Mice exposed to dim light at night exaggerate inflammatory responses to lipopolysaccharide

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ABSTRACT

The mammalian circadian system regulates many physiological functions including inflammatory responses. Appropriately timed light information is essential for maintaining circadian organization. Over the past ~120 years, urbanization and the widespread adoption of electric lights have dramatically altered lighting environments. Exposure to light at night (LAN) is pervasive in modern society and disrupts core circadian clock mechanisms. Because microglia are the resident macrophages in the brain and macrophages contain intrinsic circadian clocks, we hypothesized that chronic exposure to LAN would alter microglia cytokine expression and sickness behavior following LPS administration. Exposure to 4 weeks of dim LAN elevated inflammatory responses in mice. Mice exposed to dimly lit, as compared to dark, nights exaggerated changes in body temperature and elevated microglia pro-inflammatory cytokine expression following LPS administration. Furthermore, dLAN mice had a prolonged sickness response following the LPS challenge. Mice exposed to dark or dimly lit nights had comparable sickness behavior directly following the LPS injection; however, dLAN mice showed greater reductions in locomotor activity, increased anorectic behavior, and increased weight loss than mice maintained in dark nights 24 h post-LPS injection. Overall, these data suggest that chronic exposure to even very low levels of light pollution may alter inflammatory responses. These results may have important implications for humans and other urban dwelling species that commonly experience nighttime light exposure.

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1. Introduction

Animals have evolved in temporal niches with recurring daily events. In order to maximize survival and reproduction virtually all living organisms have developed an endogenous 24 h time keeping system termed the circadian system. Circadian rhythms govern a broad range of physiological and behavioral functions with approximately 10% of the mammalian genome under direct circadian control (Panda et al., 2002; Storch et al., 2002). In mammals, circadian rhythms are initiated in, but not limited to, the suprachiasmatic nuclei of the hypothalamus (SCN). The SCN entrains peripheral circadian clocks through neural and hormonal cues. A wide range of peripheral and central cell types express circadian oscillations (reviewed in Mohawk et al., 2012), including multiple immune cells (Arjona and Sarkar, 2005; Boivin et al., 2003; Bollinger et al., 2011; Hayashi et al., 2007; Keller et al., 2009; O'Neill and Reddy, 2011; Silver et al., 2012a).

Many immune processes fluctuate throughout the day including antigen presentation, toll-like receptor function, cytokine gene expression, and lymphocyte proliferation (Arjona et al., 2012; Scheiermann et al., 2013). Furthermore, core circadian clock proteins are involved in immune regulation. The intensity of the NF-κB response to a variety of immunomodulators is mediated by CLOCK protein, which up-regulates NF-κB transcription (Spengler et al., 2012). Cryptochrome also controls expression of pro-inflammatory cytokines by limiting cAMP production (Narasimamurthy et al., 2012). Several inflammatory diseases such as asthma (Durrington et al., 2013) and rheumatoid arthritis (Gibbs and Ray, 2013) also show daily fluctuations in severity. Moreover, there is a time of day dependent response to lipopolysaccharide (LPS) (Halberg et al., 1960); mice exhibit a more robust response to LPS challenge during the light phase compared to the dark phase of the day (Marpegan et al., 2009; Spengler et al., 2012). The relationship between the circadian and immune systems appears to be bi-directional as LPS induced sepsis results in changes in circadian rhythms in mice (O'Callaghan et al., 2012) and humans (Li et al., 2013).

The circadian system expresses approximately, but not exactly, 24 h rhythms and therefore requires external input to maintain synchrony with the environment. The primary cue for entraining the circadian system is the external light/dark cycle. Over the course of the 20th century, urbanization and the widespread adoption of electric lighting have dramatically altered lighting

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environments. 99% of the population in the US and Europe is exposed to LAN (Cinzano et al., 2001) and 20% of the population experiences high and prolonged levels of nighttime light exposure while participating in shift-work (Monk, 2000). Importantly, exposure to even very dim levels of light at night (LAN) can disrupt circadian rhythms (Fonken et al., 2013). Because the immune and circadian systems are related, it is likely that there are inflammatory consequences of nighttime light exposure. Indeed, shift-workers are at increased risk for several inflammation-related disorders including heart disease (Vyas et al., 2012), cancer (Stevens, 2009), metabolic syndrome (Pietroiusti et al., 2010), and depression (McClung, 2013).

Exposure to LAN alters peripheral inflammation in diurnal Nile grass rats (*Arvicanthis niloticus*) (Fonken et al., 2011) and Siberian hamsters (*Phodopus sungorus*) (Bedrosian et al., 2011, 2013a). Nighttime light exposure may also elevate inflammation in the central nervous system (CNS) (Bedrosian et al., 2013b). Because a circadian clock in macrophages/monocytes regulates peripheral immune responses (Keller et al., 2009) and microglia express circadian clock genes (Nakazato et al., 2011) we hypothesized that exposure to LAN would alter sickness behavior and microglia inflammatory responses. To test this hypothesis, we exposed mice to 4 weeks of dim LAN (dLAN) and then measured microglia pro-inflammatory cytokine expression and sickness responses following LPS.

2. Materials and methods

2.1. Animals

Male Swiss Webster mice ($\sim$3 months of age) were obtained from Charles River for use in this study. Mice were individually housed in propylene cages (dimensions: $33 \times 19 \times 14$ cm) 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 ($\sim$150 lux)/dark (0 lux)] for >3 weeks following arrival. After the acclimation period, mice were randomly assigned to either remain in LD or transferred to a light/dim cycle [dLAN; 14:10 light ($\sim$150 lux)/dim light (5 lux)]. All experimental procedures 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*.

2.2. Fever assessment

After 3 weeks in lighting conditions, 16 mice (n = 8 per lighting condition) were implanted intraperitoneally (i.p.) with radio-telemetric transmitters (Mini Mitters, Respironics, Bend, OR, USA) (Castanon-Cervantes et al. 2010; Bilbo et al. 2002b). Following surgeries mice were administered a 0.1 mg/kg i.p. injection of buprenorphine in sterile saline, placed in a clean home cage, and constantly monitored for 2 h. Cages were then placed on TR-4000 receiver boards that were connected to a DP-24 DataPort (Mini Mitter) and computer. Emitted temperature and activity frequencies were collected in 30 min bins and converted to temperature and locomotor activity values by interpolating from programmed calibration curves of the individual transmitters. After a ten day recovery period, mice received three consecutive injections 24 h apart, 30 min prior to lights out. Mice were injected with sterile saline for 2 days and then once with LPS (0.5 mg/kg lipopolysaccharide from *Escherichia coli* serotype 026:B6, Sigma Chemical). Home cage locomotor activity was quantified as an indication of sickness behavior during the dark phase for 4 days prior to, directly

following, and 24 h post-LPS injection. Mice were killed 48 h post-LPS via decapitation while under deep isoflurane anesthesia. Two LD mice are excluded from body temperature and activity analyses, but not sickness responses (described below), because their transmitters stopped working during the study.

2.3. Sickness behavior

Sickness behavior was monitored in the mice implanted with Mini Mitters. Food intake and consumption of sweetened condensed milk solution (Kroger brand, diluted 1:1 with tap water) were recorded for 4 days prior to, directly following, and 24 h post-LPS injection. Rodents readily consume sweetened condensed milk and lack of consumption indicates sickness. A modified bottle containing the milk solution was placed in the home cage for 5 h each day beginning with the onset of the dark phase (Zeitgeber time, ZT12–ZT17). Milk intake was quantified by subtracting the mass of the bottle at the end of the session from its initial filled mass. Food was weighed daily at ZT12 to quantify total daily food intake and mice were weighed at this time.

2.4. Microglial extractions and qPCR

A separate group of LD and dLAN mice were injected with 0.5 mg/kg LPS or sterile saline (n = 5 per group/treatment) to assess the effects of exposure to LAN on microglia activation. Microglia were extracted following a previously reported protocol 3 h after LPS injection (Wynne et al., 2010). Mice were anesthetized with isoflurane vapors, given a lethal dose of sodium pentobarbital, and perfused transcardially with ice cold sterile saline. Brains were removed and placed on ice in 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 HBSS and centrifuged for 7 min at 600*g* at 15 °C. Supernatant was discarded and cell pellets re-suspended in 70% isotonic Percoll (GE Healthcare, Uppsala, Sweden). A discontinuous Percoll gradient was then created by adding 50%, 35%, and 0% (bottom to top) isotonic Percoll layers to the cell suspension. The Percoll gradient was centrifuged for 30 min at 2000*g* at 15 °C and microglia were removed from the interphase layer between the 70% and 50% Percoll. Microglia were homogenized in Trizol and RNA was extracted according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase enzyme (Invitrogen) according to the manufacturer's protocol. Relative IL-1β, IL-6, and TNFα gene expression 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. 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.

2.5. Statistical analyses

Body temperature from the second day of saline injections was compared to body temperature following injection with LPS. Temperatures were analyzed for the 18 h following injection of LPS or saline respectively, using a repeated measures ANOVA with lighting condition (LD or dLAN) as the between subjects factor. Locomotor activity, food intake, milk intake, and body mass are all expressed as a percentage of baseline values. Baseline values are the average of the variable for the 2 days prior to saline injection collected for each individual mouse. Changes in locomotor

activity, food intake, milk intake, and body mass were assessed with repeated measures ANOVA. Relative gene expression values were analyzed using a two-way ANOVA with lighting condition (LD or dLAN) and injection (saline or LPS) as the between subjects variables. Following a significant F score, *post hoc* analyses were performed using Tukey's honestly significant differences test. Mean differences were considered statistically significant when $p < 0.05$. All statistical analyses were conducted using StatView software.

3. Results

3.1. Fever

All mice significantly altered body temperature following injection with LPS ($F_{17, 408} = 45.537$, $p < 0.05$; Fig. 1A). Both dLAN and LD mice transiently reduced body temperature in response to LPS and then heightened body temperature beginning 8 h post-injection. Furthermore, there was a significant interaction between body temperature and lighting condition over time ($F_{17, 408} = 3.513$, $p < 0.0001$); mice exposed to dimly lit nights had both a lower nadir (groups significantly differ at ZT14 and ZT15, *post hoc*, $p < 0.05$) in body temperature and a higher peak body temperature (groups significantly differ at ZT23, *post hoc*, $p < 0.05$). Overall, there was a greater range in body temperature among dLAN mice post-LPS injection as compared to mice exposed to dark nights ($F_{1,12} = 6.844$, $p < 0.05$; Fig. 1B).

3.2. Sickness behavior

In order to establish whether mice exposed to dLAN exhibit increased sickness responses following an LPS injection, sweetened condensed milk intake, food intake, home cage locomotor activity, and body mass were measured every day for 4 days prior to LPS injection, on the day of LPS injection, and 24 h post-LPS injection. All mice reduced food intake following LPS injection ($F_{2,34} = 46.271$, $p < 0.05$; Fig. 2A) and there was no effect of lighting
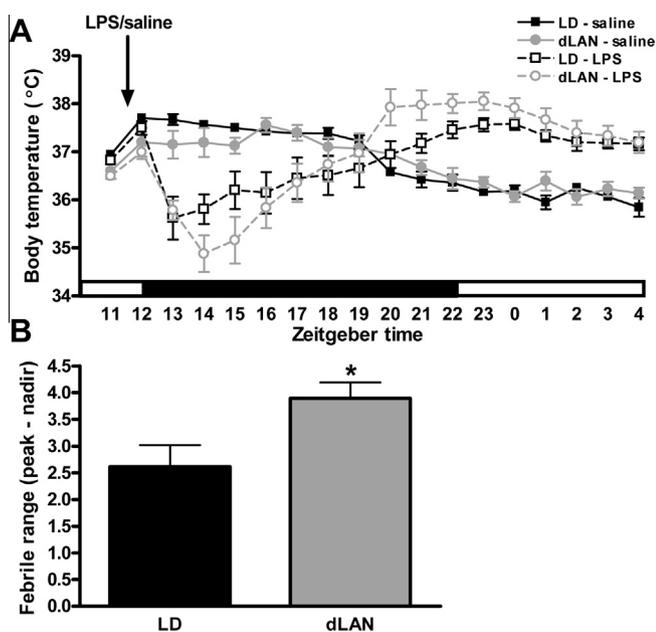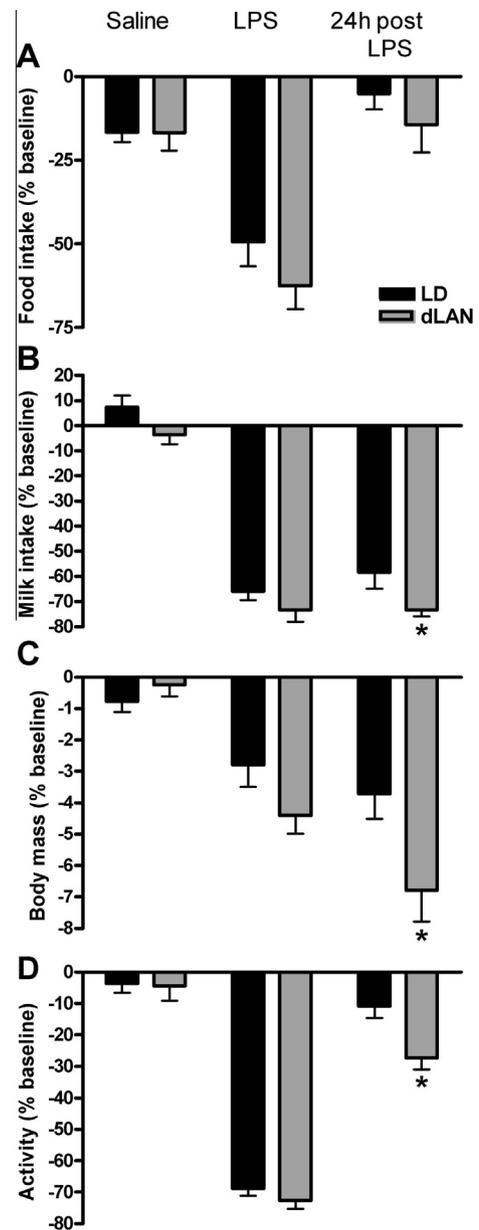
Fig. 2. (A) Food intake, (B) sweetened condensed milk intake, (C) body mass, and (D) activity following injection with LPS or saline. All values are expressed as a percentage of the baseline value established prior to injections. Data are expressed as mean ± SEM. * Main effect of lighting condition, $p < 0.05$.

condition ($p > 0.05$). There was also a significant reduction in sweetened condensed milk intake following LPS injection ($F_{2,34} = 162.795$, $p < 0.05$; Fig. 2B) and mice exposed to dLAN showed greater reductions in milk intake compared to mice exposed to dark nights ($F_{1,34} = 10.963$, $p < 0.05$). dLAN and LD mice had a comparable reduction in milk intake directly following LPS injection; however, LD mice consumed more milk than dLAN mice on the day following LPS injection (*post hoc*, $p < 0.05$).

Both groups reduced body mass following LPS injection ($F_{2,34} = 27.460$; $p < 0.05$; Fig. 2C), but mice exposed to dimly lit nights lost more weight than mice maintained in dark nights ($F_{2,34} = 3.895$; $p < 0.05$). This main effect was driven by differences in body mass on the day following LPS injections; dLAN mice continued to lose weight on the day following LPS injection, whereas LD mice did not lose additional weight at this time (*post hoc*; $p < 0.05$).

Fig. 1. (A) Body temperature following LPS or saline injection in mice exposed to dimly lit or dark night for four weeks. (B) Body temperature range following LPS injection. Data are expressed as mean ± SEM. *Main effect of lighting condition, $p < 0.05$.

Home cage locomotor activity was reduced in both groups following LPS injection ($F_{2,26} = 175.434$, $p < 0.05$; Fig. 2D). However, mice exposed to dLAN showed greater reductions in locomotor activity as compared to mice exposed to dark nights ($F_{1,26} = 8.404$, $p < 0.05$). P*ost hoc* analysis revealed that this effect was only significant on the day following LPS injection ($p < 0.01$).

3.3. Microglia gene expression

LPS injection increased microglia TNFα, ILβ, and IL6 mRNA expression ($F_{1,15} = 22.749$, 26.722, 26.818, $p < 0.05$). Furthermore, there was an interaction between lighting condition and LPS treatment on microglia TNF and IL6 mRNA expression ($F_{1,15} = 4.553$ and 5.674, $p < 0.05$). There were no differences in gene expression among saline injected mice; however, mice exposed to dLAN elevated TNF and IL6 expression following LPS as compared to mice maintained in dark nights (*post hoc*, $p < 0.05$) (see Fig. 3).

4. Discussion

The immune and circadian systems are reciprocally related with circadian disruption altering inflammatory responses (Arjona et al., 2012; Scheiermann et al., 2013). Exposure to LAN is increasingly common in modern society and can disrupts core circadian clock mechanisms (Gaston et al., 2012). Although the effects of exposure to LAN and other circadian disruptors on peripheral immune responses are well characterized (for examples see (Bedrosian et al., 2011, 2013a; Castanon-Cervantes et al., 2010; Fonken et al., 2011), the effects of circadian disruption on CNS inflammation are less understood. Here we hypothesized that chronic exposure to LAN would alter microglia cytokine expression and sickness behavior following LPS administration.

Mice injected with LPS exhibited a transient reduction in body temperature followed by a pyretic response. Multiple febrile phases typically occur following LPS administration and hypothermic responses to LPS are common in mice and vary across strain and can be effected by ambient room temperature and the dose of LPS (Romanovsky et al., 2005). Mice exposed to dimly lit nights had both a lower hypothermic body temperature 2 h post-injection and a higher peak body temperature 11 h following LPS administration. Mice exposed to dLAN also increased LPS-induced sickness behavior. dLAN mice significantly reduced locomotor activity, increased anorexic behavior, and lost more weight than mice maintained in dark nights. Although sickness behavior did not differ between groups directly following LPS challenge, dLAN mice increased sickness behavior on the day following LPS injection. This
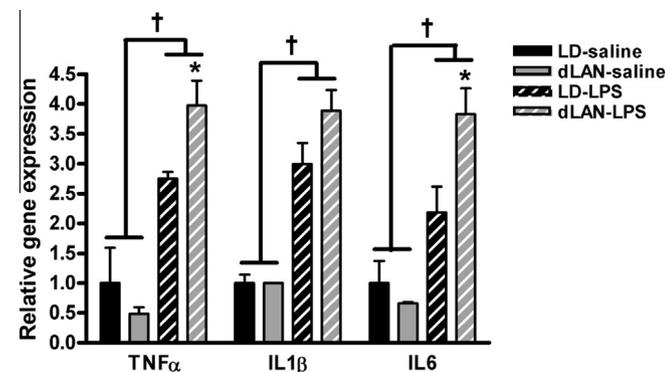

Fig. 3. Quantification of mRNA encoding TNFα, IL1β, and Il6. Values are expressed as relative abundance (mean ± SEM) after normalization to *18S* and presented as fold change relative to LD saline injected mice. *$p < 0.05$ between lighting conditions, †$p < 0.05$ between saline and LPS.

indicates mice exposed to dLAN have a prolonged sickness response compared to mice maintained in dark nights. Proper physiological and behavioral responses following pathogenic infection are critical in allowing individuals to overcome infection and regain strength (Konsman et al., 2002). While reducing food intake directly following infection appears adaptive (Adamo et al., 2010), prolonged anorexic responses can lead to cachexia and a longer recovery time following infection (Laviano et al., 2008).

Microglia are the resident immune cells in the CNS and perturbations of the microenvironment induce microglia activation (Kettenmann et al., 2011). Macrophages have an intrinsic circadian clock that controls inflammatory immune responses (Keller et al., 2009) and circadian disruption exacerbates macrophage responses to *ex vivo* LPS stimulation (Castanon-Cervantes et al., 2010). Because nighttime light exposure can disrupt circadian rhythms, we hypothesized that exposure to LAN would alter microglia response to an inflammogen. Peripheral LPS injection elevated microglia induction of TNFα and IL6 mRNA in mice exposed to dimly lit as compared to dark nights. This fits with our behavioral results, as an exaggerated sickness response is typically associated with protracted microglia activation (Norden and Godbout, 2013). There were no differences between groups in microglia cytokine expression following saline injection suggesting that LAN does not increase microglia expression of these pro-inflammatory cytokines independent of an immune challenge.

Exposure to LAN may elevate immune responses through several mechanisms. First, disruption of rhythmic organization in mice exposed to light at night may make the immune system more reactive to inflammatory challenges. Indeed, the circadian clock controls toll-like receptor mediated innate and adaptive immunity (Paladino et al., 2010; Silver et al., 2012b) and macrophages extracted from animals that undergo a chronic jet lag paradigm elevate cytokine production when stimulated *ex vivo* with LPS (Castanon-Cervantes et al., 2010). Second, exposure to light at night alters core circadian clock protein expression (Fonken et al., 2013). Circadian clock proteins are directly involved in NF-κB transcriptional regulation and downstream cytokine release (Narasimamurthy et al., 2012; Spengler et al., 2012). This suggests nighttime light exposure may alter NF-κB mediated cytokine production following LPS challenge. Finally, blunting circadian rhythms with exposure to light at night may prevent tissue recovery and clearance of potentially harmful cellular elements in circulation (reviewed in Scheiermann et al., 2013).

The observed effects of circadian disruption on the immune system are likely independent of sleep loss. We have previously demonstrated that exposure to dLAN does not disrupt sleep in mice (Borniger et al., 2013). Furthermore, our results are consistent with other work demonstrating that chronic environmental circadian disruption elevates inflammatory responses following LPS (Brager et al., 2013; Castanon-Cervantes et al., 2010). The environmental circadian disruption in the study by Brager and colleagues causes sleep loss, but the group included a control sleep disrupted group that did not show the same enhancement in immune measures. Changes in stress reactivity can also profoundly affect immune function (Frank et al., 2013); however, our previous work indicates that corticosteroids are not affected by nighttime light exposure in mice (Fonken et al., 2009, 2010, 2013). Other studies have similarly demonstrated that circadian disruption alters inflammatory responses independently of changes in glucocorticoids (e.g., Castanon-Cervantes et al., 2010).

Overall, exposure to dim nighttime lighting elevated inflammatory response in mice. Mice exposed to dimly lit nights exaggerated changes in body temperature, increased sickness behavior, and elevated microglia pro-inflammatory cytokine expression following LPS administration. The potentiated fever response and delayed recovery following LPS challenge among dLAN mice is an

especially strong index of pathology. These results suggests that chronic exposure to light pollution, even at very low intensities, may alter immunological responses. This may have important implications for humans and other urban dwelling species that commonly experience these levels of nighttime light exposure (Gaston et al., 2012).

Acknowledgments

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