hyperglycemia, and hypoinsulinemia—all markers of metabolic dysregulation (Turek et al., 2005). Manipulation of other genes in the clock gene family similarly cause metabolic abnormalities (Marcheva et al., 2010). Interactions between metabolism and the circadian system appear to be reciprocal as diet induced obesity alters the period of the central clock and dampens diurnal rhythm in locomotor activity (K. Eckel-Mahan & Sassone-Corsi, 2009; Kohsaka et al., 2007). Both
short and long duration exposure to light at night or constant light also produce symptoms of metabolic syndrome (Fonken et al., 2010; Vinogradova, 2007; Vinogradova, Anisimov, Bukalev, Semenchenko, & Zabezhinski, 2009).

Although the SCN is the dominant brain region involved in driving circadian activity, peripheral clock mechanisms are present throughout the body. Metabolic tissues such as liver, adipose, pancreas, and muscle all display independent rhythmic clock gene expression. The SCN principally regulates peripheral clock activity through neural and endocrine signaling pathways (Guo, Brewer, Champhekar, Harris, & Bittman, 2005; McNamara et al., 2001). Extra-SCN clock activity occurs in a tissue-specific manner which enables organs to cope with local physiological demands and respond to local factors. Multiple signals related to feeding and fasting entrain clock activity in metabolic tissue. Environmental cues that occur at aberrant times may lead to asynchronous activity within or between tissues which can lead to organ dysfunction (Vollmers et al., 2009). For example, changes in peripheral clock function contribute to symptoms of metabolic syndrome such as body weight gain and reduced glucose tolerance (Carvas et al., 2012; Kennaway, Owens, Voultsios, Boden, & Varcoe, 2007; Lamia, Storch, & Weitz, 2008).

We previously reported that constant light (LL) and a bright/dim light cycles (dLAN) alter metabolic parameters in mice (Fonken et al., 2010). Mice housed in LL and dLAN increase body mass and white adipose tissue, impair glucose processing, and alter food intake patterns compared to mice housed in LD. dLAN mice consume more food during the light period than at night which is atypical in nocturnal rodents (Fonken et al., 2010). Altered timing of food intake could be the mechanism by which light at night
induces weight gain as it has previously been shown to induce metabolic disorder (Arble, Bass, Laposky, Vitaterna, & Turek, 2009) and uncouple central and peripheral clock gene expression (Damiola et al., 2000). The goal of the present experiment was to determine whether or not changes in metabolism dissipate after removal of the aberrant light schedule. Studies in shift-workers offer contrasting views about whether removing circadian disruption can produce a return to baseline state. For example, current shift-workers increase systemic markers of inflammation, but former shift workers do not differ from day shift controls (Puttonen, Viitasalo, & Harma, 2011). However, former shift workers have increased risk for obesity (Puttonen, Viitasalo, & Harma, 2011). Thus, I tested whether metabolic disruption that occurs with light at night is an enduring effect following placement back in a standard light dark cycle. Mice were housed under dim light at night for 4 weeks and then transferred back to a standard light dark cycle. Placement back into dark nights ameliorated the effects of exposure to dim light at night. This suggests that changes in metabolism that occur with nighttime light exposure are not necessarily permanent.

**Methods**

**Animals**

Sixty male Swiss-Webster mice (~8 weeks of age) from Charles River Kingston were used in this study. Nocturnal rodents were used in this study to investigate the effects of nighttime light exposure independent of sleep disruption. The mice were individually housed in propylene cages (dimensions: 27.8 x 7.5 x 13 cm) at an ambient temperature of 22 ±2°C and provided with Harlan Teklad 8640 food (Madison, WI) and
filtered tap water *ad libitum.* All mice appeared healthy throughout the study and showed no signs of sickness behavior.

Mice were assigned to one of four groups: (1) a control group that remained in standard light-dark conditions [14h light (150 lux): 10h dark (0 lux); LD/LD] for the 8 weeks study, (2) a group housed in dim light at night for 8 weeks [14h light (150 lux): 10h dim (5 lux); dLAN/dLAN], (3) a group housed in LD for 4 weeks, then 4 weeks of dLAN (LD/dLAN), and (4) a group housed in dLAN for 4 weeks then 4 weeks of LD (dLAN/LD). All mice were housed in LD for one week to entrain to the local light-dark cycle and recover from the effects of shipping prior to entering the study. On day 1 of the experiment mice were weighed and transferred from an LD room to a cabinet with either an LD or dLAN light cycle. Body mass was measured weekly and glucose tolerance was evaluated after 7 weeks in experimental conditions. After 6 weeks in lighting conditions timing of food intake was measured for 4 consecutive days. At the conclusions of the study mice were anesthetized with isoflurane vapors and rapidly decapitated. Epididymal fat pads of mice that underwent the glucose tolerance test were collected and weighed and then flash frozen for qPCR analyses.
Table 3.1. Experimental design for Chapter 3.

Quantitative PCR (qPCR)

The mRNA levels of MAC1, IL6, and TNFα were assayed in epididymal fat pads as an index of peripheral inflammation (Reed et al., 2010). A small portion of the distal epididymal fat pad was collected and flash frozen at the conclusion of the study. Total RNA was extracted using a homogenizer (Ultra-Turrax T8, IKAWorks, Wilmington, NC) and an RNeasy Mini Kit (Qiagen, Austin, TX). RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Gene expression for MAC1 was determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of
individual samples run in duplicate was calculated by comparison to a relative standard curve and standardized by comparison to 18S rRNA signal.

*Intra-peritoneal glucose tolerance test (GTT)*

After 7 weeks in experimental light conditions a subset of each group of mice was administered an intra-peritoneal glucose bolus (1.5 g/kg body mass) after an 18 h fast. Blood samples of 5 µL were collected via submandibular bleed before injection and at 15, 30, 60, 120, and 180 min following injection. Blood glucose was measured immediately with a Contour blood glucose monitoring system and corresponding test strips (Bayer HealthCare, Mishawaka, IN).

*Statistical analyses*

Body mass and glucose tolerance test were compared between groups using repeated measures analysis of variance (ANOVA). Body mass gain, fat pad mass, gene expression, and food intake comparisons were analyzed using one-way ANOVA. Following a significant F score, multiple comparisons were conducted with Tukey’s HSD tests. The above statistical analyses were conducted with StatView software (v. 5.0.1, Cary, NC). In all cases, differences between group means were considered statistically significant if \( p \leq 0.05 \).

*Results*

*Body mass*

Body mass was significantly affected by light conditions over the 8 experimental weeks (\( F_{21,336} = 3.537; \ p < 0.0001 \); Fig. 1A). After 4 weeks in their respective lighting conditions, and prior to the light schedule transfer, significant differences in weight gain
were observed ($F_{3,50} = 3.710; p < 0.05$). Both groups that were initially housed under
dLAN showed elevated body mass gain compared to those housed in standard lighting
conditions (post hoc analyses; $p < 0.05$; Fig 1B). Directly following collection of week 4
body weights mice in the dLAN/LD and LD/dLAN groups were transferred to the new
light schedules. After 3 weeks of the new lighting schedules body mass gain was also
significantly affected by lighting conditions ($F_{3,50} = 3.906$ from beginning of study; Fig
1C; $F_{3,50} = 8.862$ from transfer; Fig. 1D; $p < 0.05$ respectively). LD/dLAN and
dLAN/dLAN mice gained significantly more weight throughout the study as compared to
LD/LD mice (post hoc; $p < 0.05$; Fig 1C). dLAN/LD mice did not differ from any group
with respect to weight gain when evaluating the entire 7 week period. When evaluating
body mass gain following the transfer however, dLAN/LD mice significantly reduced
weight gain compared to dLAN/dLAN and LD/dLAN mice (post hoc; $p < 0.05$; Fig 1D).

Relative epididymal fat pad mass (corrected for total body mass), a representative
measure of white adipose tissue, significantly differed by the conclusion of the study
($F_{3,31} = 4.089; p < 0.05$; Fig. 2A). Mice that were housed with dLAN for the entirety of
the study showed significantly elevated relative epididymal fat pad mass as compared to
LD/LD and dLAN/LD mice. LD/dLAN mice also had significantly elevated fat pad mass
as compared to LD/LD mice (post hoc; $p < 0.05$). These results are particularly important
because they suggest that increases in body mass among dLAN mice reflect increases in
white adipose tissue (Rogers & Webb, 1980). Furthermore, these results demonstrate that
a switch back to a standard light dark cycle after 4 weeks of housing in dLAN allows
restoration of fat levels to those of LD/LD mice. Lighting conditions did not affect paired testes, epididymides, spleen, or adrenal mass (p > 0.05 in each case).

**Gene expression**

MAC1 mRNA expression in epididymal fat pads was significantly affected by lighting conditions ($F_{3,31} = 2.948; p < 0.05$; Fig. 2B). dLAN/dLAN mice elevated MAC1 expression as compared to both LD/LD and dLAN/LD mice (post hoc; $p < 0.05$). In contrast, expression of IL6 and TNFα in the fat pads did not significantly differ between groups (data not shown, $p > 0.05$).

**Glucose tolerance test**

Three weeks after the transfer to the new lighting schedules (7 experimental weeks) mice underwent a GTT. Injection of glucose led to a rapid increase in blood glucose levels in all groups ($F_{5,155} = 267.514; p < 0.0001$; Fig 3A). Furthermore, there was a significant interaction between lighting conditions and blood glucose levels over time ($F_{15,155} = 2.427; p < 0.005$), such that dLAN/dLAN mice failed to recover glucose levels as effectively as all other groups. Single time point comparisons revealed that glucose levels were significantly elevated in dLAN/dLAN mice as compared to LD/LD mice at T60 and as compared to all other groups at T120 and T180 ($F_{3,31} = 3.285, 4.879, 4.622$, respectively; $p < 0.05$). These results suggest that dysregulation in glucose processing is secondary to increased weight gain as the LD/dLAN groups does not yet show impairments in glucose tolerance. Furthermore, the improvement in glucose processing in the dLAN/LD group suggests that impairments in glucose regulation are reversible among dLAN mice by a transfer back to standard lighting conditions.
Energy intake

Total 24 h food intake did not differ among groups (p > 0.05). There was a main effect of light cycle on timing of food intake (F\(_{3,31} = 3.912\); p < 0.05; Fig. 3B); dLAN/dLAN and LD/dLAN mice consumed significantly more of their food during the light phase as compared LD/LD mice (post hoc; p < 0.05). LD/dLAN also consumed significantly more food during the light phase than dLAN/LD mice (post hoc; p < 0.05).

Discussion

The goal of the current study was to determine whether mice returned to dark nights after dLAN exposure recover metabolic function. This study replicates previous results demonstrating that mice housed with dim light at night develop symptoms of metabolic syndrome (Fonken et al., 2010). As expected, both groups of mice housed in dLAN for the initial segment of the experiment (dLAN/dLAN and dLAN/LD) significantly increased body mass gain compared to LD mice. Half of the dLAN mice (dLAN/LD) were then transferred to LD and vice versa (LD/dLAN). Following the transfer dLAN/dLAN and LD/dLAN mice gained significantly more weight than LD/LD and dLAN/LD mice. At the conclusion of the study dLAN/LD mice did not differ from either LD/LD or dLAN/dLAN mice with respect to body mass gain. The intermediary results of the dLAN/LD mice may reflect an inability to completely recover body mass after the 4 week exposure to LAN. This would indicate permanent changes in metabolism occur after nighttime light exposure. Former shift workers show symptoms of metabolic dysfunction after return to a day shift schedule which indicates that this may be the case in humans (Puttonen, Viitasalo, & Harma, 2011). However, in the study on shift workers...
there was no specified duration of time workers were on the non-shifting schedule. An alternative explanation to the findings is that 3 weeks in LD may be insufficient to completely recover body mass to LD levels. For example, recovery of other metabolic markers can occur prior to reduction in body mass (Poudyal, Panchal, Ward, Waanders, & Brown, 2012). Food intake patterns, epididymal fat pad mass, as well as the GTT results support the latter hypothesis.

In agreement with previous findings, dLAN/dLAN mice shifted the timing of food intake compared to LD/LD controls without changing the amount consumed. dLAN/dLAN mice ate a significantly higher percentage of food during the light phase which is atypical for nocturnal rodents and may contribute to weight gain. Consuming higher amounts of food during the light phase is associated with increased weight gain in rodents (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). Moreover, restricting food intake to the dark phase can prevent weight gain in mice fed a high fat diet (Hatori et al., 2012; Sherman et al., 2012). Three weeks after dLAN/LD mice were moved back to LD, food intake no longer differed from LD/LD controls. In contrast, LD/dLAN mice shifted food intake to the light phase, with similar levels of daytime consumption as dLAN/dLAN mice. These results suggest that 3 weeks of dLAN is sufficient to induce altered timing of food intake.

Reduced glucose tolerance is a key symptom of metabolic syndrome (Kahn, Hull, & Utzschneider, 2006). Here I show that impaired glucose clearance abilities associated with dLAN are recovered by 3 weeks of exposure to LD. After 7 weeks in lighting conditions mice underwent a glucose tolerance test. dLAN/dLAN mice showed
significantly reduced glucose tolerance compared to all other groups. Importantly, the
dLAN/LD group was comparable to LD/LD controls in the GTT. This suggests that
dLAN/LD mice recover glucose processing abilities. Although dLAN/LD mice did not
show a complete reduction in body mass, the GTT results suggest that metabolic function
is restored.

Increases in body mass in dLAN are associated with increased white adipose
tissue (Fonken et al., 2010). dLAN/dLAN mice displayed significantly elevated
epididymal fat pad masses compared to LD/LD mice indicating increases in body mass in
dLAN are due to increased fat depots. At the conclusion of the study, dLAN/LD mice
had significantly reduced white adipose tissue compared to dLAN/dLAN mice.
Furthermore, LD/dLAN mice had intermediary fat pad mass compared to the LD/LD and
dlLAN/dLAN groups. Elevated fat mass is associated with widespread chronic low-grade
inflammation in peripheral metabolic tissue which led us to evaluate levels of
macrophage expression in the epididymal fat pads (Marceau et al., 1999; Plomgaard et
al., 2005). dLAN/dLAN mice significantly elevated expression of MAC1, a marker for
macrophages, in the epididymal fat pads. This indicates that there is increased
macrophage infiltration into peripheral fat tissue. Peripheral inflammation can lead to
disrupted insulin and leptin signaling further propagating fat accumulation (Hotamisligil,
2006). LD/dLAN mice had intermediary level of MAC1 expression compared to LD/LD
and dLAN/dLAN mice whereas dLAN/LD mice had comparable MAC1 expression to
LD/LD controls. This suggests that increases in peripheral inflammation are associated
metabolic dysfunction following exposure to dLAN.
This study also provides important insight into the time-course of development of metabolic syndrome in dLAN. For example, altered timing of feeding likely precede changes in glucose clearance as LD/dLAN mice did not differ in the GTT compared to LD/LD controls although they already showed a pattern of food intake similar to dLAN/dLAN mice. As discussed above, timing of food intake is a critical factor in the development of metabolic disease (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Hatori et al., 2012) and light at night likely elevates body mass gain through altering time of food intake (Fonken et al., 2010). Additionally, changes in weight gain appear to occur prior to the development of glucose intolerance because LD/dLAN mice show elevated weight gain but do not have impairments in the GTT. This would be consistent with other models of obesity in which adipose tissue releases factors (such as non-esterified fatty acids, glycerol, pro-inflammatory cytokines, etc.) that can contribute to the development of insulin resistance and β-cell dysfunction (Kahn, Hull, & Utzschneider, 2006).

Overall, these results demonstrate that re-exposure to dark nights ameliorates metabolic disruption caused by dim light at night. dLAN appears as an innocuous environmental manipulation, which may be why it was overlooked as a significant risk factor for health and disease for many years. However, because of the profound affect light has on the circadian system and upon downstream outputs such as hormone secretion, dLAN likely exerts a significant effect on many physiological processes. The circadian clock and metabolic pathways are intrinsically linked (Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012; K. Eckel-Mahan & Sassone-Corsi, 2009) with desynchrony of feeding and activity causing metabolic alterations (Arble, Bass, Laposky,
Vitaterna, & Turek, 2009; Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010). In humans even brief circadian misalignment results in adverse metabolic and cardiovascular consequences (Scheer, Hilton, Mantzoros, & Shea, 2009). The findings presented here suggest that exposure to nighttime lighting and the resulting changes in the daily pattern of food intake may be contributing factors in the current obesity epidemic. If these results apply to humans, then humans who experience weight gain in response to exposure to dim light at night may be able to help manage body weight by adjusting when they eat or by using low cost light blocking interventions such as sleep masks.
Figures

**Figure 3.1.** Return to dark nights affects body mass after exposure to dim light at night.

Body mass was differentially affected by the lighting conditions throughout the study. (A) Weekly body mass for mice over the course of the study. (B) Body mass gain after 4 weeks of lighting conditions and prior to the transfer. (C) Body mass gain at the conclusion of the study. (D) Body mass gain from the point of the transfer at experimental week 4 (different letters denote differences between groups, p≤0.05).
**Figure 3.2.** Dim light at night affects fat mass and composition.

dLAN increased relative fat pad mass and macrophage gene expression in fat pads. (A) Relative epididymal fat pad mass at the conclusion of the study. (B) Relative MAC1 mRNA expression from epididymal fat pads (different letters denote differences between groups, p≤0.05).
Figure 3.3. Dark nights reverse changes in feeding behavior and glucose processing

(A) Glucose tolerance was evaluated after 7 weeks in lighting conditions (*indicates LD/LD and dLAN/dLAN differ, †indicates dLAN/dLAN differs from LD/LD, LD/dLAN, and dLAN/LD). (B) Percentage of food consumed during the light phase (different letters denote differences between groups, p≤0.05).
CHAPTER 4

DIM LIGHT AT NIGHT EXAGGERATES WEIGHT GAIN AND INFLAMMATION ASSOCIATED WITH A HIGH FAT DIET

Obesity is a significant public health problem that reduces quality of life, increases mortality risk, and is a financial burden on society (Y. Wang, Beydoun, Liang, Caballero, & Kumanyika, 2008). Once termed a disease of western societies it is now clear that prevalence rates are increasing on a global scale (WHO, 2000). Health problems associated with obesity are replacing concerns such as under nutrition and infectious disease as the most significant contributors to global ill health (Antipatis & Gill, 2001). Although well-documented factors such as dietary choices and lethargy are known to contribute to the prevalence of obesity and metabolic disorders, additional environmental factors are now considered critical in the development and maintenance of obesity (Hill, Wyatt, Reed, & Peters, 2003).

The worldwide increase in obesity and metabolic disorders correlates with increased exposure to artificial light at night (LAN) during the 20th century. Approximately 99% of the US and Europe currently experiences light pollution that alters the natural cycle of light and dark (Navara & Nelson, 2007). Artificial lighting allows
people to extend daytime activities into the night and engage in countercyclical night time shift work. This type of aberrant light exposure can disrupt the circadian system because light is the most potent entraining signal for the mammalian biological clock (Reppert & Weaver, 2002). Many homeostatic processes are regulated by the circadian system including metabolism (Lowrey & Takahashi, 2004). For example, there are 24-hour variations in the expression of genes involved in gluconeogenesis, lipogenesis, and lipid catabolism, among others (Oishi et al., 2003; Yang et al., 2006). Disruption of both primary and secondary clock genes cause profound changes in metabolism (Marcheva et al., 2010; Paschos et al., 2012; Solt et al., 2012; Turek et al., 2005). Similarly, shifting the timing of food intake can alter weight gain independently of changes in total caloric intake (Hatori et al., 2012; Sherman et al., 2012). Even brief circadian misalignment can result in adverse metabolic and cardiovascular consequences in humans (Scheer, Hilton, Mantzoros, & Shea, 2009).

Communication between the circadian clock and metabolic system appears bi-directional as diet induced obesity is associated with behavioral and molecular circadian rhythm disturbances (Hsieh et al., 2010; Kohsaka et al., 2007). I have previously demonstrated that mice exposed to dim light at night (dLAN) significantly increase body mass and reduced glucose processing compared with mice in a standard light-dark cycle (LD), despite equivalent caloric intake and total daily activity. Nocturnal rodents typically eat substantially more food at night; however, dLAN mice consume more than half of their food during the light phase. Restricting food intake to the active phase in dLAN mice prevents body mass gain. These results suggest that low levels of light at
night disrupt the timing of food intake and other metabolic signals, leading to excess weight gain (Fonken, Kitsmiller, Smale, & Nelson, 2012; Fonken et al., 2010).

In addition to altering metabolism, disruption of circadian clock through activities such as shift work has serious health consequences including increased risk for cancer (Stevens, 2009a), tissue damage (Tunez et al., 2003), heart disease (Morris, Yang, & Scheer, 2012), and stroke (Vyas et al., 2012). A feature common to all of these pathologies is elevated inflammation. Inflammation is integrally associated with obesity, likely contributing to both the development and maintenance of metabolic syndrome (Hotamisligil, 2006). Elevated expression of tumor necrosis factor-α (TNF-α) in adipose tissue of obese mice was the first indication of inflammatory dysregulation in obesity (Hotamisligil, Shargill, & Spiegelman, 1993). Elevated fat mass is associated with widespread chronic low-grade inflammation in adipose tissue, liver, and skeletal muscle (Marceau et al., 1999; Plomgaard et al., 2005). This inflammatory response is characterized by increased levels of circulating pro-inflammatory cytokines, as well as infiltration of the tissue by immune cells such as macrophages, neutrophils, and eosinophils (Weisberg et al., 2003; H. Xu et al., 2003). Peripheral inflammation leads to disruption in insulin signaling further propagating fat accumulation. Peripheral inflammation described above is both a cause and consequence of obesity.

In contrast to the peripheral response, accumulating evidence suggests that hypothalamic inflammation resulting from a high fat diet (HFD) may occur prior to development of obesity through central leptin and insulin resistance. Acute glucose overload significantly increases NF-κB activity in the hypothalamus, but not peripheral
tissue (Zhang et al., 2008). Furthermore, hypothalamic insulin resistance occurs prior to insulin resistance in peripheral tissue (Purkayastha et al., 2010). Whereas peripheral inflammation can take weeks to develop after beginning a HFD (Kim et al., 2008), changes in hypothalamic inflammation can occur within hours of consuming high fat food (Thaler et al., 2012).

In developed and developing countries exposure to LAN and high fat diets often occur in tandem and may contribute to the increasing obesity epidemic. Thus, I hypothesized that dLAN would exaggerate metabolic dysfunction produced by a HFD in mice. Because dLAN is associated with changes in immune function (Bedrosian, Fonken, Walton, & Nelson, 2011b; Fonken, Haim, & Nelson, 2011), I also investigated whether dLAN works through a similar mechanism as HFD producing additional increases in peripheral and hypothalamic inflammation. Overall, I hypothesized that these two variables would synergistically affect metabolism through a similar mechanism.

Methods

Animals

Fifty four male Swiss–Webster mice (~8 wk of age) were obtained from Charles River Laboratories for use in this study. The mice were housed individually in propylene cages (30 x 15 x 14 cm) at an ambient temperature of 22 ± 2 °C and provided with filtered tap water ad libitum. Mice were housed in a standard light/dark cycle [14h light (150 lux): 10h dark (0 lux); LD] and provided basic rodent diet (chow; Harlan Teklad 8640, Madison, WI, USA) for one week after arrival at our facility. After the one week acclimation period, mice were randomly assigned a number and placed in one of four
groups (n=13-14 per group); mice were housed in either LD or dim light at night [14h light (150 lux): 10h dim (5 lux); dLAN] and received either a high fat diet (HFD; Research Diets D12451, New Brunswick, NJ, USA) or chow. On day 1 of the experiment mice were weighed and transferred from an LD room to a cabinet with either an LD or dLAN light cycle. At this time mice were also either maintained on chow or switched to a HFD for the duration of the study. Body mass was measured weekly at ~ZT8 and timing of food intake was measured twice daily at the onset of the light (ZT 0) and dark (ZT 14) phase, respectively, for 4 consecutive days during the final experimental week. The percentage of food consumed during the light phase was calculated for each day and averaged for each mouse to generate a single percentage value. At the conclusion of the study 6-7 mice per group were used for quantitative PCR (qPCR) and the remaining mice per group were used for immunohistochemistry.

qPCR

Between ZT 8 and 10 mice were brought into a procedure room, anesthetized with isoflurane vapors, a blood sample was collected from the retro-orbital sinus, and mice were rapidly decapitated. Brains were removed, placed in RNALater overnight and then the hypothalamus was dissected. Epididymal fat pads and liver were removed, weighed, and flash frozen. Total RNA was extracted from hypothalamic, fat, and liver tissue using a homogenizer (Ultra-Turrax T8, IKAWorks, Wilmington, NC) and an RNeasy Mini Kit (Qiagen, Austin, TX) according to manufacturer instructions. For fat extractions an additional chloroform separation step was added prior to using the kit to prevent excess fat from clogging the spin columns. RNA was then reverse transcribed into cDNA with
M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA). Gene expression for MAC1, TNFα, and POMC were determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve and standardized by comparison to 18S rRNA signal.

**Immunohistochemistry**

A subset of mice was used to assess histological evidence of microglia infiltration to the hypothalamus. Between ZT 8 and 10 mice were deeply anesthetized with isoflurane vapors, a blood sample was collected from the retro-orbital sinus, and mice were given a sodium pentobarbital overdose. Mice were then perfused transcardially with ice-cold 0.1M PBS followed by 50 mL of 4% paraformaldehyde. Brains were removed, post-fixed overnight, cryoprotected in 30% sucrose, and frozen in isopentane with dry ice. Brains were stored at -80°C and then sectioned on a cryostat at 40 μm into cryoprotectant. Sections were stored at -20°C until further processing. The sections were rinsed in phosphate buffered saline (PBS) and blocked with 4% BSA in PBS + Triton-X (TX) for 1 h with constant agitation. Sections were incubated overnight with primary rabbit-anti-Iba-1 (Wako Chemicals, Richmond, VA) diluted 1:1000 in PBS + TX. After PBS rinses the sections were subsequently incubated for 1 h at room temperature with biotinylated goat-anti-rabbit 1:1000 in PBS + TX (Vector Laboratories, Burligame, CA,
USA). Sections were then quenched for 20 min in methanol containing 0.3% hydrogen peroxide. After washing with PBS, sections were incubated for 1 hour with avidin-biotin complex (ABC Elite kit, Vector laboratories). After rinses, the sections were developed in diaminobenzidine for 2 min (Sigma, D4168). Sections were mounted on gel-coated slides, dehydrated, and coverslipped with Permount. Images were captured on a Nikon E800 microscope at 20X and analyzed using Image J software (NIH) to determine immunoreactive regions. Both sides of bilateral structures were counted in duplicate per animal.

Statistical Analyses

Comparisons between groups were conducted using a two-way analysis of variance (ANOVA) with lighting condition and diet as the between subject factors. Change in body mass over time was analyzed using a repeated measures ANOVA. Following a significant F score, multiple comparisons were conducted with Tukey’s HSD test. The above statistical analyses were conducted with StatView software (v.5.0.1, Cary, NC). In all cases, differences between group means were considered statistically significant if \( p \leq 0.05 \).

Results

Somatic measures

To determine whether dLAN exacerbates diet induced weight gain, mice were exposed to either dLAN or LD while fed HFD or chow. There was an overall increase in body mass over the 4 experimental weeks (\( F_{4,196} = 367.202; p < 0.0001 \)). Both lighting and dietary conditions affected body mass over time (Light: \( F_{4,196} = 15.525 \), Diet: \( F_{4,196} = \ldots \)
Average body mass was comparable between all groups at the start of the study (p > 0.05), but within 1 week of experimental onset both dLAN and HFD elevated body mass (Light: F_{1,49} = 17.250, Diet: F_{1,49} = 50.218; p < 0.0001). Furthermore, at the conclusion of the study relative body mass gain was increased among both dLAN and HFD groups (Light: F_{1,49} = 15.779; Diet: F_{1,49} = 136.447; p < 0.001; Fig. 1B). Increases in body mass likely reflected increases in fat mass as both dLAN and HFD increased relative epididymal fat pad mass, a reliable index of overall adiposity (Light: F_{1,21} = 9.785, Diet: F_{1,21} = 103.282; p < 0.01; Fig. 1C). Moreover, both dLAN and HFD increased blood glucose levels (Light F_{1,49} = 4.148, Diet: F_{1,49} = 5.214; p < 0.05; Fig. 1A). There were no interactions between lighting condition and diet with respect to body mass, fat pad mass, or blood glucose levels.

**Food Intake**

Despite increases in body mass among mice housed with dLAN, there were no differences in total daily food intake between lighting conditions (p > 0.05; Fig. 2A). Mice fed a high fat diet decreased food intake compared to the mice fed standard chow (F_{1,49} = 32.860; P<.0001; Fig. 2A). Decreased food intake among HFD mice was expected because the high fat food is much more calorie dense than standard chow. Although there were no differences in total food intake, dLAN and HFD both altered timing of food intake. dLAN mice increased daytime food intake as compared to mice exposed to dark nights (Light: F_{1,49} = 42.649; p < 0.0001, Fig. 2B). High daytime food intake is atypical for nocturnal rodents and changes in timing of food intake have previously been associated with changes in metabolism (Arble, Bass, Laposky, Vitaterna,
& Turek, 2009; Sherman et al., 2012; Tsai et al., 2012). HFD also caused a shift toward daytime food intake which has previously been described in (Kohsaka et al., 2007) (Diet: $F_{1,49} = 5.509; p < 0.05$).

*Peripheral Inflammation*

In metabolically related peripheral tissues (i.e., white adipose tissue (WAT), liver, and pancreas) obesity promotes a state of chronic low-level inflammation. This in turn contributes to insulin resistance, further propagating metabolic syndrome (Hotamisligil, 2006; Myers, Leibel, Seeley, & Schwartz, 2010). For this reason, I evaluated the expression of pro-inflammatory cytokines in both white adipose tissue (WAT) and liver. dLAN and HFD both elevated gene expression of MAC1, a marker for macrophages, in WAT (Light: $F_{1,20} = 9.304$, Diet: $F_{1,20} = 25.442; p < 0.01$; Fig. 3A). Additionally, dLAN and a HFD elevated expression of the pro-inflammatory cytokine TNFα in WAT (Light: $F_{1,20} = 4.649$, Diet: $F_{1,20} = 4.979; p < 0.05$; Fig. 3B). There were no differences in TNFα or MAC1 gene expression in the liver (data not shown). This is consistent with previous research as it is not uncommon for changes in peripheral inflammation to take > 4 weeks to develop in response to HFD (Kim et al., 2008).

*Hypothalamic Inflammation*

Previous research indicates that hypothalamic inflammation may occur with a HFD prior to the onset of inflammation in peripheral tissue and contribute to obesity development (Thaler et al., 2012). Because light at night is associated with changes in immune function (Bedrosian, Fonken, Walton, & Nelson, 2011b; Fonken, Haim, & Nelson, 2011), I hypothesized that hypothalamic inflammation may also be contributing
to changes in weight gain in dLAN mice. Consistent with previous reports (De Souza et al., 2005; Thaler et al., 2012; Zhang et al., 2008), HFD elevated hypothalamic TNFα expression relative to standard chow (F_{1,21} = 4.433; p < 0.05; Fig. 4A). However, no differences in MAC1 expression were apparent between light or dietary conditions (p > 0.05). This may reflect the distribution of hypothalamic microglia; with immunohistochemistry I established that the concentration of Iba1 positive cells was elevated in the arcuate nucleus of the hypothalamus in mice fed a HFD (F_{1,22} = 9.612; p < 0.01; Fig 3C,E,F), but not other hypothalamic nuclei such as the DMH (p > 0.05; Fig. 3D). There was no main effect of dLAN or interaction between lighting condition and diet with respect to Iba1 immunoreactivity. However, there was a simple effect of lighting condition within mice fed chow diet, such that dLAN increased the number of Iba1 positive cells within the chow group (F_{1,12} = 4.855; p < 0.05). Neither diet nor lighting condition affected POMC gene expression (data not shown, p > 0.05).

**Discussion**

I hypothesized that housing mice in dLAN would exaggerate metabolic changes induced by a HFD. Moreover, I predicted that dLAN and HFD would both result in elevated peripheral inflammation and upregulated inflammatory responses in the hypothalamus, indicating the manipulations worked through a similar mechanism. Although dLAN and HFD increased weight gain and peripheral inflammation, results in the central nervous system were less clear. dLAN did not alter hypothalamic TNFα, MAC1, or POMC gene expression. As anticipated, microglia staining was increased in the arcuate nucleus of mice fed a high fat diet. However, dLAN only resulted in increased
microglia staining among mice fed the chow diet. Lack of hypothalamic inflammation among both dLAN groups suggests dLAN induces weight gain through an alternative mechanism.

Both diet and lighting condition elevated body mass over the course of the study. Body mass was elevated by dLAN and HFD within the first week of experimental conditions. Among HFD mice, dLAN potentiated increases in body and fat pad mass compared to LD demonstrating that changes in environmental lighting can exacerbate the adverse effects of a HFD. HFD and dLAN also both altered timing of food intake. As previously reported, dLAN mice increase daytime food intake (Fonken et al., 2010). Whereas LD mice consume the majority of their food during the dark phase (~70%), dLAN mice show no preference for nighttime food intake. Daytime food intake is atypical for nocturnal rodents and is associated with the development of metabolic syndrome in animal models (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). In addition to dLAN shifting the daily pattern of food intake, HFD also caused subtle changes in timing of food intake. Previous work demonstrates that blocking access to food during the light phase can prevent increases in weight gain due to a HFD or dLAN (Fonken et al., 2010; Hatori et al., 2012; Sherman et al., 2012; Tsai et al., 2012). This suggests that weight gain due to both manipulations may partially result from the shift in timing of food intake.

Light at night is associated with changes in immune function in rodents (Bedrosian, Fonken, Walton, & Nelson, 2011b; Fonken, Haim, & Nelson, 2011). The effects of light at night on metabolic inflammation have not been previously
characterized. Here, I report that dLAN increases inflammation in white adipose tissue. Both TNFα and MAC1 gene expression were upregulated by dLAN and HFD. Elevated MAC1 expression is indicative of increased macrophage infiltration into fat tissue and has previously been described in models of obesity (Weisberg et al., 2003). Macrophage infiltration can increase pro-inflammatory cytokine release and result in the widespread, chronic, low-grade peripheral inflammation typical of high fat accumulation (Marceau et al., 1999; Plomgaard et al., 2005). The development of peripheral inflammation, although it propagates obesity through disrupting insulin and leptin signaling (Kim et al., 2008) generally follows the onset of obesity suggesting it is not the primary mechanism driving weight gain.

In contrast, hypothalamic inflammation may precede and contribute to the development of obesity as changes in the hypothalamic milieu are apparent within 24 h of the induction of high fat feeding (Thaler et al., 2012). Therefore, I hypothesized that hypothalamic inflammation may contribute to changes in weight gain in dLAN mice. Although HFD elevated hypothalamic TNFα expression relative to standard chow, there were no differences in TNFα expression between lighting conditions. Moreover, no differences in MAC1 expression were apparent between light or dietary conditions. Lack of significant changes in hypothalamic expression of MAC1 may be due to the specificity of the changes in hypothalamic inflammation. For example, there is a regionally specific enrichment of IKKβ and NF-κB in the mediobasal hypothalamus (includes the arcuate) that becomes potently upregulated with HFD (Zhang et al., 2008). This suggests elevated inflammation in the arcuate nucleus may be masked by the lack of change in
inflammation in other hypothalamic nuclei. In support of this hypothesis, the concentration of Iba1 positive cells was elevated in the arcuate of mice fed a HFD, but not in the dorsal medial hypothalamus. Although dLAN did not affect Iba1 positive cells among HFD mice, dLAN increased the number of Iba1 positive cells within the chow group. This suggests that hypothalamic inflammation is not essential for weight gain in dLAN as dLAN-HFD mice elevated weight gain compared to LD-HFD mice without increasing hypothalamic microglia. Continued elevation of CNS inflammatory responses combined with a long term HFD leads to gliosis and damage to proopiomelanocortin neurons (Parton et al., 2007). However, neither diet nor lighting condition affected POMC gene expression.

These results indicate that dLAN exacerbates weight gain and peripheral inflammation associated with HFD. Lack of elevated hypothalamic inflammation among dLAN mice suggests central inflammation is not the primary mechanism for light induced weight gain. Overall, these results have important implications for industrial societies in which nighttime light exposure and poor diet often co-occur. Further understanding of the mechanisms through which LAN contributes to inflammation and obesity is important for characterizing and treating metabolic disorders.
**Figures**

![Graphs](image)

**Figure 4.1.** Light at night exaggerates weight gain on a high fat diet.

Body mass and fat pad mass were elevated by dLAN and HFD. (A) Body mass throughout the experiment. (B) Final body mass gain expressed as a percentage from baseline body mass. (C) Epididymal fat pad mass corrected for final body mass. (D) Blood glucose levels at the conclusion of the study. (* p < 0.05 between lighting conditions, † p < 0.05 between dietary conditions).
Figure 4.2. High fat diet and light at night alter timing of food intake.

Total daily food intake was comparable between lighting conditions; however, dLAN mice consumed a higher percentage of food during the light phase. (A) Total daily food intake. (B) Relative daytime food intake. (* p < 0.05 between lighting conditions, † p < 0.05 between dietary conditions).
Figure 4.3. High fat diet and light at night increase adipose inflammation.

Both dLAN and HFD increased inflammatory gene expression in white adipose tissue. (A) Relative MAC1 and (B) TNFα gene expression in epididymal fat. (* p < 0.05 between lighting conditions, † p < 0.05 between dietary conditions).
Hypothalamic inflammation is elevated by HFD but not dLAN. (A) Relative TNFα and (B) MAC1 gene expression in the hypothalamus. Iba1 staining in the (C) arcuate and (D) dorsomedial nuclei of the hypothalamus. Representative photomicrographs captured at 20X from the arcuate nucleus of (E) an LD-chow mouse and (F) an LD-HFD mouse.

Figure 4.4. Hypothalamic inflammation is elevated by high fat diet but not exposure to dim light at night.
CHAPTER 5

EXERCISE ATTENUATES THE METABOLIC EFFECTS OF DIM LIGHT AT NIGHT

Over the course of the 20th century body mass rapidly increased worldwide. By the year 2000 the number of adults with excess weight surpassed those who were underweight for the first time in human history. This excess adiposity is recognized as one of the world’s leading health threats because obesity increases the risk of developing type II diabetes, cardiovascular disease, hypertension, and cancer (Caballero, 2007). The rapid growth in adiposity during the 20th century correlates with significant changes in human environment and lifestyle. In addition to changes in activity levels and dietary choices, a less appreciated environmental perturbation has been the shift in timing of daily activities. The invention of electric lighting ~150 years ago has enabled humans to illuminate their homes, hospitals, factories, and night skies and engage in activities such as countercyclical shift work (Navara & Nelson, 2007). Widespread adoption of electric lights occurred well before an understanding of circadian biology, and without any consideration of the negative biological consequences that artificial light at night (LAN) may have on physiology and behavior.
Circadian regulation of energy homeostasis is organized by an endogenous biological clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The circadian clock is entrained by light information that travels directly from light-sensitive ganglion cells in the retina to the SCN, thereby synchronizing individuals’ physiology and behavior to the external day-night cycle (Golombek & Rosenstein, 2010; Reppert & Weaver, 2002). Because light is the primary signal for the circadian clock, exposure to light at aberrant times can disrupt clock function (Navara & Nelson, 2007).

Many studies suggest a direct link between the molecular circadian clock and metabolism (Bray & Young, 2007). Mice harboring a mutation in the core circadian gene Clock are susceptible to obesity and metabolic syndrome (Turek et al., 2005). Clock mutants show dramatic changes in circadian rhythmicity, as well as altered timing of food intake and increased body mass. Serum leptin, glucose, cholesterol, and triglyceride levels are increased in Clock mutants compared to wild type (WT) mice. Mice with mutations in other clock related genes including Bmal1, Per1, Per2, Vipr2, and Rev-erba display similar metabolic outcomes (Bechtold, Brown, Luckman, & Piggins, 2008; Carvas et al., 2012; Delezie et al., 2012; Marcheva et al., 2010). Even single tissue clock gene disruptions can result in metabolic disturbances (Marcheva et al., 2010; Paschos et al., 2012). Thus, it seems reasonable to propose that disrupted circadian clock function has the potential to derange normal metabolism.

Mice housed in dim LAN (dLAN) elevate body mass and reduce glucose tolerance independent of changes in total daily food intake or home cage locomotor activity (Fonken et al., 2010). dLAN mice increase the percentage of food consumed
during the light phase as compared to mice housed in dark nights; restricting food intake
to the dark phase ameliorates weight gain among dLAN mice (Fonken et al., 2010).
Daytime food intake is associated with weight gain and metabolic disruption in mice in
other contexts (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Bray et al., 2012).
Furthermore, the relationship between the circadian clock and metabolism appears to be
bi-directional as diet induced obesity can dampen circadian rhythms (Kohsaka et al.,
2007).

As mentioned, light is the dominant entraining factor for the circadian system;
however, non-photic stimuli such as food intake and exercise can alter circadian rhythms
(Fuller, Lu, & Saper, 2008; Mistlberger & Antle, 2011). Activity is both a behavioral
output of the circadian system and an important feedback factor that can modulate
rhythms (Edgar & Dement, 1991; Mistlberger, 1991; Reebs & Stcoeur, 1994). In constant
dark conditions, timed wheel access entrains circadian rhythms in mice (Edgar &
Dement, 1991). Moreover, scheduled access to a running wheel can strengthen circadian
rhythms in mice with disrupted clock function (Power, Hughes, Samuels, & Piggins,
2010). Even under a standard light-dark cycle, ad lib access to wheels can strengthen the
power of circadian rhythms in wild type mice (Schroeder et al., 2012).

In addition to strengthening circadian rhythms, it is well established that exercise
prevents weight gain (Patterson & Levin, 2008). Therefore, I hypothesized providing
mice a running wheel for voluntary exercise would buffer against the effects of LAN on
metabolism. Specifically, I hypothesized that mice exposed to LAN would increase body
mass and alter feeding rhythms, indicating circadian system disruption. I predicted that
providing mice running wheels would strengthen circadian entrainment preventing altered timing of food intake and LAN-induced weight gain.

Materials and Methods

Animals

Forty male Swiss-Webster mice (~8 weeks of age) were obtained from Charles River Laboratories. The mice were individually housed in propylene cages (dimensions: 33 x 19 x 14 cm) at an ambient temperature of 22 ± 2° C and provided with Harlan Teklad 8640 food (Madison, WI) and filtered tap water ad libitum. Upon arrival, mice were maintained in a standard 14:10 light (150 lux) /dark (0 lux) cycle (LD; lights on at 2:00 EST) for one week in order to habituate to local lighting conditions and recover from the effects of shipping. After this period mice were randomly assigned a group, weighed, and transferred to either a cabinet with LD or dim light at night [dLAN; 14:10 light (150 lux) /dim (5 lux) light cycle]. Within each lighting condition mice received either a locked wheel or a low-profile running wheel (running surface of 15.5 cm diameter) for voluntary exercise (Med Associates, St. Albans, VT). Wheel running was constantly monitored using a wireless interface hub system which transmitted the data to a computer. Locked wheels were provided to control for the presence of a novel object in the cage. Mice were weighed every week at Zeitgeber Time (ZT) 9.

After 3 weeks in experimental conditions, food was weighed twice daily, immediately before the onset of the dark phase (ZT 14) and immediately after the onset of the light phase (ZT 0). Average food intake for the light and dark phases over three days was used to quantify percentage of daytime food intake. At the conclusion of the
study mice were individually brought into a procedure room, anesthetized with isoflurane vapors, and rapidly decapitated between ZT 9 and 11; a blood sample was then collected and epididymal fat pads were removed and weighed.

Statistical analyses

One dLAN mouse with a running wheel was removed from statistical comparisons because it did not use the wheel and one mouse was removed from the locked wheel LD group for demonstrating sickness behaviors. Effects of lighting condition and wheel access on body mass gain, fat pad mass, and percentage of daytime food intake were analyzed using two-way analysis of variance (ANOVA). A repeated measures ANOVA was used to assess change in body mass over time. Following a significant F score, multiple comparisons were conducted with Tukey’s HSD test. The above statistical analyses were performed with StatView software (v.5.0.1, Cary, NC). Running wheel activity was analyzed and actograms were generated using ClockLab Software (Coulbourn Instruments, Boston, MA). An animal was considered rhythmic when the highest peak occurred at ~1 cycle/24 h, with an absolute power of at least 0.005 mV/Hz (Kriegsfeld, et al., 2008). In all cases, differences between group means were considered statistically significant if $p \leq 0.05$.

Results

Somatic measures

Over the course of the study, body mass was elevated among all groups ($F_{4,136} = 82.814; p < 0.0001$); however, light at night potentiated increases in body mass, whereas access to a running wheel limited weight gain (Body mass over time: $F_{4,136} = 4.275$ and
Final body mass gain: \( F_{1, 34} = 7.711 \) and 8.203 respectively; \( p < 0.01 \); Fig. 1A/B). Final body mass gain did not differ between mice exposed to dLAN with a running wheel and mice housed in dark nights with either a running or locked wheel (post hoc; \( p < 0.05 \)). There were no interactions of the two variables on weight gain. Both light and access to a running wheel also affected final fat pad mass (\( F_{1,33} = 7.505 \) and 3.791; Fig. 1C); such that dLAN increased fat pad mass and presence of a running wheel reduced fat pad mass. In agreement with previous results, mice housed in dLAN increased percentage of food consumed during the light phase (\( F_{1,34} = 14.345 \); \( p < 0.001 \); Fig. 1D). In contrast to the hypothesis, wheel running also increased the percentage of food consumed during the light phase (\( F_{1,34} = 5.265 \); \( p < 0.05 \); Fig. 1D).

**Daily running wheel activity**

Total daily wheel running did not differ between mice in the LD and dLAN conditions (\( F_{1,17} = 0.144 \); \( p > 0.1 \); data not shown). All mice in LD showed a dominant rhythm of 0.042 (or 1 cycle per 24 hours) (Fig. 2A). In contrast, only 5 of the 9 dLAN mice demonstrated a dominant 24 hour wheel running rhythm (Fig. 1B).

**Discussion**

The goal of this study was to assess the effects of voluntary exercise on changes in body mass and food intake associated with exposure to dLAN in male Swiss Webster mice. I hypothesized that enhanced activity by means of optional wheel running would prevent body mass gain in mice exposed to dLAN. Specifically, I predicted that voluntary exercise would strengthen circadian organization in mice exposed to dLAN, preventing increased daytime food intake. Here I report that exercise availability limits weight gain
in mice housed under dLAN. In contrast to the hypothesis, reduced body mass occurred independently of re-establishing nighttime food intake; mice with running wheel access increased food intake during the light phase. Furthermore, although all mice maintained under dark nights had a dominant 24 hour activity rhythm, a subset of the dLAN mice showed disrupted patterns of wheel running.

These results confirm and extend previous findings (Coomans et al., 2013; Fonken, Kitzmiller, Smale, & Nelson, 2012; Fonken et al., 2010); mice exposed to LAN without access to wheel running elevated body mass gain over the course of the study. Consistent with the hypothesis, access to a running wheel reduced weight gain among mice exposed to dLAN. Final body mass gain was comparable between dLAN mice with a running wheel and both groups of LD mice. Furthermore, whereas dLAN increased relative epididymal fat pad mass, an index of overall adiposity, housing mice with a running wheel reduced fat pad mass. These results indicate that weight gain induced by dLAN is susceptible to traditional weight loss interventions, i.e. increased exercise.

As in previous studies, dLAN mice ate more food during the light phase compared to mice exposed to dark nights (Fonken et al., 2010). Eating during the light part of the day is atypical for nocturnal rodents and is associated with changes in metabolism (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Bray et al., 2012). Moreover, restricting food intake to the dark phase prevents weight gain in models of obesity (Fonken et al., 2010; Hatori et al., 2012; Mistlberger, Lukman, & Nadeau, 1998; Sherman et al., 2012). Voluntary wheel running has previously been associated with increasing the power of ambulatory activity rhythms with greater activity specifically
during the dark phase in mice housed in standard lighting conditions (Schroeder et al., 2012). Thus, I predicted that access to a running wheel would prevent the shift towards daytime food intake in dLAN mice. Contrary to my prediction, presence of a running wheel increased daytime food intake irrespective of lighting condition. These results differ from rats; rats with either voluntary wheel running or forced exercise consume more calories during the active phase (Oudot, Larue-Achagiotis, Anton, & Verger, 1996). To my knowledge the effects of voluntary wheel running on the daily pattern of food intake in mice has not been investigated. However, mice with access to running wheels have fewer, but larger, daily meals (Atalayer & Rowland, 2011).

Total daily wheel running did not differ between mice exposed to dark or dimly illuminated nights. These findings are consistent with previous research demonstrating that exposure to dLAN does not affect the amount of activity in either an open field or home cage (Fonken et al., 2009; Fonken et al., 2010). Circadian rhythms of home cage locomotor activity remain intact in mice exposed to dLAN and comparable to activity patterns in mice exposed to dark nights (Fonken et al., 2010). In the present study, however, wheel running activity was disrupted in several dLAN mice. All mice exposed to dark nights displayed a dominant 24 h rhythm in wheel running compared to 5 of 9 mice exposed to dLAN. Although I have previously asserted that dLAN does not affect sleep architecture or activity patterns, the existence of running activity during the so-called inactive periods suggests that dim light may shift circadian activity when paired with optional wheel running. Future studies should address sleep quantity and quality in mice exposed to LAN. Disparate results between wheel running and home cage activity
could reflect the different forms of behavior that the two systems monitor (Novak, Burghardt, & Levine, 2012). Home cage activity occurs for multiple reasons including grooming, feeding, or locomotor activity, whereas running wheels only monitor voluntary running. Overall, these results suggest that voluntary exercise prevents weight gain induced by dLAN without rescuing circadian rhythm disruptions.
Figures

Figure 5.1. Voluntary exercise prevents weight gain induced by dim light at night.

(A) Body mass over the course of the study. (B) Body mass gain expressed relative to baseline body mass. (C) Epididymal fat pad mass expressed relative to final body mass. (D) Percentage of food consumed during the light phase. All data are presented as mean ± SEM. * indicates dLAN-Sed differs from all other groups, † indicates main effect of lighting condition.
Figure 5.2. Dim light at night disrupts daily wheel running patterns in a subset of mice.

Representative actograph from a mouse housed in either (A) dark or (B) dim nights.
CHAPTER 6

DIM LIGHT AT NIGHT DISRUPTS MOLECULAR CIRCADIAN RHYTHMS

For >3 billion years, life outside of the highest latitudes has evolved under bright days and dark nights. Most organisms have developed endogenously driven circadian rhythms which are synchronized to this daily light/dark cycle. With the widespread adoption of electric lighting ~150 years ago, humans began brightly illuminating their nocturnal environments. Exposure to light at night is now pervasive in modern society and typically considered a mild environmental perturbation. However, the use of light at night (LAN) began prior to a deep appreciation of the importance of circadian rhythms for normal biological functions (Fonken & Nelson, 2011; Gerstner, 2012). Recent evidence suggests that exposure to unnatural light cycles increases the risk for cancer (Stevens, 2009b), sleep disturbances (Kohyama, 2009), and mood disorders (Driesen, Jansen, Kant, Mohren, & van Amelsvoort, 2010). Furthermore, exposure to light at night is increasingly associated with changes in metabolism. Shift workers who experience sustained nighttime illumination are at increased risk for cardiovascular disease and elevated body mass index (Ha & Park, 2005; Knutsson, 2003; Parkes, 2002; van Amelsvoort, Schouten, & Kok, 1999). Increases in nighttime light exposure at home
are associated with increased body mass, waist circumference and triglyceride levels, and poor cholesterol balance (Obayashi et al., 2013). Even brief exposure to altered light and food schedules can result in adverse metabolic and cardiovascular consequences (Scheer, Hilton, Mantzoros, & Shea, 2009). Moreover, I have reported that mice chronically exposed to dimly illuminated, as opposed to dark, nights elevate body mass independently of changes in total daily activity or caloric intake (Fonken, Kitsmiller, Smale, & Nelson, 2012; Fonken et al., 2010). Mice exposed to dim nights shift the timing of food intake toward the light phase and restricting food access to the dark phase prevents dLAN-associate body mass gain. The mechanism by which light at night induces these changes is not fully understood.

Here I propose that light alters metabolic homeostasis in mammals by disrupting the circadian system. As mentioned, light is the most potent synchronizing factor for the circadian system. Light information travels directly from intrinsically photosensitive ganglion cells in the retina to the master circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (S. K. Chen, Badea, & Hattar, 2011). Pacemaker neurons within the SCN thereby drive the circadian clock with an autoregulatory transcriptional-translational feedback loop of transcription activators and repressors (Albrecht, 2002). Although the SCN are the primary pacemakers in mammals, most if not all central and peripheral tissues contain the molecular machinery necessary for self-sustaining circadian oscillation (Mohawk, Green, & Takahashi, 2012). The master clock converts external light/dark information to neural and endocrine signals that synchronize
peripheral clocks (Guo, Brewer, Champhekar, Harris, & Bittman, 2005; McNamara et al., 2001).

Exposure to a pulse of light during the night can phase advance or delay the circadian clock depending on the strength and time of the light signal (Miyake et al., 2000). Exposure to constant light can alter activity rhythms and ablate circadian rhythms in glucocorticoids, two principle outputs of the circadian system (Coomans et al., 2013; Fonken et al., 2010). Moreover, specifically timed nighttime light pulses can be used to ablate the circadian system (Ruby et al., 2008). Because disruption in circadian clock genes are associated with significant changes in metabolism (Bechtold, Brown, Luckman, & Piggins, 2008; Carvas et al., 2012; Delezie et al., 2012; Marcheva et al., 2010; Paschos et al., 2012), I hypothesized that exposure to light at night alters metabolism through disrupting the circadian system. To assess the effects of light at night on the circadian clock I exposed mice to either total darkness or dim light (5 lux) during the night and then characterized the expression of several circadian clock genes and proteins in the SCN, hippocampus, liver, and adipose tissue. Five lux of nighttime light exposure was selected because (1) it is approximately five times bright than maximal moonlight (2) it is comparable to levels of light pollution found in urban areas (Kloog, Haim, & Portnov, 2009) and sleeping environments (Obayashi et al., 2013), yet (3) it is highly distinct from daytime light levels.

Methods

Animals
One hundred and twenty male Swiss Webster mice (~8 weeks of age) were obtained from Charles River for use in this study. Mice were individually housed in propylene cages (dimensions: 33 x 19 x 14cm) at an ambient temperature of 23 ±2°C and provided with Harlan Teklad 8640 food (Madison, WI) and filtered tap water ad libitum. All mice were maintained in a standard light dark cycle (LD; 14:10 light (~150 lux)/dark (0 lux)) for one week following arrival. After the 1 week acclimation period mice were randomly assigned a group and transferred to a cabinet with either LD or a light/dim light cycle (14:10 light (~150 lux)/dim light (5 lux)). Daytime lighting was provided with white LEDs on the walls of the cabinets and dim light was administered with a flexible strip of cool white LEDs wrapped around the rack on which the mouse cages were placed. The lighting intensity was measured inside the home cage and was highly consistent between cages. Mice were also assigned to 1 of 6 tissue collection time points (Zeitgeber Time (ZT) 2, 6, 10, 14, 18, 22). Mice were weighed at the start of the experimental light treatment and weekly throughout the study. After 3 weeks in lighting conditions food was weighed twice daily for four days to determine the timing of food intake. Four weeks after placement in light conditions blood and tissue were collected for either quantitative PCR (qPCR) or immunohistochemical analyses (n=5 per group/use/time point).

Quantitative PCR

Mice were anesthetized with isofluorane vapors and rapidly decapitated. Peripheral tissue was dissected out, immediately weighed on a fine balance, and flash frozen. Weighed tissues include liver, spleen, pancreas, white adipose tissue (epididymal...
and inguinal), brown adipose tissue, adrenals, heart, and skeletal muscle. Brains were collected, placed in RNAlater, and after > 24 h the hypothalamus and hippocampus were dissected out for PCR. Total RNA was extracted from liver, white adipose, hippocampal, and hypothalamic tissues using a homogenizer (Ultra-Turrax T8, IKAWorks, Wilmington, NC) and an RNeasy Mini Kit following the manufacturers protocol (Qiagen, Austin, TX). For fat extractions an additional chloroform step was added following homogenization and prior to the use of the Mini Kit. RNA concentration and purity were measured on an ND-1000 spectrophotometer (Fischer Scientific, PLACE). RNA concentrations were equalized with sterile water and RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Gene expression for Clock, Bmal1, Per1, Per2, Cry1, Cry2 and Rev-erbα were determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve and standardized by comparison to 18S rRNA signal.

Immonohistochemistry

Mice were given a lethal dose of sodium pentobarbital and perfused transcardially with ice-cold 0.1M PBS followed by 4% paraformaldehyde. Brains were removed, post-fixed overnight, cyroprotected in 30% sucrose, and frozen with dry ice. Brains were
serially sectioned at 40 μm into cryoprotectant and stored at -20°C. Sets of tissue collected at 240 μm intervals were used for immunohistochemical detection of BMAL, CLOCK, PER1, and PER2 using antibodies generously provided by David Weaver (LeSauter et al., 2012). Sections were rinsed in PBS, blocked for 1 h in 4% bovine serum albumin in 0.1 M PBS + 0.3% TX, and then incubated overnight at room temperature with primary antibody at 1:5000. The following day sections were rinsed and incubated for 1 h with biotinylated goat anti-rabbit at 1:1000 (Vector Laboratories, Burligame, CA). Endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 20 min and then the signal was amplified with avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) and tissue was developed using DAB. Tissue was then mounted onto gel coated slides, dehydrated through a series of graded ethanol washes, cleared with xylene, and coverslipped using Permount. Images of sections containing the SCN were captured at 20X using a Nikon E800 microscope. The number of immunoreactive cells in the SCN was counted in ImageJ (NIH) by a condition blind observer and averaged across section and sides of the bilateral structure to obtain one value per each mouse.

Statistical analyses

Body mass was analyzed using a repeated-measures analysis of variance (ANOVA) with time as the within subject factor and lighting condition as the between subject factor. Comparisons between lighting conditions with respect to weight gain, tissue masses, and percentage of daytime food intake were conducted using a one-way ANOVA. Blood glucose concentrations, qPCR results, and IHC results were analyzed using a two-way ANOVA with lighting condition and time as the between subjects
factors. Following a significant F score, multiple comparisons were conducted with Tukey’s HSD test. All statistical analyses were performed using StatView software (v.5.0.1, Cary, NC). In all cases, differences between group means were considered statistically significant if \( p \leq 0.05 \).

Results

Exposure to dim light at night increases body mass

Body mass increased among both groups over the course of the study (\( F_{3,354} = 304.187; p < 0.0001 \)); however, mice exposed to dim light at night significantly elevated body mass compared to mice housed in dark nights (\( F_{3,354} = 15.820; p < 0.0001 \); Fig 1A). Overall, mice exposed to dim light at night had a greater body mass gain at the conclusion of the study compared to mice exposed to dark nights (\( F_{1,118} = 15.476; p < 0.001 \); Fig 1B). There were no differences in spleen, liver, pancreas, BAT, adrenal, or heart masses between groups (\( p > 0.05 \) in all cases). Epididymal fat pad mass was elevated among mice exposed to dim light at night (\( F_{1,58} = 7.520; p < 0.01 \); Fig 1D) suggesting increases in body mass may reflect increases in white adipose tissue.

Light at night attenuates nocturnal feeding behavior

Despite increases in body mass among mice exposed to dLAN, there were no differences in total daily food intake between groups (\( p > 0.05 \); Fig S1). In agreement with previous results (Fonken, Kitsmiller, Smale, & Nelson, 2012; Fonken et al., 2010), exposure to light at night increased the percentage of food consumed during the light phase (\( F_{1,118} = 16.595; p < 0.0001 \); Fig 1D). Mice displayed a diurnal variation in blood
glucose levels ($F_{5,106} = 14.023; p < 0.0001; \text{Fig } 1E$) with no differences between lighting conditions ($p > 0.05$).

Clock gene expression is disrupted by nighttime light exposure

To test the hypothesis that nighttime light exposure affects core clock gene expression, I analyzed the diurnal expression of transcripts encoding Clock, Bmal1, Per1, Per2, Cry1, and Cry2 in the hypothalamus, hippocampus, fat, and liver every 4 h after 4 weeks of exposure to dim or dark nights. All of the core clock genes assessed in the hypothalamus displayed diurnal variation ($p < 0.05; \text{Fig. } 2A$). Expression of Clock, Bmal, and Cry1 were unaffected by lighting conditions, however, rhythmic expression of Per1, Per2, and Cry2 were all attenuated by exposure to dim light as compared to dark nights ($p < 0.05; \text{Fig } 2A$). Specifically, gene expression of Per2 and Cry1 was significantly reduced at ZT18, 6 hours after lights off, and Per1 expression was reduced at both ZT6 and ZT14.

In order to determine whether the effects of nighttime light exposure in the brain are specific to the master circadian clock I examined core clock gene expression in the hippocampus, a brain region known to show robust circadian oscillations. There was clear cycling of Clock, Bmal1, Per1, Per2, Cry1, and Cry2 in the hippocampus of mice exposed to both dark and dim nights ($p < 0.001; \text{Fig } 2B$). Overall, lighting condition had no effect on hippocampal clock gene expression ($p > 0.05$). This suggests that within the brain, changes in clock gene expression provoked by exposure to light at night may be regionally specific.
Recent work has demonstrated the importance of tissue specific clocks in regulating metabolism (e.g., Marcheva et al., 2010). Thus, I investigated the effects of nighttime light exposure on core clock gene expression in peripheral white adipose and liver tissues. All of the clock genes analyzed except for Clock displayed rhythmic variation in white adipose tissue and there was no effect of nighttime light exposure on mRNA expression levels (p > 0.05; Fig 2C). In contrast, rhythmic expression of Bmal1, Per1, Per2, Cry1, and Cry2 were all attenuated in the liver of mice exposed to dim nights (p < 0.05; Fig 2D). Taken together, these results reveal that exposure to low levels of light at night produce both tissue- and gene-specific changes in the expression levels of several core circadian clock genes.

To further explore the effects of light at night on clock transcriptional networks I studied the 24 h pattern of expression of Rev-Erb mRNA in hypothalamic, hippocampal, WAT, and liver tissues. Rev-Erb is a nuclear receptor that has prominent functions for both circadian oscillations and metabolic homeostasis (reviewed in Ribberger and Albrecht, 2012). Diurnal rhythmicity in Rev-Erb expression was observed in all tissues evaluated (p < 0.05; Fig 3). However, exposure to dim light at night reduced Rev-Erb expression levels during the light phase in both the WAT and liver (p < 0.05). Decreased Rev-Erb expression was specific to the periphery as there were no changes in hypothalamic or hippocampal Rev-Erb mRNA levels.

Core clock protein expression in the suprachiasmatic nucleus

In order to fully characterize changes in circadian clock function within the SCN after nighttime light exposure I evaluated clock protein expression every 4 h in mice.
housed under dark or dimly lit nights for 4 weeks. Rhythmic CLOCK and BMAL protein expression was observed within the SCN with no effect of nighttime light exposure (Fig 4A/B). Expression levels of both PER1 and PER2 were altered by exposure to dim light as compared to dark nights (p < 0.05; Fig 4C/D). Whereas mice exposed to dark nights had rhythmic expression of PER1, mice exposed to dim light at night showed no diurnal variation in PER1. Specifically, the peak in PER1 expression at ZT10 was abolished by exposure to dim light at night. PER2 expression was also suppressed in mice exposed to dim light at night at ZT14. These results confirm gene expression findings demonstrating dim light at night specifically targets hypothalamic Per1 and Per2.

Discussion

Exposure to electric light at night can lead to disruptions in metabolic energy homeostasis in rodents and humans (Ha & Park, 2005; Knutsson, 2003; Obayashi et al., 2013; Parkes, 2002; van Amelsvoort, Schouten, & Kok, 1999). However, it remains unclear how environmental lighting affects metabolism. Thus, I exposed mice to dim light at night and investigated changes in body mass and the circadian system. Here I establish that exposure to ecologically relevant levels of dim light during the night attenuate circadian clock gene and protein rhythms, change feeding behavior, and lead to weight gain. These observations indicate that exposure to dim light at night, a commonplace and innocuous seeming environmental manipulation, can influence the circadian system and metabolism.

Mice exposed to dim light at night showed rapid and sustained body mass elevations. Increases in body mass may reflect increases in white adipose tissue as
epididymal fat pad mass was elevated among mice exposed to dim nights. Although total daily caloric intake was comparable between groups, mice exposed to light at night consumed more food during the light period and less during the dark period than mice housed in dark nights. Disorganization in the feeding rhythm may contribute to increased body weight. Indeed, altered timing of food intake can cause weight gain and restricting feeding to specific hours prevents development of obesity (Arble, Bass, Laposky, Vitaterna, & Turek, 2009; Bray et al., 2010; Hatori et al., 2012; Sherman et al., 2012). Furthermore, mice fed a high fat diet disrupt daily feeding patterns and attenuate circadian clock gene rhythms in peripheral tissue (Kohsaka et al., 2007).

Genetic models indicate a close association between the molecular events underlying metabolism and those involved in the generation of circadian rhythms. For example, *Clock* mutant mice become overweight on a high fat diet and develop symptoms characteristic of metabolic syndrome (Turek et al., 2005). Mice with mutations in other clock related genes including *Bmal1*, *Per1*, *Per2*, *Vipr2*, and *Rev-erba* display similar metabolic outcomes (Bechtold, Brown, Luckman, & Piggins, 2008; Carvas et al., 2012; Delezie et al., 2012; Marcheva et al., 2010). These studies indicate alterations in core clock transcription factors within both the central clock and peripheral tissues alter metabolism. Importantly, these results suggest that changes in the circadian clock genes can be induced with exposure to ecologically relevant levels of dim light at night. Mice exposed to dim light at night suppress Per1 and Per2 expression at both the gene and protein level in the SCN. Importantly, there were no differences in clock gene expression
in the hippocampus, suggesting changes in central clock gene expression provoked by exposure to light at night are regionally specific.

In addition to altering clock gene expression in the hypothalamus, exposure to dim as opposed to dark nights attenuated the rhythm in all but one of the core circadian clock genes assessed in the liver. Peripheral clocks are entrained by neural and endocrine signaling from the SCN (Guo, Brewer, Champhekar, Harris, & Bittman, 2005; McNamara et al., 2001), as well as local factors such as nutritional signals (Vollmers et al., 2009). Recent work highlights the importance of peripheral clocks in regulating metabolism as single tissue clock gene deletions in the liver or fat can result in metabolic disturbances (Lamia, Storch, & Weitz, 2008; Marcheva et al., 2010; Paschos et al., 2012).

In addition to disruption in core clock mechanisms, mice exposed to dim light at night attenuated Rev-Erb expression in the liver and adipose tissue. Although previously considered an accessory feedback loop, REV-ERBs are increasingly demonstrated to be essential for circadian clock function and regulation of rhythmic metabolism. Mice deficient in both isoforms of REV-ERB show circadian rhythm adjustments and pronounced changes in metabolically related functions (Bugge et al., 2012; H. Cho et al., 2012).

Here I provide an extensive characterization of expression of circadian clock genes and proteins in both the master circadian oscillator in the brain and tissue specific clocks in mice exposed to light at night. Overall, these findings indicate that exposure to light at night attenuates core circadian clock mechanisms in the SCN at both the gene and protein level. Moreover, circadian clock function is disrupted in metabolically relevant
peripheral tissue (i.e., white adipose tissue and liver) by nighttime light exposure. These changes in circadian clock function are associated with alterations in feeding behavior and increased weight gain. These findings are significant because they provide evidence for how mild changes in environmental lighting can alter circadian and metabolic function. Exposure to light at night is pervasive in modern society and typically considered a harmless environmental perturbation; however, these results demonstrate nighttime light exposure alters homeostatic functions. Detailed analysis of temporal changes induced by nighttime light exposure may provide insight into the onset and progression of obesity and metabolic syndrome and other disorders involving sleep and circadian disruption.
Figures

**Figure 6.1.** Mice exposed to dim light at night alter somatic measures.

(A) Overall body mass (B) body mass gain and (C) epididymal fat pad mass were increased in mice exposed to dimly lit as compared to dark nights. (D) Mice exposed to dim light at night altered timing of food intake consuming more during the light phase than mice exposed to dark nights. (E) Blood glucose was rhythmic and did not differ between groups. All data are presented as (mean ± SEM). *indicates dLAN differs from LD.
Figure 6.2. Dim light at night attenuates clock gene expression.

(A) Hypothalamic, (B) hippocampal, (C) white adipose, and (D) liver tissues were collected at 4 h intervals from mice exposed to dark (black lines) or dimly lit (dotted lines) nights and Clock, Bmal1, Per1, Per2, Cry1, and Cry2 mRNA expression were quantified. Values are relative to a standard curve and normalized to 18S. * dLAN differs from LD
Figure 6.3. Dim light at night suppresses Rev-Erb expression in peripheral tissue.

Rev-Erb gene expression was quantified in the (A) hypothalamus (B) hippocampus, (C) white adipose tissue, and (D) liver. Values are expressed as relative abundance (mean ± SEM) after normalization to 18S *indicates dLAN differs from LD.
Figure 6.4. Clock protein expression is reduced in the SCN of mice exposed to dimly lit nights.
(A) PER1, (B) PER2, (C) CLOCK, and (D) BMAL immunoreactivity were analyzed in hypothalamic tissue collected every 4 h from mice exposed to either dark (black lines) or dimly lit (dotted lines) nights for 4 weeks. Images of sections containing the SCN were captured at 20X and the number of immunoreactive cells was counted and averaged across section and sides of the bilateral structure. Data are presented as (mean ± SEM). *indicates dLAN differs from LD.
Global ischemia produces high levels of central nervous system (CNS) damage that profoundly affect patient survival and long-term cognitive and psychological recovery. Minimizing this CNS injury to improve patient outcome is an important goal of current research. Importantly, the majority of damage to the CNS produced by cerebral ischemia is mediated by endogenous secondary processes (Krause, Kumar, White, Aust, & Wiegenstein, 1986; Saito, Suyama, Nishida, Sei, & Basile, 1996). Excitotoxicity, inflammation, and apoptosis develop in the days following injury and extensively contributing to outcome (Eltzschig & Eckle, 2011; Kirino, 1982; Nitatori et al., 1995; Weil, Norman, DeVries, & Nelson, 2008). The delay in CNS damage following injury provides a potential therapeutic window for influencing recovery. This suggests changes in environment may affect neural damage that develops post-ischemia.

One inconsistent environmental factor in hospitals is nighttime light exposure. Hospital intensive care units have variable levels of lighting during both day and night (Fig. 1; (Dunn, Anderson, & Hill, 2010)). These disruptive lighting conditions may influence patient recovery because light is the most potent entraining signal for the
mammalian circadian clock (suprachiasmatic nuclei; SCN). Extrinsic light information travels directly from the intrinsically photosensitive retina ganglion cells (ipRGCs) to the SCN via the retinohypothalamic tract (Hattar, Liao, Takao, Berson, & Yau, 2002) synchronizing daily physiological rhythms to the external light-dark cycle (Reppert & Weaver, 2002). Aberrant light exposure can disrupt the circadian system creating desynchrony between internal rhythms and the external environment.

Circadian rhythms are important for many homeostatic functions including those associated with the immune system (Lange, Dimitrov, & Born, 2010). There are circadian components to many immunological processes including antigen presentation, toll-like receptor function, cytokine production, and lymphocyte proliferation (Arjona & Sarkar, 2006; A. C. Silver, Arjona, Walker, & Fikrig, 2012) and many immune cells such as natural killer cells, macrophages, dendritic cells, and B cells possess molecular clock mechanisms necessary for self-sustaining oscillations (Arjona & Sarkar, 2005; Keller et al., 2009; A. C. Silver, Arjona, Hughes, Nitabach, & Fikrig, 2012). This reciprocal relationship between the circadian system and immune function has led to multiple studies evaluating the effects of chronic circadian disruption on human physiology. Shift-workers, who are chronically exposed to LAN, are at increased risk for several inflammatory disorders including heart disease (Ha & Park, 2005), cancer (Davis & Mirick, 2006; Schernhammer et al., 2001), disrupted rhythmicity of neuroendocrine function (such as corticotrophin releasing hormone, glucocorticoids, and prolactin) (Claustrat, Valatx, Harthe, & Brun, 2008; Persengiev, Kanchev, & Vezenkova, 1991), metabolic disorders and diabetes, as well as mood disorders (Dumont & Beaulieu, 2007).
Indeed, shift workers display several altered immune parameters, including elevated C-reactive protein and increased leukocyte count (Puttonen, Viitasalo, & Harma, 2011). Importantly, circadian disruption dysregulates inflammatory responses independently of sleep loss or stress (Castanon-Cervantes et al., 2010). In healthy individuals, circadian misalignment can be rapidly induced with aberrant lighting schedules, resulting in adverse metabolic and cardiovascular consequences (Scheer, Hilton, Mantzoros, & Shea, 2009).

Cardiac arrest has both seasonal and circadian (i.e., time of day) patterns of incidence and recovery (M. C. Cohen, Rohtla, Lavery, Muller, & Mittleman, 1997; Spencer, Goldberg, Becker, & Gore, 1998; Weil et al., 2009), implicating light in altering the physiological response to cerebral ischemia. Moreover, disruption of the core clock gene Per2, impairs recovery following myocardial ischemia (Eckle et al., 2012). Light exposure may be particularly salient to CA-induced neuroinflammation. Although several mechanisms contribute to damage following ischemic injury, including energetic failure, excitotoxicity, and oxidative damage (reviewed in Eltzschig & Eckle, 2011; Weil, Norman, DeVries, & Nelson, 2008), manipulation of inflammatory responses are considered a prime target for improving recovery. Following CA an inflammatory response is triggered by activation of microglia and astrocytes with a corresponding upregulation of pro-inflammatory cytokines (Stoll, Jander, & Schroeter, 1998). IL-1β and TNFα are pro-inflammatory cytokines that are upregulated within hours following global ischemia (Saito, Suyama, Nishida, Sei, & Basile, 1996) and exacerbate injury. Therefore, if dim light at night promotes inflammation, then it could be a critical factor for
consideration in cardiac arrest outcome. I hypothesized that light exposure at night would potentiate injury following cardiac arrest. Consistent with my hypotheses, light at night following CA enhanced acute cytokine responses, increased neuronal death, and resulted in higher short-term mortality. Moreover, the effects of light at night were ameliorated through inhibition of cytokines and manipulation of the light source.

**Methods**

*Animals*

8-week old male Swiss Webster mice (~30g; Charles River, Kingston, NY) were housed in a temperature- and humidity-controlled vivarium and provided *ad libitum* access to food and water. Mice were left unmanipulated for 1 week to recover from the effects of shipping and adjust to a 14:10 light/dark (LD) cycle prior to experimental manipulations.

*Cardiac arrest and cardiopulmonary resuscitation procedure*

Mice were anesthetized with 3% isoflurane in air, intubated, and maintained thereafter on 1.5% isoflurane. Mice were ventilated a tidal volume of 150 µL at a respiratory rate of 160 breaths/min. A temperature probe was inserted in the temporalis muscle on the left side of the head as an indicator of brain temperature (correlation between brain and temporalis muscle temp $r^2 = 0.942$) (Neigh, Kofler et al., 2004). A second probe monitored rectal temperature. A PE10 catheter was placed into the right jugular vein for epinephrine (EPI) and potassium chloride (KCl) administration. Blood pressure was monitored through a cannula inserted into the right femoral artery and connected to a blood pressure transducer (Columbus, Instruments). Mice were stabilized
for 10 min and blood pressure and temperature recorded at 1 min intervals (Fig. 2).

Following the 10 min acclimation, body and tail (but not head) temperature were lowered by circulating cold water through a coil system beneath the mouse to induce peripheral hypothermia restricting damage to the CNS during the CA/CPR procedure. CA was induced with an injection of KCL (50 μl, 0.5 M, 4°C) into the jugular catheter and the mouse was disconnected from the ventilator. Once a body temperature of 27°C was reached after approximately 4 min of arrest slow re-warming via a heat lamp and thermal blanket began. After 7 min 45 sec of arrest mice were reattached to the ventilator and 100 % oxygen at a tidal volume of 150 μL and a respiratory rate of 160 breaths/min was ventilated. After 8 min of arrest CPR was initiated with an injection of EPI (16 µg in 0.6 cc saline, 37°C) into the jugular catheter and chest compressions (300/min); 0.5 µg injections of EPI were administered until the mouse resuscitated (with a maximal dose of 32 µg). Mice were maintained on 100% oxygen for 25 min after return of spontaneous circulation and catheters were removed and incisions sutured.

**Tissue collection and staining**

One week following CA mice were individually brought into a procedure room, anesthetized with isoflurane vapors and a blood sample was collected via the retro-orbital sinus. Mice then received a lethal injection of sodium pentobarbital and were perfused transcardially with ice-cold 0.1M PBS followed by 50 μL of 4% paraformaldehyde. Brains were post-fixed overnight, cryoprotected in 30% sucrose, frozen on crushed dry ice, and stored at -80°C. Fourteen μm brain sections were sliced at -22°C using a cryostat and thaw mounted onto Super Frost Plus slides (Fisher, Hampton, NH). Sections were
stored at -20ºC until stained with Fluoro-Jade C (FJ-C) or Iba1 using procedures already established in our lab (Weil et al., 2009). Fluoro-Jade C. Cell death was quantified by labeling degenerating neurons with the fluorescein derivative FJ-C (Millipore, Temecula, CA). Mounted sections were thoroughly dried on a slide warmer, immersed in a basic ethanol solution (80% EtOH with 1% NaOH) and rinsed with 70% ethanol followed by water. Slides were placed in a 0.06% potassium permanganate solution for 10 min and rinsed twice. Sections were simultaneously incubated in FJ-C (0.0001% in a 1% acetic acid solution) and counterstained with DAPI (Sigma, St. Louis). Slides were rinsed 3X, aspirated, completely dried, cleared in xylene for 1 min, and coverslipped with DPX (Sigma). FJ positive cells were counted in the CA1, CA3, and DG of both hippocampal hemispheres by a condition blind experimenter using a Nikon E800 microscope at 200X magnification. Microglia. Microglia were visualized using a Iba1 directed antibody. Slides were dried, rinsed in phosphate buffered saline (PBS), and blocked with bovine serum albumin (BSA). Slides were incubated at room temperature for 24 h with rabbit anti-Iba1 antibody (Wako, Richmond, VA) diluted 1:1000 in PBS containing 0.1% Triton-X and BSA. Slides were rinsed and incubated with biotinylated goat anti-rabbit secondary antibody (1:1000; Vector Labs, Burlingame, CA) for 1 h. Sections were quenched in H₂O₂ in methanol, rinsed, and treated with Elite ABC reagent for 60 min (Vector). Sections were rinsed, developed with DAB (Vector), rinsed, dehydrated, cleared, and coverslipped. Photomicrographs of the hippocampus were taken with a Nikon E800 microscope at 20X and images were assessed using Image J software (NIH) to determine immunoreactive regions.
Cytokine expression

A separate cohort of mice that underwent CA or SHAM was used for detection of hippocampal cytokine gene expression using RT-PCR. Mice were brought individually into a procedure room, anesthetized with isoflurane vapors, blood was drawn via the retro-orbital sinus, and mice were rapidly decapitated. Brains were removed, divided sagittally along the midline, and hippocampi extracted and immersed in RNALater stabilizing solution RT-PCR. From hippocampi stored in RNALater, total RNA was extracted using a homogenizer (Ultra-Turrax T8, IKAWorks, Wilmington, NC) and an RNeasy Mini Kit (Qiagen). RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen) according to the manufacturer’s protocol. Pro-inflammatory cytokine expression for IL-1β, IL-6, and TNFα was determined using inventoried primer and probe assays (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman® Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative gene expression of individual samples run in duplicate was calculated by comparison to a relative standard curve and standardized by comparison to 18S rRNA signal.

Microglial isolation and RNA extraction

A separate cohort of mice that underwent CA or SHAM procedures was used for microglial cytokine analysis. Microglia were extracted following a previously reported protocol (Wynne, Henry, Huang, Cleland, & Godbout, 2010). Briefly, 24 h after surgery and one night of either dLAN or LD mice were anesthetized with isoflurane vapors,
blood was drawn via the retro-orbital sinus and mice were rapidly decapitated. Brains were removed and placed in ice-cold Hank’s Balanced Salt Solution (HBSS). Brains were transferred to a sterile flow hood and crushed through a 70 µm nylon cell strainer. The resulting homogenate was transferred to a 15 mL tube, topped off with additional HBSS and centrifuged for 7 min at 600g at 15°C. Supernatant was discarded and cell pellets re-suspended in 70% isotonic Percoll (GE Healthcare, Uppsala, Sweden). A discontinuous Percoll gradient was created by applying layers of 70%, 50%, 35%, and 0% isotonic Percoll (bottom to top). Percoll gradient was centrifuged for 30m at 2000g at 15°C and microglia were extracted from the interphase layer between the 70% and 50% Percoll. Microglia were then washed and RNA immediately extracted using a Sonicator (Microson XL2000, Misonix Inc., Farmingdale, NY) and an RNAeasy Micro Kit (Qiagen, Valencia CA). RNA was reverse transcribed into cDNA and processed as described above (in Real-time (RT) PCR).

*Administration of cytokine inhibitors*

An indwelling cannula was inserted into the left lateral ventricle (cannula position: +0.02 posterior and -0.95 lateral to bregma, extending 2.75 mm below the skull; Plastics One, Roanoke, VA) of anesthetized mice using a stereotaxic apparatus three days prior to CA or SHAM surgeries (Neigh et al., 2009). One hour following CA mice received a 2 µL injection of either a vehicle solution (artificial cerebral spinal fluid, aCSF), a monoclonal TNF antibody (Infliximab, IFX), an IL1 receptor antagonist (IL1-ra), or an IL6 neutralizing antibody (IL6na) based on pre-assigned group and were then
placed back in their respective lighting conditions. Cannula placement was verified on Iba1 stained tissue.

*Corticosterone radioimmunoassay*

Within 30 min of collection, blood samples were centrifuged at 3000 g for 30 min at 4°C. Plasma was collected and stored at -80°C until assayed. The samples were assayed using and 1\(^{125}\) corticosterone kit (MP Biomedicals, Solon, OH). The standard curve was run in triplicate and samples in duplicate. All samples within an experiment were run in a single assay.

*Statistical Analyses*

Statistical comparisons among groups were conducted using ANOVA. In the case of significant differences (p<0.05), a post-hoc Tukey’s test was conducted. When conditions of normality or equal variance were not met, the data were log transformed. Above statistical tests will be conducted using StatView Software v. 5.0.1. Survival plots were analyzed using Kaplan-Meier survival analysis. Differences were considered statistically significant when p < 0.05.

*Results*

*Hospital lighting levels*

In order to assess typical lighting environments for patients recovering in hospital settings, HOBO light loggers (Onset, Bourne, MA) were placed in patient rooms at The Ohio State University Wexner Medical Center Hospital. Significant nighttime light intrusions were observed in all units monitored, including the intensive care, post-surgical, and cardiac intensive care units (Fig. 1; representative patient lighting). Patients
experienced light levels as high as 100 lux several times each night between the hours of 11PM and 6AM. After confirming nighttime light disruptions in patient rooms, I used a mouse model of CA to determine whether exposure to dim light at night influences recovery following global cerebral ischemia.

*Dim light at night increases mortality and hippocampal damage following CA*

Eight week old Swiss Webster mice were acclimated to a standard light dark cycle [14h light (150 lux): 10h dark (0 lux); LD] and then underwent a CA or SHAM procedure (Fig. 2; surgical measures). I used nocturnal mice to avoid the confound of sleep disruption. Following the procedure mice either remained in LD cycle or were transferred to a bright-dim light cycle [14h light (150 lux): 10h dim light (5 lux); dLAN]. As anticipated, there was 100% survival in both SHAM groups. In contrast, CA significantly reduced survival compared to the SHAM procedure (p < 0.05; Fig 3a). Among the CA mice, mortality in the dLAN group was four-fold higher than in the LD group suggesting that modest changes in the recovery environment markedly affect cardiac arrest survival.

The reduced survival rate of mice exposed to dLAN may reflect increased neuroinflammation and hippocampal cell death. One week following CA or SHAM procedures mice were anesthetized and perfused transcardially with ice cold saline followed by 4% paraformaldehyde. Brains were cryoprotected, frozen, sectioned, and processed for Fluoro-JadeC, a marker for degenerating neurons. The Fluoro-JadeC labeled tissue was used to evaluate cell death in the hippocampus, a brain region particularly vulnerable to ischemic damage (Nikonenko, Radenovic, Andjus, & Skibo,
As expected, cell death was uniformly low among SHAM mice (Fig. 3b-d), and significantly elevated among mice subjected to CA (p < 0.05; Fig. 3b,e,f). Moreover, mice exposed to dLAN had significantly more cell death in the hippocampus one week after CA compared with mice exposed to dark nights (p < 0.05; Fig. 3b-f). Hippocampal cell death is a reliable proxy for overall recovery after global ischemia as increased hippocampal damage is associated with elevated mortality and memory deficits, as well as impaired affective responses (M. Fujioka et al., 2000; Langdon, Granter-Button, & Corbett, 2008; Neigh, Glasper et al., 2004; Neigh, Kofler et al., 2004; Zola-Morgan, Squire, & Amaral, 1986). Investigation of corticosterone concentration at 6 h intervals following CA did not reveal any significant differences between the CA-dLAN and CA-LD groups during the first 24 h of recovery (p > 0.05; Fig. 4).

**Dim light at night alters acute inflammatory status**

The inflammatory response following global ischemia is an important factor in recovery. Thus, I investigated whether dLAN alters the expression of pro-inflammatory cytokines following CA. Brain tissue was collected 24 h after CA or SHAM procedures, i.e., after only a single night of post-ischemic dLAN or LD. The brains were rapidly removed and placed in RNA later. The following day, hippocampi were dissected out and used for quantitative real time PCR (q-PCR) analyses of pro-inflammatory cytokines. As expected, TNFα, IL1β, and IL6 gene expression were elevated among CA compared to SHAM mice (p < 0.05; Fig 5a-c). Moreover, exposure to a single night of dLAN after CA was sufficient to upregulate expression of TNFα and IL1β, compared to mice exposed to a dark night (p < 0.05; Fig. 5a,b). The increase in pro-inflammatory cytokine expression
may occur very early after placement back in lighting conditions. Indeed, a difference in post-CA TNFα expression was apparent after as little as 4 h of total darkness versus dim light (p < 0.05; Fig. 5d). There were no differences in hippocampal cytokine gene expression between dLAN and LD mice that underwent the SHAM procedure (p > 0.05).

In sum, acute upregulation of inflammatory markers among CA mice housed in dLAN may contribute to the increased hippocampal neuronal death and mortality observed in this group.

Microglia are the resident immune cells in CNS and perturbations of the microenvironment can induce microglial activation, resulting in altered morphology and secretion of pro-inflammatory mediators (Graeber, 2010; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Therefore, I hypothesized that microglia increase cytokine expression 24 h after CA in dLAN mice, contributing to the overall elevation in inflammatory status in the dLAN-CA group. Microglia were extracted from whole brain tissue using a Percoll gradient 24 h after CA or SHAM procedures (a single night of dLAN or LD lighting conditions). mRNA was extracted immediately following microglial isolation and pro-inflammatory cytokine expression was evaluated. IL-1β and TNF-α mRNA expression were significantly higher in microglia isolated from CA mice as compared to SHAM mice (p < 0.05; Fig. 5e,f). Furthermore, exposure to a single night of dLAN after CA elevated microglial IL-1β and IL-6 mRNA relative to LD (p < 0.05; Fig 5f,g). These results suggest that microglia may be partially responsible for the pro-inflammatory bias observed among mice that were housed in dLAN after CA.
We also examined whether altered circulating glucocorticoid concentrations could be contributing to the impaired recovery after CA because elevated corticosterone has previously been associated with increased CA-induced neuroinflammation and neuronal death (Neigh et al., 2009). However, altered corticosteroid responses do not appear to underlie the differences in ischemic outcome between the CA-LD and CA-dLAN groups; there were no differences in corticosterone concentrations between CA or SHAM groups at 24h (p > 0.05; Fig. 5h).

*Inhibition of selective cytokines ameliorates light induced damage*

Although several mechanisms contribute to damage following ischemic injury, including energetic failure, excitotoxicity, and oxidative stress (reviewed in (Weil, Norman, DeVries, & Nelson, 2008), manipulation of inflammatory responses are considered a prime target for prevention of damage. Following ischemic brain damage both selective targeting of specific cytokines and non-selective (e.g., minocycline) inhibition of pro-inflammatory cytokines ameliorate damage improving recovery and behavioral outcomes (Craft & DeVries, 2006; Karelina et al., 2009; Mizushima et al., 2002; Neigh et al., 2009). Because the results indicate that IL-1β, TNF-α, and IL-6 mRNA expression are greater among CA-dLAN mice compared to CA-LD mice, I hypothesized that selective inhibition of these pro-inflammatory cytokines would improve outcome following CA-dLAN. Three days prior to the CA procedure, mice were implanted with a cannula directed at the lateral ventricle. Two hours following CA, mice were administered a single 2μL ICV injection of either vehicle (artificial cerebrospinal fluid; aCSF), mouse IL6 neutralizing antibody (IL6-na), TNF monoclonal antibody
(infliximab; IFX), or recombinant mouse IL-1 receptor antagonist (IL1-ra). Hippocampal cell death was evaluated using Fluoro-JadeC as described above. IL1-ra and IFX decreased hippocampal cell death compared to aCSF treatment among CA-dLAN mice (p < 0.05; Fig. 6b), producing levels of neuronal death that were similar to CA-LD mice treated with the vehicle (p>0.05). In contrast, treatment with IL6-na did not ameliorate hippocampal neuronal damage associated with CA-dLAN.

A similar pattern was apparent for microglial activation, which is often used as an index of neuroinflammation (Amantea, Nappi, Bernardi, Bagetta, & Corasaniti, 2009). Brain tissue was labeled with Iba-1, an antibody directed against microglia; increased Iba-1 surface area suggests microglial activation (Donnelly, Gensel, Ankeny, van Rooijen, & Popovich, 2009). Significantly greater microglial activation in the CA1, CA2, and CA3 subfields of the hippocampus was apparent among the CA-dLAN mice treated with the vehicle (aCSF) relative to the CA-LD mice treated with the vehicle (p < 0.05; Fig. 6c-i). Furthermore, treatment of CA-dLAN mice with IL1-ra or IFX reduced microglia activation in the CA1, CA2, and CA3 (p < 0.05; Fig. 6c-i) relative to the CA-dLAN mice treated with vehicle. Iba1 expression in CA-dLAN mice treated with IL1-ra or IFX did not differ from CA-LD mice treated with the vehicle in any of the hippocampal subfields quantified. In contrast, CA-dLAN mice treated with IL6-na had levels of microglial activation in the CA1 that were comparable to CA-dLAN mice treated with the vehicle, while levels of microglial activation in the CA2 and CA3 regions were intermediate between vehicle treated CA-dLAN and CA-LD mice. Thus, inhibiting IL-1 and TNF-α signaling in CA-dLAN mice normalized the microglial and
neurodegenerative responses. Furthermore, the results suggest IL1 and TNFα cytokine pathways may be more involved than IL6 in inducing hippocampal damage.

*Alternative spectra of lighting minimize light induced damage*

The circadian system is not equally responsive to all wavelengths of lighting. The intrinsically photosensitive retinal ganglion cells (ipRGCs) that project to the master circadian pacemaker in the SCN contain melanopsin and are most responsive to the blue region of the visible light spectrum ranging from 450 to 485 nm. These wavelengths are present in broad spectrum white light such as natural sunlight and the majority of indoor lighting. Longer wavelengths of lighting, such as red light, do not activate ipRGCs and therefore minimally influence the circadian system (Brainard et al., 2008; Figueiro & Rea, 2010). Thus, I hypothesized that the circadian system is involved in dLAN induced damage following CA with ipRGCs communicating the light information. To test this hypothesis I examined whether mice exposed to red light at night would more closely resemble the LD phenotype than the dLAN phenotype. Following CA, mice were placed in LD, dLAN [14 h light (150 lux): 10 h dim light (5 lux; 6500K cool white light-containing blue wavelengths)] or a bright-dim red light cycle [rLAN; 14 h light (150 lux): 10 h dim red (5 lux; 636 nm)]. Tissue was collected either seven days later for analysis of neuronal damage (assessed by Fluoro-JadeC) and microglial activation (via Iba-1) or after 24 h for evaluation of pro-inflammatory cytokine expression.

Unlike full spectrum light at night, dim red light at night did not increase mortality following CA. There were no differences in mortality between CA mice exposed to rLAN versus LD (p > 0.05; Fig. 7a). As in the first experiment, CA-dLAN
increased hippocampal cell death compared to CA-LD (p < 0.05; Fig. 7b,d-f). In contrast, rLAN did not exacerbate ischemic cell death. Hippocampal neuronal damage among CA-rLAN mice resembled that of CA-LD mice (p > 0.05) and there was significantly less damage among rLAN mice compared to dLAN mice following CA (p < 0.05; Fig. 7b,d-f). I similarly replicated the findings that CA-dLAN mice exhibited increased microglia activation in multiple hippocampal subfields compared to CA-LD conspecifics (p < 0.05; Fig. 7c,g-i), whereas rLAN did not increase post-ischemic microglia activation relative to CA-LD (p > 0.05; Fig. 7c,g-i).

Because the previous results indicate that light at night affects recovery by altering acute changes in the inflammatory response I evaluated hippocampal pro-inflammatory cytokine expression 24 h after CA and a single night of dark, dim white light or dim red light. Again, dLAN mice elevated hippocampal TNFα and IL6 expression compared to LD mice 24 h post-ischemia (p < 0.05; Fig. 7j,l). Red light did not elevate pro-inflammatory cytokine expression compared to LD controls (p > 0.10); dLAN mice had significantly elevated TNFα, IL6 and IL1β expression compared to rLAN mice (p < 0.05; Fig 7j-l). These results indicate that standard indoor lighting could potentiate neuroinflammation and neuronal damage following CA, whereas alternative lighting using wavelengths greater than ~600 nm are not likely to produce the same detrimental biological responses.

Discussion

Cardiovascular disease is the leading causes of death in the US (CDC, 2009). The survival rate for cardiac arrest is very low, and the majority of patients who survive live
with extensive physical, cognitive, and affective disabilities (Elliott, Rodgers, & Brett, 2011; Keuper, Dieker, Brouwer, & Verheugt, 2007; Lim, Alexander, LaFleche, Schnyer, & Verfaellie, 2004). However, the results presented in this dissertation indicate that adjusting environmental lighting could prove to be an inexpensive and effective way to improve patient outcome in cardiac intensive care units. Because of patient safety concerns and the need for monitoring, hospital ICU rooms are rarely completely dark and it is not uncommon for patients to be exposed to bright lights (100 lux) several times per night (Figure 1; Dunn, Anderson, & Hill, 2010). Indeed, even patients whose eyelids are closed may be affected by the light intrusion (Robinson, Bayliss, & Fielder, 1991).

Here I show that exposing mice to as little as 5 lux of dim light at night after resuscitation from CA exacerbates neuroinflammation and neuronal damage, and increases short-term mortality four-fold relative to mice that are maintained in a consistent light-dark cycle. The effects on neuronal damage and mortality appear to be mediated by increased neuroinflammation among CA mice exposed to dLAN. Indeed, TNF-α mRNA expression in the hippocampus is elevated as early as 4 h after exposure to dLAN, and by 24 h the CA-dLAN group has significantly greater TNF-α, IL-1β, and IL-6 mRNA expression compared to the CA-LD group (Fig. 2). These pro-inflammatory cytokines are known to contribute to damage after cerebral ischemia (Betz, Schielke, & Yang, 1996; Hurn et al., 2007). Thus, early changes in the inflammatory response caused by light at night may alter the trajectory of recovery resulting in higher mortality and increased neuronal damage characterized after one week.
Inhibition of TNF-α, IL-1, or IL-6 signaling among CA-dLAN mice produced 7-day survival rates that approximated or exceeded the survival rate for the CA-LD group (Fig. 3a), although only treatment with IL-1ra or IFX significantly reduced microglial activation and neuronal damage relative to the CA-dLAN mice. Thus, two bodies of evidence point to a role of increased inflammatory responses in mediating elevated neuronal damage among the CA-dLAN mice: (1) both proinflammatory cytokine gene expression and neuronal damage were elevated after exposure to dLAN relative to LD and (2) treatment with IL1ra or IFX prevented the exacerbation of neuronal damage and microglial activation observed among vehicle treated CA-dLAN mice.

Although pharmacological intervention clearly reduced the detrimental effects of dLAN on CA-induced microglial activation, neuronal damage, and short-term mortality, a far simpler approach to improving CA outcome is to modify the physical qualities of the nighttime light to prevent increased neuroinflammation. For example, night time red light of the same illuminance as the dim white light did not exacerbate CA-induced neuronal damage or microglial activation in mice. Indeed, the CA-rLAN mice did not differ significantly from CA-LD mice in either of these measures (Fig. 3). These data are consistent with studies reporting that red light at night does not affect other aspects of physiology and behavior in humans or other animals to the same extent as broad spectrum white light that contains blue wavelengths (Figueiro, Wood, Plitnick, & Rea, 2011). The effects of night time light are likely mediated by the suprachiasmatic nucleus or “master circadian clock”, which receives input from melanopsin containing ipRGCs in the retina. The ipRGCs are activated by blue light (~480nm, found in outdoor and most
indoor lighting, especially fluorescent lights), but are unaffected by long wavelength light, such as red light. The minimal influence of red light compared to white light on CA recovery suggests that light recognition by the circadian system is involved in dim white light induced exacerbation of CA damage. Importantly, exposure to dim light at night is an equally potent facilitator of inflammation in diurnal rodents (Fonken, Haim, & Nelson, 2011).

In sum, the mouse data presented here suggest that exposure to light at night, a common occurrence in hospital rooms, increases short-term mortality and compromises recovery from cerebral ischemia by exacerbating neuroinflammation. Using red lights at night in hospital rooms or having patients wear goggles that filter lower wave length light could be inexpensive solutions that allow visibility without priming the immune system of the patients. If the effects of white light at night are replicated in cardiovascular patients, then these results could have important implications for the design of lighting in clinical settings and could apply to a broad number of conditions and medical procedures that involve ischemia and inflammation, such as stroke, cardiovascular artery bypass graft, sickle cell disease, sleep apnea, and organ transplant.
Figure 7.1. Ambient lighting in cardiac intensive care unit patient rooms.
**Figure 7.2.** Cardiac arrest and cardiopulmonary resuscitation surgical parameters.

Mean arterial blood pressure, temporalis temperature, and core body temperature across the CA/CPR procedure.
Figure 7.3. Dim light at night impairs cardiac arrest recovery.

(A) dLAN increases short-term mortality following CA in a rodent model. (B-F) dLAN exacerbates CA induced hippocampal cell death as indicated by Fluoro-JadeC staining. Representative Fluoro-JadeC stained sections from the CA1 of (C) LD-SH (D) dLAN-SH (E) LD-CA and (F) dLAN-CA mice one week following the CA procedure.
Figure 7.4. Corticosterone concentrations in the 24 h following cardiac arrest.
**Figure 7.5.** Cytokine expression is elevated in the hippocampus and microglia of mice exposed to dim light at night following cardiac arrest.
Hippocampal (A) TNFα, (B) IL1β, and (C) IL6 gene expression are upregulated 24 h following CA and placement in dLAN. (D) TNF-α expression is elevated as early as 6 hours post-CA and only 4 hours of dLAN. Microglial (E) TNFα, (F) IL1β, and (G) IL6 gene expression are also altered 24 h following CA and placement in dLAN. (h) Serum corticosterone concentrations are unaffected by light at night.
Figure 7.6. Selective inhibition of specific cytokines attenuates inflammation and neuronal cell death following cardiac arrest and exposure to dim light at night.

(A) Percent survival following CA and treatment with different cytokine inhibitors. (B) Neuronal damage in the hippocampus as indicated by Fluoro-JadeC staining. (C) Proportional area of Iba1 staining in the CA1. Representative photomicrographs from the CA1 of mice treated with (D) LD-Veh (E) dLAN-Veh (F) dLAN-IL1ra (G) dLAN-IL6ab (H) dLAN-IFX. (I) Proportional area of Iba1 staining throughout the hippocampus.
Figure 7.7. Manipulation of lighting wavelength minimizes light at night induced damage following CA.

(A) Percent survival following CA and placement in LD, dLAN, or rLAN. (B) Neuronal damage in the hippocampus as indicated by Fluoro-JadeC staining. (C) Proportional area
of Iba1 staining in the CA1. Representative photomicrographs of Flourojade staining from the CA1 of (D) LD (E) dLAN and (F) rLAN mice. Representative photomicrographs of Iba1 staining from the CA1 of (G) LD (H) dLAN and (I) rLAN mice. (J) Proportional area of Iba1 staining throughout the hippocampus. Compared to LD controls (K) TNFα, (L) IL1β, and (M) IL6 gene expression are elevated 24 h following CA and placement in dLAN but not rLAN (*dLAN significantly differs from both rLAN and LD, #dLAN
Most organisms possess an endogenous biological clock that is synchronized by a very reliable exogenous cue: the daily cycle of light and dark produced by the rotation of the Earth about its axis. This biological clock is adaptive as it helps to maintain both daily and seasonal rhythms that allow animals to anticipate changes in the external environment (Hut & Beersma, 2011). The natural light-dark cycle, however, is now disrupted for many humans and nonhuman animals. With the advent of electric lights, light exposure is no longer limited to the natural pattern. Instead of aligning the circadian system with a stable cyclical factor, individuals currently experience a variety of lighting schedules. This divergence from the natural environment is not without repercussions. Disruptive lighting affects many physiological and behavioral functions (Fonken & Nelson, 2011; Navara & Nelson, 2007). For example, individuals exposed to altered light cycles are at increased risk for heart disease (Ha & Park, 2005), cancer (Davis & Mirick, 2006; Kloog, Portnov, Rennert, & Haim, 2011; Schernhammer et al., 2001), sleep disturbances (Deboer, Detari, & Meijer, 2007; Kohyama, 2009), circadian rhythm
dysfunctions (Borugian, Gallagher, Friesen, Switzer, & Aronson, 2005), disrupted rhythmicity of neuroendocrine function (Claustrat, Valatx, Harthe, & Brun, 2008; Persengiev, Kanchev, & Vezenkova, 1991), mood disorders (Dumont & Beaulieu, 2007; Fonken et al., 2009), metabolic dysfunction (Fonken et al., 2010; Reiter, Tan, Korkmaz, & Ma, 2011), and reproductive dysfunction (Fiske, 1941; Thomas, Oommen, & Ashadevi, 2001). One common factor for many of these pathologies is altered immune function.

Circadian timing in mammals is organized by a hierarchy of oscillating tissues, at the top of which are the suprachiasmatic nuclei (SCN) of the hypothalamus (Reppert & Weaver, 2002). Light information is the primary entraining cue for this master circadian clock. Light travels from the external environment through the intrinsically photosensitive retinal ganglion cells (ipRGCs) to the SCN. The SCN then influences downstream “slave” oscillators via the autonomic nervous system and control of the sleep-wake cycle. SCN driven sympathetic innervation of the pineal gland regulates the release of melatonin; nighttime sympathetic neural stimulation leads to the production of melatonin from its precursor serotonin in the pineal gland. This nocturnal melatonin signal provides time of day information to cells throughout the body and is the most reliable peripheral marker of central clock activity (Blask, 2009). Nocturnal lighting, if sufficiently bright, disrupts the synthesis of melatonin (Brainard et al., 1985; Brainard, Richardson, Petterborg, & Reiter, 1982; Dauchy et al., 2010). Importantly, modulation of both the circadian system and melatonin alters immunological measures.
Multiple immune markers such as interleukin 2 (IL2), IL10, IL6, IL1β, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and chemokine receptor 2 (CCR2) are expressed in a circadian pattern (Lundkvist, Robertson, Mhlanga, Rottenberg, & Kristensson, 1998; Young et al., 1995). Disruption of the circadian system through jet-lag, genetic mutations, or light exposure, changes the normal pattern of immune parameters. For example, mice with a loss of function mutation in the clock gene *Period 2* (Per2) have irregular production of IL10 and IFNγ in response to lipopolysaccharide (LPS) injection (Arjona & Sarkar, 2006). Mice deficient in Bmal1, another critical clock component, show early signs of aging such as sarcopenia, cataracts, organ shrinkage and elevated reactive oxygen species (ROS) in the kidney and spleen. The lifespans of Bmal1 deficient mice are also reduced (Kondratov, Kondratova, Gorbacheva, Vykhovanets, & Antoch, 2006). Cry 1 and 2 knockout mice have exacerbated cytokine and joint swelling after arthritic induction (Hashimoto et al., 2010). Furthermore, using a phase advancing chronic jet-lag (CJL) protocol causes persistent hypothermia and reduced survival following LPS administration in mice, and macrophages extracted from these mice have increased cytokine response to LPS (Castanon-Cervantes et al., 2010).

The nighttime increase in pineal melatonin production and secretion correlates with reduced innate immune responses (Markus, Ferreira, Fernandes, & Cecon, 2007). NF-κB, a pleiotropic transcription factor involved in the regulation of genes encoding for immune related enzymes, displays daily variation (Cecon, Fernandes, Pinato, Ferreira, & Markus, 2010; Z. Chen, Gardi, Kushikata, Fang, & Krueger, 1999). NF-κB induces multiple pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), and
cyclooxygenase-2 (COX-2). Melatonin blocks NF-κB nuclear translocation in leukocytes and endothelial cells, suppressing immune gene transcription activity (Gilad et al., 1998; Tamura, Cecon, Monteiro, Silva, & Markus, 2009). Darkness induced suppression in NF-κB transcription is blocked by propranolol (a drug that inhibits melatonin production in addition to altering catecholaminergic function). \textit{In vitro} work has further supported the role of melatonin in NF-κB signaling as melatonin reduces NF-κB in cultured pineal glands (Cecon, Fernandes, Pinato, Ferreira, & Markus, 2010). In a rat model of diabetes increased expression of NF-κB and pro-inflammatory cytokines including TNFα and IL6 were reduced with melatonin treatment (Negi, Kumar, & Sharma, 2011). Melatonin also impairs capacity for rolling and adhesion among leukocytes (Lotufo, Lopes, Dubocovich, Farsky, & Markus, 2001). Furthermore, communication between the circadian and immune system is bidirectional with multiple studies characterizing an immune-pineal axis (Couto-Moraes, Palermo-Neto, & Markus, 2009; Lopes, Mariano, & Markus, 2001; Markus, Ferreira, Fernandes, & Cecon, 2007). For example, administration of LPS inhibits norephinephrine (NE) induced N-acetylserotonin (NAS) production resulting in decreased nocturnal melatonin production (da Silveira Cruz-Machado et al., 2010).

Taken together, these studies suggest that nighttime lighting may affect immune function through disruption of the circadian system and suppression of melatonin. Recently our lab demonstrated that Siberian hamsters (\textit{Phodopus sungorus}) exposed to dim nighttime lighting (dLAN) reduce immunological capabilities (Bedrosian, Fonken, Walton, & Nelson, 2011a). Housing female Siberian hamsters under dLAN for 4 weeks reduces delayed-type hypersensitivity responses, decreases bactericidal capacity of blood,
and prevents fever-associated reductions in locomotor activity. Because Siberian hamsters are nocturnal, and in the laboratory are typically exposed to light at night during their active period, I asked whether light at night would evoke similar responses in a diurnal species. Thus, I investigated the effects of nighttime light exposure on immune parameters in Nile grass rats (*Arvicanthis niloticus*), a diurnal rodent species. Male grass rats were exposed to either a standard light-dark cycle or dim light at night for 3 weeks and then tested for delayed type hypersensitivity, bacteria killing capacity, and antibody production.

**Methods**

**Animals**

Male grass rats (*Arvicanthis niloticus*) used in this study were bred in the Nelson lab colony at the Ohio State University from a wild stock obtained by Dr. Laura Smale, Michigan State University, from the Masai Mara reserve in Kenya. Grass rats were bred under a light-dark (LD) cycle (14:10 light (~150 lux)/dark (0 lux); lights illuminated at 7:00 Eastern Standard Time [EST]) and all animals were provided food (ProLab RMH 2000, LabDiet) and water *ad libitum*. Experimental grass rats were weaned between 21 and 24 days of age and housed with same sex siblings in polypropylene cages (40 cm x 20 cm x 20 cm) with straw bedding. Colony rooms were maintained at a temperature of 20 ± 4º C and a relative humidity of 50% ± 10%. At approximately 3 months of age grass rats were singly housed, randomly assigned a number, and were either maintained in LD (n=9) or placed in dLAN (n=9; 14:10 light (~150 lux)/ dim (~5 lux); lights illuminated at
7:00 EST) for the remainder of the study. Immunological testing began after 3 weeks in the lighting conditions.

*Delayed-type hypersensitivity (DTH)*

DTH is a cell mediated response that provides information about the primary immune reaction to invading pathogens. After 3 weeks in lighting conditions grass rats were assessed for DTH response to the chemical antigen 2,4-dinitro-1-fluorobenzene (DNFB; Sigma, St. Louis, MO). Grass rats were individually brought into a procedure room between 14:00 and 15:00 EST, lightly anesthetized with isoflurane vapors, weighed, and a blood sample was collected from the retro-orbital sinus for use in the bacteria killing and corticosterone assays (see below). Following blood collection a 1 x 2 cm patch of fur was shaved on the dorsum and 25 μl of DNFB in a 0.5% solution (wt/vol) of 4:1 acetone to olive oil (prepared fresh daily) was applied to the dorsal skin in the same location on two consecutive days. To obtain a baseline measurement, both right and left pinna were measured during sensitization with a constant loading dial micrometer (Mitutoyo, America Corp., Aurora, IL, USA). Grass rats were then left undisturbed for 1 week, after which they were again anesthetized, pinna thickness measured, and challenged on the surface of the right pinna with 20 μl of 0.2% (wt/vol) DNFB in 4:1 acetone to olive oil. Left pinna was treated with the vehicle solution and both pinnae were measured every 24 h for 7 days. Pinna swelling values obtained on each day were expressed as a percentage of baseline thickness. All measurements occurred between 14:00 and 15:00 EST. DTH is an *in vivo* measure of cell mediated immune responses that is characterized by swelling at the site of DNFB challenge. Swelling of the right pinna is
due to infiltration of leukocytes into the epidermis and dermis (Vadas, Miller, Gamble, & Whitelaw, 1975). This immune measure was previously validated; pinna swelling is positively correlated to the intensity of the immune reaction (Phanuphak, Moorhead, & Claman, 1974).

*Keyhole limpet hemocyanin (KLH)*

Two weeks following the conclusion of DTH measures humoral immune function was assessed by injecting grass rats with 140 µg KLH suspended in 0.2 mL sterile saline. KLH is a respiratory protein from the giant keyhole limpet (*Megathura crenulata*) that produces a robust antigenic response without inducing fever or a long-term inflammatory response. KLH production has not previously been assessed in grass rats, therefore, this dose was determined based on other arvicoline rodents and resulted in similar patterns of plasma immunoglobulin G (IgG) production (Klein & Nelson, 1999; Weil, Martin, & Nelson, 2006). Blood was drawn from the retro-orbital sinus at the time of injection and 5, 10, and 15 days post injection in order to capture peak immunoglobulin production (Demas, Chefer, Talan, & Nelson, 1997). All blood sampling occurred between 14:00 and 15:00 EST. Blood samples were centrifuged at 4°C for 30 min at 3.3 g and plasma was pulled off and stored in microcentrifuge tubes at -80°C.

*KLH ELISA*

Plasma concentrations of anti-KLH IgG were determined using an enzyme-linked immunosorbent assay (ELISA) as previously described (Demas, Chefer, Talan, & Nelson, 1997). Plates were coated overnight with dialyzed KLH antigen, washed, and blocked the subsequent night with a milk blocking buffer. Plates were then washed and
150 µL of plasma diluted 1:80 in PBS+Tween was added in duplicated to the wells. Following a 3 h incubation plates were again washed and incubated for 1.5 h with a secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG). Plates were then treated with the enzyme substrate (p-nitrophenyl phosphate) before determining the optical density of each well with a plate reader (Benchmark Microplate Reader, Biorad, Hercules, CA) and 405 nm wavelength filter. To minimize intra-assay variability optical density was averaged over duplicate wells and expressed as a percentage of the plate-positive control value for statistical analyses.

**Bactericidal capacity of blood plasma**

Blood samples collected directly prior to DTH sensitization were used in this assay. Blood was centrifuged at 3300 g for 30 min at 4° C and plasma was pulled off and stored at -80° C. Plasma samples were diluted 1:20 in L-glutamine CO₂-independent media (Gibco, Carlsbad, CA, USA). A standard number of colony-forming units (CFUs) of *Escherichia coli* (Epower 0483E7, Fisher Scientific) were added to each sample and samples were incubated for 30 min at 37° C. Using sterile techniques 75 µL of each sample was plated in duplicate on tryptic soy agar plate. Two positive controls of diluted bacteria alone and two negative controls of CO₂-independent media were also plated. Plates were inverted and incubated overnight and total CFUs were counted and expressed as a percent of the positive control.

**Radioimmunoassay Procedure (RIA)**

Blood samples were collected for RIA of corticosterone from the retro-orbital sinus of grass rats on the first day of DTH sensitization. Blood samples were centrifuged
at 4° C for 30 min at 3.3g and plasma was pulled off and stored in sealable polypropylene microcentrifuge tubes at -80° C until assayed. Total plasma corticosterone concentrations were determined in duplicate using an ICN Diagnostics 125I double antibody kit (Costa Mesa, CA, USA). The high and low limits of detectability of the assay were 1200 and 3 ng/ml, respectively. The intra-assay coefficient of variation was 11%. All procedures followed the manufacturer guidelines.

**Activity analyses**

A separate cohort of grass rats were implanted intraperitoneally (i.p.; under sterile conditions) with telemeters (PDT-4000; Minimitter, Bend, OR) while under isoflurane anesthesia. Surgical wounds were treated topically with Betadine (Sigma Chemical, St. Louis, MO) to discourage infection, and grass rats were injected (i.p.) with buprenorphine (0.1mg/kg; Sigma Chemical) in sterile saline to alleviate pain during recovery. Following surgery, grass rats were placed in a clean cage, which was placed on a receiver (Minimitter) connected to a computer. Receivers collated emitted body temperature and movement activity frequencies continuously over 30 min intervals and converted them to raw data based on pre-programmed calibration curves for each transmitter.

**Data analyses**

Tissue weight, body mass, and total surviving CFU’s were compared between lighting conditions using a one-way analysis of variance (ANOVA). Delayed-type hypersensitivity swelling and anti-KLH were analyzed with a repeated measures ANOVA with lighting condition as the between-subject factor and time as the within subject variable. Following a significant result on repeated measures ANOVA, single
time point comparisons were made. Plasma corticosterone concentrations were analyzed using a one tailed t-test based on a priori hypotheses (Keppel & Wickens, 2004). Fourier analysis was used to determine whether locomotor activity was rhythmic and followed 24 h periodicity using Clocklab software from Actimetrics (Wilmette, IL). Grass rats were considered rhythmic when the highest peak occurred at ~1 cycle per day with an absolute power of at least 0.005 mV/Hz as previously described (Kriegsfeld et al., 2008). FFT power values for 0.083 cycles per day were compared between lighting conditions by one way ANOVA. Percentage of daytime activity and total daily activity were also analyzed by one way ANOVA. Nonlinear regression analysis was used in GraphPad Prism software (v. 4 La Jolla, CA). In all cases, differences between group means and correlation coefficients were considered statistically significant if p ≤ 0.05.

**Results**

*Reproductive and somatic measures*

There were no differences in body, reproductive tissue, adrenal, spleen, or thymus mass between groups (p > 0.05; Table 1).

*Circadian activity pattern*

There were no differences between grass rats housed with dLAN and those in LD with respect to circadian pattern in activity, total daily activity, or percentage of activity occurring during the light phase (p>0.05; Fig. 1).

*Plasma corticosterone concentrations*

Samples for corticosterone analysis were collected directly prior to DTH sensitization. Plasma corticosterone concentrations were elevated among grass grass rats
exposed to dLAN as compared to those exposed to dark nights ($t_{16} = 1.815; p ≤ 0.05$; Fig. 2a).

**DTH swelling responses**

One dLAN grass rat did not develop a swelling response and was excluded from comparisons. The remaining grass rats all exhibited robust swelling of the right pinna over the measurement period ($F_{5,75} = 19.58, p ≤ 0.05$; Fig. 2b). There was a main effect of lighting condition, such that grass rats exposed to dLAN had significantly elevated swelling of the right pinna as compared to conspecifics housed with dark nights ($F_{5,75} = 4.30, p ≤ 0.05$). dLAN grass rats had greater swelling on days 2 and 3 post challenge than grass rats exposed to dark nights ($F_{1,15} = 5.62$ and $5.19$, respectively, $p ≤ 0.05$). Furthermore, there was a positive association between plasma corticosterone concentrations and pinna swelling on days 2, 3, and 5 post challenge ($r^2 = 0.327, 0.219, 0.391, p ≤ 0.05$; day 2 shown; Fig. 2c).

**KLH antibody production**

Two grass rats, one per group, produced no antibody in response to the KLH injection and were excluded from the analyses. Grass rats significantly elevated antibody production following KLH injection ($F_{3,42} = 105.60, p ≤ 0.05$). There was an interaction between light condition and time ($F_{3,42} = 5.21, p ≤ 0.05$), such that grass rats exposed to dLAN increased anti-KLH IgG production 10 and 15 days following injection ($F_{1,14} = 13.11, 7.67, p ≤ 0.05$; Fig. 3a).

**Bacteria colony killing**
Grass rats housed with dLAN decreased the percentage of surviving CFUs compared to grass rats housed in standard LD conditions ($F_{1,16} = 8.155, p \leq 0.05$; Fig. 3b).

**Discussion**

Light at night influenced immune function in male Nile grass rats. Rats exposed to dLAN elevated bacteriacidal capacity, and humoral and cell-mediated immune responses. Increased immune activity occurred independently of overt changes in circadian locomotor activity. dLAN grass rats increased plasma corticosterone concentrations during the active phase after three weeks in lighting conditions which may have affected immunological measures. These results contrast with previously reported results in nocturnal rodents undergoing similar experimental nighttime light exposure. The results suggest that male and female, as well as diurnal and nocturnal, rodents may respond differently to the effects of nighttime light exposure.

Rats exposed to dLAN increased pinna swelling compared to LD rats in response to the antigen DNFB. DTH is a measure of cell-mediated immunity that demonstrates primary somatic immune response to an invading pathogen. Pinna swelling is caused by increased infiltration of macrophages and lymphocytes into the epidermis and dermis (Vadas, Miller, Gamble, & Whitelaw, 1975) and has previously been positively correlated to the intensity of the immune reaction (Phanuphak, Moorhead, & Claman, 1974). Functionally, elevated swelling in DTH testing is indicative of increased resistance to viruses, bacteria, and fungi (Bilbo et al., 2002).
Increased pinna swelling in dLAN grass rats contrasts with previously reported results in which swelling was suppressed in Siberian hamsters exposed to light at night (Bedrosian, Fonken, Walton, & Nelson, 2011a). DTH responses may vary over the course of the day. In the previous study, Siberian hamsters underwent DTH testing during the light phase when they are generally inactive. In the present study DTH testing also occurred during the light phase, however, grass rats are diurnal and active at this time. Other factors that vary in a circadian pattern such as immune cells and hormones may contribute to the equivocal t-cell mediated results (Bollinger, Bollinger, Naujoks, Lange, & Solbach, 2010). For example, exposure to dLAN elevates glucocorticoid concentrations in grass rats but not Siberian hamsters (Bedrosian, Fonken, Walton, Haim, & Nelson, 2011a). Furthermore, glucocorticoid concentration can alter diurnal rhythms in T-cell mediated inflammatory responses, an effect which may be partially mediated by melatonin. Adrenalectomy abolishes the diurnal rhythm in BCG inflammation; however, the rhythm can be recovered with exogenous administration of melatonin (Lopes, Mariano, & Markus, 2001). In the previous study in Siberian hamsters (6) DTH was also assessed in female Siberian hamsters as compared to male grass rats in the current experiments. Although female Siberian hamsters were ovariectomized, varying levels of sex steroids may have contributed to divergent results (Kanda & Watanabe, 2005). Overall, assessing DTH responses during the active phase in this study is more ecologically relevant because it is more likely that grass rats would encounter a pathogen while awake and interacting with the external environment.
Exposure to light at night suppresses melatonin production in both rodents and humans (Brainard et al., 1985; Brainard, Richardson, Petterborg, & Reiter, 1982; Dauchy et al., 2010). Even very low levels of light exposure can alter melatonin concentrations in rodents (Evans, Elliott, & Gorman, 2007). Although I did not measure melatonin concentrations in this study, it is likely that they were decreased with exposure to light at night. It is unlikely, however, that changes in DTH response reflect suppression of melatonin among dLAN rats. Melatonin is positively associated with DTH responses in diurnal and nocturnal rodent species (Drazen & Nelson, 2001; Haldar & Singh, 2001).

Glucocorticoids both increase and suppress DTH responses depending on the type and duration of the stressor (Dhabhar, 2002; Dhabhar & McEwen, 1999). Typically, acute stress increases DTH, whereas chronic stress suppresses DTH reactions, indicating that changes in DTH are in some cases related to altered glucocorticoid concentrations (Dhabhar & McEwen, 1999). During acute stress, blood leukocytes redistribute to the skin, mucosal linings, lung, liver, and lymph nodes, key areas in preventing breaching of immune defenses. In this study the positive association between corticosterone concentrations and pinna swelling suggests that the two may be related. It is possible that a long-term stressor such as light at night induces a state of functional glucocorticoid resistance. Previous work has demonstrated that psychosocial stressors can cause splenic macrophages to become resistant to the suppressive effects of glucocorticoid hormones (Avitsur, Stark, Dhabhar, Padgett, & Sheridan, 2002; Avitsur, Stark, & Sheridan, 2001; M. T. Bailey, Avitsur, Engler, Padgett, & Sheridan, 2004). Alternatively, the elevation in glucocorticoids among dLAN rats may be sufficiently low to exert an atypical effect on
DTH swelling. Glucocorticoids exert a U-shaped influence on multiple factors; for example, basal or low stress levels of corticosterone enhance glucose utilization, hippocampal synaptic excitability, hippocampal-dependent learning, and cerebral perfusion rate whereas higher physiological levels of corticosterone exert opposite effects (Sapolsky, 2004). Moreover, melatonin and glucocorticoids interact in modulating immunological processes. Acute and chronic stress increase plasma melatonin concentrations in rodents (Couto-Moraes, Palermo-Neto, & Markus, 2009; Dagnino-Subiabre et al., 2006). This may be a compensatory mechanism as melatonin protects against some effects of chronic stress (Brotto, Gorzalka, & LaMarre, 2001). This interaction has important implications for this study because it suggests that glucocorticoids may increase melatonin concentrations partially compensating for the light induced suppression in melatonin.

Rats exposed to dLAN enhanced antibody production following injection with KLH. Anti-KLH production is a general indicator of B cell activity. Previous studies have reported no differences in anti-KLH production or enhanced production in melatonin treated rodents (Demas, Chefer, Talan, & Nelson, 1997; Drazen & Nelson, 2001). Furthermore, primary and secondary antibody production is decreased in mice treated with propranolol (Maestroni, Conti, & Pierpaoli, 1986). Thus, differences in antibody production between dLAN and LD rats may be independent of putative changes in melatonin. Elevated concentrations of glucocorticoids following social defeat are associated with enhanced lymphocyte release of IFNγ and IL6. However, changes in anti-KLH production are not apparent (Merlot, Moze, Dantzer, & Neveu, 2004). Furthermore,
in another model of social defeat elevated glucocorticoid concentrations were associated with impairment in antiviral immunological memory (de Groot, Boersma, Scholten, & Koolhaas, 2002). Again this indicates that changes in anti-KLH production may occur independently of changes in corticosteroids. Alternatively, light at night may be an atypical stressor affecting the glucocorticoid system in a different manner than other chronic stressors.

Bactericidal capacity was enhanced in dLAN rats as compared to those housed in standard lighting conditions. Bacteria killing is a low-cost nonspecific immune response predominately mediated by plasma proteins (L. B. Martin, 2nd, Weil, & Nelson, 2007). Plasma bactericidal capacity increases with immune challenge and represents an enhanced ability to clear a bacterial infection (Weinrauch, Abad, Liang, Lowry, & Weiss, 1998). Elevated glucocorticoids concentrations have been associated with enhanced bactericidal capacity in spleen cells from mice that underwent social disruption stress (M. T. Bailey, Engler, Powell, Padgett, & Sheridan, 2007). Melatonin, however, generally enhances bactericidal capacity (Terron et al., 2009).

Overall, nighttime light exposure increased immunocompetence in grass rats exposed to light at night. Light at night may exert its effects through changes in the hypothalamic-pituitary adrenal axis as corticosteroid concentrations were elevated among dLAN rats. It is widely accepted that stress affects immune responses, with chronic stress generally exerting an immunosuppressive effect. The role of glucocorticoids in immunological processes are complex however (Sorrells, Caso, Munhoz, & Sapolsky, 2009); depending on the type of stress, glucocorticoids can have opposite effects. Light at
night may also disrupt circadian processes leading to changes in immune function. Although gross changes in locomotor activity were not apparent, disruption of the circadian system at the molecular level may have occurred. Previous work has indicated disruption of clock function can lead to exacerbated immune activity (Arjona & Sarkar, 2006; Castanon-Cervantes et al., 2010; Hashiramoto et al., 2010). Melatonin can also be directly produced by immune cells (Pontes, Cardoso, Carneiro-Sampaio, & Markus, 2006). Importantly, this study did not evaluate all aspects of immune function; it is possible that other arms of the immune system could be differentially affected by nighttime light exposure (L. B. Martin, 2nd, Weil, & Nelson, 2007).

Enhanced immune responses are not always favorable. Immunological processes are energetically costly and a delegation of energy to immune responses in unnecessary situations can reduce fitness (L. B. Martin, Weil, & Nelson, 2008). Moreover, enhancement of the immune response in cases such as allergic asthma is detrimental (Wills-Karp, 1999). Because of the contrasting results obtained in this study and another study conducted in a nocturnal rodent species, further research on the effects of light at night on immune function are warranted. Nighttime light exposure is currently experienced by over 99% of the population of the US and Europe. Light pollution has significant ecological consequences for animals living in urban and suburban areas and may contribute to species loss (Navara & Nelson, 2007). It is important to understand the physiological implications of this exposure in order to work toward preventing ecologically related complications.
### Tables

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<td>93.55 +/- 6.66</td>
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<tr>
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<td>Testes (mg)</td>
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<td>1713 +/- 48</td>
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<td>Adrenal (mg)</td>
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<td>Thymus (mg)</td>
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<tr>
<td>Spleen (mg)</td>
<td>142 +/- 9</td>
<td>148 +/- 12</td>
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**Table 8.1.** Tissue masses in Nile grass rats exposed to dimly lit or dark nights
Figure 8.1. Nile grass rats exposed to dimly nights elevate corticosterone concentrations and delayed-type hypersensitivity swelling responses.

Rats exposed to dim light at night (A) elevated plasma corticosterone concentrations compared to rats with dark night. (B) Dim light rats exhibited increased pinna swelling 2 and 3 days following challenge with the antigen 2,4-dinitro-1-fluorobenzene. (C) There was an association between peak pinna swelling and corticosterone concentrations 2 days after challenge. Data are presented as mean ± SEM. *p ≤ 0.05.
Figure 8.2. Rats exposed to light at night enhance humoral immune function and plasma bactericidal capacity.

(A) Anti-KLH IgG responses were elevated in dLAN rats 10 and 15 days following inoculation. (B) Plasma obtained from dLAN grass rats caused fewer surviving bacterial colonies. Data are presented as mean ± SEM. *p ≤ 0.05.
Figure 8.3. Grass rats exposed to dark and dimly lit nights have comparable activity.

Representative actograph of a grass rat housed in (A) dark or (B) dimly lit nights.
CHAPTER 9

DIM NIGHTTIME LIGHT IMPAIRS COGNITION AND PROVOKES
DEPRESSIVE-LIKE RESPONSES IN A DIURNAL RODENT

Biological rhythms are highly adaptive, aligning individuals to daily fluctuations in the external environment, as well as synchronizing internal homeostatic processes. The master mammalian circadian clock is located in the suprachiasmatic nuclei (SCN) and regulates timing of subordinate oscillators throughout the central nervous system and periphery. External lighting is important in synchronizing the circadian system and maintaining daily temporal organization. Prior to the widespread adoption of electric lighting, individuals’ biological clocks were entrained to a consistent pattern of light and dark; in contrast, modern light exists in several temporal patterns. Moreover, shift-work, trans-meridian travel, and inconsistent sleep schedules have rapidly increased during the past century. Because the change in nighttime lighting has occurred so rapidly in terms of evolutionary history, it is likely that significant physiological and ecological perturbations have resulted. For example, disruption of the circadian system results in adverse health conditions such as heart disease (Ha & Park, 2005), cancer (Davis &
Mirick, 2006; Schernhammer et al., 2001), and metabolic dysfunction (Reiter, Tan, Korkmaz, & Ma, 2011).

Circadian disruption and light at night are implicated in impaired cognition. Rats housed in constant illumination perform poorly in the Morris water maze (A. Fujioka et al., 2011; Ling et al., 2009; Ma et al., 2007). Constant light causes tau hyperphosphorylation, increased expression of endoplasmic reticulum (ER) stress-related proteins, thinner synapses, and increased superoxide dismutase and monoamine oxidase (Ling et al., 2009). Similarly, rats housed in constant light display impaired spatial learning in the Morris water maze with accompanying changes in long-term depression in the CA1 area of the hippocampus (Ma et al., 2007). Constant light also impairs learning and memory in mice, which may be related to decreased neurogenesis (A. Fujioka et al., 2011). Furthermore, mice undergoing experimental jet lag decrease neurogenesis and have prolonged deficits in learning and memory as evaluated in a conditioned place preference task (Gibson, Wang, Tjho, Khattar, & Kriegsfeld, 2010).

In addition to influencing learning and memory, circadian disruption changes mood (Monteleone, Martiadis, & Maj, 2010). Seasonal lighting, abnormalities in the circadian clock (Benedetti et al., 2008), and sleep disorders are associated with depression (Bunney & Bunney, 2000). Constant light alters anxiety and depressive-like behaviors in mice (Fonken et al., 2009; Martynhak et al., 2011). Furthermore, Siberian hamsters exposed to dim light during the dark phase increase depressive-like responses and have reduced spine density in the CA1 area of the hippocampus (Bedrosian, Fonken, Walton, Haim, & Nelson, 2011a).
In all of these studies, only nocturnal rodents were used. Both circadian influences on behavior and masking effects of light are very different in nocturnal and diurnal species. Thus, in the present experiment, I examined behavioral and brain responses of diurnal male Nile grass rats to dim light at night (dLAN).

**Methods**

**Animals**

Male grass rats (*Arvicanthis niloticus*) used in this study were bred at The Ohio State University from a wild stock obtained from LS. Grass rats were bred under a standard light-dark (LD) cycle (14:10 light (~150 lux) / dark (0 lux)). All animals were provided food (ProLab RMH 2000, LabDiet) and filtered tap water *ad libitum*. Experimental grass rats were weaned between 21 and 24 days of age and housed with same sex siblings in polypropylene cages (40 cm x 20 cm x 20 cm) with straw bedding. Colony rooms were maintained at a temperature of 20 ± 4º C and a relative humidity of 50% ± 10%.

At 10 weeks of age grass rats were singly housed, randomly assigned a number, and either maintained in LD or placed in dLAN (14:10 light (~150 lux)/ dim (~5 lux)). Blood samples were collected at Zeitgeber Time (ZT) 6 via retro-orbital bleed after two weeks for corticosterone analysis and one week later grass rats underwent behavioral testing to assess cognitive and affective behaviors. Testing occurred in the following order: Barnes maze, sucrose anhedonia, and forced swim test. The sucrose anhedonia test occurred between ZT 8-13; all other tests were conducted between ZT 1-ZT 6.
Retro orbital blood samples (~0.20 ml) were collected from grass rats anesthetized with isoflurane vapors for RIA of corticosterone concentrations, prior to the onset of behavioral testing. Blood samples were centrifuged at 4°C for 30 min at 3.3 g and plasma aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at -80°C until assayed for corticosterone concentrations using a radioimmunoassay (RIA). Total plasma corticosterone concentrations for grass rats were determined in duplicate in an assay using an ICN Diagnostics 125I double antibody kit (Costa Mesa, CA, USA). The high and low limits of detectability of the assay were ~1000 and 5 ng/ml, respectively. All procedures followed those described by the manufacturer guidelines.

**Behavioral testing**

*Barnes maze.* The Barnes maze is a brightly lit arena with 18 evenly spaced holes, one leading to dark box and the others blocked off with black inserts (Sunyer, 2007). On the first day animals were acclimated to the maze; a bright light and loud fan were turned on as they were guided from the center of the maze to the target hole. After entering, the bright light and fan were turned off and the grass rat were left undisturbed for 30 sec. Animals then underwent four days of training consisting of 3, 90 sec trials separated by 10 min intervals in the home cage. One day after the last training trial animals were given a 60 sec probe trial in which the escape box was blocked off. Latency to find the target hole and number of errors were scored during all trials. *Sucrose Anhedonia.* Consumption of a 2% sucrose solution between ZT 8 and ZT 13, was recorded in all grass rats to measure sucrose anhedonia (Willner, Muscat, & Papp, 1992). Before presentation of the sucrose solution, grass rats were administered water in
modified water bottles for three consecutive days, to control for novelty of the bottles. The bottles were weighed before and after the 5 h sample time; the next day animals were provided a choice between a 2% sucrose solution and water. Sucrose consumption was normalized to water consumption. *Forced Swim Test.* To assess depressive-like responses, grass rats were placed in ~17 cm water (22 ±1°C), within an opaque, cylindrical tank (diameter = 24 cm, height = 53 cm). Swimming behavior was videotaped for 5 min and scored by a condition-blind observer with the Observer software (Noldus Corp, Leesburg, VA, USA). Latency to float and time spent floating served as dependent measures; both are used in rodents, including grass rats, to assess depressive-like response (Ashkenazy-Frolinger, Kronfeld-Schor, Juetten, & Einat, 2010; Porsolt, Bertin, & Jalfre, 1977).

*Hippocampal Morphology*

Grass rats were killed between ZT 3 and ZT 5, and brains were removed, and processed for Golgi impregnation using the FD Rapid GolgiStain™ Kit (FD NeuroTechnologies Inc., Ellicott City, MD) according to the manufacturer’s instructions. Brains were sliced at 100μm, thaw mounted onto gelatin coated slides counterstained with cresyl violet (Sigma), dehydrated, and coverslipped. Brains were assessed for hippocampal cell morphology and spine density in the dentate gyrus (DG), CA1, and CA3 using a Nikon E800 brightfield microscope. Tracings were done with Neurolucida software (MicroBrightField, Burlington, VT, USA) at a magnification of 200× for neuronal morphology and 1000× for spine density. Six representative neurons were selected per area, per animal. Whole cell traces were analyzed using NeuroExplorer.
software, for cell body size and perimeter, and dendritic length (MicroBrightField, Burlington, VT). Sholl analysis defines dendritic complexity by the number of dendritic branch points at fixed intervals from the cell bodies (Sholl, 1956) and was conducted on apical and basilar dendrites. From each neuron >20 µm were selected in the apical and basilar areas respectively (except in the DG where granule cells lack bidirectional projections). All spine segments selected were at least 50 µm distal to the cell body. Spine density (spines per 1 µm) was calculated for each trace and averaged per cell, per area, and per animal.

Statistical Analyses

Comparisons for behavior analyses and hormone concentrations were conducted using a one-way ANOVA. Neuronal characteristics and spine densities were averaged per animal and then analyzed using a one-way ANOVA. Sholl analyses were also averaged per animal and then analyzed using a repeated measures ANOVA with lighting condition as the between subject factor and distance from the cell body as the with-in subject factor. For each Barnes maze session (1–4) latencies and error rates, respectively, were averaged per session for each grass rat; data were subject to repeated-measures ANOVA (lighting condition as the between subject factor and session as the with-in subject factor). Fourier analysis was used to determine whether locomotor activity was rhythmic and followed 24 h periodicity using Clocklab software from Actimetrics (Wilmette, IL). Grass rats were considered rhythmic when the highest peak occurred at ~1 cycle per day with an absolute power of at least 0.005 mV/Hz as previously described (Kriegsfeld et al., 2008). FFT power values for 0.083 cycles per day were compared
between lighting conditions by one way ANOVA. Percentage of daytime activity and total daily activity were also analyzed by one way ANOVA. The above statistical analyses were conducted with StatView software (v. 5.0.1, Cary, NC). Nonlinear regression analysis was used in GraphPad Prism software (v. 4 La Jolla, CA). In all cases, differences between group means were considered statistically significant if $p \leq 0.05$.

**Results**

**Somatic Measures**

There were no differences in body or reproductive tissue mass ($p > 0.05$; data not shown).

**Learning and Memory**

Grass rats exposed to dLAN decreased learning abilities in the Barnes maze. dLAN grass rats had an increased latency to reach the target hole and a higher error rate as compared to grass rats housed under dark nights. There was a main effect of lighting condition, such that dLAN increased latency to reach the target hole over the course of training trials ($F_{1,42} = 6.064$, $p < 0.05$; Fig. 1A). Post-hoc analysis revealed dLAN increased latency to reach the target hole on days 2 and 4 of the training trials ($p < 0.05$). Furthermore, there was a main effect of lighting condition with respect to error rate; grass rats housed under dLAN increased errors over the course of training trials ($F_{1,42} = 8.184$, $p < 0.05$; Fig. 1B). On day two of the training trials dLAN grass rats showed increased error rate compared to grass rats housed under standard LD conditions ($p < 0.05$).
Twenty-four hours after the final training session, memory retention was assessed with a probe trial. During the probe trial the target box was removed and behavior on the maze was recorded for 60 sec. dLAN grass rats spent a lower percentage of time investigating the target hole compared to LD grass rats; these data indicate decreased memory retention (F$_{1,14}$ = 7.084, p < 0.05; Fig. 1C). Our lab has previously found no differences in spontaneous locomotor activity between grass rats housed under dLAN and LD in a 5 min brightly lit open field task (unpublished observations). This indicates Barnes maze differences are due to changes in learning and memory and not differences in motivation when exposed to a brightly lit open space.

**Affective Responses**

Depressive-like responses were evaluated using a sucrose anhedonia and forced swim task. Grass rats exposed to dLAN increased depressive-like responses in the sucrose anhedonia test. One grass rat was excluded from analyses because of a leaky water bottle. dLAN grass rats reduced consumption of a sucrose solution demonstrating an anhedonic-like response (F$_{1,15}$ = 4.711, p < 0.05; Fig. 2A).

Grass rats exposed to dLAN also increased behavioral despair in the forced swim task. dLAN grass rats reduced latency to first float indicating they more rapidly reach a state of behavioral despair (F$_{1,16}$ = 4.774; p<0.05; Fig. 2B). No differences, however, were observed between groups with respect to float duration which is the primary depressive-like response evaluated in the forced swim test (p > 0.10).

**Corticosterone Concentrations**
Grass rats displayed elevated corticosterone concentrations 2 weeks after placement in dLAN compared to LD (F_{1,16} = 4.521, p < 0.05; Fig. 2D).

**Hippocampal Neuronal Morphology**

Exposure to dLAN is associated with changes in neuronal morphology in the CA1 and DG regions of the hippocampus. Rats housed under dLAN reduced dendritic length in the DG and CA1 basilar dendrites (F_{1,11} = 4.875, 7.357 respectively, p < 0.05; Fig 3). Furthermore, there was a positive association between dendritic length in the dentate gyrus and sucrose consumption in the sucrose anhedonia test (r = 0.455, p = .016; Fig. 3C). Groups did not differ with respect to spine density, cell body area, or cell body perimeter in any area (Table 1).

**Discussion**

This study investigated the effect of dim nighttime light exposure on depressive-like responses and learning and memory in Nile grass rats, a diurnal rodent. Here I show that exposing grass rats to light at night impaired their spatial learning and memory as evaluated by the Barnes maze (Sunyer, 2007). Animals were trained to find a target hole on the maze over the course of 4 days and then evaluated in a probe trial. During the training trials, dLAN impaired performance as compared to LD. dLAN increased the latency for grass rats to reach the target hole and increased the number of visits to incorrect holes. Spatial memory was similarly impaired by dLAN. Twenty-four hours after the final training session, memory retention was assessed with a probe trial. dLAN decreased the percentage of time spent investigating the target hole, indicating decreased memory retention.
These results confirm and extend previous findings (A. Fujioka et al., 2011; Ling et al., 2009; Ma et al., 2007) indicating that housing nocturnal rodents under light at night impairs spatial learning and memory. The results demonstrate that nighttime light exposure also impairs spatial learning and memory in diurnal rodents. Furthermore, previous studies have used 24 h lighting of the same intensity while in this study grass rats were exposed to a distinctly darker phase. Grass rats exposed to dark or dimly lit nights display maintain a diurnal activity pattern and have equivalent levels of total daily locomotor activity and similar locomotor activity rhythms (Fonken, Haim, & Nelson, 2011; McElhinny, Smale, & Holekamp, 1997). Constant lighting conditions used in previous studies can result in an arrhythmic activity rhythm (Cambras, Castejon, & Diez-Noguera, 2011). These results demonstrate that cognitive impairments occur in animals with light at night in the absence of disruption in locomotor activity rhythm.

The effects of light at night on other cognitive functions remain unspecified (Castro et al., 2005). It is possible that nighttime light exposure specifically targets hippocampal dependent learning and memory through disruption of circadian processes (Ruby et al., 2008). Alternatively, dLAN may represent a mild chronic stressor producing deficits in learning and memory via reduced neurogenesis, changes in hippocampal architecture, or both processes (A. Fujioka et al., 2011; Gould & Gross, 2002; McEwen & Sapolsky, 1995). Studies in nocturnal rodents have reported glucocorticoid concentrations to be both elevated or unaffected by nighttime light exposure (Abilio, Freitas, Dolnikoff, Castrucci, & Frussa-Filho, 1999; Fonken et al., 2009; Fonken et al., 2010; Van der Meer, Van Loo, & Baumans, 2004). Grass rats housed under dLAN
elevated plasma corticosterone concentrations. dLAN may be a stronger stressor to diurnal as compared to nocturnal rodents. Circulating glucocorticoid concentrations are predictive of hippocampal atrophy and memory deficits in both humans and rodents (Bodnoff et al., 1995; Lupien et al., 1998). Because corticosterone concentrations were only measured at a single time point however, conclusions drawn from the results must be constrained.

Grass rats exposed to dLAN increased depressive-like responses in a sucrose preference test. dLAN reduced consumption of a sucrose solution in the sucrose anhedonia test. LD rats consumed a higher percentage of sucrose than water, whereas dLAN grass rats showed no preference for the sucrose solution. This implies that the sucrose solution had diminished hedonic valence for grass rats exposed to dLAN which models a key feature of human depression (Willner, Muscat, & Papp, 1992). Results in the forced swim test were equivocal. Increased floating time in the forced swim test is considered “behavioral despair” because rodents putatively stop searching for an escape mechanism (Porsolt, Bertin, & Jalfre, 1977). There were no differences in total floating time in the forced swim test between LD and dLAN grass rats, but grass rat housed under dLAN reduced latency to first float. The forced swim test has not been extensively used in grass rats, although one study reported increases in floating duration in grass rats housed in short photoperiods (Ashkenazy-Frolinger, Kronfeld-Schor, Juetten, & Einat, 2010). Grass rats are poor swimmers, which suggests the forced swim test may not be a reliable behavioral measure (Duplantier & Ba, 2001).
The depressive-like phenotype of the dLAN grass rats is consistent with my predictions based on depressive disorders related to both stress (Willner, 1997) and circadian dysfunction (Turek, 2007). Furthermore, nighttime light exposure increases depressive-like responses in nocturnal rodents (Bedrosian, Fonken, Walton, Haim, & Nelson, 2011b). Light at night may increase depressive-like responses through changes in hippocampal circuitry. dLAN decreased dendritic length in DG and CA1 basilar dendrites. Furthermore, there was a positive association between dendritic length in the dentate gyrus and sucrose consumption in the sucrose anhedonia test. The hippocampus is a critical structure in the pathophysiology of depressive disorders. Depression is associated with changes in glucocorticoids and hippocampal atrophy (Sapolsky, 2000; Sheline, Wang, Gado, Csernansky, & Vannier, 1996). Moreover, changes in hippocampal morphology are associated with chronic stress and depressive-like responses in rodents (Hajszan et al., 2009; Magarinos et al., 2011; Magarinos, McEwen, Flugge, & Fuchs, 1996).

In summary, these results suggest that exposure to dim nighttime lighting can alter hippocampal neuronal morphology, impair learning and memory, and increase depressive-like responses in a diurnal rodent. Opportunities for exposure to light at night have rapidly increased during the past century. The present results suggest that this exposure may have accompanying maladaptive effects. Finding an appropriate model to test whether changes in environmental lighting are related to mood disorders is critical. Many animal models are potentially confounded by the use of nocturnal rodents that may not experience the same form of disruption as diurnal animals when exposed to light at
night. The responses to light at night seen in the present study are comparable to those
described in previous reports using nocturnal rodents (e.g., Bedrosian et al., 2011a),
which increases confidence that nocturnal rodents are appropriate subjects of study of this
issue. In addition, these results raise questions about the use of rodent vivaria that have
windows in the doors of animal rooms and continuous lighting in the halls.
Tables

Table 1. Morphological characteristics of neurons in the CA1, CA3, and DG of the hippocampus. Represented as mean ± SEM micrometers; AD = apical dendrite; BD = basilar dendrite.

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<tr>
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Table 9.1. Characteristics of hippocampal neurons in grass rats exposed to dark or dimly lit nights.

Characteristics of neurons in the CA1, CA3, and DG of the hippocampus of grass rats exposed to dark or dimly lit nights. Represented as mean ± SEM micrometers; AD = apical dendrite; BD = basilar dendrite.
Grass rats exposed to dim light at night show impairments in learning and memory. Dim light at night impaired spatial learning and memory in the Barnes maze. (A) Latency to reach the target hole in the Barnes maze by day (average of 3 trials). (B) Number of visits to false holes by session. (C) Relative visits to the target hole versus false holes during the probe trial.
Figure 9.2. Exposure to dim light at night increases depressive-like responses in grass rats.

(A) Amount of sucrose solution versus total liquid consumed in a sucrose anhedonia task. (B) Latency to first float the forced swim test. (C) Correlation between percentage of sucrose consumed and dendritic length in the dentate gyrus of the hippocampus. (D) Corticosterone concentrations after two weeks in lighting conditions. Data are expressed as mean ± standard error of the mean (SEM). *p<0.05 between groups.
Figure 9.3. Exposure to dim light at night alters hippocampal morphology in grass rats.

(A) Total dendritic length was reduced in CA1 basilar dendrites and (B) in the dentate gyrus (DG). (C) Representative tracing from the CA1 of an LD grass rat (black) compared to a dLAN grass rat (red). (D) Representative tracing from the DG of an LD grass rat (black) and dLAN grass rat (red). Data are expressed as mean ± SEM. *p<0.05 between groups.
CONCLUSIONS

Exposure to light at night is generally considered an innocuous environmental manipulation. The perceived harmlessness of nocturnal illumination likely persists because the invention and widespread adoption of the electric lighting occurred prior to an understanding of circadian biology. The goal of this dissertation was to determine the physiological consequence of exposing nocturnal (Swiss Webster mice) and diurnal (Nile grass rats) rodents to ecologically relevant levels of dim (~5 lux) light at night. In this dissertation, I demonstrate that exposure to light at night has significant metabolic, immunological, and behavioral repercussions.

Summary

The global increase in the prevalence of obesity and metabolic disorders coincides with the increase of exposure to light at night and shift work. Circadian regulation of energy homeostasis is controlled by an endogenous biological clock that is synchronized by light information (Reppert & Weaver, 2002). To promote optimal adaptive functioning, the circadian clock prepares individuals for predictable events such as food availability and sleep, and disruption of clock function causes circadian and metabolic disturbances (Green, Takahashi, & Bass, 2008). To determine whether a causal relationship exists between nighttime light exposure and obesity I examined the effects of exposure to light at night on body mass in male mice (Chapter 2). Mice exposed to dim
light at night or continuous lighting increase body mass and impair glucose processing as compared to mice exposed to dark nights. Changes in body mass occur independently of changes in total daily food intake or activity. However, mice attenuate the daily pattern in food intake, eating more during the rest phase than mice exposed to dark nights. Blocking daytime food intake in mice with light at night prevents weight gain. Furthermore, placing mice back in dark nights following exposure to 4 weeks of dim light at night prevents changes in glucose tolerance and partially restores body mass (Chapter 3).

In industrialized societies, exposure to light at night and more typical obesogenic factors such as a high fat diet and sedentary lifestyle, often occur in tandem and may contribute to the increasing obesity epidemic. Thus, I examined the effects of a high fat diet and exercise availability on dim light at night associated weight gain in Chapters 4 and 5, respectively. Dim light at night exaggerates weight gain in mice fed a high fat diet (Chapter 4). Moreover, both high fat feeding and dim light at night increase daytime food intake and elevate peripheral inflammation. Exposure to a high fat diet but not dim light at night elevates hypothalamic inflammation suggesting that these two factors may work through different physiological mechanisms to affect weight regulation. As anticipated, access to a functional running wheel prevents body mass gain in mice exposed to dim light at night (Chapter 5). Voluntary exercise suppresses weight gain in mice exposed to dimly lit nights without rescuing changes to the circadian system; increases in daytime food intake induced by exposure to dim light at night are not diminished by exercise availability. Furthermore, exposure to light at night disrupts wheel running behavior in a subset of mice exposed to dim light at night.
Because changes in circadian clock mechanisms affect metabolism (Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007; Lamia, Storch, & Weitz, 2008; Marcheva et al., 2010; Oishi et al., 2006; Paschos et al., 2012; Turek et al., 2005) and exposure to light at night affects circadian rhythms (Albrecht, Sun, Eichele, & Lee, 1997; Schwartz, Tavakoli-Nezhad, Lambert, Weaver, & de la Iglesia, 2012; Shigeyoshi et al., 1997), in Chapter 6, I investigated the effects of exposure to light at night on both central and peripheral core circadian clock mechanisms. Mice exposed to dim light at night attenuate core circadian clock rhythms in the SCN at both the gene and protein levels. Circadian clock rhythms are also perturbed in the liver of mice exposed to dimly lit as compared to dark nights.

In addition to affecting metabolism, circadian system disruptions are linked to alterations in immune function. For example, many of the pathologies associated with exposure to light at night such as cancer (Stevens, 2009b), obesity (X. S. Wang, Armstrong, Cairns, Key, & Travis, 2011), and mood disorders (Driesen, Jansen, Kant, Mohren, & van Amelsvoort, 2010) involve changes in inflammation. Moreover, multiple immune related parameters show circadian oscillations (Lange, Dimitrov, & Born, 2010) (Arjona & Sarkar, 2006; Narasimamurthy et al., 2012; A. C. Silver, Arjona, Walker, & Fikrig, 2012) (Arjona & Sarkar, 2005; Keller et al., 2009; A. C. Silver, Arjona, Hughes, Nitabach, & Fikrig, 2012). Therefore, Chapter 7 of this dissertation addressed the effects of exposure to light at night on recovery from cardiac arrest in mice. Following either a cardiac arrest or a sham procedure, mice were exposed to dark or dimly lit nights. Mice exposed to dimly illuminated as compared to dark nights elevate mortality in the week
following cardiac arrest. Furthermore, mice exposed to dim light at night post-cardiac arrest increase neuroinflammation and hippocampal cell death. Dim light at night likely affects cardiac arrest recovery by elevating inflammation; selective inhibition of IL-1β or TNFα ameliorates the effects of dim light at night on cardiac arrest recovery. Additionally, restricting the wavelength of the nighttime light exposure to ~640 nm eliminates the detrimental effects of nighttime light exposure on cardiac arrest outcome.

The experiments described in the first 7 chapters of this dissertation were conducted using nocturnal rodents in order to examine the effects of nighttime light exposure without disrupting sleep. However, in diurnal species many hormones and immune parameters vary with secretion patterns 180° out of phase to those of nocturnal rodents. Furthermore, light can have very different effects on activity and masking in diurnal versus nocturnal rodents. Thus, in the final two chapters of this dissertation (Chapters 8 and 9) I investigated the effects of nighttime light on behavior, hippocampal connectivity, and immune related parameters, in grass rats. Rats exposed to dim light at night increase delayed-type hypersensitivity pinna swelling which is consistent with enhanced cell-mediated immune function. Similarly, rats exposed to dimly lit as compared to dark nights increase antibody production following inoculation with keyhole lymphocyte hemocyanin (KLH) and increase bactericidal capacity. In contrast to nocturnal rodents, daytime corticosterone concentrations are elevated in grass rats exposed to nighttime lighting.

In addition to influencing immune function, three behavioral effects are apparent in grass rats exposed to dim light at night: (1) decreased preference for a sucrose solution,
(2) increased latency to float in a forced swim test, and (3) impaired learning and memory in the Barnes maze. Light at night also reduces dendritic length in dentate gyrus and basilar CA1 dendrites. In agreement with findings in nocturnal rodents (Fonken et al., 2010), nighttime light exposure does not disrupt the pattern of circadian locomotor activity in grass rats.

**Mechanisms**

There are several mechanisms by which nighttime light exposure can affect physiology. In this dissertation, I specifically focused on how light at night may influence metabolism through disruption of the circadian system. Exposure to unnatural light at night can also affect physiological processes through melatonin suppression, alterations in glucocorticoids, and changes in sleep architecture.

Melatonin is an endogenously synthesized molecule that is secreted by the pineal gland during the night in both nocturnal and diurnal mammals (Reiter, 1991). Melatonin secretion is potently inhibited by exposure to sufficient levels and durations of nighttime lighting in both rodents and humans (Brainard, Rollag, & Hanifin, 1997). For example, exposing humans to one hour of 45 lux of nighttime light exposure decreases plasma melatonin by ~60% (Brainard, Richardson, Petterborg, & Reiter, 1982). Similar to circadian clock gene disruption, suppressing melatonin secretion is associated with increased risk for developing cancer (Blask, 2009; Blask et al., 2005), obesity (Mantele et al., 2012; Tan, Manchester, Fuentes-Broto, Paredes, & Reiter, 2011), and mood disorders (Srinivasan, De Berardis, Shillcutt, & Brzezinski, 2012). Although there is compelling evidence that suppression of melatonin secretion can contribute to weight gain, I
specifically did not focus on melatonin for several reasons: (1) pineal melatonin suppression requires high and sustained levels of nighttime light exposure (Brainard, Rollag, & Hanifin, 1997), (2) nighttime light exposure below the threshold for melatonin suppression is associated with changes in metabolism (Obayashi et al., 2013), and (3) multiple strains of laboratory mice that lack pineal melatonin demonstrate changes in metabolism with nighttime light exposure (Coomans et al., 2013; Fonken et al., 2010).

Diurnal variations in other hormones may be disrupted by exposure to light at night. Glucocorticoids are of particular interest in the context of nighttime light exposure because (1) light at night may be interpreted as a stressor (Ma et al., 2007) and (2) glucocorticoids are a primary output of, and feedback signal for the circadian system (Kiessling, Eichele, & Oster, 2010; Sage et al., 2004). Importantly, I did not focus on glucocorticoids as a primary mechanism for physiological changes associated with nighttime light exposure because exposure to light at night appears to affect glucocorticoid secretion in only a subset of mammals. For example, Nile grass rats show elevations in serum corticosterone concentrations after chronic exposure to dim light at night, but mice do not (Fonken, Haim, & Nelson, 2011; Fonken et al., 2010). Despite disparate effects of light at night on glucocorticoids, Swiss Webster mice and Nile grass rats show similar changes in physiology and behavior following exposure to light at night (Fonken et al., 2009; Fonken, Kimsmill, Smale, & Nelson, 2012; Fonken & Nelson, 2013). This suggests that alterations in glucocorticoids are not critical for dim light at night-associated changes.
Finally, exposure to light at night may affect metabolism through disturbing sleep architecture. Sleep disruptions can profoundly affect physiology and contribute to multiple pathological conditions including metabolic syndrome (Mullington, Haack, Toth, Serrador, & Meier-Ewert, 2009; Spiegel, Tasali, Leproult, & Van Cauter, 2009). In order to dissociated the effects of nighttime light exposure from sleep disruptions I specifically worked with nocturnal Swiss Webster mice in the majority of the studies in this dissertation. These studies indicate that light at night causes changes in metabolism independently of sleep disruptions. However, due to the synergistic effects of light at night and disrupted sleep on metabolism, future research should address whether these variables act through similar mechanisms to affect metabolism.

Overall, it is difficult to tease apart which mechanism is the greatest contributing force to the negative effects of nighttime light exposure. Melatonin suppression, circadian disruption, changes in the HPA axis, and sleep disturbances likely all contribute to the deleterious outcomes associated with exposure to light at night.

**Implications**

Over 99% of the population in the US and Europe is exposed to light at night (Cinzano, Falchi, & Elvidge, 2001). In addition to experiencing urban light pollution, many people bring light into their homes by turning on electric lights after sunset, watching TV late into the night, or using computers directly prior to bed. It is estimated that two-thirds of the population experience this form of “social jet lag” (Roenneberg, Allebrandt, Merrow, & Vetter, 2012). Moreover, shift workers make up approximately 20% of the populations and are exposed to high and prolonged levels of light at night.
It is also important to note that exposure to light at night is not just a human issue. Many plant and animal species are affected by nighttime light exposure, as lighting from infrastructure strays into the atmosphere creating a general nighttime glow termed “light pollution” (Navara & Nelson, 2007). The results presented in this dissertation suggest that unnatural exposure to light at night may have significant ecological implications. Indeed, exposure to light at night is known to affect mating (Dominoni, Quetting, & Partecke, 2013; Kempenaers, Borgstrom, Loes, Schlicht, & Valcu, 2010), foraging and predation (Davies, Bennie, & Gaston, 2012; Dwyer, Bearhop, Campbell, & Bryant, 2012; Rydell, 1992; Stone, Jones, & Harris, 2009), and migration in multiple species (Z. Wang et al., 2011).

**Prevention and Interventions**

Preventing the general population from excessive exposure to light at night can be achieved with relatively low-cost manipulations, such as using curtains to block out street lights, turning off hallway lights, and removing all light sources, including televisions and computers, from bedrooms. Furthermore, adhering to a consistent schedule and avoiding rapid phase shifts can minimizing “social jet lag” (Roenneberg, Allebrandt, Merrow, & Vetter, 2012).

In shift-working populations, avoiding phase shifts and nighttime light exposure is often unavoidable. However, not all nocturnal illumination equally affects the circadian system. The intrinsically photosensitive retinal ganglion cells that project to the SCN are most responsive to the blue region of the visible spectrum (ranging from 450 to 485 nm) with longer wavelengths of lighting minimally influencing the circadian system (Brainard
et al., 1985; Brainard, Richardson, Petterborg, & Reiter, 1982). Manipulation of lighting wavelength may prove effective in blocking out light-induced physiological changes. To that end, ongoing research is investigating the effectiveness of preventing exposure to blue wavelength light with specially designed goggles and light fixtures.

**Future Directions**

Future studies should confirm the importance of the circadian system in mediating light at night-associated physiological changes. This could be accomplished by several means including (1) examining the effects of exposure to red light at night, (2) using a mouse strain lacking ipRGCs such as *Opn4<sub>aDTA/aDTA</sub>* mice (LeGates et al., 2012), or (3) by ablating ipRGCs with saporin conjugated to a melanopsin polyclonal antibody (Ingham, Gunhan, Fuller, & Fuller, 2009). Additionally, these studies should particularly focus on potential interventions to limit deleterious changes associated with exposure to light at night.

Because the research presented in this dissertation may have important implications for human health, future studies should directly investigate the effects of exposure to light at night on humans. First, conducting comparative work in the Amish, who are minimally exposed to light at night, may provide insight into some of the unintended consequences of exposure to light at night. For example, Amish have reduced risk compared to the general population for developing breast cancer and metabolic syndrome, two conditions associated with exposure to light at night (Fonken & Nelson, 2011). Second, the effects of nighttime light exposure on patient recovery should be evaluated. This could be achieved by introducing altered light fixtures into hospital and
retroactively monitoring variables such as length of patients stay. Finally, the effects of dim light at night on human physiology should be investigated in a controlled laboratory setting. Exposing people to a single night, or week, of dim light at night and monitoring various circadian and metabolic outputs may highlight the mechanism by which exposure to electril light at night influences human health
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